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The cooperative roles of the dopamine receptors, D_1R and D_5R, on the regulation of renal sodium transport

John J. Gildea, PhD[1], **Ishan T. Shah, BS**[1], **Robert Van Sciver, BS**[1], **Jonathan A. Israel, MD**[1], **Christoph Enzensperger, PhD**[2], **Helen E. McGrath, BA**[1], **Pedro A. Jose, MD, PhD**[3], and **Robin A. Felder, PhD**[1]

[1]The University of Virginia Health System, Department of Pathology, Charlottesville, VA

[2]Institut für Pharmazie, Lehrstuhl für Pharmazeutische/Medizinische Chemie, FriedrichSchiller-Universität Jena, Jena, Germany

[3]University of Maryland School of Medicine, Departments of Medicine and Physiology, Baltimore, MD

Abstract

Determining the individual roles of the two dopamine D_1-like receptors (D_1R and D_5R) on sodium transport in the human renal proximal tubule has been complicated by their structural and functional similarity. Here we used a novel D_5R-selective antagonist (LE-PM436) and D_1R or D_5R-specific gene silencing to determine second messenger coupling pathways and heterologous receptor interaction between the two receptors. D_1R and D_5R co-localized in renal proximal tubule cells and physically interact, as determined by co-immunoprecipitation and FRET microscopy. Stimulation of renal proximal tubule cells with fenoldopam (D_1R/D_5R agonist) led to both adenylyl cyclase and phospholipase C (PLC) activation using real-time FRET biosensors ICUE3 and CYPHR, respectively. Fenoldopam increased cAMP accumulation and PLC activity and inhibited both NHE3 and NaKATPase activities. LE-PM436 and D_5R siRNA blocked the fenoldopam-stimulated PLC pathway but not cAMP accumulation, while D_1R siRNA blocked both fenoldopam-stimulated cAMP accumulation and PLC signaling. Either D_1R or D_5R siRNA, or LE-PM436 blocked the fenoldopam dependent inhibition of sodium transport. Further studies using the cAMP-selective D_1R/D_5R agonist SKF83822 and PLC-selective D_1R/D_5R agonist SKF83959 confirmed the cooperative influence of the two pathways on sodium transport. Thus, D_1R and D_5R interact in the inhibition of NHE3 and NaKATPase activity, the D_1R primarily by cAMP, while the D_1R/D_5R heteromer modulates the D_1R effect through a PLC pathway.

Correspondence to be sent to: Robin A. Felder, Ph.D. The University of Virginia, P.O. Box 801400, Charlottesville, VA 22908, rfelder@virginia.edu, Ph 434-466-1131, Fax 434-924-5718.

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Introduction

Dopamine produced in the renal proximal tubule from circulating L-DOPA acts as a paracrine and autocrine hormone to regulate greater than 50% of sodium excretion in animals and humans on moderately high salt intake.[1-3] Dopamine exerts its natriuretic effect by acting with cell surface receptors and intracellular pathways to stimulate intracellular adenylyl cyclase (AC) and phospholipase C (PLC) activities and inhibit sodium transport. There are 5 dopamine receptors expressed in renal proximal tubule cells (RPTCs): the D_1-like (D_1R and D_5R) and the D_2-like (D_2R, D_3R, D_4R) receptors, that interact with other systems including the renin angiotensin system to regulate renal sodium excretion.[4, 5] In mice, a deficiency in local production of dopamine results in hypertension and a decrease in longevity.[6]

The D_5R is of particular interest because it has a 10-fold higher affinity for dopamine than D_1R but has an 80% homology in the transmembrane domain and a 30% homology in the N and C termini.[7] Both D_1R and D_5R are linked to $G_{\alpha S}$.[7] The D_1R, but not D_5R, also couples to G_O[8] and G_{Olf}[9] while the D_5R but not D_1R, couples to G_Z[10] and $G\alpha_{12/13}$.[11] In the rat forebrain, D_1-like receptors couple to both $G_{\alpha S}$ and $G_{\alpha q}$ while in the rat hippocampus and amygdala, D_1-like receptors couple only to $G_{\alpha q}$.[12] In contrast, in hippocampal and brain cortical and striatal tissues of $D_5R^{-/-}$ mice, PLC is not activated in the brain following stimulation with dopamine, the non-selective D_1R/D_5R agonist SKF38393, or the PLC-selective D_1R/D_5R agonist SKF83959.[13, 14] This suggests that in specific areas in the brain, D_1R couples preferentially to AC and the D_5R couples to PLC.[15]

Which D_1-like receptor subtype is linked to Gαq and PLC in cells in which both D_1-like receptors are expressed is not clear. While the D_1R and D_5R may have different anatomical distributions in the brain,[16] both the renal D_1R and D_5R are found in specific nephron segments (proximal convoluted and straight tubules, thick ascending limb of Henle, distal convoluted tubule and cortical collecting duct), suggesting a possible interaction between these receptors.[17] We have reported that in renal cortical membranes, D_1-like receptors are linked to Gαq and that PLC activity can be stimulated by D_1-like receptors independent of AC.[18] Others have reported that the linkage of renal D_1-like receptors to PLC may be observed to a greater extent in rats fed a high salt diet.[19] We have previously shown that both D_1R and D_5R are expressed in RPTCs.[20] However, little is known about their physical association and the relative contribution of each receptor on AC and PLC signaling in a single cell type. Studying the D_1R and D_5R in a single cell type is important because similarities and differences in signaling pathways may be tissue specific (vide supra).

Deletion of either D_1R ($D_1R^{-/-}$)[21] or D_5R ($D_5R^{-/-}$)[22] gene in mice results in elevation of blood pressure. Increased salt intake further increases the high blood pressure of $D_5R^{-/-}$mice.[23, 24] The effect of salt intake on blood pressure in $D_1R^{-/-}$ mice has not been reported. Since both the D_1R and D_5R are important in the control of blood pressure,[3-5, 17, 24-26] in part by regulation of renal sodium transport, we further examined the AC and PLC pathways and their relationship to NHE3 and NaKATPase using agonists that have selectivity to D_5R, AC, and PLC and silencing of D_1R or D_5R (see our model display of these pathways and agonists). These studies were performed using human RPTCs with

normal D_1R and AC coupling, since a fully functional D_1R is necessary for D_1R/D_5R agonist-mediated inhibition of NHE3 and NaKATPase.[27]

Results

We demonstrated that the D_1R and D_5R were physically associated in the same macromolecular protein complex using a co-immunoprecipitation method (Figure 1). We further demonstrated that the amount of D_5R protein pulled down using the D_1R antibody was increased following fenoldopam (FEN, 1 μmol/L, 30-min) stimulation ($P<0.001$, N=6, one immunoblot is shown in Figure 1 inset). Further evidence of association is seen by reciprocal co-immunoprecipitation with D_1R and D_5R, and adequate washing is demonstrated by inclusion of the appropriate non-specific IgG antibody controls in the initial immunoprecipitation (Figure S1).

Confocal microscopy was used to determine if the two receptors co-localize in renal cortical proximal tubules. The confocal immunofluorescence images demonstrated that the D_1R and D_5R were both highly expressed in the proximal tubule and showed a similar sub-cellular localization, i.e., sub-apical position at the base of the microvilli (Figure S2). The composite images of both human and rat tubules demonstrated extensive co-localization in the sub-apical region. This sub-apical localization of the two receptors was verified by co-staining with a sub-apical marker α-adaptin (data not shown).

FRET analysis of these experiments was not possible because the two antibodies that work well on fixed tissue bind to either intracellular (D_1R) or extracellular (D_5R) epitopes that are on opposite sides of the phospholipid bilayer and are therefore outside the distance measurable by FRET. We therefore investigated their possible heterooligomerization using live-FRET microscopy. Figure S3 demonstrates live-cell sensitized emission-corrected FRET analysis of two different color-GFP fusion proteins of D_1Rs and D_5Rs heterologously expressed in HEK293 cells. A positive corrected FRET signal suggests that the two receptors are within 10 nm of each other. We verified these findings in lightly fixed non-permeabilized human RPTCs (Figure 2), using two different extracellular epitope-specific fluorescently-labeled antibodies that should bind to both epitopes either on the D_1R or the D_5R. These two antibodies should be found on the same side of the phospholipid bilayer and therefore can be used for FRET analysis. The photomicrographs in Figure 2 demonstrated a clear cell surface FRET signal. The addition of FEN (1μmol/L, 30-min) increased the FRET signal and the amount of D_1R at the cell surface.

A new D_5R antagonist (LE-PM436)[28] was utilized to distinguish signaling of D_5Rs from D_1Rs. To validate its selectivity, we used HEK293 cells that were stably transfected with either D_1R or D_5R cDNA and expressed these receptors at normal physiological concentrations, measured at 3 pmoles/mg membrane protein.[29] cAMP production was measured following SKF38393 (non-selective D_1R/D_5R receptor agonist, 1 μmol/L, 30-min) stimulation and blocked with LE300 (non-selective D_1R/D_5R receptor antagonist) and LE-PM436 (selective D_5R antagonist) (Figure 3). The addition of LE300 (10 μmol/L) inhibited SKF-stimulated intracellular cAMP production in both D_1R and D_5R-expressing HEK293 cells, confirming that it is not selective to either D_1R or D_5R. LE-PM436 (1 nmol/L)

inhibited SKF-stimulated cAMP production by 84.0 ±1.2% in D_5R -expressing HEK293 cells, but did not inhibit the SKF-stimulated cAMP production in D_1R-expressing HEK293 cells, confirming that LE-PM436 is a D_5R-selective antagonist ($P<0.001$ vs SKF+VEH, N=8 per group). These studies also confirm the coupling of either D_1R or D_5R to AC, however the amount of cAMP produced by D_5R-transfected HEK293 cells in response to SKF was 26.4 ±0.5% the amount measured from the D_1R-transfected cells (N=18, $P<0.001$, data not shown), despite similar receptor expression (using Bodipy630 SKF83566 binding, N=12, data not shown). Basal cAMP levels were 1.04±0.08 pmoles/mg protein in D_1R-expressing HEK293 cells, and 1.13±0.03 pmoles/mg protein in D_5R-expressing HEK293 cells. Induced levels for D_1R-expressing HEK293 cells were 15.52±1.07 pmoles/mg protein following SKF exposure (30-min, 1 μmol/L) and induced levels of D_5R-expressing HEK293 cells were 4.01±0.08 pmoles/mg protein.

Intracellular calcium, cAMP, and PLC levels were measured in RPTCs in response to FEN with and without the antagonists LE300 (D_1R and D_5R), U73122 (PLC) and LE-PM436 (D_5R) (Figure 4). The left Y-axis depicts the ICUE3 (measure of intracellular cAMP accumulation) and CYPHR (measure of intracellular PLC activation state), normalized to vehicle (VEH). The right Y-axis depicts the intracellular ratiometric Ca^{++} concentration measured by FURA-2. Stimulation with FEN (1 μmol/L, 20-min) increased the cAMP levels, PLC activity, and Ca^{++} levels in RPTCs ($^{\#}P<0.001$ vs VEH, N=6 for ICUE3/CYPHR, N=11 for FURA-2). The addition of the D_1R/D_5R antagonist LE300 (10 μmol/L, 20-min) blocked the FEN-mediated increase of all 3 variables (*$P<0.001$ vs FEN, N=6 for ICUE3/CYPHR, N=17 for FURA-2). Both the PLC inhibitor U73122 (10 μmol/L, 20 min) and the D_5R-specific inhibitor LE-PM436 (1 nmol/L, 20-min) blocked the FEN-mediated PLC activation but did not affect cAMP levels (*$P<0.001$ vs FEN, N=3-14). The D_1R/D_5R receptor AC-specific activator SKF83822 (10 μmol/L, 20-min) caused an increase only in cAMP levels and not Ca^{++} levels or PLC activity ($^{\#}P<0.001$ vs VEH, N=3-17). By contrast, the PLC-activator SKF83959 (10 μmol/L, 20-min) increased PLC activity and Ca^{++} levels but not cAMP ($^{\#}P<0.001$ vs VEH, N=4-17) levels. When these two agonists (SKF83822 and SKF83959) were combined there was no additive effect (data not shown).

We next utilized siRNA technology. The efficacy of the siRNA is demonstrated in Figure S4, using immunofluorescence staining of transfected RPTCs. RPTCs that were transfected with D_1R or D_5R siRNA had a marked decrease in their respective expression levels compared to scrambled (SCR) RNA control, using the same timing and format as the sodium assays ($P<0.001$ vs SCR, N=4). Figure 5 shows that D_1R siRNA blocked the FEN-stimulated increase in cAMP accumulation and PLC activity (*$P<0.05$ vs SCR, N=16 for ICUE3, N=4 for CYPHR). By contrast, the D_5R siRNA blocked only the FEN-mediated increase in PLC activity (*$P<0.05$ vs SCR, N=4), while the combination of the D_1R and D_5R siRNAs blocked both signaling pathways (*$P<0.05$ vs SCR, N=14 for ICUE3, N=4 for CYPHR). These data indicate that in RPTCs, the D_1R is coupled to both AC and PLC whereas the D_5R is linked mainly to PLC. This is in contrast to the linkage of D_5R to AC in HEK293 cells heterologously expressing D_5R.

We next determined how selective D_1R and/or D_5R stimulation or reduction with siRNA would ultimately affect sodium influx via NHE3 or sodium efflux via NaKATPase (Figure

6). The D_1R/D_5R agonist FEN (1 μmol/L, 30-min) inhibited both NHE3-mediated sodium influx and NaKATPase-mediated sodium efflux in immortalized RPTCs (*P<0.0001 vs VEH, N=6, Figure 6, Panel A). The addition of U73122 (10 μmol/L), LE-PM436 (1 nmol/L), or LE300 (10 μmol/L), by themselves did not affect sodium influx or efflux (data not shown) but their co-incubation with FEN returned the sodium influx and efflux to VEH levels. The D_1-like receptor AC-specific activator SKF83822 (10 μmol/L, 20-min) inhibited both NHE3 and NaKATPase ($^{\#}$P<0.001 vs VEH, N=6). By contrast, the PLC-activator SKF83959 (10 μmol/L, 20-min) did not inhibit either NHE3 or NaKATPase activity. This suggests that the primary inhibitory signaling pathway for NHE3 and NaKATPase is via AC. The combination of SKF83822 and SKF83959 inhibited both NHE3 and NaKATPase (*P<0.0001 vs VEH, N=6), and to a greater extent than SKF83822 alone. As shown in Figure 6 (Panel B), the FEN-mediated inhibition of sodium influx and efflux were reversed by siRNA specific to D_1R, D_5R, or both D_1R and D_5R (*P<0.05 vs SCR, **P<0.001 vs SCR, N=6). These data indicate that both the cAMP and PLC pathways are necessary for inhibition of sodium influx and efflux, involving both D_1R and D_5R.

In order to control for the effects of the antagonists alone (U73122, LE-PM436 and LE300), each one was included in both the sodium influx and efflux assays and showed no effect in either assay (data not shown). Antisense oligos to the D_1R or D_5R[20] recapitulated the effects we observed with the specific inhibitors of either D_1R or D_5R (data not shown).

Discussion

The interplay between the D_1R and D_5R, two closely related cell surface receptors that share the same endogenous agonist (dopamine), is important in understanding the role of dopamine in cell function. The D_1R and D_5R are expressed in different areas of the brain, which suggests that the D_5R has a distinct role in neurotransmission when compared to the D_1R.[16] However, the direct interaction of endogenously expressed D_1R and D_5R has not been well studied. Dopamine receptors outside the central nervous system are often expressed within the same cells. For example, the D_1R and D_5R are both expressed in the tunica media of systemic arteries[30] and both are expressed in almost all segments of the nephron where active sodium transport occurs.[17] D_1R and D_5R heterologously co-expressed in HEK293 cells have been found to hetero-oligomerize.[31]

In our current studies, we demonstrated that the D_1R and D_5R were not only present in RPTCs, but they co-localized, co-immunoprecipitated, and directly physically interacted, as demonstrated by FRET microscopy. The co-localization of the D_1R and D_5R was seen in a compartment at the base of the microvilli in the proximal tubule in human and rat kidneys. Microvilli are key structures in the renal proximal tubule that serve to increase the luminal membrane surface area for protein and peptide uptake and catabolism and ion transport. Microvilli also serve as sensors for sampling and adjusting the concentration of various solutes within the glomerular filtrate in response to mechanotransduction.[32] The D_5R and D_1R were found clustered in a tight band in the sub-microvilliary space, where they could be rapidly recruited to the plasma membrane in response to cell surface receptor stimulation. We demonstrated in the current report that the D_1R/D_5R agonist FEN caused recruitment of

the D_1R to the cell surface plasma membrane, corroborating our previous report using surface biotinylation and western blot analysis.[33]

We have reported that salt-sensitive hypertension in humans correlates with defective recruitment of D_1R to the cell surface of urinary RPTCs when their intracellular sodium levels were increased.[34] In contrast, we found that the D_5R was not recruited to the cell surface but appears to have a constant cell membrane concentration not altered by agonist stimulation.[35] We further found that this constant level of D_5R at the cell membrane was lower in RPTCs whose D_1R coupling to AC was defective.[35] The D_5R has not been demonstrated to stimulate cAMP accumulation in the kidney but has been shown to couple to AC in heterologous overexpression systems such as the HEK293 cells shown in this report. Overexpression of proteins can result in promiscuous associations and therefore, we studied D_1R and D_5R endogenously expressed in the same cell, i.e., RPTC. These studies are the first demonstration of the blocking of cellular D_5R-coupled second messenger activity using a selective D_5R antagonist. LE-PM436 and siRNA to the D_5R demonstrated similar blocking effects in all experimental assays further confirming the selectivity of LE-PM436 to the D_5R. However, LE-PM436 may have an important advantage over siRNA in studying the D_5R because the long-term silencing of the D_5R expression by siRNA cannot be readily reversed. Our current studies showed for the first time in RPTCs that endogenously express both D_1R and D_5R that the D_5R preferentially stimulated PLC activity and increased intracellular Ca^{++}, effects that were inhibited by the D_5R selective antagonist LE-PM436. The independence of these pathways is highlighted by the data that demonstrated that SKF83822 stimulated AC but not PLC, while SKF83959 stimulated PLC but not AC. Also, neither SKF83822 nor SKF83959 inhibited the pathway that it does not stimulate. This distinction is important because there is a PKA and PKC cross-talk following D_1R[36] and D1-like receptor [37] stimulation. Indeed in the current study, PLC stimulation was blocked when the D_1R was silenced. It has been shown that dopaminergic inhibition of NaKATPase via PLC and PKC is Na^+ dependent.[38]

Previous reports have demonstrated that D_1-like receptor stimulation inhibited various ion transporters and exchangers in the renal proximal tubule luminal membrane (NHE3, Na^+Pi co-transporter, and the Cl^-/HCO_3^- exchanger) and ion exchanger and sodium pump in the basolateral membrane (NaKATPase, and the Cl^-/HCO_3^- exchanger).[26] However, it is not known which D_1-like receptor subtype, D_1R or D_5R, mediates these effects. In D_5R deficient mice, distal tubular sodium transporters/channels ($Na^+K^+2Cl^-$co-transporter, Na^+Cl^- co-transporter, and the α and γ subunits of the epithelial sodium channel) were increased,[39] suggesting a tonic inhibition of the activity of these proteins via the D_5R. In the current experiments, we showed that in addition to a functional D_1R, D_5R stimulation was also necessary for the inhibition of NHE3-mediated sodium influx and NaKATPase-mediated sodium efflux following D_1R/D_5R receptor stimulation. Taken together with our previous studies demonstrating that the D_5R is responsible for the tonic down-regulation of the anti-natriuretic angiotensin II type 1 receptor (AT_1R) in rat and human RPTCs, these data suggest that the D_1R is the mediator, while the D_5R as part of the D_1R/D_5R heteromer and PLC signaling acts as the modulator, of the inhibition of sodium transport. Our proposed model for these pathways and their roles in sodium transport (NHE3 and NaKATPase) regulation is shown in Figure 7.

Previous studies on the physiological effects of dopamine and synthetic dopamine agonists and antagonists have been confounded by the lack of a selective antagonist to either receptor. The recent availability of LE-PM436 will now enable the in vivo study of the selective roles of the renal D_1R and D_5R receptors. An overactive renal renin angiotensin system (RAS) and diminished function of the renal dopaminergic system has been associated with hypertension and salt-sensitivity *in-vivo* in mouse, rats, and humans.[40] One class of anti-hypertensive pharmaceutical drugs that affect these systems and consequently blood pressure in all three species are AT_1R blockers. One of the many beneficial effects of AT_1R blockers may be mediated through the pathways described in this paper. Higher expression of angiotensinogen in high salt conditions, as well as numerous SNPs in the RAS system such as ACE, angiotensinogen or AT_1R, could lead to overactive AT_1R stimulation, downregulation of the D_5R and subsequent loss of D_5R-PLC specific natriuresis.[41] Use of AT_1R blockers under high salt conditions would allow the increased conversion of L-dopa to dopamine in the proximal tubule to stimulate both the D_1R and D_5R to aid in natriuresis and lower blood pressure since there would be diminished counter-regulation by the AT_1R. The implications of this paper may also suggest reasons why AT_1R blockers are not always effective; patients with proximal tubule D_1R -AC uncoupling may have diminished high salt D_5R-PLC induction of natriuresis. In animal models, the drug LE-PM436 would allow testing of these hypotheses in acute studies that otherwise would be difficult with siRNA or knockout models where gene compensation could alter the proper interpretation of the results.

Methods

RPTC Culture

RPTCs were obtained from normal tissue from nephrectomies in human subjects, under an institutional review board-approved protocol according to the Declaration of Helsinki, Title 45, Part 46, and U.S. Code of Federal Regulations. Cell lines were immortalized as previously described[33, 42, 43] and demonstrated only proximal tubule-specific characteristics as extensively detailed in our previous publication.[27] We have identified two distinct sub-populations of RPTCs, those that have a D_1R with normal AC coupling, and those with defective D_1R to AC coupling (uncoupled). We utilized only the normally-coupled RPTC lines i7448, i16, and i22 in the current studies, since a fully functional D_1R is necessary in our sodium transport assays.[27]

HEK293 Cell Culture and Transfection

Human embryonic kidney (HEK293) cells were obtained from ATCC (Manassas, VA) and grown in DMEM high glucose with 10% FBS. They were stably transfected with human D_1R and D_5R as previously described[23, 44] and these cells were used to confirm the specificity of LE-PM436 for the D_5R. For transient transfection of GFP fusion constructs used in the live FRET analysis, the D_1R and the D_5R were PCR-amplified from cDNA clones obtained from the Missouri S&T cDNA Resource Center (www.cdna.org) and subcloned into the pEGFP-N1 vector (Clontech) that had the EGFP replaced with either CyPet for D_5R or YPet for D_1R.

Pharmacological Agents

The D_1-like (D_1R/D_5R) receptor agonists, FEN and SKF38393, were used along with D_1-like (D_1R/D_5R) receptor antagonist LE300 and the novel selective D_5R antagonist LE-PM436. PLC inhibitor U73122, cAMP-specific activator SKF83822, and PLC-specific activator SKF83959 were also used. Details about these and the ouabain and EIPA used in the sodium transport assays (including source information) can be found in Table S1 in the Online Supplement.

Co-Immunoprecipitation of D_1R and D_5R

Co-immunoprecipitation of the two receptors was performed as previously described [33] using rabbit D_1R antibody (1:50 dilution, sc-14001, Santa Cruz Biotechology). The detection step used goat polyclonal anti-D_5R (1:100 dilution, sc-1440 Santa Cruz Biotechology), followed by a donkey anti-goat IR Dye 800 secondary antibody (1:15,000, LI-COR Biosciences). Non-specific rabbit IgG (Sigma) was used as a negative control immunoprecipitating antibody and no D_5R-specific signal was detected. Lack of cross reactivity with the respective antibodies was verified using the stably transfected D_1R-HEK293 and D_5R-HEK293 cells. The FEN effect was measured after 30-min (1 μmol/L FEN).

Immunofluorescence

The basic immunofluorescent staining procedure has been reported.[27] The D_1R antibody used on fixed tissue was from Dr. Jose's laboratory.[45] The 3rd extracellular loop-specific D_1R antibody used for cell surface labeling was from Dr. Carey's laboratory.[46,47] The 3rd extracellular loop-specific D_5R antibody used in fixed tissue and for cell surface labeling was from Millipore (AB 9509). Details about both staining procedures are in the Online Supplement.

Sensitized Emission FRET Microscopy

Sensitized emission Fluorescent Resonance Energy Transfer (FRET) analysis of the physical association of D_1R and D_5R was performed using two different systems. One system utilized live-cell microscopy of HEK293 cells transiently transfected with D_1R-YPet (yellow fluorescent protein) and D_5R-CyPet (cyan fluorescent protein) fluorescent protein fusion constructs. The second set of experiments was performed on fixed non-permeablized RPTCs using extracellular epitope-specific and directly-fluorescently-labeled antibodies to the D_1R and D_5R using Alexa 488 and Alexa 555 dyes, respectively. Live-cell imaging was performed using a Bioptechs Delta T stage heating system and Bioptechs objective heater. Spectral bleedthrough calculations and corrected FRET (cFRET) imaging was performed according to the FRET Module of Slidebook 4.2. Verification of proper microscope setup and calculations was provided by using both positive (ICUE3 FRET biosensor for CyPet/YPet and ICUE-YR for 488/555) and negative controls (co-transfected CyPet and YPet non-fusion fluorescent proteins). Details about filters and FRET analysis are in the Online Supplement.

Fluorescent Biosensors for cAMP Accumulation (ICUE3) and PLC Activation (CYPHR)

cAMP accumulation and PLC activation were measured by intracellular real-time kinetic assays using the fluorescent biosensors ICUE3 and CYPHR, respectively. This involved transfecting RPTCs with a plasmid containing a novel fluorescence resonance energy transfer sensor for cAMP (ICUE3) or Addgene plasmid 14864 for PLC activation (CYPHR) according to the methods of Violin et al.[48,49]

The ICUE biosensor contains a cAMP binding domain of EPAC1, a known cAMP target in proximal tubule cells necessary for NHE3 inhibition.[50] RPTCs were electroporated according to our previously optimized protocol.[33] They were allowed to recover in culture media for 24-hr before imaging. Cells were treated with combinations of agonists and antagonists and imaged with confocal microscopy every 30 sec for 20-min. The final read was used to generate the data in Figures 4 and 5.

siRNA

100 nmol/L D_1R siRNA (target sequence: 5′ CAUCUCAUCCUCUGUAAUA 3′), D_1R scrambled control (5′ GCAUCUUUACACCACUUAU 3′), D_5R siRNA (target sequence: 5′ CCCUUCUUCAUCCUUAACU 3′) or D_5R scrambled control (5′ CUCCAUUCCCAUCUUCUAU 3′) was transfected for 24-hr into RPTCs by electroporation as previously published.[33]

NHE3-mediated Sodium Influx Assay

Details and specificity of this assay, where we use ouabain to inhibit NaKATPase and measure sodium accumulation in RPTCs, was previously published[27] and is described in the Online Supplement. The figures were made using data collected at the 30-min time point.

NaKATPase-mediated Sodium Efflux Assay

The detailed method used to measure the rate of sodium efflux was previously published[33] and is outlined in the Online Supplement. The figures were made using data collected at the 30-min time point.

Calcium Imaging

RPTCs were labeled with fura-2 AM (Invitrogen) using identical conditions established for the SBFI labeling as stated in the NHE3-mediated sodium influx assay. The fura-2 here simply replaces the SBFI used in the influx assay. The filter set and imaging parameters are also identical for the two assays. Data for this figure was collected at the 20-min time point.

cAMP Assay

cAMP accumulation was measured after 30-min of agonist stimulation (SKF38393, see Table S1) with IBMX (500 μmol/L) using a commercial ELISA kit (Cayman Chemical) as previously described.[33] Antagonists LE-PM436 and LE300 (see Table S1) were added 10-min prior to the agonist incubation.

Statistical Analysis

The data are expressed as mean ± SE. Comparisons within and among groups were made by repeated measures or factorial ANOVA, respectively, followed by Holm-Sidak or Duncan's test. Student's t-test was used for two-group comparisons. P values of <0.05 were considered significant.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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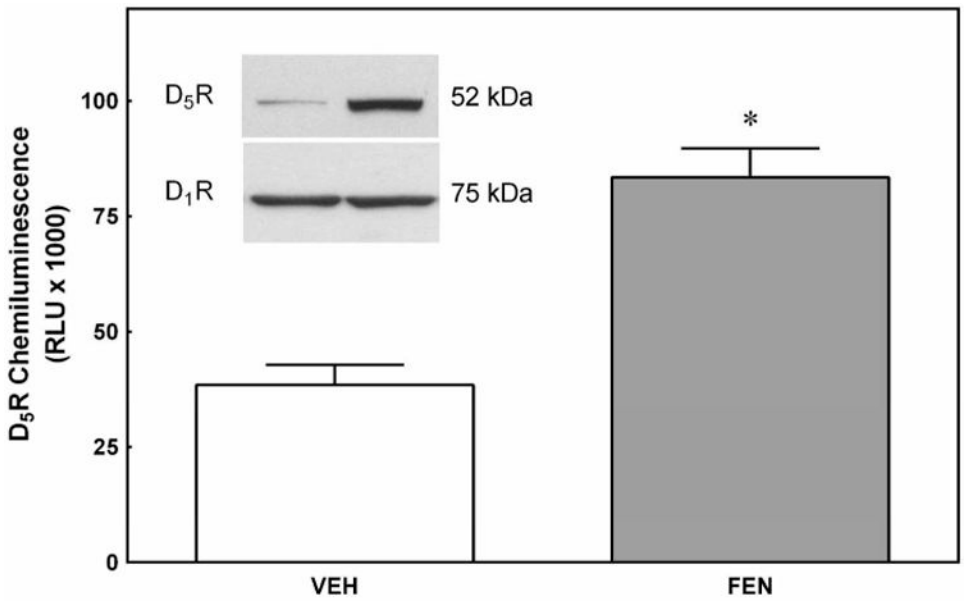

Figure 1. Co-Immunoprecipitation of D_1R and D_5R

Immortalized human RPTCs were incubated with and without fenoldopam (FEN, D_1R/D_5R agonist, 1 μmol/L, 30 min) and the physical interaction between the D_1R and D_5R was determined by co-immunoprecipitation and western blotting. FEN induced an 111.7±9.4% increase in D_1R and D_5R protein-protein interaction using a rabbit anti-D_1R immunoprecipitating antibody and a goat anti-D_5R detection antibody ($P<0.001$, N=6 per group).

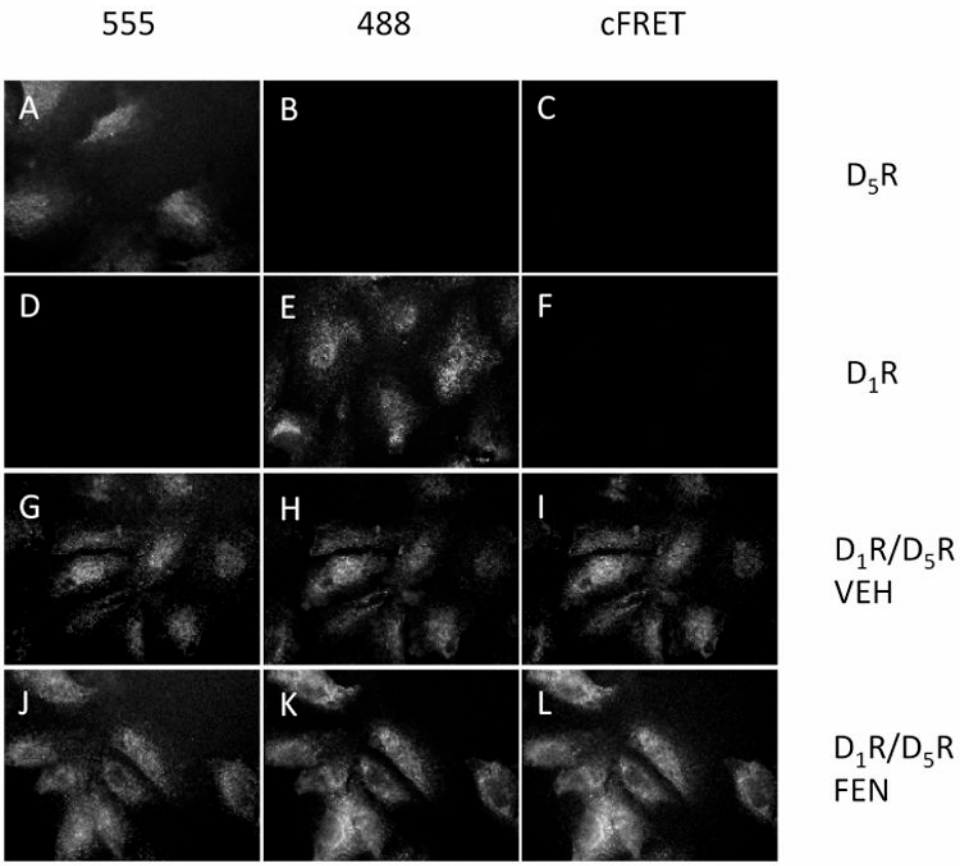

Figure 2. Fixed cell surface D_1R / D_5R FRET measurement in non-permeabilized human RPTCs

The first row of images indicates that when Alexa 555-labeled D_5R antibody was added to the RPTCs, a clear signal was measured in the Alexa 555 channel (A), and there was no spectral bleedthrough into the Alexa 488 filter channel (B) and no measurable signal in the corrected FRET (cFRET) channel (C), as expected. The second row of images indicates that the same was true when the Alexa 488-labeled D_1R antibody was added to RPTCs. There was a clear signal measured in the Alexa 488 channel (E), no spectral bleedthrough into the Alexa 555 channel (D) and no measurable signal in the cFRET channel (F). In the third row, RPTCs were first incubated with vehicle (VEH) and then with Alexa 555-labeled D_5R and Alexa 488-labeled D_1R antibodies. A clear signal was measured in the Alexa 555 (G) and Alexa 488 channels (H), and a clear cFRET signal was detected in the cFRET channel (I). In the bottom row, RPTCs were incubated with the D_1R/D_5R agonist fenoldopam (FEN, 1 μmol/L, 30 min) before fixing and staining with the two antibodies. An increase in the mean intensity of the Alexa 488 channel indicates that the D_1R was recruited to the cell surface (K compared to H). Additionally there was an increase in cFRET signal upon addition of FEN (L compared to I). Images were collected using a 60× water immersion objective lens.

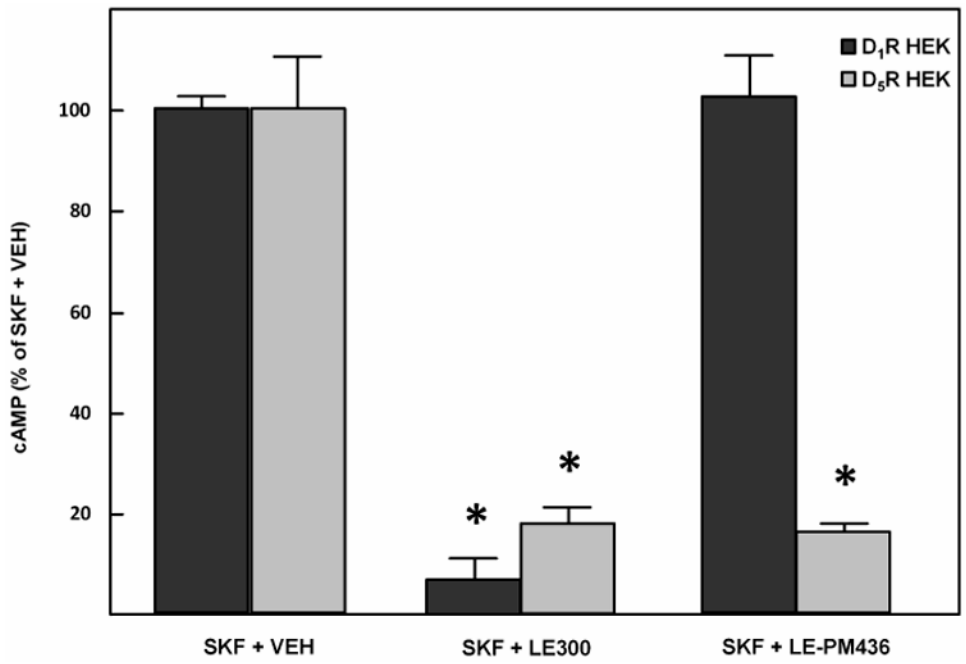

Figure 3. Selective Inhibition of D_5R-transfected HEK293 cells with LE-PM436

The D_1R/D_5R antagonist LE300 (10 μmol/L, 30 min) inhibited the stimulatory effect of SKF38393 (D_1R/D_5R agonist, 10 μmol/L, 30 min) on intracellular cAMP production in both D_1R- and D_5R- transfected HEK293 cells. LE-PM436 (1 nmol/L, 30 min) only inhibited SKF-induced cAMP production in the D_5R-transfected HEK293 cells (84.0 ±1.2% decrease, *P<0.001 vs VEH, N=8 per group).

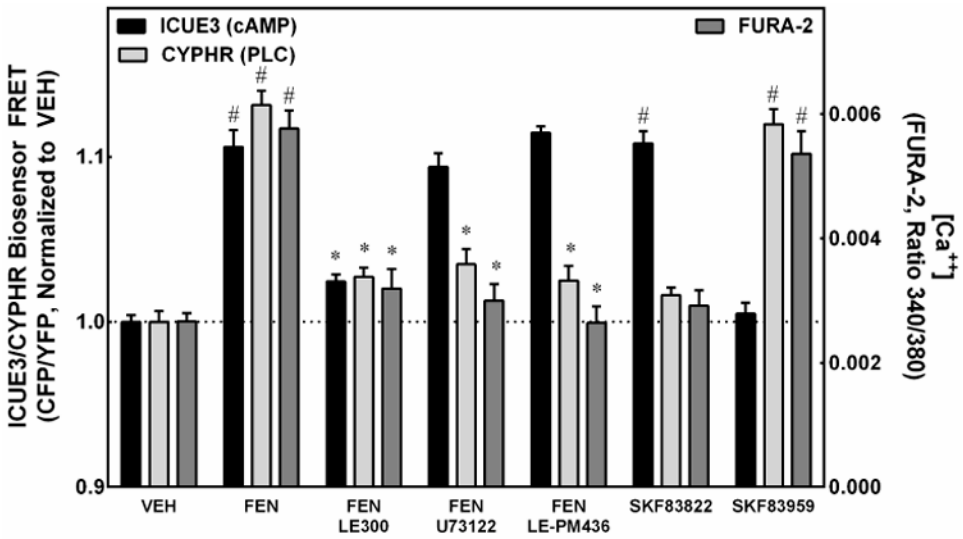

Figure 4. D_1R and D_5R Specific Signaling to AC or PLC

ICUE3 (measure of intracellular cAMP accumulation) and CYPHR (measure of intracellular PLC activation state) are depicted on the left Y-axis, normalized to vehicle (VEH). The right Y-axis depicts the intracellular Ca^{++} using FURA-2. Stimulation with the D_1R/D_5R agonist fenoldopam (FEN, 1 μmol/L, 20 min) increased intracellular Ca^{++} and cAMP levels and activated PLC ($^{\#}P<0.001$ vs VEH, N=6 for ICUE3/CYPHR, N=11 for FURA-2). Addition of the D_1R/D_5R antagonist LE300 (10 μmol/L, 20 min) blocked the FEN-mediated increase of all 3 variables (*P<0.001 vs FEN, N=6 for ICUE3/CYPHR, N=17 for FURA-2). The PLC inhibitor U73122 (10 μmol/L, 20 min) and the D_5R-specific inhibitor LE-PM436 (1 nmol/L, 20 min) both blocked the FEN-mediated increase in intracellular Ca^{++} and PLC activity but did not affect cAMP levels (*P<0.001 vs FEN, N=3-14). The AC-specific activator SKF83822 (10 μmol/L, 20 min) caused an increase only in cAMP ($^{\#}P<0.001$ vs VEH, N=3-17) and the PLC-specific activator SKF83959 (10 μmol/L, 20 min) increased PLC activity and Ca^{++} levels ($^{\#}P<0.001$ vs VEH, N=4-17).

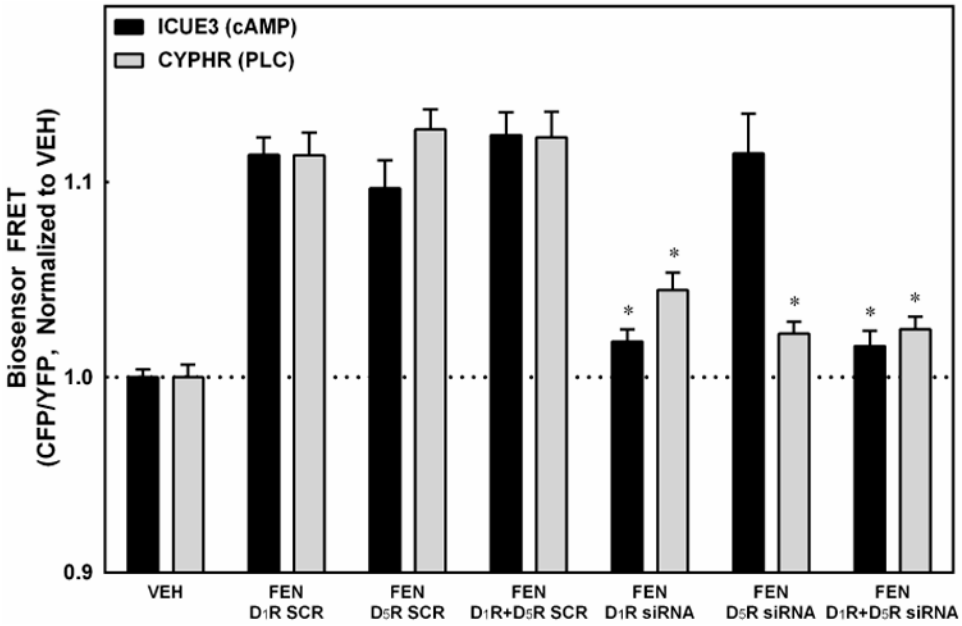

Figure 5. D_1R vs. D_5R siRNA Effects on AC and PLC pathways

D_1R siRNA blocked the fenoldopam (FEN, D_1R/D_5R agonist) -stimulated increases in cAMP level and PLC activity (*P<0.05 vs SCR, N=16 for ICUE3, N=4 for CYPHR). D_5R siRNA blocked only the PLC effect (*P<0.05 vs SCR, N=4), and the combination of the D_1R and D_5R siRNAs blocked both pathways (*P<0.05 vs SCR, N=14 for ICUE3 and N=4 for CYPHR).

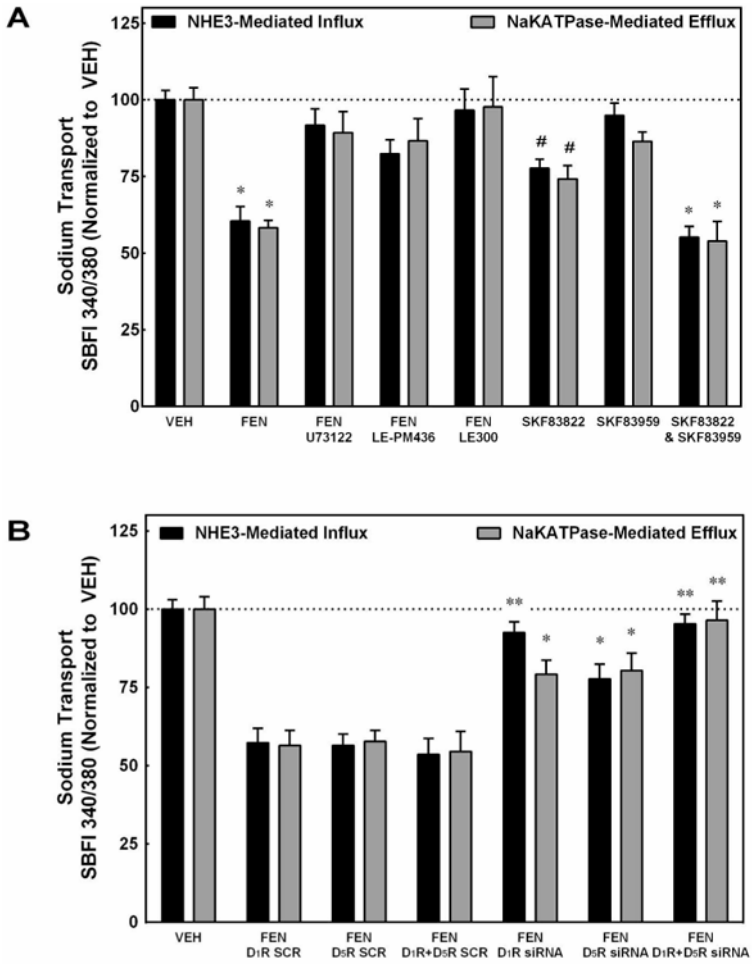

Figure 6. Sodium Transport Assays

PANEL A: The D_1R/D_5R agonist fenoldopam (FEN, 1 μmol/L, 30 min) inhibited both NHE3-mediated sodium influx and NaKATPase-mediated sodium efflux in immortalized RPTCs (*P<0.0001 vs VEH, N=6). The co-addition of U73122 (PLC inhibitor, 10 μmol/L), LE-PM436 (D_5R-specific inhibitor, 1 nmol/L), or LE300 (D_1R/D_5R antagonist, 10 μmol/L) returned the sodium influx and efflux to VEH levels. The AC-specific activator SKF83822 (10 μmol/L, 20 min) inhibited both NHE3 and NaKATPase ($^{\#}$P<0.001 vs VEH, N=6). The PLC-specific activator SKF83959 (10 μmol/L, 20 min) did not inhibit either NHE3 or NaKATPase but the combination of SKF83822 and SKF83959 inhibited both NHE3 and NaKATPase and to a greater extent than SKF83822 alone (*P<0.0001 vs VEH, N=6).

PANEL B: The FEN-mediated inhibition of sodium efflux can be partially reversed by siRNA specific to D_1R or D_5R, or fully reversed by the combination of D_1R and D_5R siRNAs (*P<0.05 vs SCR, **P<0.001 vs SCR, N=6). D_1R siRNA alone reversed sodium influx as effectively as the combination of the D_1R and D_5R siRNAs.

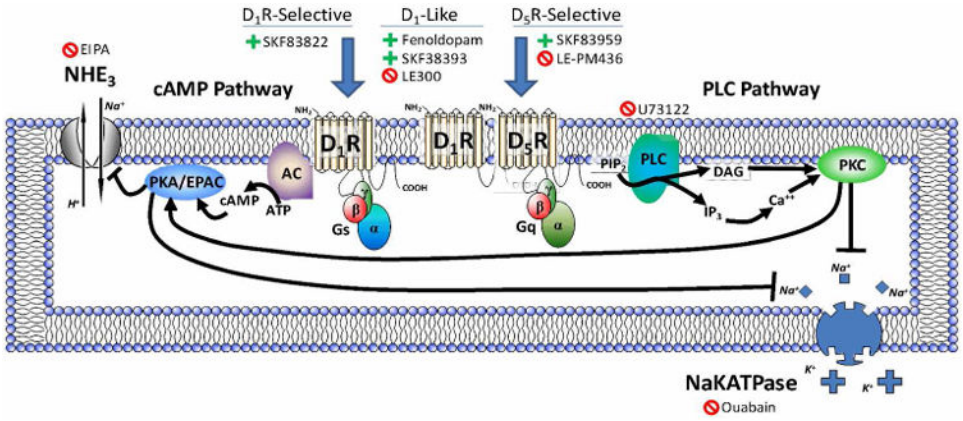

Figure 7.

This model lists the D_1R and D_5R agonists and inhibitors and depicts our proposed pathways and heteromeric structure in relation to signaling and regulation of sodium transport in a human renal proximal tubule cell (RPTC). D_1R signaling through adenylyl cyclase (AC) and cAMP and PKA or EPAC (exchange protein activated by cAMP) can inhibit both NHE3 and NaKATPase, while the heteromeric D_1R/D_5R complex signaling through PLC acts to positively modulate the D_1R activity. In RPTCs, SKF83822, which stimulates adenylyl cyclase (AC), may be a D_1R selective agonist while SKF83959, which stimulates phospholipase C (PLC), may be a D_5R selective agonist and inhibited by LE-PM436, the newly described D_5R-selective antagonist. Fenoldopam and SKF38393 stimulate both D_1R and D_5R while LE300 inhibits both D_1R and D_5R. U73122 inhibits PLC activity, ouabain inhibits NaKATPase activity and EIPA inhibits NHE3 activity.