Caveolin-1 and Dopamine-Mediated Internalization of NaKATPase in Human Renal Proximal Tubule Cells

John J. Gildea, Jonathan A. Israel, Andrew K. Johnson, Jin Zhang, Pedro A. Jose, Robin A. Felder

Abstract—In moderate sodium-replete states, dopamine 1–like receptors (D1R/D5R) are responsible for regulating > 50% of renal sodium excretion. This is partly mediated by internalization and inactivation of NaKATPase, when associated with adapter protein 2. We used dopaminergic stimulation via fenoldopam (D1-like receptor agonist) to study the interaction among D1-like receptors, caveolin-1 (CAV1), and the G protein–coupled receptor kinase type 4 in cultured human renal proximal tubule cells (RPTCs). We compared 2 groups of RPTCs, 1 of cell lines that were isolated from normal subjects (nRPTCs) and a second group of cell lines that have D1-like receptors that are uncoupled (uncoupled RPTCs) from adenyl cyclase second messengers. In nRPTCs, fenoldopam increased the plasma membrane expression of D1R (10.0-fold) and CAV1 (1.3-fold) and markedly decreased G protein–coupled receptor kinase type 4 by 94 ± 8%; no effects were seen in uncoupled RPTCs. Fenoldopam also increased the association of adapter protein 2 and NaKATPase by 53 ± 9% in nRPTCs but not in uncoupled RPTCs. When CAV1 expression was reduced by 86.0 ± 8.5% using small interfering RNA, restimulation of the D1-like receptors with fenoldopam in nRPTCs resulted in only a 7 ± 9% increase in association between adapter protein 2 and NaKATPase. Basal CAV1 expression and association with G protein–coupled receptor kinase type 4 was decreased in uncoupled RPTCs (58 ± 5% decrease in association) relative to nRPTCs. We conclude that the scaffolding protein CAV1 is necessary for the association of D1-like receptors with G protein–coupled receptor kinase type 4 and the adapter protein 2–associated reduction in plasma membrane NaKATPase. (Hypertension. 2009;54:1070-1076.)

Key Words: dopamine receptors ■ caveolin ■ proximal tubule ■ hypertension ■ GRK4 ■ NaKATPase ■ FRET

The mechanisms by which renal dopamine, in concert with other sodium regulator pathways, regulates > 50% of renal sodium excretion are not completely understood. Intra-renal administration of a dopamine 1 (D1)-like receptor antagonist (SCH23390) decreased sodium excretion by 57% in conscious uninephrectomized dogs1-2 and in anesthetized volume-loaded rats.3-4 Disruption of D1-like receptor (D1R or D1R) genes in mice increases salt-sensitive blood pressure.5 Dopamine and D1-like receptor agonists are natriuretic in experimental animals6 and humans,7,8 and ecopipam, a selective D1-like receptor antagonist, increases blood pressure in humans9 (presumably by preventing sodium excretion).

Dopamine inhibits sodium transport in several segments of the nephron, causing increased sodium excretion.5 In the renal proximal tubule (RPT), dopamine inhibits the sodium/hydrogen exchanger 3 (NHE3)10,11 and NaKATPase.12,13 The decrease in sodium/hydrogen exchanger 3 from the plasma membrane involves binding to adapter protein 2 (AP2), followed by endocytosis. Dopamine stimulation and/or sodium concentration associate AP2 with NaKATPase, cause NaKATPase translocation from the plasma membrane into early and late endosomes, and decrease rubidium uptake, an index of NaKATPase activity in opossum and rodent cells.15-18 However, the effect of dopamine on NaKATPase internalization, activity, and association with AP2 has not been shown in human RPT cells (RPTCs). There may be species differences in the regulation of sodium transport. For example, in humans, the α subunit of NaKATPase expressed in the kidney is more sensitive to the inhibition by ouabain compared with rodents.19,20

Caveolin, especially caveolin-1 (CAV1), has been shown to be important in the NaKATPase internalization process.21,22 Caveolins, localized in lipid rafts of plasma membranes, tether and regulate signaling complexes into functional units (eg, G protein–coupled receptors).23,24 We have reported that fenoldopam (FEN; D1-like receptor agonist) stimulates caveolin 2 (CAV2) and D1R association in human embryonic kidney 293 cells, which do not express CAV1.25 Furthermore, FEN-stimulated membrane D1R recruitment and cAMP accumulation were greater in membranes with CAV2-containing lipid rafts than in those depleted of CAV2 with antisense oligonucleotides or in nonlipid rafts without CAV2.26

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From the Department of Pathology (J.J.G., J.A.I., A.K.J., R.A.F.), University of Virginia Health System, Charlottesville, Va; Children’s National Medical Center (P.A.J.), Center for Molecular Physiology, Department of Pediatrics, George Washington University, Washington, DC; Department of Pharmacology and Molecular Sciences, Johns Hopkins School of Medicine (J.Z.), Baltimore, Md.
Correspondence to Robin A. Felder, University of Virginia, PO Box 801400, Charlottesville, VA 22908. E-mail rfelder@virginia.edu
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G protein–coupled receptor kinase 4 (GRK4) is 1 of 7 members of the GRK family and has been shown to be specifically located in RPTCs, where it constitutively phosphorylates and desensitizes D1R in specific plasma membrane microdomains.27,28 However, the role of the interaction among GRK4, CAV1, and D1-like receptors and NaKATPase internalization in human RPTCs has not been described. We tested the hypothesis that CAV1, interacting with GRK4, is necessary for D1R-mediated inhibition of NaKATPase activity in human RPTCs through binding with AP2. Because D1R signaling is impaired in hypertensive humans and rats,6,8,23,29,30 this interaction may be aberrant in essential hypertension.

Materials and Methods
Please see the online Data Supplement at http://hyper.ahajournals.org for the Expanded Methods section.

Cell Lines
We have generated RPTCs from institutional review board–approved normal human subjects (nRPTCs) and RPTCs from subjects in which D1R is uncoupled from adenylyl cyclase stimulation (uRPTCs)28,31–33 (see details in the online Data Supplement Methods section), as described previously.27,28,31,34–36

Determination of cAMP Accumulation
cAMP was measured both by a commercial ELISA (Cayman Chemical) and an intracellular real-time kinetic assay, which involves transfecting RPTCs with a plasmid containing a novel fluorescence resonance energy transfer (FRET) cAMP sensor (ICUE3) according to the method of Violin et al.37 Details of both methods are outlined in the online Data Supplement.

Measurement of Plasma Membrane and Total Cell Protein Expression
Detergent-free plasma membrane sheet isolation was performed using sulfo-NHS-SS-biotin, as reported previously.35 Total cell lysates (20 μg; 4°C) were loaded per lane for electrophoresis and immunoblotting.35 The proteins of interest were detected using rabbit polyclonal anti-GRK4 (1:200 dilution; Santa Cruz; sc-13079), followed by a goat antirabbit infrared dye (IR Dye 800; Li-Cor) secondary antibody and imaged on an Odyssey infrared imaging system (Li-Cor).

Measurement of the Association of GRK4 and CAV1
GRK4 and CAV1 association was measured by coimmunoprecipitation of 1 mg of cellular protein in 1 mL of lysis buffer (M-PER; Pierce) and 2 μg of CAV1 monoclonal antibody (BD Biosciences). Detection used rabbit polyclonal anti-GRK4 (1:200 dilution; Santa Cruz; sc-13079), followed by a goat antirabbit infrared dye (IR Dye 800; Li-Cor) secondary antibody and imaged on an Odyssey infrared imaging system (Li-Cor).

Small Interfering RNA to CAV1 and GRK4
CAV1 small interfering RNA (siRNA; target sequence: 5′CCGCACTCAACCTTGCAAGA3′) and scrambled control: 5′CCGCAACTGTCCGACAAACA3′)18 was designed and ordered prehybridized (Sigma-Genosys). The most effective GRK4 siRNA (target sequence: 5′AATACAAAGAGAAAGTCA3′ and scrambled control: 5′AGAAGATAAGAACATAAC3′) was chosen among 10 candidate target sequences by Western blotting. Details of transfection are provided in the online Data Supplement.

NaKATPase Internalization Assay
Measurement of NaKATPase internalization with AP2 was adapted from previously published techniques,15,15 which showed that the internalization of and decrease in NaKATPase activity are associated with AP2. Menomins was shown to be necessary to measure the effect of dopamine.17,18 Details are provided in the online Data Supplement.

NaKATPase Confocal Microscopy and Plasma Membrane Localization
Plasma membrane–localized NaKATPase expression was performed on RPTCs using similar treatments as for the NaKATPase experiments, with subsequent fixation and staining with a monoclonal antibody to NaKATPase. Details are outlined in the online Data Supplement.

NaKATPase Activity
We have established a sodium eflux assay that measures NaKATPase activity in cultured cells as ouabain-sensitive reduction in sodium export. Details are included in the online Data Supplement.

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 test. A t test was used for 2-group comparisons. A P<0.05 was considered significant.

Results
Our cell lines were well characterized for their RPT origin.27,28 We measured cAMP in 2 nRPTC and 2 uRPTC cell lines, determined by the response to fenoldopam (FEN; 1 μmol/L; 30 minutes) or dimethyl sulfoxide (DMSO) vehicle control. We compared our ELISA with a novel FRET–based method (Figure 1). Figure 1A shows the >2-fold increase in cAMP accumulation (ELISA) induced by FEN in nRPTCs (but not in uRPTCs) that was blocked by the D1-like receptor antagonist, L6300 (10 μmol/L), indicating that the stimulatory effect was via D1-like receptors. A cAMP FRET biosensor, ICUE3,37 showed that D1-like stimulation with FEN caused a significant rise in the intracellular cAMP level in nRPTCs, which reached a plateau by 7 minutes (Figure 1B). Minimal effect was seen in uRPTCs, confirming our previous reports with older methods.28,32

We next studied the plasma membrane expression of D1R, GRK4, and CAV1 in nRPTCs and uRPTCs using Western blotting techniques (Figure 2). Figure 2A shows that D1R plasma membrane recruitment was increased (10.73±1.70-fold; P<0.05; n=4) over DMSO vehicle (VEH) control after FEN (1 μmol/L; 30 minutes) stimulation in nRPTCs. In uRPTCs, there was no significant increase in FEN-induced D1R recruitment to the plasma membrane. Basal levels of plasma membrane GRK4 expression were lower in uRPTCs than in nRPTCs (58.4±8% decrease; P<0.05; n=4). Figure 2B shows that FEN stimulation decreased plasma membrane GRK4 abundance in nRPTCs (94±8.0; P<0.05; n=4) but had no effect in uRPTCs. CAV1 basal plasma membrane expression was 40.0% (±7.0%; P<0.05; n=4) lower in uRPTCs than in nRPTCs and increased (1.3±0.09-fold; P<0.05; n=4) after FEN stimulation only in nRPTCs (Figure 2C). Figure 2D shows β-tubulin and Ponceau S loading controls.

To prove that the reduced recruitment of D1R to the plasma membrane in uRPTCs with FEN stimulation was because of GRK4, we silenced GRK4 gene expression with GRK4 siRNA (supplemental Figure S1, available in the online Data
Supplement). The addition of GRK4-specific siRNA reduces GRK4 expression by 70.6±5.6 (P<0.05; n=3) by Western blotting (Figure S1A). We have reported that, in uRPTCs, GRK4 is constitutively active, and the D₁R is phosphorylated, desensitized, and internalized. When siRNA to GRK4 was added to uRPTCs and compared with scrambled siRNA (SCR) and DMSO VEH controls, the FEN-mediated (1 μmol/L for 30 minutes) D₁R plasma membrane expression was partially restored (P<0.05; n=6; Figure S1B).

The total cellular CAV1 expression level was increased by 47±16% (P<0.05; n=8) with FEN stimulation (1 μmol/L; 4 hours) when compared with DMSO VEH (Figure 3). Angiotensin (ANG) II stimulation (10 nM; 4 hours) reduced CAV1 expression by 67±8% (P<0.05; n=8).

Because CAV1 is a known negative regulator of GRKs, we studied the association of GRK4 and CAV1 in nRPTCs and uRPTCs with coimmunoprecipitation experiments. CAV1 was immunoprecipitated using a monoclonal anti-CAV1 antibody and immunoblotted with GRK4 rabbit polyclonal antibody (Figure 4). A 62-kDa immunoreactive band was observed corresponding with the predicted size of GRK4. CAV1/GRK4 coimmunoprecipitation was 58±5% less in uRPTCs compared with nRPTCs (P<0.05, t test; n=4 per group).

The inverse of this experiment is shown in Figure S2, where we immunoprecipitated GRK4 and detected CAV1 and GRK4. There was a 55.6±14.1% decrease in uRPTCs compared with nRPTCs (P<0.05; n=4), further validating the difference in GRK4/CAV1 association between the cell groups.

Treatment with β-methyl cyclodextrin (βMCD; 2 mmol/L; 1 hour), an agent that reduces cholesterol and CAV1 in the plasma membrane, decreased CAV1 membrane expression in nRPTCs (52±4%; P<0.05 versus PBS VEH, nRPTCs; ANOVA, Holm-Sidak test, n=8) but not in uRPTCs (Figure 5). The depletion of plasma membrane CAV1 by βMCD reduced CAV1 expression in nRPTCs to the basal levels seen in uRPTCs. Basal plasma membrane expression of CAV1 was lower in uRPTCs by 56±11% (P<0.05; n=8) relative to nRPTCs.

We used another approach to reduce CAV1 expression. siRNA to CAV1 reduced total cellular CAV1 expression by 86±8.5% (P<0.05, t test; n=8) in nRPTCs and 89±4% (P<0.05, t test; n=8) in uRPTCs compared with SCR-transfected control cells (Figure S3). Total cellular expression of CAV1 was once again lower (25.2±3.0%; P<0.05; n=8) in uRPTCs than in nRPTCs.

Internalization of NaKATPase was measured by immunoprecipitating AP2 with a rabbit polyclonal antibody and Western blotting with NaKATPase and AP2 monoclonal antibodies, producing single bands of the correct molecular weight for both AP2 and NaKATPase (Figure S4). Because there was only a single band for both proteins, further analysis was continued by immuno-dot blot (Figure 6). FEN stimulated the AP2-mediated NaKATPase internalization in nRPTCs (□), a response that was not seen in uRPTCs (□). In nRPTCs, there was a 53±9% (P<0.05; n=6) increase in association between AP2 and NaKATPase with FEN (1 μmol/L for 30 minutes). This effect was completely blocked by βMCD or siRNA to CAV1. Control SCR had no effect on FEN-stimulated internalization of NaKATPase in nRPTCs. In uRPTCs, FEN had no effect on AP2-NaKATPase coimmunoprecipitation and was not affected by CAV1 siRNA or CAV1 SCR. Other controls used were βMCD alone, CAV1 siRNA alone, CAV SCR alone, or treatment without monensin; these controls had no effect on the basal association of AP2 and NaKATPase (Figure S5). Controls without monensin showed no change in cell-surface NaKATPase expression after FEN stimulation in nRPTCs in Figure S6. Thus, an increase in intracellular sodium concentration is necessary for NaKATPase internalization.

Confocal images of NaKATPase displayed a predominantly plasma membrane expression pattern in control (VEH) cells, which was reduced after FEN stimulation when an increase in cytoplasmic accumulation was seen (P<0.05, t test; n=3; Figure S7). uRPTCs displayed an identical expression pattern as control nRPTCs but displayed no...
We measured NaKATPase activity as ouabain-inhibitable (P<0.01; n=3 versus VEH) sodium efflux (Figure S8). FEN reduced the sodium efflux in nRPTCs by 50.2±4.6% versus VEH (P<0.01; n=3), which was CAV1 dependent, because it was blocked by MCD (P<0.05 vs VEH, nRPTCs). FEN had no effect in uRPTCs.

**Discussion**

These current studies demonstrate a CAV1 dependence on the D1R-like receptor coupling to NaKATPase in human RPTCs. The current study, along with our previous report, indicates that regulation of the D1R by CAV1 and/or CAV2 may depend on the surrounding microenvironment (lipids and associating receptors) in human RPTCs versus human embryonic kidney 293 cells.

CAV1 can also directly interact with GRKs 1, 2, and 5 in A431, NIH-3T3, and COS-1 cells, but a CAV1 interaction with GRK4 has not been reported. In human embryonic kidney 293 cells, GRK2 physically interacts with CAV2; CAV1 is not expressed in human embryonic kidney 293 cells. Thus, our demonstration of GRK4 coimmunoprecipitation with CAV1 is also novel, although not surprising. The lower GRK4/CAV1 association in uRPTCs is in keeping with the negative modulatory role of CAV1 on GRK4; GRK4 is constitutively active in uRPTCs.

Our experiments demonstrate that CAV1 is also involved with GRK4 in the proper recycling, resensitization, and desensitization of the D1R. In contrast, in uRPTCs, basal membrane expression of CAV1 is decreased compared with nRPTCs. In uRPTCs, short-term FEN stimulation does not increase CAV1 plasma membrane protein expression. GRK4 plasma membrane protein does not change, and the D1R is not recruited to the plasma membrane. These studies suggest...
that genetic defects in GRK4 that interrupt its association with CAV1 lead to desensitization and impaired function of the D1R. The role of ANG II in this process is currently unknown, although it is possible that the decreased expression of CAV1 may be attributed to the lack of negative regulation of the uncoupled D1R on the ANG II type 1 receptor.43 The expression of the ANG II type 1 receptor is not different in the nRPTC and uRPTC cell lines examined (data not shown). However, we have demonstrated that ANG II type 1 receptor expression and function are increased in mice overexpressing human GRK4 142V and 486V genes, relative to mice overexpressing human GRK4 wild-type genes.44,45

In genetic hypertension, the ability of dopamine and D1-like receptor agonists to increase sodium excretion is impaired. RPTs from hypertensive rats have an impaired ability to decrease sodium transport (eg, sodium/hydrogen exchanger 3,46–49 NaHCO3 cotransporter,50 Cl/HCO3 exchanger,51 and NaKATPase6,52–56). Dopamine inhibits sodium transporter activity, in part by increasing its internalization, an action that is also impaired in hypertension.16,57 An increased activity of NaKATPase in RPTs has been shown in several rodent genetic models of hypertension.56,58 Our studies presented here in human RPTCs demonstrate that the scaffolding protein CAV1 is a mediator in the association of the D1R with its kinase, GRK4, and its role in AP2-associated reduction in plasma membrane NaKATPase. Ultimately, the transregulation of CAV1, D1R, and GRK4 will influence sodium transport. We are studying NaKATPase and sodium/hydrogen exchanger 3–mediated Na/H flux, which will be reported in a future article.
Perspectives
There is a growing appreciation for the role of the dopaminergic system in regulating sodium balance and, hence, blood pressure. Genetic variants in a protein, GRK4, have been linked to essential hypertension and salt sensitivity in both humans and rodent models for these conditions. In these studies, we showed that CAV1, a principal membrane scaffolding protein, is involved in the organization of the D1R-like receptor signaling pathway and intracellular sodium. These studies further decipher the mechanisms of how dopamine, GRK4, and CAV1 control the sodium pump. The ubiquitous expression of CAV1 may limit its usefulness as a potential therapeutic target. However, selective targeting of GRK4, which regulates only a few G protein–coupled receptors in a limited number of organs, may be a new useful antihypertensive therapeutic approach.

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Disclosures
R.A.F. and P.A.J. were awarded a US Patent (No. 6 660 474) on “GRK Mutants in Essential Hypertension,” which has been assigned to Hyponex, Inc.

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1John J. Gildea, 1Jonathan A. Israel, 1Andrew K. Johnson, 3Jin Zhang, 2Pedro A. Jose, and 1Robin A. Felder

1The University of Virginia Health System, Department of Pathology, Charlottesville, VA
2Children’s National Medical Center, Center for Molecular Physiology, Department of Pediatrics, George Washington University, Washington, DC
3Johns Hopkins School of Medicine, Baltimore, MD.

Short title: Dopamine receptor internalization of NaKATPase

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
Expanded Materials and Methods

Cell Lines:
University of Virginia Institutional Review Board (IRB)-approved normal tissue from nephrectomized kidneys was used to isolate RPTC verified by the following criteria: cAMP accumulation upon parathyroid hormone stimulation; positive plasma membrane binding of lotus tetragonobolus agglutinin; expression of γ-glutamyl transpeptidase, aminopeptidase N and NHE3 (1). The hybridoma producing monoclonal antibody 452 against CD13 (aminopeptidase N) was kindly donated by Dr. Meenhard Herlyn. We also verified the lack of expression of Tamm-Horsfall protein, sodium potassium 2Cl co-transporter, sodium chloride transporter, and epithelial sodium channel, which are markers of potentially contaminating tubule cells from the thick ascending limb of Henle, distal convoluted tubule and collecting duct.

Determination of cAMP accumulation:
ELISA: nRPTC and uRPTC were grown to 80% confluence, serum-starved overnight, and then washed in phosphate-buffered saline (PBS) with calcium and magnesium. 1 mM isobutyl-1-methyl-xanthine (to inhibit phosphodiesterase activity) was added along with the D1-like receptor agonist fenoldopam (1 μM) or vehicle control (dimethyl sulfoxide, DMSO) and incubated for 30 minutes at 37°C. The D1-like receptor antagonist LE300 (10 μM) was added 10 minutes prior to the addition of fenoldopam, to show D1-like receptor specificity of the measured end points. Commercial ELISA kit (Cayman Chemical) was performed according to manufacturer’s specifications.

ICUE3: The optimized transfection protocol for human RPTC was found to be a square wave pulse with 250 mV, 2000 μF capacitance, 1000 ohms resistance, 15 ms duration, two sets of 2 pulses, and 0.1 ms between pulses using 5x10⁵ cells per well and 0.4 μg/well plasmid. These settings produced between 60% and 90% transfection efficiencies depending on the cell line, passage number, and confluence of the cells at the time of harvest. Transfected cells were plated in 96-well collagen coated glass-bottomed plates (Matrical, Fisher Scientific), and CFP/YFP ratiometric imaging was performed following fenoldopam (1 μM) treatment. An increase in cAMP decreases the association of CFP and YFP producing an increase in the normalized CFP/YFP ratio. Images were acquired from 10 cells per well, 3 wells per group, on an Olympus IX81 spinning disk multi-well automated microscope and analyzed using the ratiometric imaging module of Slidebook 4.2.

Measurement of the association of GRK4 and CAV1:
GRK4 and CAV1 association was measured by co-immunoprecipitation. Cells were lysed in M-PER (Pierce), and 1 mg protein in 1 ml lysis buffer was immunoprecipitated by adding 2 μg CAV1 monoclonal antibody (BD Biosciences) for two hours. Protein A/G agarose (Pierce) was subsequently added, and then incubated for 30 minutes. The beads were washed three times in lysis buffer and protein was eluted from the column with lithium dodecyl sulfate (LDS) loading buffer with reducing agent (Invitrogen) and western-blotted as above, using rabbit polyclonal anti-GRK4 (1:200 dilution, Santa Cruz, sc-13079). The HA antigen is not present in these cells and therefore the 12CA5 monoclonal anti-HA antibody (gift from Tanya Watts University of Toronto) was used as a negative control. The association was also shown by immunoprecipitation with monoclonal GRK4 antibody conjugated to agarose (Santa Cruz, sc-9985AC) and detected with rabbit polyclonal CAV1 followed by goat anti-rabbit IR Dye 800 secondary antibody and imaged on Odyssey infrared imaging system.

SiRNA to CAV1 and GRK4:
Cells were grown in six-well plates (60% confluence), and 50% fresh complete media changed 2 hours before transfection. siRNA (50 nM) against CAV1 and GRK4, or scrambled control siRNA, was incubated with 6 μl oligofectamine (Invitrogen) in Optimem media (Invitrogen) and added to the cells for 4 hours, then FBS (fetal bovine serum) was added to a 2% final concentration and incubated overnight. The next day the cell culture media were switched to growth factor-free media with 0.5%
FBS for another 24 hours before lysing for western blotting or using in the NaKATPase internalization assay.

**NaKATPase Internalization Assay:**
RPTC were growth factor-starved overnight in 0.5% FBS, then monensin (5 µM) was added for 30 minutes to increase the intracellular sodium concentration to 20 mM. β-methyl cyclodextrin (βMCD, 2 mM) was added to deplete membrane cholesterol and CAV1 (2, 3). Two µg rabbit anti-AP2 antibody (α-adaptin, Santa Cruz, sc-10761) or a non-specific rabbit IgG (Sigma) were added to 500 µg lysate in 1 ml lysis buffer (2 hours). Protein A sepharose was incubated for 30 minutes with the lysate, washed 3X in lysis buffer and eluted with LDS loading buffer with reducing agent (Invitrogen). The eluate was immuno dot-blotted for NaKATPase using a 1:50 dilution of hybridoma culture supernatant (clone A5, Developmental Studies Hybridoma Bank). Non-specific rabbit IgG did not show any signal above background when examined by western blot (Figure S4). The NaKATPase signal was normalized to the amount of AP2 pulled down using a replicate well probed with a mouse anti-AP2 monoclonal antibody (α-adaptin, Santa Cruz, sc-17771). Following the addition of both NaKATPase and AP2 mouse monoclonal antibodies, goat anti-mouse IR Dye 800 secondary antibody was added, and the cells were imaged using an Odyssey infrared imaging system.

**NaKATPase confocal microscopy and plasma membrane localization:**
Human RPTC were plated on 25 mm collagen-coated glass coverslips in a 6 well dish. Treatments were the same as for the NaKATPase internalization assay, except that after the 30 minute treatment period, the cells were washed with PBS three times and fixed with 4% paraformaldehyde in PBS for 5 minutes, washed with PBS, and residual paraformaldehyde quenched with 50 mM glycine for 5 minutes. Cells were washed, then permeabilized with PBS containing 0.2% Triton X 100 for 5 minutes, and blocked with 1% BSA (bovine serum albumin) and 5% normal goat serum in PBS with 0.2% Tween 20 for 1 hour. NaKATPase α-1 monoclonal (clone C464.6, Millipore) culture supernatant was diluted 1:500 in blocking buffer and incubated overnight at 4°C. Cells were washed in PBS with 0.2% Tween 20 and secondary goat anti-mouse Alexa 488 was diluted 1:500 and incubated in blocking buffer for one hour at room temperature, then washed three times in PBS and mounted in Fluoromount G (Chemicon). Images were acquired with a Zeiss 510 Meta confocal microscope using a 60X water immersion objective at 1 micron z-axis intervals for a total z-axis height of 10 microns. A mid z-axis plane was taken at 5 microns from the most basal plane in both VEH control and FEN (1 µM, 30 min.) All images were acquired using the exact same settings, which were set such that the secondary only control showed no staining and the VEH control displayed strong plasma membrane specific staining. Plasma membrane localized NaKATPase expression was quantified using the ImageJ version 1.39o image processing software program. The confocal image and the DIC image were opened in two separate windows and the “sync windows” macro (courtesy of Patrick Kelly) was run. A 5 µ box was selected and placed at the plasma membrane chosen using the DIC image, and the integrated intensity of the confocal image was measured. The box was then moved 10 µ toward the cell interior and the integrated intensity re-measured. This was repeated 20X around the perimeter of each cell, and 10 cells per well quantified. Each cell was studied three times. The plasma membrane to cytoplasm ratio was the average surface intensity divided by the average cytoplasmic intensity. Background intensity was measured from a cell free region and subtracted prior to calculating the intensity ratio.

**Sodium Efflux Assay:**
RPTC were cultured in 96-well glass bottom collagen coated Matrical plates (Spokane, WA) at 37°C until they reached 80% confluence. Cells were serum-starved overnight prior to loading with a sodium ion indicator, sodium benzofuran isophthalate (SBFI, 5µM) (Molecular Probes, Eugene, OR) with Pluronic 127 for 2 hours in PBS with calcium and magnesium. Cells were allowed to recover at
37°C in serum free media for 30 minutes, then washed twice with room temperature potassium free HEPES media (20 mM HEPES pH 7.4, 130 mM NaCl, 1 mM CaCl, 1 mM MgCl) and incubated at room temperature for 30 minutes to raise the internal sodium concentration. VEH, FEN, βMCD or OUB (Ouabain, a cardiac glycoside, known to bind to and inhibit NaKATPase) were added to respective wells for 10 minutes and placed on the stage of an automated microscope for time lapse multiwell ratiometric imaging. Directly before imaging, EIPA (10 uM final concentration) and KCl (2.7 mM final concentration) were added to all wells as 10X stocks, and then imaged for 20 minutes. EIPA (5-(N-ethyl-N-isopropyl) amiloride) is a selective inhibitor of the Na+/H+ exchanger 3 (NHE3), which is the exclusive sodium hydrogen exchanger in human RPTC. Each measurement shown is the change in 340/380 ratio from time 0 in 3 separate well with 20 cells per well measured.

Microscopy:
Images were collected on an Olympus IX-81 inverted epifluorescence microscope with a UV-specific 20X UAPO objective and Hamamatsu ER CCD camera. Slidebook software package 4.2 (Intelligent Imaging Innovations, Inc., Denver, CO) integrated all of the hardware as well as provided for image acquisition and processing. An automated XY stage and piezo-Z allowed us to precisely capture images from multiple wells of a 96-well microplate under the same conditions. We can simultaneously capture time lapse data from up to 3 fields of view per well, in 12 separate wells per experiment.
References


FIGURE S1

A.

Figure S1 A. Western blot analysis of reduction of GRK4 expression by GRK4 siRNA. GRK4 immunoreactivity was detected by western blot in total cell lysates from RPTC cells transfected with siRNA directed against GRK4, or scrambled control. *P<0.05 vs control, n=3/group.

B.

Figure S1 B. Western blot analysis of D₁R plasma membrane expression in uRPTC cell lines transfected with GRK4 siRNA or scrambled (SCR) siRNA control. GRK4 siRNA increased D₁R receptor plasma membrane expression in response to fenoldopam (1 μM, 30 min) in uRPTC. (*P<0.05 vs. others, ANOVA, Holm-Sidak test, n=6 per group).
Figure S2. Association of CAV1 with GRK4 in nRPTC and uRPTC. CAV1 and GRK4 association was measured as the amount of immunoreactive CAV1 by western blot of immunoprecipitated GRK4 (shown in top insert) using a monoclonal antibody. Immunoreactive CAV1 associated with GRK4 was decreased in uRPTC compared to nRPTC (*P<0.05 vs nRPTC, t-test, n=4/group).
Figure S3. Reduction of CAV1 total cell expression using CAV1 siRNA in nRPTC and uRPTC. siRNA to CAV1 or scrambled siRNA control was transfected into RPTC. Basal CAV1 expression was lower in uRPTC than in nRPTC (# P<0.05 nRPTC scr con vs uRPTC scr con, n=8/group). CAV1 siRNA significantly inhibited CAV1 expression in both nRPTC and uRPTC cell lines (*P<0.01 CAV1 siRNA vs scr con, n=8/group). β-Tubulin (βTUB) was used as a control protein for loading.
**Figure S4. Western Blot Showing Specificity of Immunoprecipitating Antibodies to NaKATPase and AP2.** Lysates from nRPTC and uRPTC with and without Fenoldopam (FEN) are immunoprecipitated with a rabbit polyclonal antibody to AP2 or Non-specific rabbit immunoglobulin G (NS IgG) and detected by western immunoblot using monoclonal antibodies to NaKATPase depicted in the upper panel, and a monoclonal antibody to AP2 in the lower panel.
**Figure S5. AP2-mediated internalization of NaKATPase.** Immunoprecipitated AP2 followed by NaKATPase dot blot is expressed as % of vehicle (VEH) in nRPTC (open bars) and uRPTC (striped bars). Additional controls, cells without monensin (w/o MON), beta methyl cyclodextrin alone (βMCD), CAV1 siRNA alone (CAV1 siRNA), and CAV1 scrambled control siRNA (CAV1 SCR), showed no significant change from vehicle control treated cells in either nRPTC or uRPTC.
Figure S6. The effect of monensin (MON: 5μM, 30 min) and fenoldopam (FEN: 1mM, 30 min) on the cell surface expression of NaKATPase. nRPTC were incubated with MON or VEH for 30 minutes, and then either FEN or VEH was added for an additional 30 minutes. Cell surface membranes were isolated and the amount of NaKATPase was measured by Western blot using a specific monoclonal antibody. FEN reduced the amount of immunoreactive NaKATPase only in nRPTC treated with MON (*P<0.05 vs. others, ANOVA, Holm-Sidak test, n=3/group).
Figure S7. Confocal microscopy of NaKATPase in nRPTC treated with a D1R agonist, fenoldopam (FEN), or vehicle (VEH). nRPTC cells are imaged simultaneously for differential interference contrast (DIC, panels A and B) and immunoreactive NaKATPase by confocal immunofluorescence microscopy (panels C and D). Cells were treated with DMSO vehicle control (VEH, panel A and C) or fenoldopam (FEN, panels B and D, 1 μM, 30 min). Cells were plated to match experimental conditions used throughout the paper, showing non-polarized flat morphology and distinct nucleus in the DIC images. A predominantly plasma membrane NaKATPase expression pattern is seen in VEH treated cells. FEN treatment caused a marked decrease in plasma membrane NaKATPase expression, accompanied by an increase in its accumulation in the cytoplasm. Images are representative of the experiment performed three times. Ten cells per well in three wells were analyzed per experiment (*P<0.05 vs VEH, t-test, n=3/group). White bar equals 20 microns.
Figure S8. FEN-induced reduction in NaKATPase-dependent sodium efflux in nRPTC is fully reversed by βMCD. Cells were SBFI sodium dye loaded, then sodium loaded by incubation in potassium free HEPES buffered saline to reversibly inhibit NaKATPase, then sodium efflux was initiated by co-addition of 2.7 mM KCl (to stimulate NaKATPase) and addition of EIPA (10 μM, to inhibit sodium entry through NHE3). The amount of intracellular sodium, as measured by SBFI ratiometric fluorescence, decreases when VEH control is added to both nRPTC (open diamond) and uRPTC (closed diamond). The addition of Ouabain (OUB, 100 μM) completely inhibits this time dependent decrease in intracellular sodium concentration in both nRPTC (open triangle) and uRPTC (closed triangle). FEN (1 μM) inhibits the sodium efflux only in nRPTC (open square, *P<0.05 vs. others after the 7 min. time point, ANOVA, Holm-Sidak test, n=6/group) not uRPTC (closed square), and the FEN dependent inhibition in nRPTC is completely reversed by co-incubation with βMCD (open circle).