ONLINE SUPPLEMENT

The HK-2 human renal proximal tubule cell as a model for GRK4-mediated dopamine-1 receptor uncoupling

John J. Gildea[1], Ishan Shah[1], Ryan Weiss[1], Nick Casscells[1], Helen E. McGrath[1], Jin Zhang[2] and Robin A. Felder[1]

[1]The University of Virginia Health System, Department of Pathology, Charlottesville, VA, [2]Johns Hopkins School of Medicine, Baltimore, MD.

Short title:
Aberrant GRK4-mediated adenylyl cyclase coupling in HK-2

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

Determination of cAMP accumulation:

Transfection of the ICUE3 sensor was performed on a Gene Pulser MXcell 96-well electroporation system (Bio-Rad).

Immunofluorescent Staining of HK-2 and human RPTC for Proximal Tubule Characteristics:

Markers of proximal tubules used were: lotus tetragonolobus agglutinin (LTA), γglutamyl transpeptidase (GGT), megalin (MEG), aminopeptidase A (APA), aminopeptidase N (APN, also known as CD13), NHE3 (which is also found in the medullary thick ascending limb (mTAL)) and villin. We also stained for caveolin-1(CAV1), which is present in the proximal tubule but also stains in other regions of the nephron (see Table S1 below). Markers of cells from other nephron segments which served as negative controls were Tamm-Horsfall protein (THP) (found in the mTAL) and sodium chloride co-transporter (NCC) (expressed in the distal convoluted tubule (DT).
Controls for non-specific fluorescent streptavidin binding and fluorescent secondary antibody staining were performed for each cell type and threshold levels for background staining established. Exposure times were identical for each cell type across each individual antibody or lectin, and the fluorescence levels shown in the image are above established background levels. The specifics of the staining procedure were: cells were placed on collagen IV-coated glass bottom 96-well plates and washed twice with phosphate-buffered saline (PBS). They were fixed in PBS containing 4% paraformaldehyde and 1% Triton-X100 for 5 minutes and washed with tris-buffered saline (TBS) three times for 5 minutes. The fixed cells were then blocked overnight in PBS containing 5% non-fat dry milk that had been heated, sonicated and spun at 9000x*g* for 30 minutes. Cells to be used with biotinylated lectin were blocked first with 2 µg/ml unlabelled streptavidin and washed in PBS. 4 µmol/L biotin was added, and cells were washed and blocked with bovine serum albumin. Lectin (biotinylated LTA, 20 µg/ml) staining was performed in bovine serum albumin and detected with 2 µg/ml Alexa 488-labeled streptavidin. Other primary antibodies were added at the concentrations listed below in Table 1. All incubations were done overnight with gentle rocking at 4°C, followed by washing three times (5 minutes each) in PBS-T (PBS plus 0.2% Tween 20). The cells were incubated in PBS with 2% milk with the corresponding Alexa-674 conjugated secondary antibodies (2 µg/ml, Invitrogen) for 90 minutes at room temperature, washed three times in PBS-T and imaged using an Olympus IX81 automated multi-well spinning disk confocal microscope.

NHE3 mediated Na+ Accumulation Assay:

Cell and Drug Preparation:

RPTCs were cultured in 96-well glass bottom collagen-coated Matrical plates (Spokane, WA) at 37°C until they reached 50% confluence. Cells were serum-starved overnight prior to loading with a sodium ion indicator, sodium benzofuran isophthalate (SBFI, 5 µmol/L) (Molecular Probes, Eugene, OR) with 0.04% Pluronic F-127 for 2 hours in PBS with calcium and magnesium. Cells were washed twice and allowed to recover at 37°C in serum free media for 30 minutes. They were then washed two more times with PBS and incubated at room temperature. Cells were placed under the microscope and XYZ positions marked for imaging. A single baseline image was acquired, and then ouabain

(100 μmol/L), a cardiac glycoside which increases intracellular sodium concentration by blocking sodium efflux via inhibition of NaKATPase activity, was added to each well with and without fenoldopam (FEN, 1 μmol/L, D_1-like receptor agonist) or vehicle (VEH). Other drugs used were LE300 (10 μmol/L, D_1-like receptor antagonist), EIPA (10 μmol/L, NHE inhibitor), S3226 (10 μmol/L, NHE3 inhibitor), and cariporide (HOE-642, 10 μmol/L, NHE1 inhibitor).

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 (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 precise capture of images from multiple wells of a 96-well microplate under the same conditions. Time-lapse data were simultaneously captured from up to 12 wells per experiment, which allowed the comparison of data from several cell lines with several drug treatment combinations. Cells with sufficient SBFI were selected during image analysis in order to obtain quantifiable data. Cells with poor loading were deemed unhealthy and thus not representative of normal sodium transport activity. Less than 10% of cells in our experiments were poorly-loaded with dye. Time-lapse ratiometric images were acquired every three minutes, starting from time 0 in 3 separate wells per group with 20 cells per well measured.

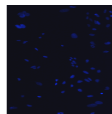
TABLE S1

ANTIBODY / LECTIN	REGION OF KIDNEY	CONCENTRATION	SOURCE
Biotinylated-LTA	PT	1:100	Vector Labs B-1325
GGT	PT	1:100	Neomarkers clone 138H11
MEG rabbit polyclonal	PT	1:50	Santa Cruz Biotechnology sc-25470
APA rabbit polyclonal	PT	1:50	Santa Cruz Biotechnology sc-18065
APN / CD13 clone 452 monoclonal	PT	1:20 hybridoma culture supernatant	Dr. Meenhard Herlyn (The Wistar Institute of Anatomy and Biology, Philadelphia, PA).
NHE3 rabbit polyclonal	PT and mTAL	1:100	Millipore AB3085
Villin monoclonal	PT	1:100	Beckman Coulter IM0258
CAV1 rabbit polyclonal	PT, DT, collecting duct, glomerulus	1:100	Santa Cruz Biotechnology sc-894
THP rabbit polyclonal	mTAL	1:200	Santa Cruz Biotechnology sc-20631
NCC rabbit polyclonal	DT	1:500	Millipore AB3553

FIGURE S1

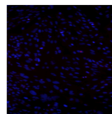
	Uncoupled			Coupled		Primary Cell Cultures		
	HK2	i2	i25	i14	i16	P1	P2	P3
LTA	1	3	3	3	3	4	4	4
GGT	4	3	3	4	4	3	4	3
MEG	2	3	3	3	3	4	4	4
APA	2	3	3	3	3	3	3	3
APN	0	4	3	3	3	4	4	4
Villin	3	3	3	3	3	3	3	3
NHE3	3	3	3	3	3	4	4	4
CAV1	3	3	3	4	4	3	4	3
THP	0	0	0	0	0	0	0	0
NCC	0	0	0	0	0	0	0	0

2°Rab Alexa 647



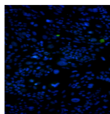
VEH

2°Goat Alexa 594



VEH

2°SA Alexa 488



VEH

Figure S1. Immunofluorescent staining of HK-2 and human RPTC for proximal tubule (PT) characteristics. All cell lines (including three control primary cell lines P1, P2, P3) were stained with known markers for renal proximal tubules: LTA, GGT, MEG, APA, APN and Villin. They were also stained for CAV1, which is present in PT as well as DT, collecting duct and glomerulus, and for NHE3, which is in PT and mTAL. Negative controls included THP (marker for medullary thick ascending limb), and NCC (marker for distal tubule). Images were acquired at 100x magnification using identical camera exposures for each antibody. The numbers inset in each photomicrograph indicate relative intensity of staining, scored on a 0-4 scale. HK-2 was negative for APN and showed remarkably reduced LTA staining.

FIGURE S2

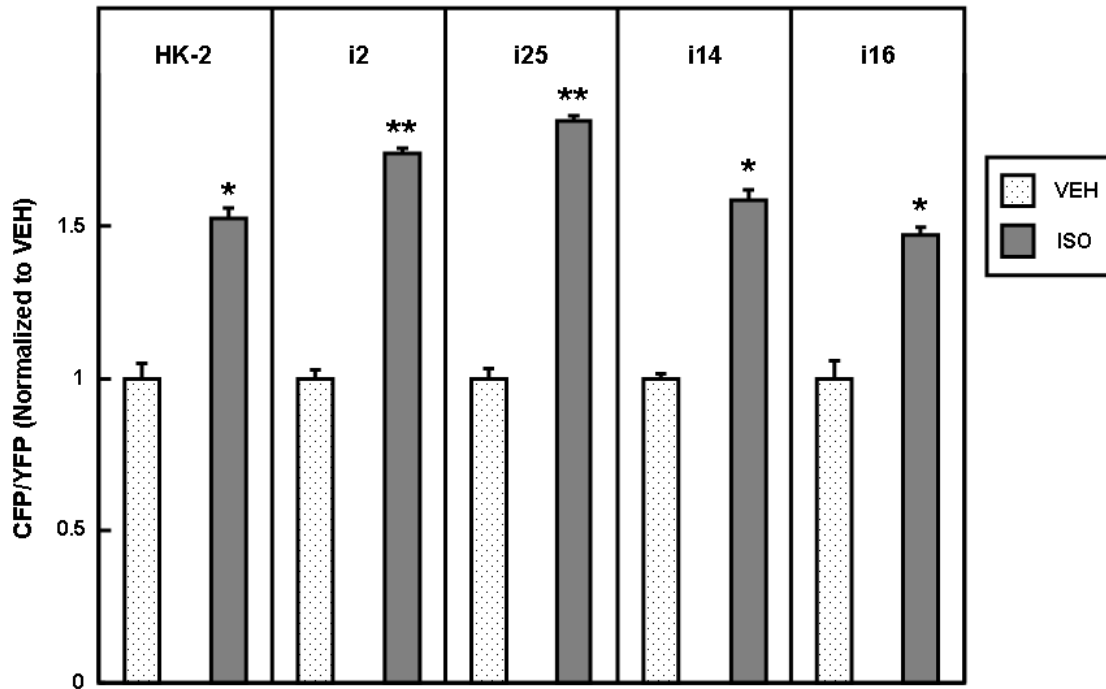


Figure S2. cAMP response of HK-2, uRPTC (i2 and i25) and nRPTC (i14 and i16) to isoproterenol. Intracellular cAMP accumulation was measured using a cAMP FRET Biosensor, ICUE3. The beta-adrenergic receptor agonist isoproterenol (ISO, 1 µmol/L, 30 min) significantly increased cAMP accumulation in each of the cell lines (*P<0.001 vs VEH, N=10). The response of the uRPTCs i2 and i25 is significantly higher than that seen in their normally coupled counterparts, i14 and i16, or the HK-2 (**P<0.01, N=10 vs the other 3 cell lines, P< 0.001 from VEH).

FIGURE S3

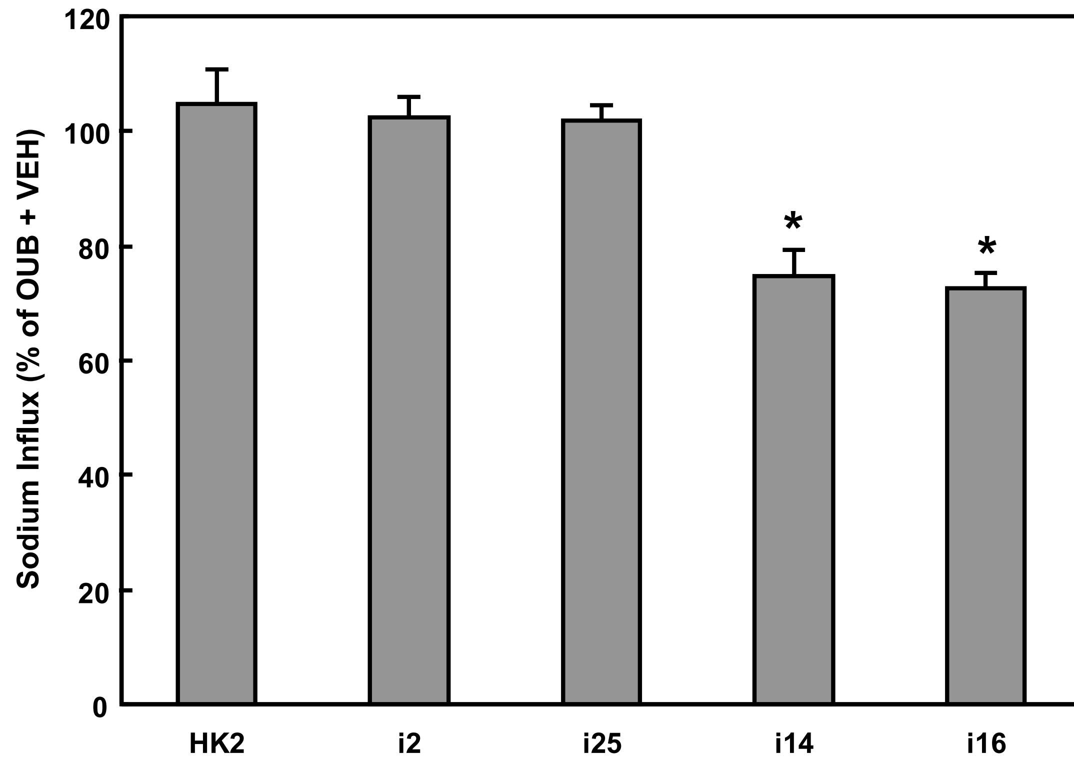


Figure S3. Summary of sodium influx (NHE3 assay) data from Figure 4 (of text). The data for the 30 minute time point that was shown in Figure 4 are summarized here. The OUB + FEN groups are depicted as a percentage of the OUB + VEH control for each cell line: HK-2 and uncoupled RPTC i2 and i25, and coupled RPTC i14 and i16 (*P<0.001, N=6).

FIGURE S4

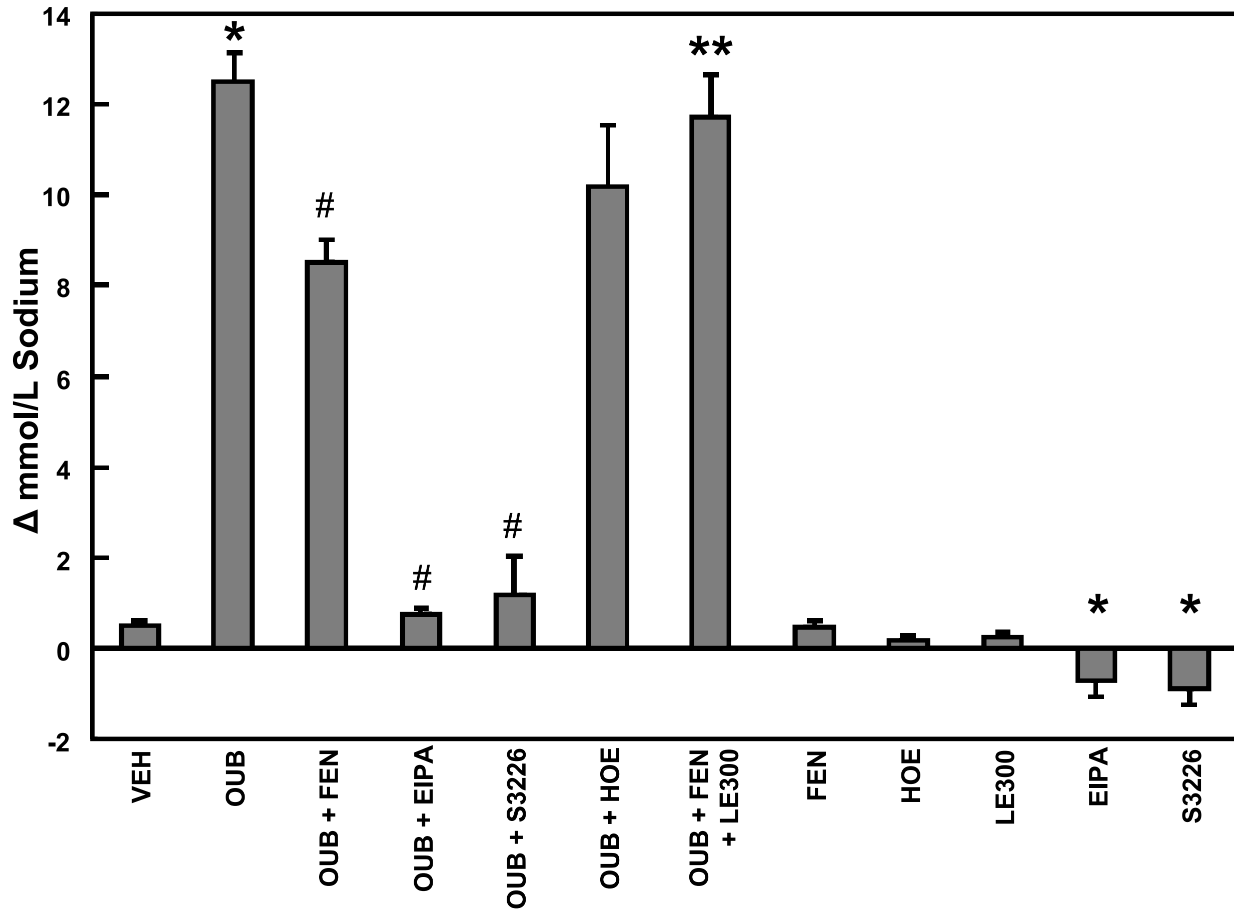


Figure S4. Change in intracellular sodium accumulation in the coupled nRPTC cell line i16, at 30 minute time point. The ouabain (OUB)-mediated increase in intracellular sodium (2nd bar) was inhibited by FEN (D_1-like receptor agonist) and was blocked by the NHE inhibitor EIPA and the NHE3 specific inhibitor S3226, but not significantly reduced by the NHE1 specific inhibitor, HOE. The FEN reduction in intracellular sodium accumulation was reversed by LE300 (D_1-like receptor antagonist). The addition of LE300, FEN and HOE, by themselves, did not cause a significant increase in intracellular sodium concentration over the initial baseline reading (line at 0). However EIPA and S3226, by themselves, caused a significant decrease in intracellular sodium concentration from baseline (*P<0.05 vs VEH, N=6; #P<0.05 vs OUB, N=6; **P<0.05 vs OUB + FEN, N=12).