SUPPLEMENTAL MATERIAL

EVIDENCE THAT BINDING OF CYCLIC GMP TO THE EXTRACELLULAR DOMAIN OF NA^+/K^+-ATPASE MEDIATES NATRIURESIS

Brandon A. Kemp, BA
Nancy L. Howell, BA
John J. Gildea, PhD
Josh D. Hinkle, PhD
Jeffrey Shabanowitz, PhD
Donald F. Hunt, PhD
Mark R. Conaway, PhD
Susanna R. Keller, MD
Robert M. Carey, MD

Running Title: Na^+/K^+ATPase Binds Cyclic GMP

From the Department of Medicine-Division of Endocrinology and Metabolism (BAK, NLH, SRK, RMC), the Department of Pathology (JJG) the Department of Chemistry (JDH, JS, DFH) and the Division of Translational Research and Applied Statistics, Department of Public Health Sciences (MRC), University of Virginia, Charlottesville, VA

Correspondence: Dr. Robert M. Carey, P.O. Box 801414, University of Virginia Health System, Charlottesville, VA 22908-1414; telephone: 434-924-5510; fax 434-982-3626; email: rmc4c@virginia.edu.

METHODS

Confocal Microscopy:

Stained tubules imaged under epifluorescence illumination use a custom-built automated Olympus IX81 spinning disk confocal microscope and a 60X UIS2 UPlanSApo NA 1.2 water immersion objective. The microscope is controlled using Slidebook 5.5 software (3i, Denver CO) and 5 micron thick z-stack images captured using a Hamamatsu Electron Multiplier CCD C9100-02 camera camera at 0.25 micron intervals and post-processed identically using AutoQuant 3D spinning disk blind deconvolution. Exposure times are between 100 and 800 milliseconds. Applied Scientific Instruments automated excitation and emission filters wheels house hard coated Semrock filters and a multipass dichroic filter cube are in a Sedat configuration (DA/FI/TR/Cy5/Cy7-5X5M-B-000). Applied Scientific Instruments automated x-y-z piezo stage is enclosed inside a custom built temperature and humidity-controlled CO_2 incubator and mounted on a 3'x5' nitrogen driven floating air table.

Total RPTC Membrane Preparation and Western Blot Analysis:

Slices of kidney cortex (approx. 100 mg per kidney) were homogenized in Mammalian Protein Extraction Reagent (MPER; Thermo Scientific) lysis buffer with Halt protease and phosphatase inhibitor cocktail (Thermo Scientific) and centrifuged at 900 x g (Eppendorf Centrifuge 5810 R with A-4-62 rotor with 18 cm max radius for 10 min at 4ºC to remove cellular debris. The supernatant was collected, and total protein measured using a bicinchoninic acid (BCA) assay (Pierce). Sodium dodecylsulfate (SDS) samples were prepared, separated by SDS-PAGE (10% Tris-HCl polyacrylamide gels; 40 µg of protein loaded per lane), and transferred onto a nitrocellulose membrane by electroblotting. Membranes were blocked in 5% milk in TBS with 0.1% Tween-20 (TBST) for 2h at 4°C and then incubated overnight at 4ºC with the following primary antibodies in 5% milk TBST: Src (Cell Signaling; 1:1,000; Cat. #2109), and Erk 1/2 [Cell Signaling; 1:1,000; Cat. #4695] or 5% BSA in TBST: phospho-Src^{Tyr416} (pSrc ^{Tyr416}) [Cell Signaling; 1:1,000; Cat. #6943] and phospho-Erk 1/2$^{Thr202/Tyr204}$ (pErk 1/2 $^{Thr202/Tyr204}$) [Cell Signaling; 1:1,000; Cat. #4370]. After 3 washes in TBST, membranes were incubated with HRP-conjugated anti-rabbit secondary antibody in 5% milk TBST (GE Healthcare; 1:2,500; Cat. #NA934V) for 2h at room temperature (RT). Signals were detected using chemiluminescence and band densities were measured with Image J software (NIH). The membranes were stripped for 20 min (Restore Western blot stripping buffer; Thermo Scientific) and blocked in 5% milk for 1h at 4ºC. The membranes were then incubated with the primary antibody β-tubulin (Millipore; 1:1,000; Cat. #05-661) in 5% milk for 2h at RT and subsequently with the HRP-conjugated anti-mouse secondary antibody (GE Healthcare; 1:2,500; Cat. #NXA931V). All blots were normalized to β-tubulin expression.

RPT Isolation and Experimentation:

The night before isolation, 100 µL Poly-l-lysine (Sigma; Cat. #P4832, 1:10 diluted in water) was added to wells in a 96-well glass bottom plate and kept at 4ºC. The day of isolation, 12-week-old female Sprague Dawley rats were anesthetized with ketamine

(60 mg/kg) and xylazine (4 mg/kg) via an IP injection. Following a midline laparotomy, the kidneys were removed. The cortex was excised, sliced into small pieces, and placed in 15 mL Opti-MEM media (Gibco) with collagenase A (Roche; 2 mg/mL). The mixture was placed in an orbital shaker for 90 min at 37ºC. The mixture was then passed through 2 successive sieves (212 μm and 106 μm) and the eluate spun at 600 rpm for 3 min to collect RPTs. The RPTs were washed in 20 mL PBS, spun at 600 rpm for 3 min (Eppendorf Centrifuge 5810 R with A-4-62 rotor with 18 cm max radius), and then resuspended in a final volume of 2 mL Opti-MEM. After washing the Poly-l-lysine-coated 96-well plate 3 x with PBS, 100 μL resuspended RPTs diluted in Opti-MEM (25 μl RPTs + 75 μL Opti-MEM per well) were added. After 30 min at RT vehicle (Opti-MEM) or pharmacological agent in Opti-MEM was added for 1h at RT in the dark. The RPTs were then washed 3 x with PBS. Following these washes, for studies involving BIO-cGMP, BIO-cAMP, or Azido-BIO-cGMP, RPTs were incubated with streptavidin Alexa Fluor 488 (Invitrogen; 1:2,000; Cat. #S32354) for 30 min at RT in the dark. After another set of 3 washes, RPTs were imaged. The experiment was repeated 4 separate times using new fresh kidneys every time for N=4. Within each experiment, for each condition 3 separate wells in a 96-well plate were averaged.

Cross-linking and Immunoprecipitation Experiments:

The digestion and preparation of RPTs was performed as described above under **RPT Isolation and Experimentation** with minor modifications. No coating of wells with Poly-l-lysine was done and after the final wash with PBS, isolated RPTs were reconstituted in 8 mL Opti-MEM. Two mL aliquots of this mixture was added to 4 separate wells of a 6-well plastic plate and let sit for 30 min at RT. Vehicle (Opti-MEM) or pharmacological agent(s) in Opti-MEM were added to the wells for 1h at RT in the dark. The tubules were removed from the plate and washed 3 x in 4 mL Opti-MEM and pelleting at 600 rpm (Eppendorf Centrifuge 5810 R with A-4-62 rotor with 18 cm max radius) for 3 min. The final Opti-MEM supernatant was removed, the RPTs were resuspended in 2 mL fresh Opti-MEM and added back to the 6-well plate to sit for 5 min at RT in the dark. Plates to be cross-linked were placed into a Spectrolinker XL-1000 UV crosslinker (Spectronics Corporation) for 30 sec at a distance of approximately 15 cm from the UV light source. RPTs in wells were collected, spun at 600 x rpm for 3 min, and the supernatant was removed. RPTs were solubilized in 1.5 mL MPER with Halt protease and phosphatase inhibitor cocktail, and total protein was determined by BCA assay. Magnetic streptavidin beads (40 μL) [Invitrogen] were added to solubilized RPTs (1 mg protein/mL). After incubation of samples on a 360º rocker for 2h at RT, beads were washed 3 x in 1 mL PBS. Following the last wash, 150 μL 2% SDS with 3 mM biotin in PBS was added and samples heated to 99ºC on an orbital shaker. Twenty μL of the final supernatant was mixed with 20 μL 2x sample buffer and samples were separated on a 10% Tris-HCl polyacrylamide gel. Western blot analysis was performed as described above. Membranes were incubated with α-1 NKA (αNKA; Millipore; 1:5,000; Cat. #05-369). The experiments in **Figure 1**, **Panels A**, **B**, and **C**, were repeated 3, 8, and 6 times, respectively, using new fresh kidneys every time.

Mass Spectrometry Preparation and Analysis:

Three cross-linked Azido-BIO-cGMP samples were separated in a 10% Tris-HCl polyacrylamide gel. The gel was washed 3 x in ultrapure water for 5 min and stained with Coomassie SimplyBlue SafeStain (Invitrogen) for 2h at RT. The gel was then washed for 2h in ultrapure water and the area at approx. 110 kDa (corresponding to NKA molecular weight) was excised and processed for mass spectrometry. Excised gel pieces were suspended in water, transferred to a 1.5 mL Sarstedt Low-Bind Tube and segmented into approx. 2 x 2 mm pieces. The pieces were incubated for 3 x 10 min in HPLC grade water followed by an incubation in 50% water, 40% ethanol, and 10% acetic acid for 30 min on a shaker to remove Coomassie SimplyBlue SafeStain within the gel bands. If pieces were still stained, the solution was replaced and the process repeated. Darkly stained gel pieces were left to destain in this solution overnight. Gel pieces were then incubated in 100 µL 100 mM ammonium bicarbonate for 2 x 5 min and dehydrated in 100% acetonitrile for 10 min. The acetonitrile supernatant was removed, and gel pieces were dried in a vacuum concentrator. The pieces were rehydrated in 50 µL 10 mM dithiothreitol in 100 mM ammonium bicarbonate for 1h at 37ºC to reduce any disulfide bonds. After incubating for approx. 5 min at RT, the supernatant was removed, and the solution was replaced with with 50 µL 55 mM iodoacetamide and shaken in the dark for approx. 45 min to alkylate the free sulfhydryl groups formed by reduction of the disulfide bonds. The pieces were dried in a vacuum concentrator for approx. 20 min and then rehydrated with 25 µL cold 12.5 ng/µL trypsin in 100 mM ammonium bicarbonate. After incubating for 45 min, excess trypsin was removed (generally a negligible amount, most was absorbed by the gel pieces) and 25 µL 100 mM ammonium bicarbonate was added. Proteins in gel pieces were digested overnight while shaking at RT. After addition of 25 µL ammonium bicarbonate, the digest was incubated for 15 min and transferred to a clean 1.5 mL collection tube. After addition of 50 µL 5% formic acid, 25% acetonitrile, and 70% water the digest was incubated for 15 min. The supernatant containing the eluted peptides was transferred to the collection tube. Fifty µL of 5% formic acid, 50% acetonitrile, and 45% water was added to the gel pieces and incubated for 15 min. The supernatant was transferred to the collection tube. The samples were rehydrated with 50 µL water for 10 min and the supernatant was collected. 50 µL 5% formic acid, 75% acetonitrile, and 20% water was added to the bands and incubated for 15 min. The supernatant was collected. The collection tube containing all the peptides eluted from the gel pieces was dried in the vacuum concentrator for approx. 1h and dried peptides were stored at -40ºC until analysis. Isolated peptides were reconstituted in 0.1% acetic acid. Internal standards, angiotensin I (100 fmol) and vasoactive intestinal peptide (100 fmol) were loaded onto an 8 cm reverse-phase preparatory column 360 µm outer diameter x 75 µm inner diameter packed with 15 µm C18 packing material (YMC). The preparatory column was rinsed with Solvent A (0.1% acetic acid in water) for 30-40 min at RT. It was then connected to an 8 cm reverse-phase analytical column 360 µm OD x 50 µm ID packed with 5 µm C18 packing material (YMC). Peptides were eluted from the column using a gradient transitioning from 100% Solvent A to 0%-60%-100% solvent B (0.1% acetic acid in 66% acetonitrile, 33% water) at 0-60-65 min respectively while in-line with an Thermo Scientific™ Orbitrap Fusion™ Tribrid™ mass spectrometer. The mass spectrometry data acquisiton method was as follows: FT (Orbitrap) MS1 (60k

resolution), IT (Ion Trap) MS1, data-dependent top-speed (2 sec) IT ETD-CID (Electron transfer dissociation – collision activated dissociation) toggle. Files were searched using Byonic through Proteome Discover using NCBI's RefSeq protein database to identify the peptides within the analysis. Peptides identified at a false discovery rate of 1% which corresponded to the sequence of αNKA were manually annotated to verify correct assignment.

Sodium Efflux Assay:

SV40 large T-antigen immortalized Wistar Kyoto (WKY) rat RPTCs, passage 23, were obtained from Ulrich Hopfer[25] and cultured in DMEM F-12 media (Gibco), supplemented with 5% fetal bovine serum (Genesee Scientific), ITS (Invitrogen), EGF (Sigma-Aldrich), and 1% penicillin/streptomycin (Genesee Scientific) at 37°C and 5% CO_2. Experiments were conducted in a 96-well glass bottom collagen coated Matrical plates (Spokane, WA) at 37°C after cells reached 80% confluence. Cells were serum-starved overnight prior to loading with a Na^+ ion indicator, Na^+ benzofuran isophthalate (SBFI, 5 μM) (Molecular Probes, Eugene, OR) with Pluronic 127 for 2h in PBS with calcium and magnesium. Cells were allowed to recover at 37°C in serum free media for 30 min, washed 2 x with RT potassium free HEPES media (20 mM HEPES pH 7.4, 130 mM NaCl, 1 mM CaCl, 1 mM MgCl), and then incubated in media without K^+at RT for 30 min to allow intracellular Na^+ to accumulate. VEH (PBS), cGMP (2 μM), Azido-BIO-cGMP (2 μM), or fenoldopam (FEN; 1 μM) were added to respective wells (6 wells per condition) for 10 min and plates placed on the stage of an automated fluorescent microplate reader (BMG Pherastar FS) for time lapse multiwell ratiometric measurements. Directly before imaging, 5-(N-ethyl-N-isopropyl)-amiloride (EIPA; 10 uM final concentration) and KCl (2.7 mM final concentration) were added to all wells as 10X stocks, and then cells were imaged for 20 min. Measurements shown represent the changes in the 340/380 ratio relative to time 0 in 6 separate wells at 20 locations per well measured.

***In Silico* Docking of cGMP to NKA:**

The structure of the high-affinity NKA-Ouabain complex was used for docking of cGMP (PDB ID code 4HYT).[26] Ouabain was removed and polar hydrogens were added using AutoDock Tools.[27] Gasteiger charges and hydrogen atoms were assigned to cGMP (PDB ligand: PCG) using AutoDock Tools. A grid box was centered around the ouabain binding site and docking performed using AutoDock Vina.[28] Results were inspected and visualized using UCSF ChimeraX.[29]

Human NKA ATPase Activity Assay:

For ATPase activity measurements, cGMP powder was dissolved in 100 mM MOPS/Tris pH 7.4 and cGMP concentration in the solution verified by absorbance measurement at 252 nm (ε=13,500/M/cm). The stock solution was further diluted to working concentrations in ATPase assay medium (120 mM NaCl, 20 mM KCl, 1 mM ATP).[2] NKA activity was measured by supplementing ATPase assay medium with indicated concentrations of cGMP, digoxin or equivalent volumes of buffer for control. Assays were started by adding 3 nM purified human NKA (NKA:human α1β1FXYD purified from K. phaffii solubilized in mixed $C_{12}E_8$/18:0-18:1

phosphatidylserine/cholesterol micelles[30]) and activity was measured over 1h at 37°C in a 96-well PCR plate while shaking. The amount of released phosphate was determined spectrophotometrically according to the method of Baginski[31] and activity normalized to the untreated control sample.

Power Calculations:

The number of animals required to reach statistical significance was calculated using the G*Power 3.1 program[32] with the following assumptions. An α error probability of 0.05, a power (1-β) of 0.2, estimations of variance (std dev) based on preliminary data or previous publications, correlation among repeated measures held at 0.5 and medium effect size (0.25). Based on our extensive experience with most of the experimental approaches used in this paper, we expected to obtain statistically significant differences, if present, using the numbers of rats stated under each protocol.

SUPPLEMENTAL FIGURES

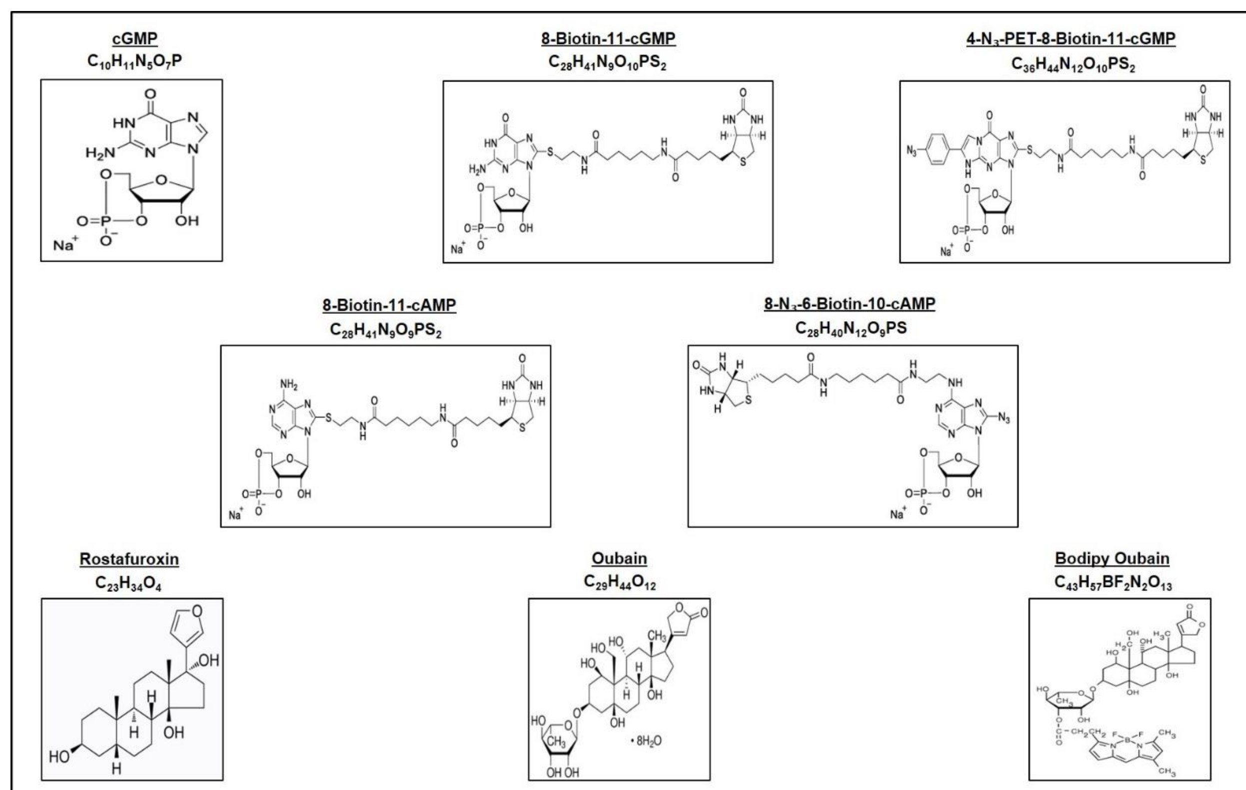

Figure S1. Molecular structures for all compounds used in studies.

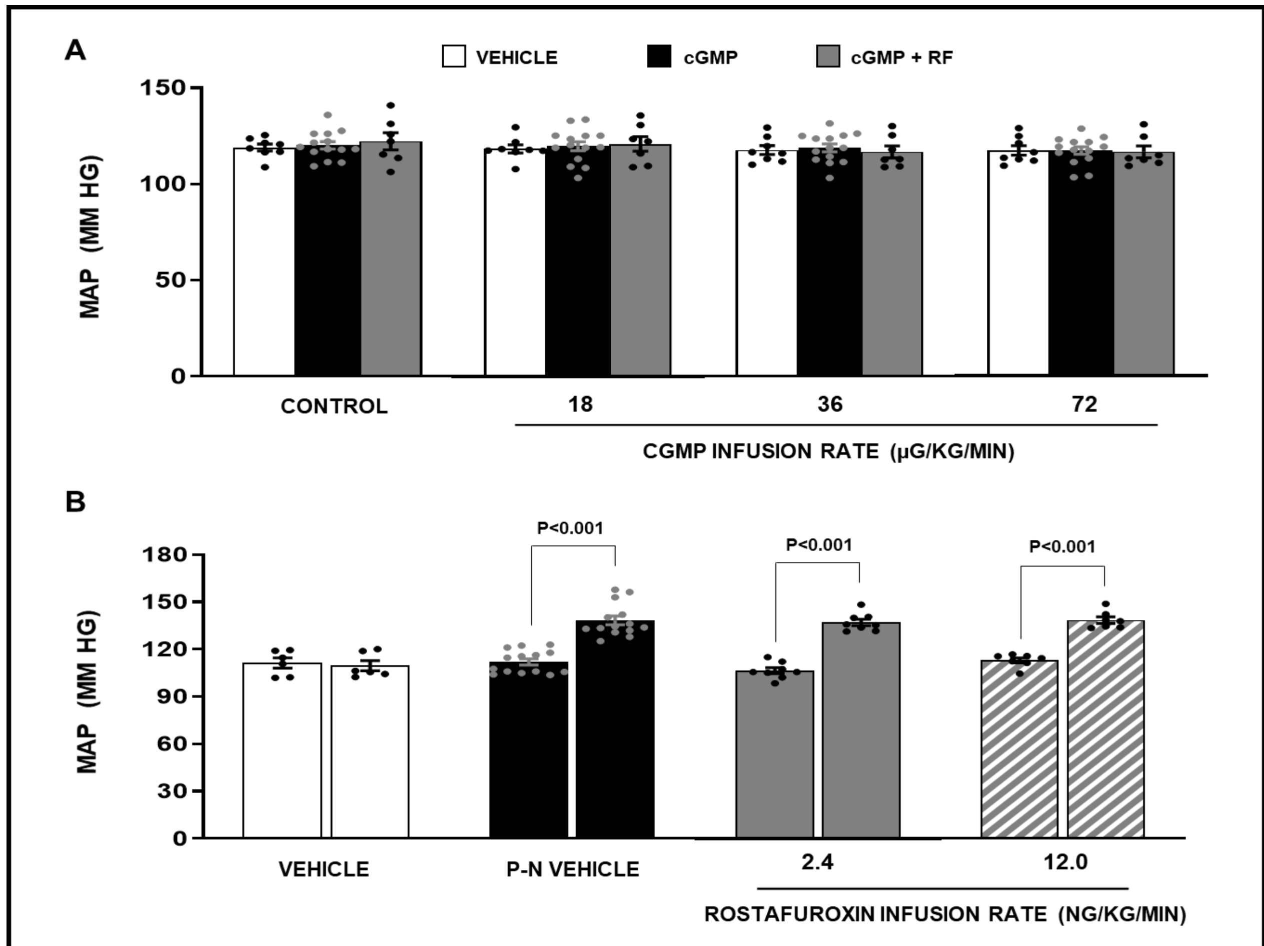

Figure S2. Panel A. Mean arterial pressure (MAP) response to the following conditions corresponding to **Figure 1, Panel A**: (□) Time Control (N=8): rats received renal interstitial (RI) infusion of vehicle (VEH) D_5W for the entire 2h study. (■) cGMP (N=14): rats received RI infusion of VEH for 30 min during the control period followed by cumulative RI infusions of cGMP (18, 36, and 72 μg/kg/min; each dose for 30 min) during the experimental periods. (■) cGMP + Rostafuroxin (RF) (N=7): rats received RI infusion of VEH for 30 min during the control period followed by the RI co-infusion of cGMP + RF (12 ng/kg/min) during the experimental periods. Results are reported as mmHg. Data represent mean ± 1 SE. Overall 2-way ANOVA analysis for **Panel A**; F=6.72, $P=4.9x10^{-6}$. **Panel B.** MAP in response to the following conditions corresponding to **Figure 1**, **Panel B**: (□) Vehicle (VEH) Control (N=6): rats received renal interstitial (RI) infusion of VEH D_5W during both 30 min periods. (■) Pressure-natriuresis (P-N) VEH (N=14): rats received RI infusion of VEH during both the 30 min control and 30 min high renal perfusion pressure periods. (■) P-N + Rostafuroxin (RF; 2.4 ng/kg/min) (N=8): rats received RI infusion of RF during both the 30 min control and 30 min high renal perfusion pressure periods. (▨) P-N + RF (12 ng/kg/min) (N=7): rats received RI infusion of RF during both the 30 min control and high renal perfusion pressure periods. Results are reported as mmHg. Data represent mean ± 1 SE. Statistical significance was determined by using the repeated measures analysis with an unstructured covariance matrix in SAS PROC MIXED program. The ANOVA with

permutation P value was based on 2,000 permutations of group assignment to individual N values and a repeated measures analysis with an unstructured covariance matrix.

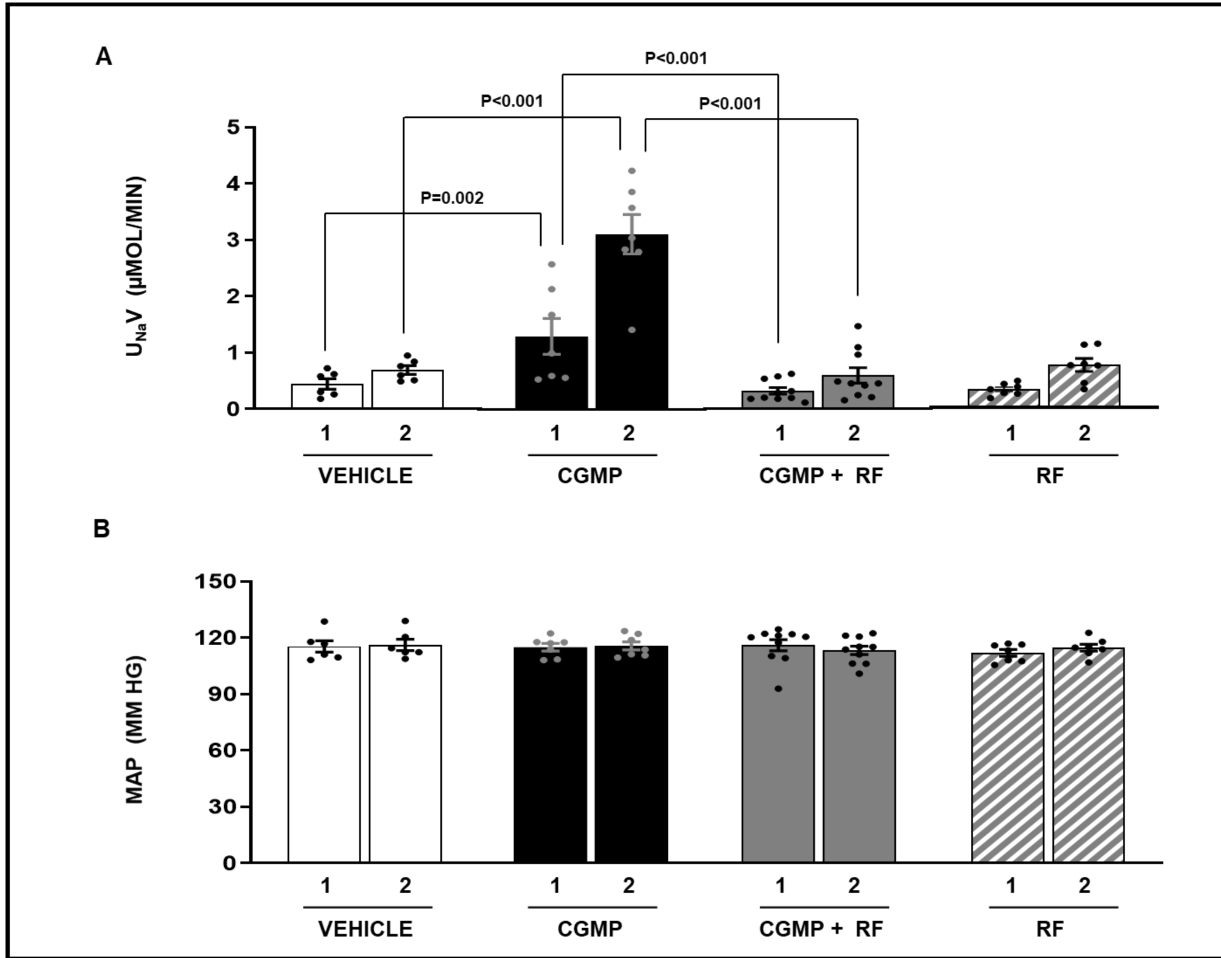

Figure S3. Panel A. Urine sodium (Na^+) excretion ($U_{Na}V$) in response to the following conditions: (□) Vehicle (VEH) (N=6): rats received renal interstitial (RI) infusion of VEH D_5W for two 1h periods. (■) cGMP (N=7): rats received RI infusion of cGMP (72 µg/kg/min) for two 1h periods. (■) cGMP + Rostafuroxin (RF) (N=10): rats received RI co-infusion of cGMP + RF (12 ng/kg/min) for two 1h periods. (▨) RF (N=8): rats received RI infusion of RF for two 1h periods. Results are reported as µmol/min. **Panel B.** Mean arterial pressure (MAP) in response to the conditions in **Panel A**. Results are reported as mmHg. Data represent mean ± 1 SE. Statistical significance was determined by using the repeated measures analysis with an unstructured covariance matrix in SAS PROC MIXED program. The ANOVA with permutation P value was based on 2,000 permutations of group assignment to individual N values and a repeated measures analysis with an unstructured covariance matrix.

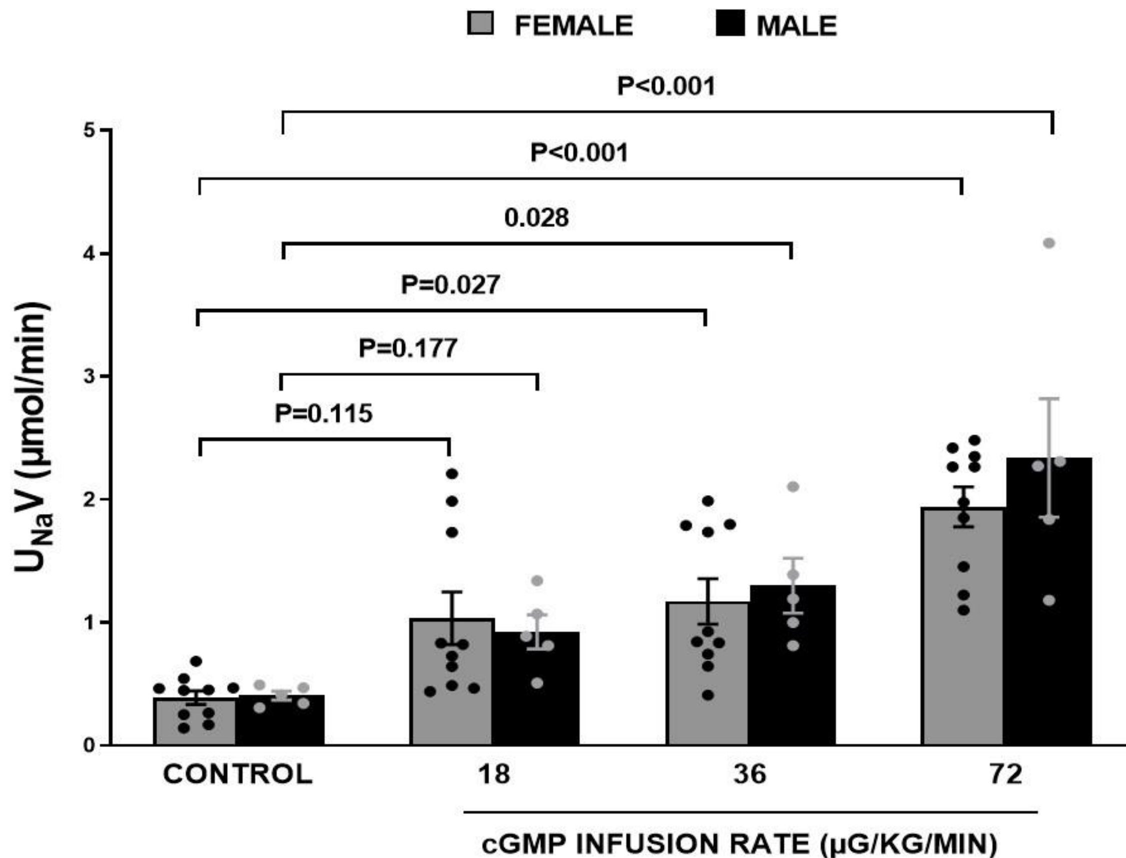

Figure S4. Urine sodium (Na^+) excretion ($U_{Na}V$) of male (N=5) and female (N=8) rats in response to renal interstitial (RI) infusion of vehicle (VEH) control or cGMP. Rats received RI infusion of VEH for 30 min during the control period followed by cumulative RI infusions of cGMP (18, 36, and 72 μg/kg/min; each dose for 30 min) during the experimental periods. Statistical significance was determined by using the repeated measures analysis with an unstructured covariance matrix in SAS PROC MIXED program. The ANOVA with permutation P value was based on 2,000 permutations of group assignment to individual N values and a repeated measures analysis with an unstructured covariance matrix.

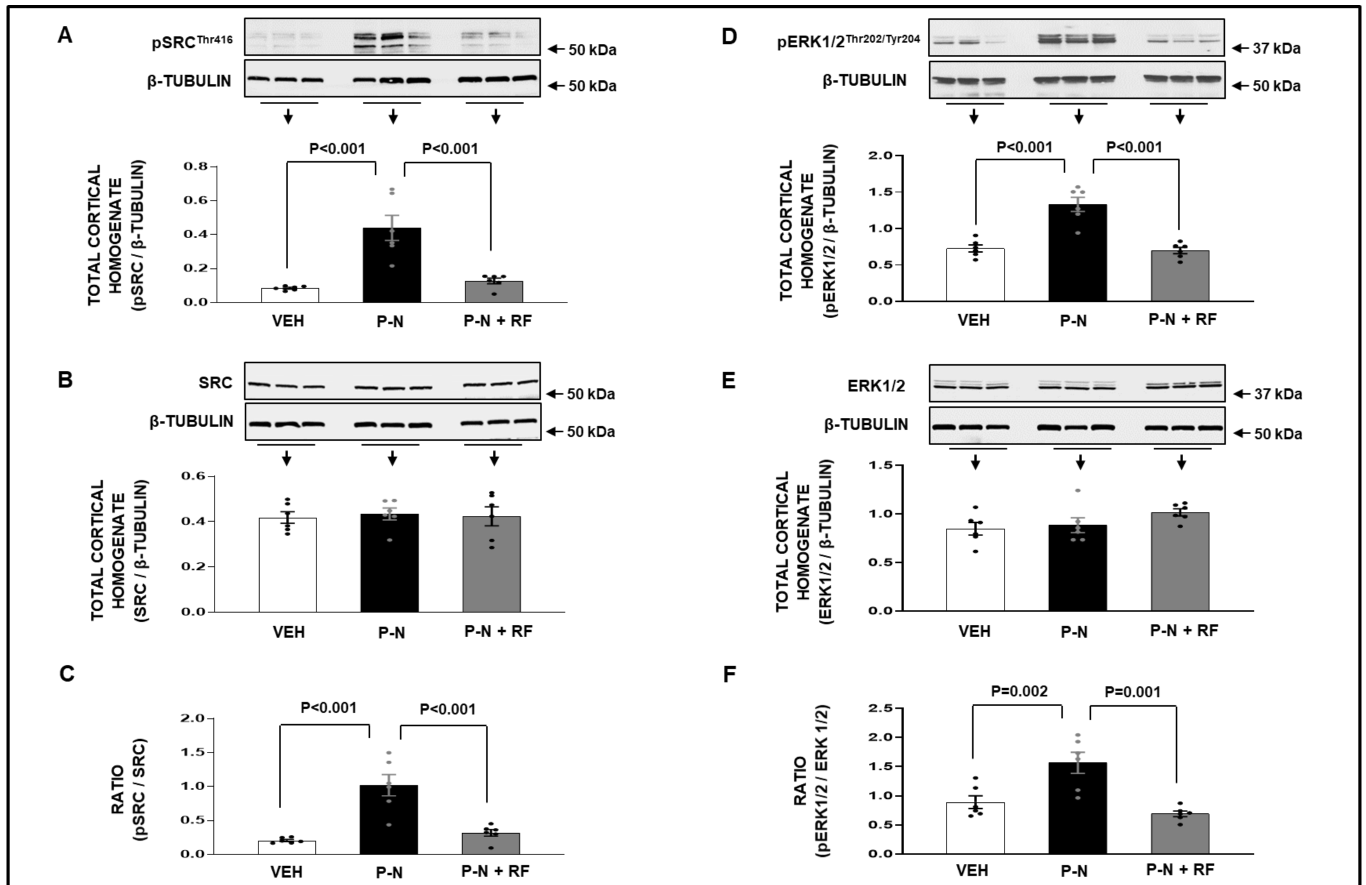

Figure S5. Panel A. Western blot analysis of total cortical homogenate phospho-Src^{Thr416} (pSrc^{Thr416}) protein levels in response to the following conditions: (□) Time Control Vehicle (VEH; N=6): rats received renal interstitial (RI) infusion of VEH D_5W during both 30 min periods. (■) Pressure-Natriuresis (P-N) VEH (N=6): rats received RI infusion of VEH during both the 30 min control and 30 min high renal perfusion pressure periods. (■) P-N + Rostafuroxin (RF; 12 ng/kg/min) (N=8): rats received RI infusion of RF during both the 30 min control and 30 min high renal perfusion pressure periods. **Panel B.** Western blot analysis of total Src protein levels in response to the same conditions in **Panel A**. **Panel C.** Ratio of (pSrc^{Thr416} / β-Tubulin) / (total Src / β-Tubulin) in response to the same conditions in **Panel A**. **Panel D.** Western blot analysis of phospho- Erk1/2$^{Thr202/Tyr204}$ (pErk 1/2 $^{Thr202/Tyr204}$) protein levels in response to the same conditions in **Panel A**. **Panel E.** Western blot analysis of total Erk 1/2 protein levels in response to the same conditions in **Panel A**. **Panel F.** Ratio of (pErk 1/2 $^{Thr202/Tyr204}$ / β-Tubulin) / (total Erk 1/2 / β-Tubulin) in response to the same conditions in **Panel A**. Data represent mean ± 1 SE. Statistical significance was determined by using the repeated measures analysis with an unstructured covariance matrix in SAS PROC MIXED program. The ANOVA with permutation P value was based on 2,000 permutations of group assignment to individual N values and a repeated measures analysis with an unstructured covariance matrix.

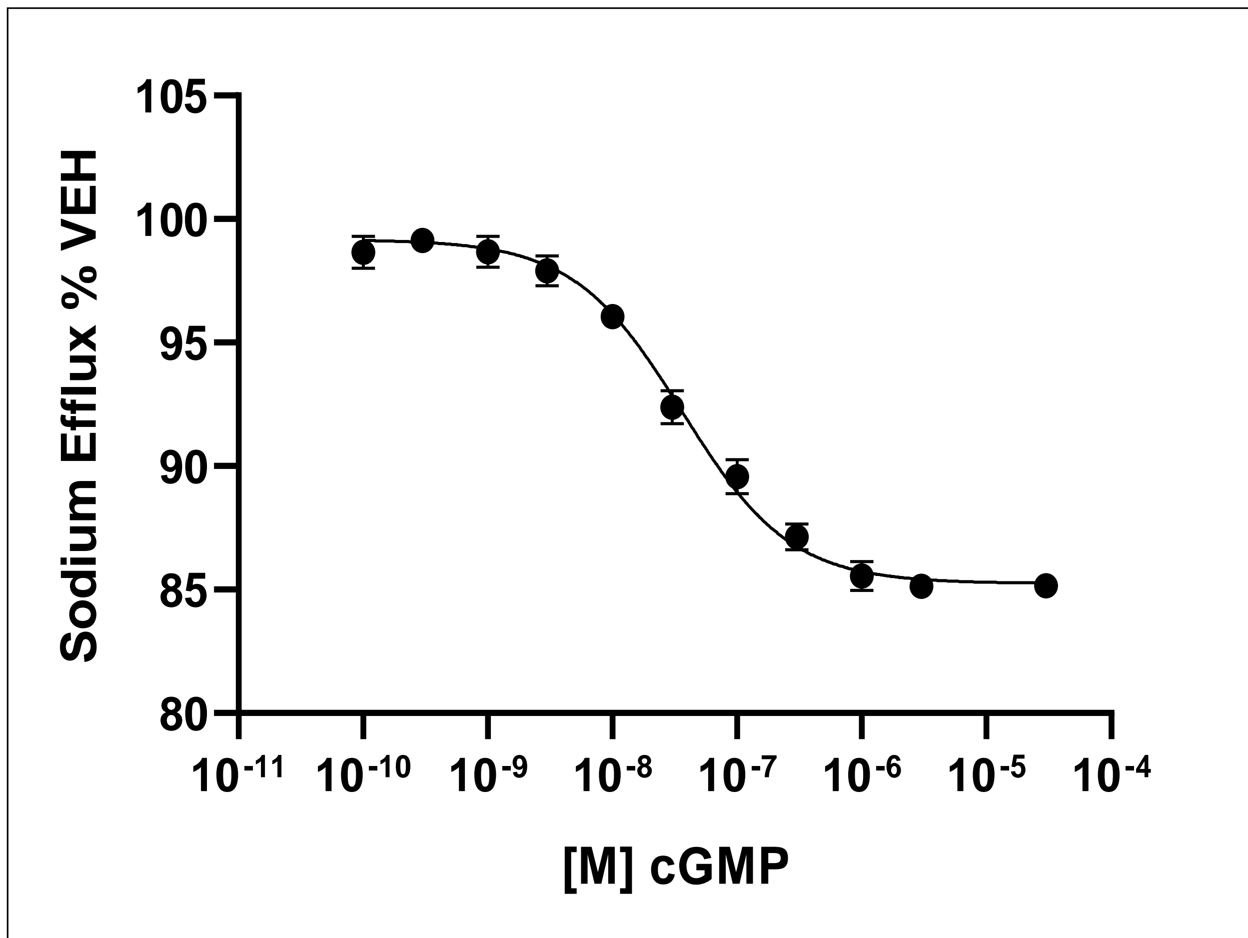

Figure S6. In cultured rat renal proximal tubule cells, cGMP reduces sodium efflux in a concentration-dependent manner (IC_{50} 36 nM). Intracellular sodium concentration was determined using the sodium sensitive dye SBFI. For each cGMP concentration measurements were taken from 6 separate wells in a 96-well plate with 20 locations measured per well.

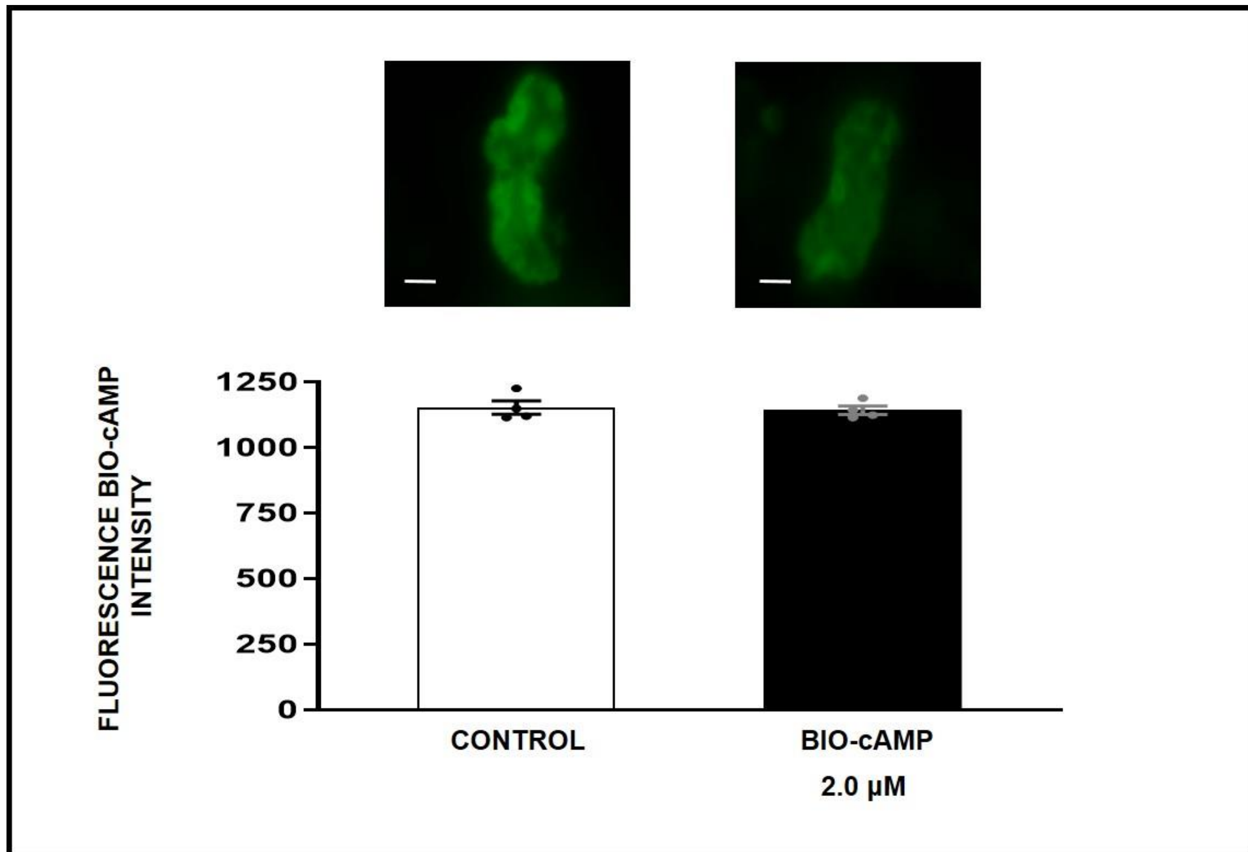

Figure S7. Confocal microscopy experiments with renal proximal tubules (RPT) isolated from normal rats (N=4 for each experiment). For each experiment and each condition, measurements from 3 separate wells in a 96 well plate were averaged. (□) Control: Opti-MEM or (■) Biotinylated-cAMP (BIO-cAMP): 2 µM. Control fluorescence values represent background, and all other results are reported as BIO-cAMP fluorescence intensity above background. Data represent mean ± 1 SE. Scale bars represent 10 µm. Statistical significance was determined by using the repeated measures analysis with an unstructured covariance matrix in SAS PROC MIXED program. The ANOVA with permutation P value was based on 2,000 permutations of group assignment to individual N values and a repeated measures analysis with an unstructured covariance matrix. These results demonstrate that BIO-cAMP exhibits no fluorescence signal over background in RPTs.

Rank	ProteinName	LogProb	# Spectra	Unique Peptides	Coverage	# Amino Acids
1	>NP_075580.2 unconventional myosin-Ic [Rattus norvegicus]	272.0570432	191	54	54.21451	1044
2	**>NP_036636.1 sodium/potassium-transporting ATPase subunit alpha-1 precursor [Rattus norvegicus]**	241.6833866	171	50	47.21403	1023
3	>NP_036866.1 hexokinase-1 [Rattus norvegicus]	186.7203059	143	42	45.86052	918
4	>NP_001013175.1 NAD(P) transhydrogenase, mitochondrial [Rattus norvegicus]	157.5178402	107	33	35.54325	1086
5	>sp|K2C1_HUMAN|(Common contaminant protein)	139.929609	100	27	34.52561	643
6	>sp|K1C9_HUMAN|(Common contaminant protein)	130.070912	60	22	48.95658	623
7	>sp|K1C10_HUMAN|(Common contaminant protein)	127.8357381	88	28	41.82118	593
8	>NP_037115.2 unconventional myosin-Id [Rattus norvegicus]	127.3698695	77	33	33.79718	1006
9	>XP_006231272.1 PREDICTED: glycine dehydrogenase (decarboxylating), mitochondrial isoform X1 [Rattus norvegicus]	124.108992	73	29	32.03122	1024
10	>NP_036876.2 pyruvate carboxylase, mitochondrial precursor [Rattus norvegicus]	103.3433557	42	20	25.2122	1178
11	>NP_476480.2 heterogeneous nuclear ribonucleoprotein U [Rattus norvegicus]	88.39059367	85	25	28.82202	798
12	>XP_006240311.1 PREDICTED: C-1-tetrahydrofolate synthase, cytoplasmic isoform X1 [Rattus norvegicus]	85.15185223	35	16	18.59722	941
13	>sp|K22E_HUMAN|(Common contaminant protein)	80.30560615	45	18	31.7829	645
14	>NP_001292806.1 myosin-9 [Rattus norvegicus]	58.10458394	25	13	8.265302	1960
15	>NP_001120921.1 actin, cytoplasmic 2 [Rattus norvegicus]	49.75817185	20	11	34.66657	375
16	>XP_008764648.1 PREDICTED: unconventional myosin-VI isoform X6 [Rattus norvegicus]	47.83856181	26	15	13.48762	1253
17	>NP_001007146.1 catenin alpha-1 [Rattus norvegicus]	44.04546928	21	12	15.85901	908
18	>NP_596895.1 lon protease homolog, mitochondrial precursor [Rattus norvegicus]	40.77382882	23	12	13.57893	950
19	>NP_036921.1 dipeptidyl peptidase 4 [Rattus norvegicus]	38.78123159	20	12	14.60233	767
20	>NP_446418.1 laminin subunit gamma-1 precursor [Rattus norvegicus]	38.50573665	12	6	4.35594	1607
21	>NP_058686.2 alpha-adducin [Rattus norvegicus]	37.13589388	10	6	11.02039	735
22	>XP_017452344.1 PREDICTED: collagen alpha-3(VI) chain isoform X7 [Rattus norvegicus]	33.69769794	10	6	2.493158	3289
23	>NP_001178544.1 LIM domain and actin-binding protein 1 [Rattus norvegicus]	30.13082449	15	8	10.19866	755
24	>NP_476443.1 ADP/ATP translocase 2 [Rattus norvegicus]	27.43086524	12	8	26.17441	298
25	>NP_112624.1 cadherin-1 precursor [Rattus norvegicus]	27.0492134	4	3	7.336335	886
26	>XP_006229092.1 PREDICTED: ras-interacting protein 1 isoform X1 [Rattus norvegicus]	26.15141672	6	3	5.520828	960
27	>sp|TRYP_PIG|(Common contaminant protein)	21.52369791	29	9	25.10812	231
28	>NP_036783.2 band 3 anion transport protein [Rattus norvegicus]	14.72848059	5	3	5.06465	928
29	>NP_001102160.1 ER membrane protein complex subunit 1 precursor [Rattus norvegicus]	12.28397748	5	3	4.833832	993
30	>NP_001017461.1 2-oxoglutarate dehydrogenase, mitochondrial precursor [Rattus norvegicus]	12.04365984	5	3	5.669594	1023
31	>NP_036792.2 elongation factor 1-alpha 2 [Rattus norvegicus]	8.160001747	2	1	2.375805	463
32	>NP_446443.1 VIP peptides preproprotein [Rattus norvegicus]	7.914221478	15	1	7.058782	170
33	>NP_001292372.1 ubiquitin-40S ribosomal protein S27a [Rattus norvegicus]	7.567819091	2	1	10.25634	156
34	>NP_542150.1 AP-2 complex subunit beta [Rattus norvegicus]	7.287381956	2	1	1.577285	951
35	>NP_001178538.1 laminin subunit alpha-5 precursor [Rattus norvegicus]	6.755143007	1	1	0.727175	3713

Figure S8. Mass spectrometry peptide analysis listing the 35 most prominent proteins identified from a crosslinked azido-biotinylated-cGMP (Azido-BIO-cGMP) sample. Na^+/K^+ATPase (NKA) has the second most abundant number of peptides identified in the sample.

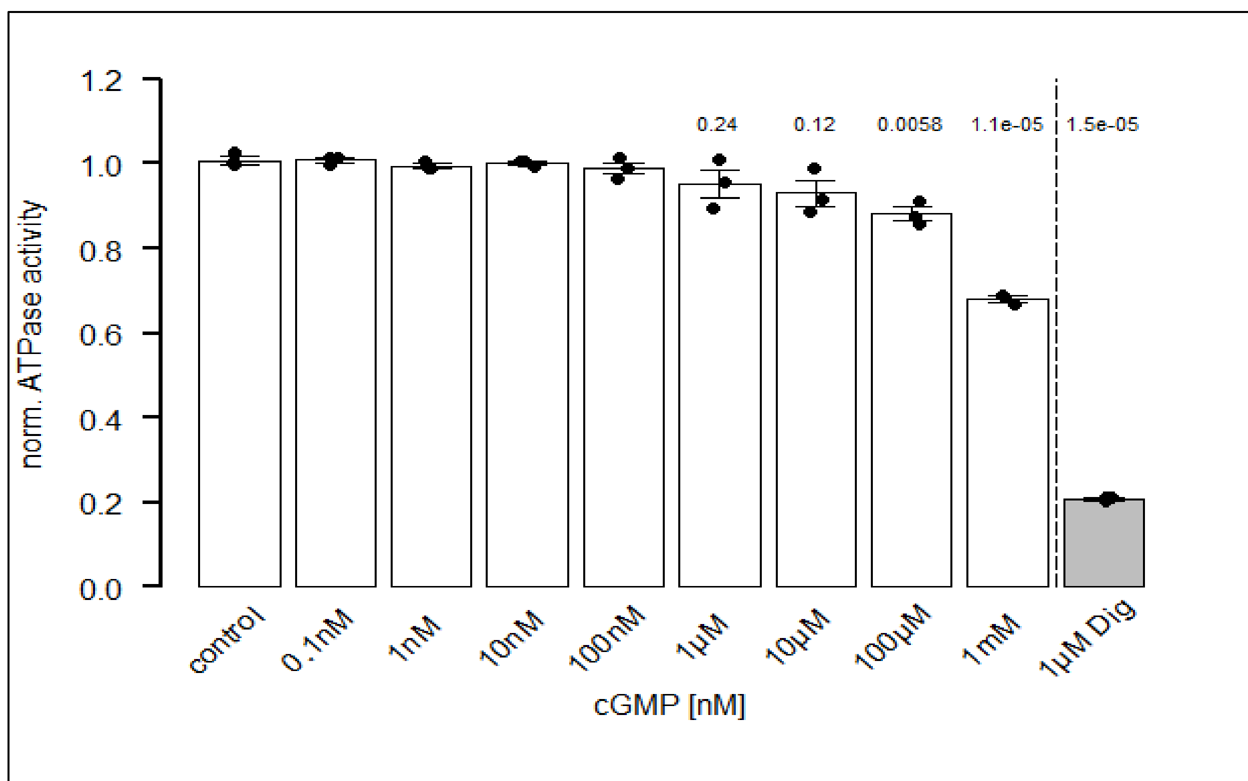

Figure S9. ATPase activity of human NKA in the presence of cGMP. ATPase activity in the presence of indicated concentrations of cGMP was normalized to an untreated control sample. Inhibition by 1 µM digoxin is provided for reference. p-values of samples with respect to the control sample (as determined using Student's *t*-tests) are indicated (N=3, data represent mean ± SE).