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ANP Differentially Modulates Marinobufagenin-Induced Sodium Pump Inhibition in Kidney and Aorta

Olga V. Fedorova, Natalia I. Agalakova, Christopher H. Morrell, Edward G. Lakatta, Alexei Y. Bagrov

Abstract—NaCl loading and plasma volume expansion stimulate 2 natriuretic systems, vasoconstrictor, digitalis-like Na/K-ATPase inhibitors and vasorelaxant ANP peptides. Several hormones, including ANP, regulate activity of the Na/K-ATPase by modulation of its phosphorylation state. We studied effects of ANP on Na/K-ATPase phosphorylation and inhibition by an endogenous sodium pump ligand, marinobufagenin, in the aorta and renal medulla from male Sprague–Dawley rats. Marinobufagenin dose-dependently inhibited the Na/K-ATPase in renal and vascular membranes at the level of higher (nanomolar) and lower affinity (micromolar) binding sites. Marinobufagenin (1 nmol/L) inhibited Na/K-ATPase in aortic sarcolemma (18%) and in renal medulla (19%). prepro-ANP 104 to 123 (ppANP) and α -human ANP ($[\alpha$ -hANP] both 1 nmol/L) potentiated marinobufagenin-induced Na/K-ATPase inhibition in the kidney, but reversed the effect of marinobufagenin in the aorta. Similarly, ppANP and α -hANP modulated the sodium pump (ouabain-sensitive ^{86}Rb uptake) inhibitory effects of marinobufagenin in the aorta and renal medulla. In renal medulla, ppANP and α -hANP induced α -1 Na/K-ATPase phosphorylation, whereas in aorta, both peptides dephosphorylated Na/K-ATPase. The effect of ppANP on Na/K-ATPase phosphorylation and inhibition was mimicked by a protein kinase G activator, 8-Br-PET-cGMP (10 $\mu\text{mol/L}$), and prevented by a protein kinase G inhibitor, KT5823 (60 nmol/L). Our results suggest that α -1 Na/K-ATPase inhibition by marinobufagenin in the kidney is enhanced via Na/K-ATPase phosphorylation by ANP, whereas in the aorta, ANP exerts an opposite effect. The concurrent production of a vasorelaxant, ANP, and a vasoconstrictor, marinobufagenin, potentiate each other's natriuretic effects, but ANP peptides may offset the deleterious vasoconstrictor effect of marinobufagenin. (*Hypertension*. 2006;48:1160-1168.)

Key Words: sodium-potassium exchanging adenosinetriphosphatase ■ natriuretic hormones ■ ANP ■ ouabain ■ marinobufagenin ■ cGMP

The search for the identity of natriuretic factors that become activated under conditions of sodium retention and that promote natriuresis via inhibition of the Na/K-ATPase (NKA) in the renal tubules evolved from the concept of a natriuretic hormone.^{1–4} According to this concept, in hypertensive patients, excessive response of natriuretic sodium pump ligands produces inhibition of the sodium pump in vascular smooth muscle sarcolemma, potentiating vasoconstriction.³ Another group of endogenous natriuretics, members of ANP family, are sensitive to NaCl loading and exhibit natriuretic properties, but, unlike digitalis-like factors, natriuretic peptides relax vascular smooth muscle.^{5,6}

An endogenous bufadienolide NKA inhibitor, marinobufagenin (MBG), is stimulated after acute^{7–9} and chronic^{10–12} Na loading, in contrast to endogenous ouabain, which does not exhibit sustained elevations during chronic high NaCl intake¹³ but, rather, becomes elevated during sodium restriction.¹⁴ MBG exhibits greater affinity to rodent ouabain-resistant α -1 isoform of the NKA, the main sodium pump isoform in the renal epithelium and major isoform in vascular sarco-

lemma,^{15,16} than to ouabain-sensitive α -3 NKA isoform.¹⁷ In vitro, at concentrations comparable to its in vivo levels, MBG acts a vasoconstrictor¹⁸ and inhibits renal NKA.⁹ Administration of anti-MBG antibody to rats with NaCl-sensitive hypertension lowers arterial pressure^{10,12} and reduces sodium excretion.^{11,19}

Two humoral natriuretic systems, NKA inhibitors and natriuretic peptides, both exist in mammals. Members of the ANP family exhibit natriuretic effects and vasorelaxation,^{6,20} whereas MBG acts as a natriuretic and a vasoconstrictor.^{10,12} Both ANP²¹ and MBG¹⁰ become elevated in NaCl-sensitive volume-expanded hypertension. Recently, in hypertensive patients with congestive heart failure, we demonstrated that plasma levels of α -human ANP (α -hANP) and that of MBG exhibit a strong positive correlation.²²

Based on the above observations, we hypothesized that simultaneous stimulation of ANP and MBG in NaCl-loaded volume-expanded states would potentiate each other's natriuretic effects, whereas ANP would offset the potentially deleterious vasoconstrictor effect of MBG. Activation of the

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guanylyl cyclase–cGMP–protein kinase G (PKG) pathway underlies both renal and vascular effects of ANP peptides.^{23–26} Because cGMP has been reported to regulate activity of the NKA by modulation of its phosphorylation state,^{27,28} we also hypothesized that ANP would affect MBG sensitivity of the NKA via modulation of its PKG-dependent phosphorylation. Previously, ANP has been shown to inhibit renal sodium pump,^{25,29,30} and both direct and indirect evidence suggest that ANP and/or cGMP can stimulate the sodium pump in the cardiovascular system.^{24,31–33} We, therefore, further hypothesized that, whereas both ANP and MBG exhibit synergistic interaction on renal α -1 NKA, ANP would antagonize MBG-induced inhibition of the NKA in vascular sarcolemma.

Previous studies have shown that prepro-ANP (ppANP) becomes stimulated in patients with congestive heart failure^{34,35} and that, *in vivo*, in humans this peptide induces a natriuretic effect.³⁵ In the present study, we compared the effects of α -hANP and ppANP (H-Ser-Ser-Asp-Arg-Ser-Ala-Leu-Leu-Lys-Ser-Lys-Leu-Arg-Ala-Leu-Leu-Thr-Ala-Pro-Arg-OH; human ppANP 104 to 123, or pro-hANF 79 to 98, or kaliuretic peptide) on MBG-induced inhibition of the NKA and the sodium pump in rat aorta and renal medulla and studied effects of natriuretic peptides on phosphorylation of the NKA in these tissues.

Methods

Protocol

The protocol was approved by the Animal Care and Use Committee of the National Institute on Aging at the National Institutes of Health. Twenty-four Sprague Dawley rats (males, 8 to 10 weeks old; Charles River Laboratories, Inc, Wilmington, DE) and time-pregnant Sprague–Dawley rats at day 21 of gestation ($n=5$; for preparation of NKA from fetal brain) were euthanized with an overdose of sodium pentobarbital (65 mg/kg, IP). Aortae and kidneys were collected and used immediately for *in vitro* measurements of Na pump activity. Both tissues and fetal brains were also used for membrane preparations.

Preparation of Vascular, Renal, and Brain Membranes

Membranes from aortae were prepared as described previously.³⁶ Aortic rings were minced by scissors, processed with Polytron 20S homogenizer (Kinematica) in 250 mmol/L of sucrose and 5 mmol/L of histidine solution (4°C; pH 7.4), and centrifuged (6000g; 15 minutes; 4°C) in a Sorvall RC-5B centrifuge (DuPont Instruments). The pellet was homogenized in a glass-Teflon homogenizer and combined with the supernatant, which was respun at 20 000g for 30 minutes at 4°C, and the resultant supernatant centrifuged (Beckman L8-N; 148 000g; 90 minutes; 4°C). The pellet was suspended in a homogenizing medium; applied to discontinuous sucrose gradients consisting of 0.32, 0.8, 1.0, 1.2 and 1.4 mol/L layers buffered with 5 mmol/L of histidine (pH 7.4); and centrifuged at 148 000g for 90 minutes (Beckman L8-N SW28; 4°C). The fraction containing sarcolemma, appearing at 0.8 mol/L, was sedimented by the centrifugation at 148 000g for 90 minutes and the pellets resuspended in a homogenizing medium to a protein concentration of 1 mg/mL and stored in liquid nitrogen (LN₂).

Renal NKA was partially purified from outer medulla as described by Jorgensen³⁷ with modifications as reported in detail.⁹ Medulla slices were homogenized in a solution, containing (in mM) sucrose 250, histidine 30, imidazole 5, and EDTA 1 (4°C; pH 7.4), then centrifuged (6000g; 15 minutes; 4°C) in a Sorvall RC-5B centrifuge (DuPont Instruments). The supernatant was respun at 15 000g for 30 minutes at 4°C and the resultant supernatant centrifuged (Beckman

L8-N, 148 000g; 90 minutes; 4°C). The pellet was suspended in a homogenizing medium, applied to discontinuous sucrose gradients, consisting of 0.32 to 1.2 mol/L of layers buffered with 30 mmol/L of histidine and 5 mmol/L of imidazole (pH 7.4), and centrifuged at 148 000g for 90 minutes (Beckman L8-N SW28; 4°C). The pellet appearing at the 0.8 mol/L fraction was aspirated, resuspended in a homogenizing medium to a protein concentration 3 to 4 mg/mL, and stored in LN₂.

NKA from fetal brain, enriched in α -3 and α -2 NKA isoforms, was prepared as described previously with minor modifications.³⁸ Brains were homogenized in a glass-Teflon homogenizer in a solution of 250 mmol/L of sucrose and 5 mmol/L of histidine (4°C; pH 7.4) and centrifuged (6000g; 15 minutes; 4°C) in a Sorvall RC-5B centrifuge (DuPont Instruments). The supernatant was centrifuged at 148 000g for 90 minutes at 4°C (Beckman L8-N; 148 000g; 90 minutes; 4°C). The resultant pellet was suspended in a homogenizing medium to a protein concentration of 2 mg/mL and stored in LN₂.

NKA activity was measured as reported previously.³⁶ To increase the permeability of membranes vesicles, membranes were pretreated with alamethicin (0.5 mg/mg of protein). Aliquots of aortic sarcolemma or renal medulla (1 μ g protein/100 μ L) were preincubated for 30 minutes at 37°C with compounds as described and then incubated for 1 hour (for aorta) or 15 minutes (for renal medulla) at 37°C in NUNC low-binding polystyrene plates in medium containing (in mM): NaCl 100, KCl 4, MgCl₂ 3, EDTA 1, Tris 50, ATP 3, and NaN₃ 5 (pH 7.4). The reaction was stopped by quenching solution containing 1.25 N H₂SO₄. NKA activity was estimated as the difference between total ATPase activity in the presence and absence of 5 mmol/L of ouabain. The NKA activity in aortic sarcolemma was 12.6 \pm 0.5 μ mol of inorganic phosphate per milligram of protein per hour and comprised 15% of the total ATPase activity. The NKA activity in renal medulla was 28.6 \pm 0.9 μ mol of inorganic phosphate per milligram of protein per hour and comprised 35% of the total ATPase activity.

NKA Phosphorylation

NKA phosphorylation was assessed as described previously.^{36,39} Membranes were pretreated with alamethicin (0.5 mg/mg of protein) and preincubated in the presence of compounds studied for 30 minutes at 32°C in a buffer containing (in mM): Tris phosphate 10, magnesium acetate 5, and CaCl₂ 0.5. Phosphorylation was initiated by the addition of [γ -³²P]ATP (1000 counts per minute/pmol) to a final concentration of 70 μ mol/L. The reaction was quenched with an equal volume of Novex Tri-Glycine SDS sample buffer. Electrophoresis was performed as described below (Western blotting). Phosphoproteins were visualized by a 12- to 24-hour exposure of nitrocellulose membranes on Kodak XAR-5 film at 4°C. The optical density of bands, corresponding to NKA (112 kDa), was quantified via laser densitometry using Bio-Rad Gel Doc 1000 software (Bio-Rad Laboratories). To verify the localization of α -1 NKA, the same nitrocellulose membranes were further exposed to the mouse monoclonal α -1 NKA antibody (Upstate Biotechnologies; 1:2000; Western blotting, below). Because basal levels of NKA phosphorylation in aortic sarcolemma were very low, phosphorylation has been enhanced by preincubation of membranes with 50 nmol/L phorbol 12,13-diacetate for 3 minutes before the addition of the studied compounds, as reported previously.^{36,40}

Western Blotting

Solubilized protein from aortic sarcolemma, renal medulla, and fetal brain as a standard (where appropriate) was separated by 12% Tris-glycine polyacrylamide gel electrophoresis and transferred to a nitrocellulose membrane.²⁶ The proteins were visualized using monoclonal mouse anti- α -1 NKA (Upstate Biotechnologies, Lake Placid, NY; 1:2000), polyclonal rabbit anti- α -2 NKA (Upstate Biotechnologies; 1:500), monoclonal mouse anti- α -3 NKA (Affinity BioReagents Inc, Golden, CO; 1:500) antibody, polyclonal rabbit anti-PKG1 (cGPK1 β) antibody (Stressgen, Victoria, British Columbia, Canada; 1:2000), or polyclonal goat anti-PKG2 (cGKII) antibody (Santa Cruz Biotechnology, Inc, Santa Cruz, CA; 1:2,500)

followed by incubation with peroxidase-conjugated anti-mouse or anti-rabbit antiserum (Amersham Corp; 1:1000) or anti-goat antiserum (Santa Cruz Biotechnology, Inc; 1:1000). NKA bands were visualized by 1- to 5-minute exposure of nitrocellulose membrane on Kodak XAR5 film.

Sodium Pump in Aortic Rings

The transport activity of sodium pump in the thoracic aorta was estimated by measurement of ouabain-sensitive ^{86}Rb uptake, as reported previously.¹² Vascular rings 2 to 2.5 mm in diameter were equilibrated for 60 minutes in 5-mL flasks in a medium containing (in mM): NaCl 120, KCl 4, CaCl_2 2.5, MgCl_2 2.0, NaH_2PO_4 1.1, NaHCO_3 24, and glucose 5.6 gassed with 95% O_2 and 5% CO_2 at 32°C (pH 7.4). Then ^{86}Rb (0.1 $\mu\text{Ci}/\text{sample}$; NEN Life Science Products) was added, and the vessels were incubated in the presence and in the absence of 2 mmol/L of ouabain for 60 minutes at 37°C. The aortic rings were then rinsed 3 times in ice-cold medium, blotted with filter paper, weighed, and counted in a gamma counter (Cherenkov radiation). Total ^{86}Rb uptake was determined on a wet weight basis, and the activity of the sodium pump was estimated as the difference between the total uptake of ^{86}Rb and the uptake in the presence of 2 mmol/L of ouabain and expressed in nanomoles of ^{86}Rb per gram tissue per minute.

Sodium Pump in Renal Medulla

The activity of the sodium pump in the suspension of outer medullary tubules, in which fragments of the thick ascending limb comprise $\approx 90\%$ of tissue mass, was estimated by measurement of ouabain-sensitive ^{86}Rb uptake measured as reported previously in detail,⁴¹ with minor modification. Slices of outer medulla were rinsed in ice-cold modified Eagle's minimal essential medium, containing (in mM) NaCl 137, KCl 4, MgSO_4 1, CaCl_2 1, Na_2HPO_4 0.33, NaH_2PO_4 0.44, NaHCO_3 4, glucose 5, essential and nonessential amino acids 4, and HEPES 15 (pH 7.4). Medullary fragments were incubated in the rinsing solution containing 0.1% collagenase (weight/volume; CLS 2; 215 U/mg; Worthington Biochemical Corp) and 0.1% BSA for 60 minutes at 37°C under aeration with 95% O_2 -5% CO_2 . The suspension of tubular fragments was obtained on ice by pouring tissue through graded filters (150 to 100 μm). After centrifugation at 100g for 3 minutes at 4°C, tubular fragments were washed with ice-cold solution, resuspended in incubation medium to 2 to 4 mg of protein per milliliter, and preincubated at 37°C for 30 minutes. Then 200 μL of tubular fragments suspension were incubated for 1 hour in oxygenated solution in the absence and presence of 5 mmol/L of ouabain. ^{86}Rb uptake was determined after the addition of 10 μL of incubation solution containing $^{86}\text{RbCl}$ (0.1 $\mu\text{Ci}/\text{sample}$; NEN Life Science Products) for 10 minutes. Tubular fragments were then washed by ice-cold medium, centrifuged, and lysed in 1% sodium deoxycholate. The radioactivity was measured by liquid scintillation. Protein was measured by Lowry method. ^{86}Rb uptake was expressed as nanomoles of ^{86}Rb per milligram of protein per minute.

Reagents

MBG (99.5% purity) was purified from parotid glands of *Bufo marinus* toads as reported previously in detail.⁴² ppANP (104 to 123) was purchased from Bachem Bioscience Inc. α -hANP was purchased from Sigma Chemicals. A stable cGMP analog, 8-Br-PET-cGMP, and a PKG inhibitor, KT5823, were purchased from BioLog Life Science.

Statistical Analyses

Data are presented as mean \pm SEM. Statistical analyses used 2-way or 1-way ANOVA followed by multiple-comparisons Bonferroni test (GraphPad Instat and GraphPad Prism, GraphPad Software Inc) and by 3-way ANOVA (SAS 9.1).

Results

Figure 1 demonstrates the abundance of α -1, α -2, and α -3 NKA isoforms in aortic sarcolemma, in renal medulla, and in

Na/K-ATPase isoforms in rat tissues

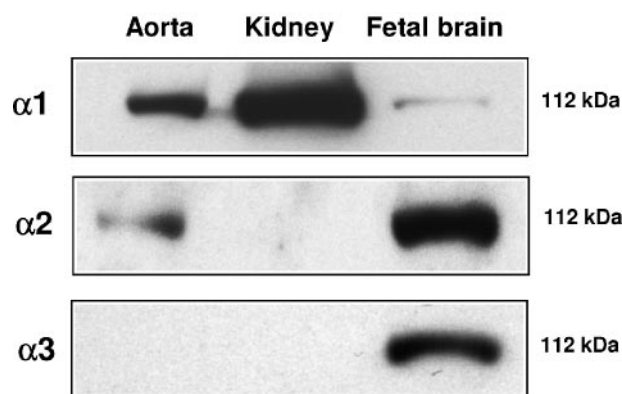


Figure 1. Western blots of the α -1, α -2, and α -3 isoforms of the NKA in aortic sarcolemma, renal medulla, and fetal brain membranes (10 μg protein per lane).

the reference membrane preparation, rat fetal brain. The α -1 NKA was abundant in the kidney and in aortic sarcolemma but was almost undetectable in fetal brain membranes. α -2 NKA was present in brain membranes, less in aortic sarcolemma, and was undetectable in the kidney. α -3 NKA was present in rat fetal brain membranes but was absent in renal medulla and in aortic sarcolemma.

Figure 2 presents concentration-response curves of MBG-induced inhibition of the sodium pump in aortic rings and in the suspension of outer medullary tubules (Figure 2A and 2B) and inhibition of the NKA in aortic sarcolemma and membranes from renal medulla (Figure 2C and 2D). The inhibitory effects of MBG were studied in the presence and the absence of a low concentration of ppANP (1 nmol/L). MBG potently, and in a concentration-dependent manner, inhibited the sodium pump and NKA from both tissues. In both tissues, inhibition occurred at the level of higher- and lower-affinity binding sites (Table). In aorta, ppANP markedly attenuated the NKA- and sodium pump-inhibitory effects of MBG at the level of high-affinity binding sites, and analysis of concentration-response curve revealed inhibition occurring at the level of low-affinity binding sites only (Table). The effects of ppANP on renal sodium pump and NKA were opposite of those in aorta, and ppANP potentiated sodium pump- and NKA-inhibitory effects of MBG. For example, whereas in the absence of ppANP 1 nmol/L MBG inhibited renal NKA by 19%, in its presence NKA became inhibited by 49%.

Using 3-way ANOVA (tissue, MBG concentration, and activity of NKA and sodium pump in the absence and in the presence of ppANP), we assessed the effect of tissue (aorta versus kidney) on the modulation of MBG-induced inhibition of the sodium pump and NKA by ppANP. The 3-way interaction term was very significant for both the sodium pump ($F=273$; $P<0.001$) and NKA ($F=74$; $P<0.001$). To determine whether the effect of ppANP on MBG is the same in aorta and kidney, a set of linear contrasts were constructed that looked at the difference in the mean activity for aorta between MBG and MBG+ppANP and tested whether this difference is the same for kidney, simultaneously for all concentrations. The tests were highly statistically significant for both

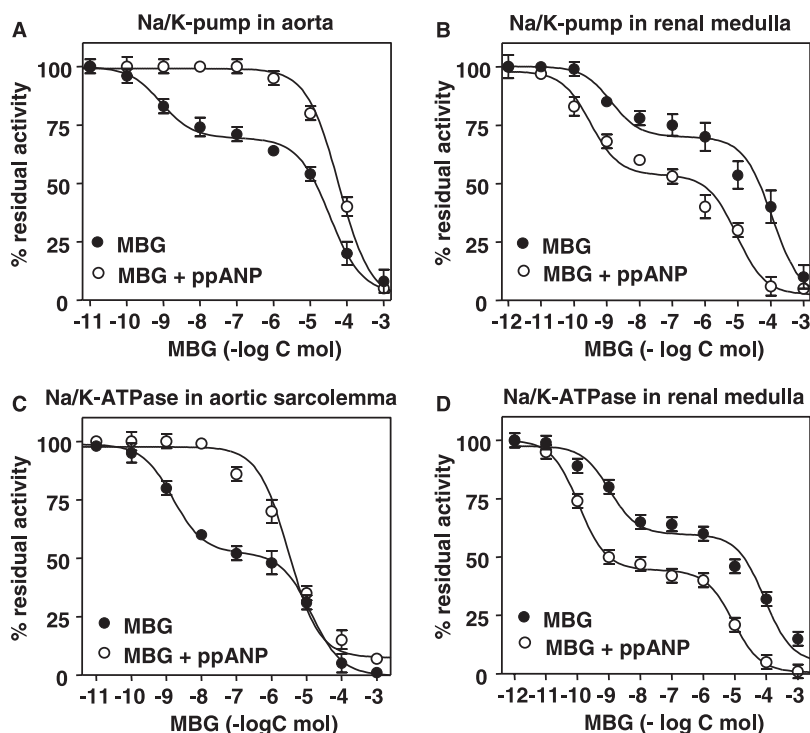


Figure 2. The sodium pump activity in aorta (A) and outer medullary tubules (B) and Na/K-ATPase activity in aortic sarcolemma (C) and renal medulla (D) after inhibition by MBG in the absence and in the presence of 1 nmol/L of ppANP. For each group, concentration-response curves were determined 5 to 8 times. These were averaged to give mean ± SEM. The mean values of IC₅₀ for the 1- and 2-site competition models are listed in the Table.

the sodium pump activity (CHISQ=892.03; PCHI<0.0001) and for NKA (CHISQ=574.08; PCHI<0.0001), indicating that ppANP differentially modifies the effect of MBG in the aorta and kidney.

Next, we studied the sodium pump inhibitory effect of 1 nmol/L MBG, a concentration that has been observed in plasma of hypertensive rats, in the presence of ppANP in the physiologically relevant concentration (1 nmol/L), a stable cGMP analog that activates PKG, 8-Br-PET-cGMP (10 μmol/L), and a PKG inhibitor, KT5823 (60 nmol/L). In aortic rings (Figure 3A), MBG alone inhibited sodium pump by 18%. A total of 1 nmol/L of ppANP did not affect aortic sodium pump activity, but in the presence of ppANP, MBG failed to induce sodium pump inhibition. Likewise, 8-Br-PET-cGMP fully antagonized MBG-induced sodium pump inhibition. A PKG

inhibitor, KT5823 alone did not affect the sodium pump activity in rat aorta, but in the presence of KT5823 and ppANP, MBG inhibited sodium pump by 42%, that is, KT5823 not only reversed the effect of ppANP but also potentiated MBG-induced sodium pump inhibition.

In outer medullary tubules (Figure 3B), 1 nmol/L of MBG alone inhibited the sodium pump by 18%. A total of 1 nmol/L of ppANP alone had no significant effect on the sodium pump activity, but it markedly potentiated the sodium pump-inhibitory effect of 1 nmol/L of MBG. The effect of a PKG activator, 8-Br-PET-cGMP, in renal medulla was more substantial than that of ppANP; in its presence, MBG inhibited sodium pump by 45%. In the presence of KT5823, ppANP and MBG together did not affect sodium pump activity.

The reference compound ouabain (1 nmol/L) alone did not significantly alter the sodium pump activity in either aortic rings or outer medulla (Figure 3A and 3B). In both tissues studied, 1 nmol/L of ppANP did not alter the effects of ouabain (data not shown).

Data on the effect of ppANP on NKA phosphorylation are summarized in Figure 4. In renal medulla, 1 nmol/L of ppANP markedly enhanced NKA phosphorylation, and this effect was further potentiated by 8-Br-PET-cGMP, whereas a PKG inhibitor, KT5823, reduced ppANP-induced phosphorylation. The effects of ppANP on phosphorylation of aortic sarcolemma NKA were opposite from those in renal medulla. In aortic sarcolemma, ppANP reduced the level of NKA phosphorylation. 8-Br-PET-cGMP, a PKG activator, reduced NKA phosphorylation and potentiated ppANP-induced NKA dephosphorylation. A PKG inhibitor, KT5823, reversed ppANP-induced dephosphorylation of the NKA from aortic sarcolemma. As demonstrated in Figure 5, PKG 1 isoform was detected in the aorta, but not the kidney, whereas PKG 2 isoform was abundant in the kidney and undetectable in aorta.

Interaction of MBG With the Sodium Pump and Na/K-ATPase From Aorta and Renal Medulla in the Absence and in the Presence of 1 nmol/L ppANP

Compounds and Tissue	High-Affinity IC ₅₀ (nM)	Low-Affinity IC ₅₀ (μM)
Sodium pump in aorta, ppANP (-)	1.6±0.4	13.9±3.8
Sodium pump in aorta, ppANP (+)	Undetectable	3.1±1.5
Sodium pump in renal medulla, ppANP (-)	1.4±1.8	23.9±6.9
Sodium pump in renal medulla, ppANP (+)	0.3±0.2	9.1±6.1
Na/K-ATPase from aorta, ppANP (-)	0.90±0.50	34±12
Na/K-ATPase from aorta, ppANP (+)	Undetectable	63±11
Renal Na/K-ATPase, ppANP (-)	1.1±4.1	20.0±2.5
Renal Na/K-ATPase, ppANP (+)	0.11±0.04	8.9±2.4

Data are means ± SEM from 5–8 inhibitory curves. For individual analysis of concentration-effect curves for MBG: nonlinear regression using 2-site or 1-site competition models.

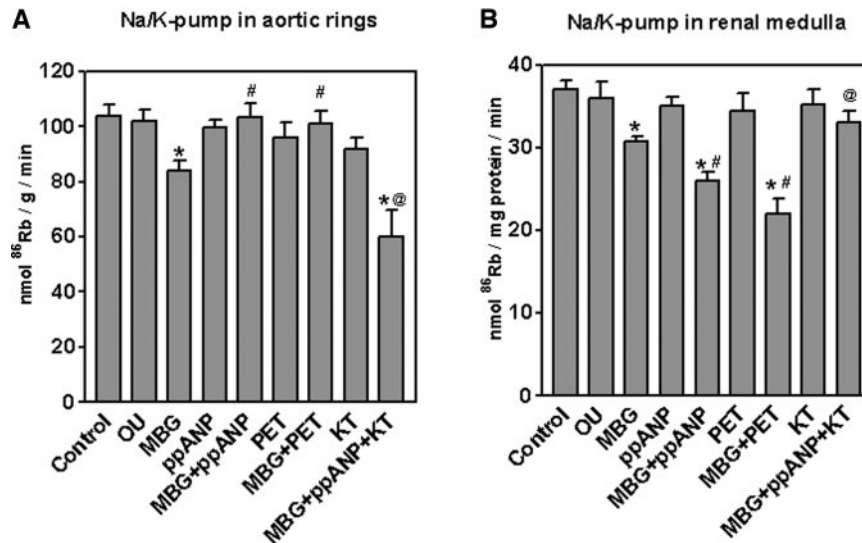


Figure 3. The sodium pump inhibition by 1 nmol/L of MBG in the absence and in the presence of 1 nmol/L of ppANP, 10 μ mol/L of 8-Br-PET-cGMP (PET), 60 nmol/L of KT5823 (KT), and their combinations in the rings of rat aorta and in outer medulla. Ouabain ([OU] 1 nmol/L) has been tested as reference compound. Each bar represents the mean \pm SEM of 6 to 12 measurements. By 1-way ANOVA followed by Bonferroni test: * P <0.01 vs control; # P <0.01 vs effect of MBG alone; @ P <0.01 vs combined effect of MBG and ppANP. By 2-way ANOVA: Effect of MBG in renal medulla in the absence and in the presence of ppANP (MBG: P <0.001; ppANP: P <0.001; interaction: P =0.03); effect of MBG in renal medulla in the presence and in the absence of PET (MBG: P <0.001; PET: P <0.001; interaction: P =0.01); effect of MBG and ppANP together in the absence and in the presence of KT in renal medulla (MBG and ppANP: P <0.001; KT: P =0.14; interaction: P =0.01). Effect of MBG in aorta in the absence and in the presence of ppANP (MBG: P <0.01; ppANP: P =0.02; interaction: P =0.001); effect of MBG in aorta in the presence and in the absence of PET (MBG: P <0.02; PET: P =0.10; interaction: P =0.001); and effect of MBG and ppANP together in the absence and in the presence of KT in aorta (MBG and ppANP: P <0.001; KT: P <0.005; interaction: P =0.006).

The main observations obtained with ppANP were confirmed using α -hANP at a concentration of 1 nmol/L (Figure 6). In rat aortic rings, α -hANP alone did not affect the sodium pump activity, but reversed sodium pump inhibition induced by 1 nmol/L of MBG (Figure 6A). In aortic sarcolemma, α -hANP stimulated NKA activity by 20% and also antagonized MBG-induced NKA inhibition (Figure 6B). Similar to ppANP, α -hANP reduced the level of phosphorylation of α -1 NKA from rat aortic sarcolemma (Figure 6C). In renal medulla, 1 nmol/L of α -hANP, unlike that of ppANP, alone inhibited the sodium pump and NKA (Figure 6D and 6E). However, 1 nmol/L of α -hANP potentiated inhibition of renal NKA and sodium pump by 1 nmol/L of MBG to a lesser extent than ppANP. Figure 6F demonstrates that 1 nmol/L of α -hANP potentiated phosphorylation of the NKA from renal medulla.

Discussion

The main findings of the present study are that ANP, via a cGMP-dependent mechanism, dephosphorylates NKA from vascular sarcolemma and markedly reduces its sensitivity to an endogenous digitalis-like ligand, MBG. In renal medulla, ANP exhibits an opposite effect, that is, it induces NKA phosphorylation and sensitizes the sodium pump to the inhibitory effect of MBG. Because aortic sarcolemma and renal medulla express PKG1 and PKG2 isoforms, respectively,⁴³ we hypothesize that these 2 PKG isoforms mediate the opposing effects of ANP on NKA phosphorylation and MBG sensitivity.

Our present findings demonstrate that the inhibitory effect of low concentrations of MBG in renal NKA and sodium

pump may be markedly enhanced by ppANP and by α -hANP. Thus, 1 nmol/L of MBG, a concentration that is present in plasma in hypertensive rats and in patients with congestive heart failure,^{10,22} inhibits renal sodium pump and NKA by \approx 20%. In the presence of a low concentration of ppANP, 1 nmol/L, the NKA-inhibitory effect of MBG becomes dramatically potentiated and achieves 49% (Figure 2B). Such a degree of inhibition in vivo should substantially influence renal sodium transport. In the present study, we investigated mechanisms of modulation of MBG-induced NKA/sodium pump inhibition using ppANP at the concentration 1 nmol/L. Plasma levels of this peptide in healthy individuals comprise \approx 0.1 nmol/L.^{34,35} In patients with congestive heart failure, plasma concentrations of ppANP exceed 1 nmol/L.³⁵ Previously, in the kidney, physiologically relevant concentrations of this peptide have been shown to act via a guanylyl-cyclase/cGMP-dependent mechanism and to inhibit renal NKA.^{23,29}

Clinical data demonstrate that after administration to patients, ppANP exhibits high natriuretic activity but does not significantly affect the arterial pressure.³⁵ In humans, α -hANP lowers the blood pressure²⁰ but produces a more modest natriuretic effect as compared with that of ppANP.³⁵ This pattern is consistent with a pattern of modulation of MBG-induced inhibition of the NKA by 2 natriuretic hormones observed in the present study (Figures 2D and 6B): whereas ppANP more potently potentiated MBG-induced inhibition of NKA from renal medulla, α -hANP more potently reversed MBG-induced inhibition of NKA from aortic sarcolemma. However, in the ⁸⁶Rb transport experiments performed in aortic rings and in medullary tubular fragments, potency of ppANP and α -hANP did not differ with respect to

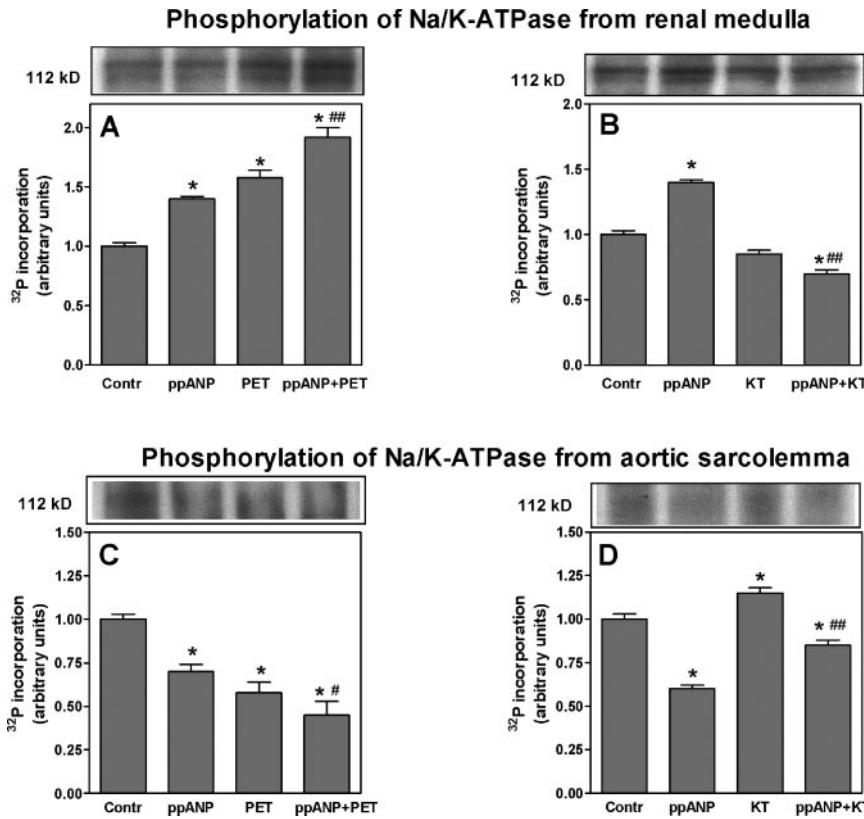


Figure 4. The effects of 1 nmol/L of ppANP, 10 μmol/L of 8-Br-PET-cGMP (PET), and 60 nmol/L of KT5823 (KT) on phosphorylation of α-1 NKA from renal medulla (A and B) and aortic sarcolemma (C and D). Top, representative autoradiograms of ³²P-labeled proteins after SDS gel electrophoresis and transfer of the samples to nitrocellulose. Bottom, optical density of the bands; each bar represents mean ± SEM from 4 to 6 measurements. By 1-way ANOVA followed by Bonferroni test: **P*<0.01 vs control; *P*<0.05, ##*P*<0.01 vs effect of ppANP. By 2-way ANOVA: Renal medulla, effect of ppANP in the absence and in the presence of PET (ppANP: *P*<0.001; PET: *P*<0.001; interaction: *P*=0.03) and effect of ppANP in the absence and in the presence of KT (ppANP: *P*<0.001; KT: *P*<0.001; interaction: *P*=0.004). Aorta, effect of ppANP in the absence and in the presence of PET (ppANP: *P*<0.001; PET: *P*<0.001; interaction: *P*=0.02) and effect of ppANP in the absence and in the presence of KT (ppANP: *P*<0.001; KT: *P*<0.001; interaction: *P*=0.02).

modulation of MBG-induced inhibition of the sodium pump (Figures 3A, 3B, 6A, and 6D). We hypothesize that biologically active compounds known to affect the NKA activity and produced by renotubular, vascular, and endothelial cells (endothelin-1, angiotensin II, and NO)¹⁶ might have interacted with the effects of natriuretic peptides. Further studies of NKA modulation by ANP are required to explain the above difference in potency.

Previous studies demonstrated the importance of regulation of renotubular NKA by protein kinases with regard to fine tuning of the mechanisms controlling renal sodium excretion.^{30,44} Stimulation of vascular NKA as a consequence of the cGMP–PKG pathway activation has been described in previous experiments.^{24,31–33} Our present results demonstrate that the effects of both ppANP and a stable cGMP analog, 8-Br-PET-cGMP, are associated with a dephosphorylation of α-1 NKA from aortic sarcolemma and with a loss of its sensitivity to an endogenous ligand, MBG. In the present experiment, a PKG inhibitor, KT5823, partially reversed ppANP-induced dephosphorylation of the NKA from aortic sarcolemma, but the level of phosphorylation remained reduced (Figure 4D). Nevertheless, the inhibitory effect of MBG on the sodium pump in aortic rings in the presence of

both ppANP and KT5823 was augmented (Figure 3A). This observation suggests that other factors, such as protein kinase A and protein phosphatases,^{25,27,30} may be involved in the regulation of NKA phosphorylation by ANP.

The mechanisms underlying the effect of PKG on the levels of NKA phosphorylation have been investigated in several previous studies. Blanco et al²⁷ have shown that α-NKA isoforms differentially respond to PKG activation: whereas cGMP inhibited activity of α1/β1 and α3/β1 isozymes, it did not affect the activity of α2/β1. Subsequently, it was demonstrated that PKG phosphorylates α-isoform of the NKA from various species.²⁸ In this study, unlike phosphorylation sites for protein kinases A and C, located in the NKA α-subunit C and A terminus, respectively, the site for PKG phosphorylation was found to be located in the central part of the α-subunit polypeptide chain. The above results²⁸ agree with the earlier data of Scavone et al,²⁵ who demonstrated that low ANP concentrations, via a cGMP-dependent mechanism, inhibit the NKA from rat renal medulla. Because this cGMP-dependent effect has been mimicked by a protein phosphatase inhibitor, okadaic acid, it has been concluded that NKA phosphorylation is a potential target for cGMP-dependent effects of ANP.²⁵ More recently, in rabbit cardiomyocytes,

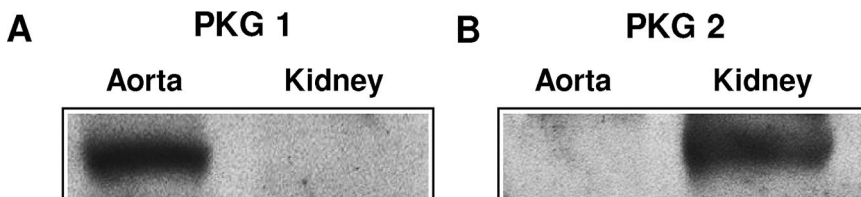


Figure 5. Western blots of PKG 1 and PKG 2 isoforms in rat renal medulla and aortic sarcolemma.

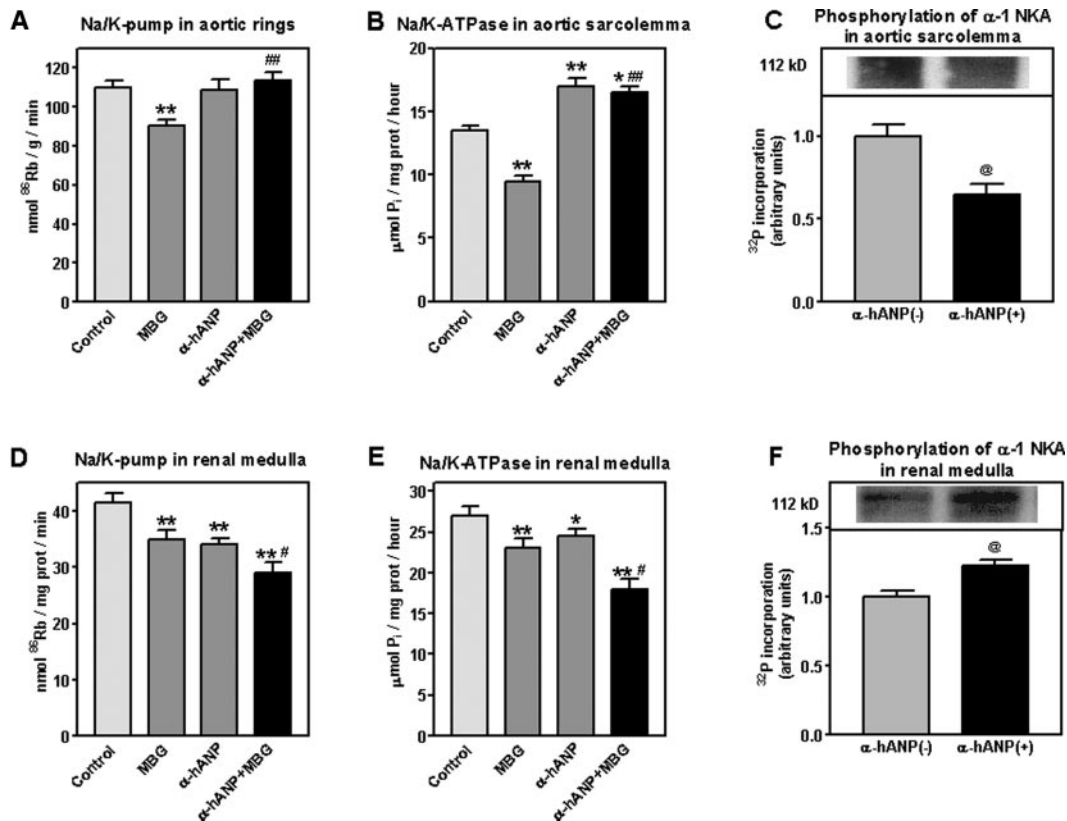


Figure 6. The sodium pump (A and D) and NKA (B and E) inhibition by 1 nmol/L MBG in the absence and in the presence of 1 nmol/L of α -hANP. Each bar represents the mean \pm SEM of 6 to 12 measurements. By 1-way ANOVA followed by Bonferroni test: * P <0.05, ** P <0.01 vs control; # P <0.05, ## P <0.01 vs effect of MBG alone. By 2-way ANOVA: Inhibition of renal sodium pump by MBG in the absence and in the presence of α -hANP (MBG: P <0.01; α -hANP: P <0.01; interaction: P =0.001). Inhibition of renal NKA by MBG in the absence and in the presence of α -hANP (MBG: P <0.01; α -hANP: P <0.01; interaction: P =0.05). Inhibition of aortic sodium pump by MBG in the absence and in the presence of α -hANP (MBG: P <0.01; α -hANP: P <0.01; interaction: P =0.09). Inhibition of the NKA from aortic sarcolemma by MBG in the absence and in the presence of α -hANP (MBG: P <0.001; α -hANP: P <0.01; interaction: P =0.004). C and F, effects of 1 nmol/L of α -hANP on the level of phosphorylation of α -1 NKA in rat aortic sarcolemma and renal medulla; By 2-tailed t test: @ P <0.01 vs α -hANP (-).

sodium nitroprusside was shown to stimulate the sodium pump, but, unlike the above results obtained by Scavone et al,²⁵ okadaic acid in this experiment abolished, rather than potentiated, cGMP-dependent sodium pump stimulation.³³ Studies of the impact of PKG isoforms on its opposing effects on the NKA are required to elucidate the mechanism of action of PKG on the sodium pump activity and phosphorylation status.

In conclusion, our results demonstrate that α -1 NKA inhibition by MBG is enhanced via NKA phosphorylation by ANP in the kidney, whereas in the aorta, ANP exerts an opposite effect. Thus, the concurrent production of a vasorelaxant ANP and a vasoconstrictor, MBG, may potentiate each other's natriuretic effects, but ANP peptides can offset the deleterious vasoconstrictor effect of MBG.

Perspectives

Our present observations may have pathophysiological implications. Under conditions of sodium loading and fluid retention, when both MBG and ANP peptides are stimulated, ANP would potentiate an MBG-induced adaptive natriuretic effect but would offset its maladaptive action, vasoconstriction. Aging, hypertension, diabetes mellitus, and congestive heart failure are all associated with heightened levels of

digitalis-like sodium pump ligands^{10,22,45,46} and with a decline in cGMP-PKG signaling in the kidney and cardiovascular tissues.^{20,21,47-49} Such a decline may shift the balance between effects of MBG and ANP on renal and cardiovascular NKA toward lesser inhibition of renal sodium pump and greater inhibition of vascular NKA and, thus, contribute to the pathogenesis of hypertension. In accord with the above notion, blockade of elevated levels of circulating MBG with a specific anti-MBG antibody produces antihypertensive effects in NaCl-loaded Dahl-S rats,¹⁰ pregnant rats with preeclampsia-like symptoms,¹² and hypertension in chronic renal failure.¹¹ All the above conditions are associated with the impairment of protective endothelial cGMP-dependent mechanisms.⁵⁰⁻⁵² Moreover, recent evidence indicates that nanomolar MBG concentrations stimulate generation of reactive oxygen species and induce hypertrophic signaling and fibrosis in cardiomyocytes.^{53,54} Therefore, ANP could also counterbalance growth-promoting effects of NKA inhibitors, in agreement with the growing evidence of tissue-protective effects of ANP peptides.⁵⁵

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Disclosures

None.

References

- Dahl LK, Knudsen KD, Iwai J. Humoral transmission of hypertension: Evidence from parabiosis. *Circ Res*. 1969;24(suppl):21–33.
- Buckalew VM Jr, Nelson DB. Natriuretic and sodium transport inhibitory activity in plasma of volume-expanded dogs. *Kidney Int*. 1974;5:12–22.
- Blaustein MP. Sodium ions, calcium ions, blood pressure regulation and hypertension: a reassessment and a hypothesis. *Am J Physiol*. 1977;232:C167–C173.
- de Wardener HE, Clarkson EM. Concept of natriuretic hormone. *Physiol Rev*. 1985;65:658–759.
- de Bold AJ, Borenstein HB, Veress AT, Sonnenberg H. A rapid and potent natriuretic response to intravenous injection of atrial myocardial extract in rats. *Life Sci*. 1981;28:89–94.
- Zeidel ML. Renal actions of atrial natriuretic peptide: regulation of collecting duct sodium and water transport. *Annu Rev Physiol*. 1990;52:747–759.
- Bagrov AY, Fedorova OV, Dmitrieva RI, French AW, Anderson DE. Plasma marinobufagenin-like and ouabain-like immunoreactivity during acute saline volume expansion in anesthetized dogs. *Cardiovas Res*. 1996;206:296–305.
- Fedorova OV, Doris PA, Bagrov AY. Endogenous marinobufagenin-like factor in acute plasma volume expansion. *Clin Exp Hypertens*. 1998;20:581–591.
- Fedorova OV, Lakatta EG, Bagrov AY. Endogenous Na,K pump ligands are differentially regulated during acute NaCl loading of Dahl rats. *Circulation*. 2000;102:3009–3014.
- Fedorova OV, Talan MI, Agalakova NI, Lakatta EG, Bagrov AY. An endogenous ligand of α -1 sodium pump, marinobufagenin, is a novel mediator of sodium chloride dependent hypertension. *Circulation*. 2002;105:1122–1127.
- Periyasamy SM, Liu J, Tanta F, Kabak B, Wakefield B, Malhotra D, Kennedy DJ, Nadoor A, Fedorova OV, Gunning W, Xie Z, Bagrov AY, Shapiro JJ. Salt loading induces redistribution of the plasmalemmal Na/K-ATPase in proximal tubule cells. *Kidney Int*. 2005;67:1868–1877.
- Fedorova OV, Kolodkin NI, Agalakova NI, Namikas AR, Bzhelyansky A, St-Louis J, Lakatta EG, Bagrov AY. Antibody to marinobufagenin lowers blood pressure in pregnant rats on a high NaCl intake. *J Hypertens*. 2005;23:835–842.
- Balzan S, Nicolini G, Iervasi A, Di Cecco P, Fommei E. Endogenous ouabain and acute salt loading in low-renin hypertension. *Am J Hypertens*. 2005;18:906–909.
- Manunta P, Messaggio E, Ballabeni C, Sciarrone MT, Lanzani C, Ferrandi M, Hamlyn JM, Cusi D, Galletti F, Bianchi G. Salt Sensitivity Study Group of the Italian Society of Hypertension. Plasma ouabain-like factor during acute and chronic changes in sodium balance in essential hypertension. *Hypertension*. 2001;38:198–203.
- Blanco G, Mercer RW. Isozymes of the Na,K-ATPase: heterogeneity in structure, diversity in function. *Am J Physiol*. 1998;275:F633–F650.
- Feraïlle E, Doucet A. Sodium-potassium-adenosinetriphosphatase-dependent sodium transport in the kidney: hormonal control. *Physiol Rev*. 2001;81:345–418.
- Fedorova OV, Bagrov AY. Inhibition of Na/K ATPase from rat aorta by 2 Na/K pump inhibitors, ouabain and marinobufagenin: evidence of interaction with different alpha-subunit isoforms. *Am J Hypertens*. 1997;10:929–935.
- Bagrov AY, Fedorova OV. Effects of 2 putative endogenous digitalis-like factors, marinobufagenin and ouabain, on the Na/K pump in human mesenteric arteries. *J Hypertens*. 1998;16:1953–1958.
- Fedorova OV, Agalakova NI, Talan MI, Lakatta EG, Bagrov AY. Brain ouabain stimulates peripheral marinobufagenin via angiotensin II signalling in NaCl loaded Dahl-S rats. *J Hypertens*. 2005;23:1515–1523.
- Florkowski CM, Richards AM, Espiner EA, Yandle TG, Frampton C. Renal, endocrine, and hemodynamic interactions of atrial and brain natriuretic peptides in normal men. *Am J Physiol*. 1994;266:R1244–R1250.
- Sterzel RB, Luft FC, Gao Y, Lang RE, Ruskoaho H, Ganten D. Atrial natriuretic factor in sodium-sensitive and sodium-resistant Dahl rats. *J Hypertens*. 1987;5:17–24.
- Fridman AI, Matveev SA, Agalakova NI, Fedorova OV, Lakatta EG, Bagrov AY. Marinobufagenin, an endogenous ligand of α -1 Na/K-ATPase, is a marker of congestive heart failure severity. *J Hypertens*. 2002;20:1189–1194.
- Vesely DL, Bayliss JM, Sallmann AL. Human prepro natriuretic factors 26–55, 56–92, and 104–123 increase renal guanylate cyclase activity. *Biochem Biophys Res Commun*. 1987;143:186–193.
- Hedge SS, DeFeo ML, Jandhyala BS. Effects of atrial natriuretic factor on cyclic GMP content in the rat aortic smooth muscle: studies on the role of membrane Na,K+ATPase. *Clin Exp Pharmacol Physiol*. 1989;16:623–629.
- Scavone C, Scanlon C, McKee M, Nathanson JA. Atrial natriuretic peptide modulates sodium and potassium activated adenosine triphosphatase through a mechanism involving cyclic GMP and cyclic GMP-dependent protein kinase. *J Pharmacol Exper Ther*. 1995;272:1036–1043.
- Rybalkin SD, Yan C, Bornfeldt KE, Beavo JA. Cyclic GMP phosphodiesterases and regulation of smooth muscle function. *Circ Res*. 2003;93:280–291.
- Blanco G, Sanchez G, Mercer RW. Differential regulation of Na,K-ATPase isozymes by protein kinases and arachidonic acid. *Arch Biochem Biophys*. 1998;359:139–150.
- Fotis H, Tatjanenko LV, Vasilets LA. Phosphorylation of the alpha-subunits of the Na,K-ATPase from mammalian kidneys and *Xenopus* oocytes by cGMP-dependent protein kinase results in stimulation of ATPase activity. *Eur J Biochem*. 1999;260:904–910.
- Chiou S, Vesely DL. Kaliuretic peptide: the most potent inhibitor of Na-K-ATPase of the atrial natriuretic peptides. *Endocrinology*. 1995;136:2033–2039.
- Aperia A, Holtback U, Syren ML, Svensson LB, Fryckstedt J, Greengard P. Activation/deactivation of renal Na(+),K(+)-ATPase: a final common pathway for regulation of natriuresis. *FASEB J*. 1994;8:436–469.
- Rapoport RM, Waldman SA, Schwartz K, Winquist RJ, Murad F. Effects of atrial natriuretic factor on cyclic GMP levels and relaxation in rat aorta. *Eur J Pharmacol*. 1985;115:219–229.
- Chen KH, Chen SJ, Wu CC. Regulation of Na+K+ATPase in rat aortas: pharmacological and functional evidence. *Chin J Physiol*. 2005;48:86–92.
- William M, Vien J, Hamilton E, Garcia A, Bungaard H, Clarke RJ, Rasmussen HH. The nitric oxide donor sodium nitroprusside stimulates the Na-K pump in isolated rabbit cardiac myocytes. *J Physiol*. 2005;565:815–825.
- Macaulay Hunter EF, Kelly PA, Prowse C, Woods RJ, Lowry PJ. Analysis of peptides derived from pro atrial natriuretic peptide that circulate in man and increase in heart disease. *Scand J Clin Lab Invest*. 1998;58:205–216.
- Nasser A, Dietz JR, Siddique M, Patel H, Khan N, Antwi EK, San Miguel GI, McCormick MT, Schocken DD, Vesely DL. Effects of kaliuretic peptide on sodium and water excretion in persons with congestive heart failure. *Am J Cardiol*. 2001;88:23–29.
- Fedorova OV, Dorofeeva NA, Lopatin DA, Lakatta EG, Bagrov AY. Phorbol diacetate potentiates Na/K-ATPase inhibition by a putative endogenous ligand, marinobufagenin. *Hypertension*. 2002;39:298–302.
- Jorgensen PL. Purification and characterization of the Na-K ATPase from outer medulla of mammalian kidney. *Biochem Biophys Acta*. 1974;336:36–52.
- Urayama O, Shutt H, Sweadner KJ. Identification of three isozyme proteins of the catalytic subunit of the Na,K-ATPase in rat brain. *J Biol Chem*. 1989;264:8271–8280.
- Feschenko MS, Sweadner KJ. Phosphorylation of Na,K-ATPase by protein kinase C at Ser18 occurs in intact cells but does not result in direct inhibition of ATP hydrolysis. *J Biol Chem*. 1997;272:17726–17733.
- Fedorova OV, Talan MI, Agalakova NI, Droy-Lefaux MT, Lakatta EG, Bagrov AY. Myocardial PKC beta2 and the sensitivity of Na/K-ATPase to marinobufagenin are reduced by cicletanine in Dahl hypertension. *Hypertension*. 2003;41:505–511.
- Kiroytcheva M, Cheval L, Carranza ML, Martin PY, Favre H, Doucet A, Feraïlle E. Effect of cAMP on the activity and the phosphorylation of

- Na⁺,K⁽⁺⁾-ATPase in rat thick ascending limb of Henle. *Kidney Int.* 1999;55:1819–1831.
42. Bagrov AY, Roukoyatkina NI, Pinaev AG, Dmitrieva RI, Fedorova OV. Effects of 2 endogenous Na,K-ATPase inhibitors, marinobufagenin and ouabain, on isolated rat aorta. *Eur J Pharmacol.* 1995;274:151–158.
 43. Smolenski A, Burkhardt AM, Eigenthaler M, Butt E, Gambaryan S, Lohmann SM, Walter U. Functional analysis of cGMP-dependent protein kinases I and II as mediators of NO/cGMP effects. *Naunyn Schmiedebergs Arch Pharmacol.* 1998;358:134–139.
 44. Aperia A. Regulation of sodium/potassium ATPase activity: impact on salt balance and vascular contractility. *Curr Hypertens Rep.* 2001;3:165–171.
 45. Takahashi H, Matsusawa M, Okabayashi H, Suga K, Ikegaki I, Nishimura M, Yoshimura M. Endogenous digitalislike substance in an adult population in Japan. *Am J Hypertens.* 1988;1:168S–172S.
 46. Bagrov YY, Manusova NB, Egorova IA, Fedorova OV, Bagrov AY. Endogenous digitalis-like ligands and Na/K-ATPase inhibition in experimental diabetes mellitus. *Front Biosci.* 2005;10:2257–2262.
 47. Scavone C, Munhoz CD, Kawamoto EM, Glezer I, de Sa Lima L, Marcourakis T, Markus RP. Age-related changes in cyclic GMP and PKG-stimulated cerebellar Na,K-ATPase activity. *Neurobiol Aging.* 2005;26:907–916.
 48. Lin KY, Ito A, Asagami T, Tsao PS, Adimoolam S, Kimoto M, Tsuji H, Reaven GM, Cooke JP. Impaired nitric oxide synthase pathway in diabetes mellitus: role of asymmetric dimethylarginine and dimethylarginine dimethylaminohydrolase. *Circulation.* 2002;106:987–992.
 49. Schrier RW, Abraham WT. Hormones and hemodynamics in heart failure. *N Engl J Med.* 1999;341:577–585.
 50. He H, Kimura S, Fujisawa Y, Tomohiro A, Kiyomoto K, Aki Y, Abe Y. Dietary L-arginine supplementation normalizes regional blood flow in Dahl-Iwai salt-sensitive rats. *Am J Hypertens.* 1997;10:89S–93S.
 51. Clark BA, Ludmir J, Epstein FH, Alvarez J, Tavera L, Bazul J, Franco A, Maradiegue E, Ludmir A. Urinary cyclic GMP, endothelin, and prostaglandin E2 in normal pregnancy and preeclampsia. *Am J Perinatol.* 1997;14:559–562.
 52. Schmidt RJ, Baylis C. Total nitric oxide production is low in patients with chronic renal disease. *Kidney Int.* 2000;58:1261–1266.
 53. Priyadarshi S, Valentine B, Han C, Fedorova OV, Bagrov AY, Liu J, Periyasamy SM, Kennedy D, Malhotra D, Xie Z, Shapiro JI. Effect of green tea extract on cardiac hypertrophy following 5/6 nephrectomy in the rat. *Kidney Int.* 2003;63:1785–1790.
 54. Kennedy DJ, Vetteth S, Sankaridrug M, Periyasamy SM, Kanj M, Fedorova L, Khouri S, Kahaleh B, Xie Z, Malhotra D, Kolodkin NI, Lakatta EG, Fedorova OV, Bagrov AY, Shapiro JI. Central role for the cardiotonic steroid, marinobufagenin, in the pathogenesis of experimental uremic cardiomyopathy. *Hypertension.* 2006;47:488–495.
 55. Rosenkranz AC, Woods RL, Dusting GJ, Ritchie RH. Antihypertrophic actions of the natriuretic peptides in adult rat cardiomyocytes: importance of cyclic GMP. *Cardiovasc Res.* 2003;57:515–522.