BIOCHEMISTRY
including biophysical chemistry & molecular biology

Article

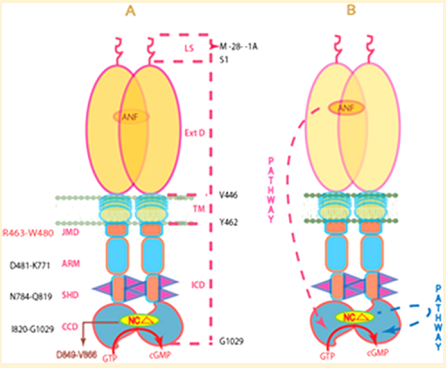
pubs.acs.org/biochemistry

Ca^{2+} Modulation of ANF-RGC: New Signaling Paradigm Interlocked with Blood Pressure Regulation

Teresa Duda,* Alexandre Pertzev, and Rameshwar K. Sharma*

Research Divisions of Biochemistry and Molecular Biology, The Unit of Regulatory and Molecular Biology, Salus University, Elkins Park, Pennsylvania 19027, United States

ABSTRACT: ANF-RGC is the prototype receptor membrane guanylate cyclase that is both the receptor and the signal transducer of the most hypotensive hormones, ANF and BNP. It is a single-transmembrane protein. After binding these hormones at the extracellular domain, ANF-RGC at its intracellular domain signals the activation of the C-terminal catalytic module and accelerates the production of the second messenger, cyclic GMP, which controls blood pressure, cardiac vasculature, and fluid secretion. At present, this is the sole transduction mechanism and the physiological function of ANF-RGC. Through comprehensive studies involving biochemistry, immunohistochemistry, and blood pressure measurements in mice with targeted gene deletions, this study demonstrates a new signaling model of ANF-RGC that also controls blood pressure. In this model, (1) ANF-RGC is not the transducer of ANF and BNP, (2) its extracellular domain is not used for signaling, and (3) the signal flow is not downstream from the extracellular domain to the core catalytic domain. Instead, the signal is the intracellular Ca^{2+}, which is translated at the site of its reception, at the core catalytic domain of ANF-RGC. A model for this Ca^{2+} signal transduction is diagrammed. It captures Ca^{2+} through its Ca^{2+} sensor myristoylated neurocalcin δ and upregulates ANF-RGC activity with a $K_{1/2}$ of 0.5 μM. The neurocalcin δ-modulated domain resides in the ^{849}DIVGFTALSAESTPMQVV^{866} segment of ANF-RGC, which is a part of the core catalytic domain. Thereby, ANF-RGC is primed to receive, transmit, and translate the Ca^{2+} signals into the generation of cyclic GMP at a rapid rate. The study defines a new paradigm of membrane guanylate cyclase signaling, which is linked to the physiology of cardiac vasculature regulation and possibly also to fluid secretion.



The discovery of ANF-RGC (atrial natriuretic factor receptor guanylate cyclase), the first member of the membrane guanylate cyclase family, was a landmark event in the field of cellular signaling.[1−9] It established a new field of membrane guanylate cyclases. With the inclusion of two other members, CNP-RGC, the receptor of C-type natriuretic peptide (CNP),[10,11] and STa-RGC, the receptor of heat stable enterotoxin, guanylin, and uroguanylin,[12,13] it demonstrated that the membrane guanylate cyclase family is a surface receptor family. The sequential developments in the field disclosed three branches of the family: (1) the original, surface receptor; (2) the Ca^{2+}-modulated ROS-GC, with two members, ROS-GC1 and ROS-GC2; and (3) the odorant (uroguanylin) surface receptor and Ca^{2+}-modulated with one member, ONE-GC.[8] ROS-GC primarily exists in the vision-linked sensory neurons. There it is a central component of phototransduction. It is also present in the olfactory bulb neurons, but its physiological linkage with olfaction has not been established.[14] ONE-GC is the olfactory receptor of the odorant uroguanylin[15,16] and, indirectly, of atmospheric CO_2.[17,18]

Common structural traits of the membrane guanylate cyclase family are that its members are single-transmembrane-spanning proteins, composed of modular blocks.[8] Functionally, they are homodimeric. In each monomeric subunit, the transmembrane module divides the protein into two roughly equal portions, extracellular and intracellular. Their core catalytic domains are conserved, all residing in the intracellular region of their respective cyclases.

A striking topographical difference in the orientation of the core catalytic domain between the subfamilies of the surface receptor and ROS-GC and ONE-GC exists. This is caused by the C-terminal extension (CTE) tails of ROS-GC and ONE-GC, which are absent in the surface receptor. Thus, in the ROS-GC and ONE-GC subfamilies, the catalytic domain flows into CTE, which is not the case with the surface receptor subfamily.

The core catalytic domain of ROS-GC and ONE-GC is modulated differently than that of surface receptor subfamily members. ROS-GC and ONE-GC sense Ca^{2+} signals via their Ca^{2+} sensor domains, which reside in the intracellular region on the N- and C-terminal sides. There are four Ca^{2+} sensors of ROS-GC1, GCAP1, GCAP2, neurocalcin δ, and S100B, each bound to its respective domain.[19] Importantly, the GCAP2 and S100B domains overlap and reside on CTE, and the GCAP1-binding domain is located N-terminally with respect to the catalytic domain. ONE-GC is modulated by three Ca^{2+} sensors: GCAP1, neurocalcin δ, and hippocalcin.[20−22] Their targeted domains reside on the core catalytic domain of ONE-GC.[20−22] Intriguingly, the GCAP1-modulated Ca^{2+} signal stimulates

Received: August 31, 2012
Revised: October 12, 2012
Published: October 22, 2012

ONE-GC activity,[20] in contrast to ROS-GC1 where it inhibits cyclase activity.

There is one prominent difference between the transduction mechanisms of ROS-GC and ONE-GC. While ROS-GC is solely modulated by the Ca^{2+} signals generated inside the sensory neurons, the ONE-GC transduction mechanism is more complex. Being a uroguanylin receptor, it generates the uroguanylin signal at its extracellular domain and via its Ca^{2+} sensors amplifies the signal at its intracellular domain.[23] Additionally, ONE-GC is also modulated in a Ca^{2+}-independent fashion. It transduces the atmospheric CO_2 signal via the carbonic anhydrase enzyme.[17] The enzyme generates bicarbonate that, in turn, binds and stimulates the ONE-GC core catalytic domain, amino acids 880−1028.[18]

Noting these mechanistic complexities and similarities, these authors proposed a unified signaling theme of the ROS-GC and ONE-GC subfamilies in which "Ca^{2+}-sensors and ROS-GC are interlocked sensory transduction elements".[19] Our study extends this theme to ANF-RGC. It discloses a new ANF-RGC transduction mechanism. In this mechanism, the receptor domain where ANF and BNP bind is not involved in signaling. In fact, the extracellular domain, the transmembrane domain, and the ATP-regulated domain are bypassed and the core catalytic domain is directly stimulated and generates cyclic GMP. The signal for the stimulation is Ca^{2+}. In mice, the disabling of the Ca^{2+} signaling mechanism through genetic modification leads to hypertension. The findings define a new signal transduction model of the membrane guanylate cyclase family and link it with blood pressure regulation.

EXPERIMENTAL PROCEDURES

Expression in COS Cells. COS cells maintained in DMEM medium supplemented with 10% fetal bovine serum and antibiotics were transfected with ANF-RGC cDNA using the calcium phosphate coprecipitation technique.[24] Sixty-four hours after being transfected, cells were washed with 50 mM Tris-HCl (pH 7.4)/10 mM Mg^{2+} buffer and homogenized, and the particulate fraction was pelleted by centrifugation.

ANF-RGC Soluble Construct of Amino Acids 788−1029. A full-length ANF-RGC cDNA in expression vector pcDNA3 was used for polymerase chain reaction (PCR) amplification. The amplified fragment encoding the ANF-RGC region of amino acids 788−1029 was cloned into the pFastBac vector (Bac-to-Bac Baculovirus expression system, Invitrogen system), yielding a six-His tag at the N-terminus. The plasmid was sequenced to confirm its identity. Using DH10Bac cells, the recombinant bacmid was generated and transfected into Sf-9 cells to produce recombinant baculoviruses. For protein expression, the suspension of Sf-9 cells was infected at a multiplicity of infection of 6−10 at a cell density of $\sim 1 \times 10^6$ cells/mL. Cells were harvested 70−80 h after infection and lysed, and the protein was purified on a Ni-NTA column and via fast-performance liquid chromatography (FPLC) on a Superdex 75 column.

Guanylate Cyclase Activity Assay. The membrane fraction was incubated in an ice bath with or without neurocalcin δ in the assay system containing 10 mM theophylline, 15 mM phosphocreatine, 20 μg of creatine kinase, and 50 mM Tris-HCl (pH 7.5). Appropriate Ca^{2+} concentrations were adjusted with precalibrated Ca^{2+}/EGTA solutions of a Ca^{2+} buffer kit (Molecular Probes/Invitrogen). The total assay volume was 25 μL. The reaction was initiated by addition of the substrate solution (4 mM $MgCl_2$ and 1 mM GTP, final concentrations) and maintained by incubation at 37 °C for 10 min. The reaction was terminated by the addition of 225 μL of 50 mM sodium acetate buffer (pH 6.2), and then the mixture was heated in a boiling water bath for 3 min. The amount of cyclic GMP formed was determined by a radioimmunoassay.[25]

Expression and Purification of Neurocalcin δ. Myristoylated neurocalcin δ was expressed and purified according to the protocol described previously.[26] Nonmyristoylated neurocalcin δ was expressed and purified following the same protocol except that the cells expressing neurocalcin δ were not cotransfected with *N*-myristoyltransferase and myristic acid was not added to the culture.

Antibodies. Antibodies against ANF-RGC and neurocalcin δ were raised in rabbits. Their specificities were described previously.[20,26] The antibodies were affinity-purified. The PDE2A antibody was purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Secondary antibodies conjugated to a fluorescent dye (DyLight 488 and DyLight 549) were purchased from Jackson ImmunoResearch Laboratories, Inc. (West Grove, PA).

Protein Quantification for the Determination of ANF-RGC Catalytic Efficiency. The antigen for the ANF-RGC antibody (ANF-RGC fragment of amino acids 486−661) was expressed and purified as described previously.[27] Its concentration was determined using purified bovine serum albumin standards. Aliquots of the antigen (from 10 to 0.1 ng) were diluted in a Laemmli sodium dodecyl sulfate sample buffer and loaded next to 50 μg (total protein) of COS cell membranes expressing ANF-RGC. After electrophoresis in a 7% sodium dodecyl sulfate−polyacrylamide gel, the proteins were transferred onto a PVDF membrane. The membrane was blocked overnight in blocking solution (2% BSA in Tris-buffered saline containing Tween 20), immunostained with an anti-ANF-RGC polyclonal antibody, and developed using a Pierce/Thermo-Fisher Scientific SuperSignal reagent kit and goat anti-rabbit peroxidase conjugates. The images were collected by exposing the membrane to Kodak X-ray film. The intensities of the signals were quantified from the calibration curve produced by the antigen standards.

Immunohistochemistry. Mice were sacrificed by lethal injection of ketamine/xylazine (the protocol approved by the Salus University IUCAC) and perfused through the heart, first with a standard Tris-buffered saline (TBS) and then with freshly prepared 4% paraformaldehyde in TBS. The adrenal glands were removed and fixed for 1−4 h in 4% paraformaldehyde with TBS at 4 °C, cryoprotected in 30% sucrose overnight at 4 °C, and cut into 20 μm sections using a Hacker-Bright OTF5000 microtome cryostat (HACKER Instruments and Industries Inc., Winnsboro, SC). The sections were washed with TBS, blocked in 10% normal donkey serum in a TBS/0.5% Triton X-100 (TBST) mixture for 1 h at room temperature, washed with TBST, incubated with the respective antibody in blocking solution overnight at 4 °C, washed with TBST, incubated with DyLight (488 or 549)-conjugated donkey anti-rabbit (or as necessary donkey anti-goat) antibody (200:1) for 1 h, and again washed with TTBS. Images were acquired using an inverted Olympus IX81 microscope/FV1000 spectral laser confocal system and analyzed using Olympus FluoView FV10-ASW. Digital images were processed using Adobe Photoshop.

Genetically Modified Mice. Care of the experimental animals conformed to the protocols approved by the IACUC at

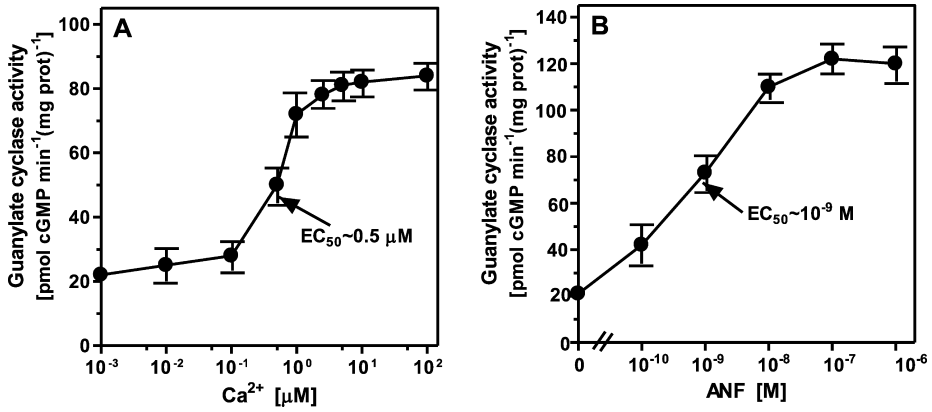


Figure 1. ANF-RGC is responsive to ANF and Ca^{2+} signals. COS cells were transfected with ANF-RGC cDNA. Sixty hours after transfection, the membrane fraction was prepared and assayed for guanylate cyclase activity in the presence of increasing concentrations of Ca^{2+} and 2 μM recombinant myristoylated neurocalcin δ (A) or the indicated concentrations of ANF and 0.8 mM ATP. Ca^{2+} or EGTA was not added to the assay mixture. (B) The experiments were conducted in triplicate and repeated two times for reproducibility. The results presented are averages ± SD from these experiments. The EC_{50} values were determined graphically.

Salus University and was in strict compliance with National Institutes of Health guidelines.

Neurocalcin $\delta^{+/-}$ (NC$\delta^{+/-}$). Two mouse genomic fragments of neurocalcin δ were amplified by PCR: first, from intron 14 to the codon for V^{12} (exon 15); second, within intron 15. The genomic distance between these two fragments was ~1000 bp. They were cloned individually into the multiple cloning sites, separated by the PGK-neo cassette of the pPNT vector. The linear DNA consisting of both neurocalcin δ fragments separated by the PGK-neo cassette was released from the vector and electroporated into mouse ES cells. The clones with homologous recombination were injected into C57BL/6 blastocytes. Male chimera was bred to C57BL/6 females. NC$\delta^{+/-}$ heterozygotes were obtained. The attempts (1 year) to breed homozygous NC$\delta^{-/-}$ mice have to date been unsuccessful.

Membrane Preparation. Adrenal glands were removed from the wild-type mice (control) or the genetically modified mice. The tissues were powdered using a pestle and mortar under liquid nitrogen, homogenized in a buffer consisting of 250 mM sucrose, 10 mM Tris-HCl (pH 7.4), and 1 mM EGTA (or 10 μM Ca^{2+}), containing a protease inhibitor cocktail, and centrifuged at 1000*g* and then at 100000*g* to pellet the membrane fraction. This fraction was suspended in 50 mM Tris-HCl (pH 7.4)/10 mM $MgCl_2$ buffer and used for the guanylate cyclase activity assay.

Blood Pressure Measurements. Systolic blood pressures of neurocalcin δ gene-targeted mice (NC$\delta^{+/-}$) and their isogenic controls (wild type, NC$\delta^{+/+}$) were measured every day for 1 week by the noninvasive computerized tail-cuff method with CODA (Kent Scientific) according to the manufacturer's protocol. An average blood pressure level of 10 sessions per day was calculated for analysis after mice had been trained for 3 days. The mice were maintained on normal chow and drinking water available *ad libitum*.

Statistical Analysis. The blood pressure data are expressed as means ± the standard deviation. Differences between the two groups analyzed, NC$\delta^{+/+}$ (wt, control) and NC$\delta^{+/-}$, were compared using a Student's *t* test. A *P* value of <0.05 was considered significant.

RESULTS

ANF-RGC Is a Bimodal Transduction Switch. ANF-RGC is the receptor and the transducer of the signals generated by the peptide hormones ANF and BNP.[9] Binding of these hormones at the ANF-RGC extracellular domain signals activation of the C-terminal catalytic module and accelerates the production of their second messenger, cyclic GMP, which controls blood pressure, cardiac vasculature, and fluid secretion. The mechanism of ANF-RGC signal transduction is one in which ligand binding to its receptor domain triggers a chain of structural changes. These changes are carried through the transmembrane domain to the intracellular domain where, by ATP-dependent changes within the ARM domain, they are processed further. Ultimately, they are sensed by the core catalytic module, which translates them into the production of cyclic GMP.[9] This is the traditional and the only established ANF-RGC signal transduction mechanism.

The myristoylated (myr) form of neurocalcin δ (NCδ) is a Ca^{2+} sensor protein of ROS-GC1 signaling.[19,26] In the course of mapping its domain on ROS-GC1 to which it binds and transmits the Ca^{2+} signal for its translation into the generation of cyclic GMP, these authors made a remarkable observation, that it binds directly to the core catalytic domain and, thereby, activates ROS-GC1.[28] A protein database search indicated sequence conservation of the catalytic domain in the membrane guanylate cyclase family. Therefore, to test whether ANF-RGC is also linked with its Ca^{2+} signaling,[20] recombinant ANF-RGC expressed in COS cells was exposed to varying concentrations of Ca^{2+} in the presence of 2 μM myr-NCδ. The results, presented in Figure 1A, show that Ca^{2+} in a dose-dependent fashion with an EC_{50} of 0.5 μM stimulated ANF-RGC activity, V_{max} occurring at ~1 μM Ca^{2+}. Thus, Ca^{2+} via its sensor NCδ signals ANF-RGC activation. As expected, a side-by-side experiment showed that ANF in the presence of ATP stimulated ANF-RGC with an EC_{50} of 1 nM (Figure 1B). These results demonstrate that ANF-RGC is a bimodal signal transduction switch: one mode transducing the ANF signal and the other transducing the Ca^{2+} signal into the production of cyclic GMP.

To determine the liaison between these two signaling modes, the membranes of COS cells expressing ANF-RGC were exposed first to 1 μM Ca^{2+} in the presence of 2 μM myr-NCδ

dx.doi.org/10.1021/bi301176c | *Biochemistry* 2012, 51, 9394−9405

and then to increasing concentrations, ranging from 10^{-11} to 10^{-6} M, of ANF and a constant ATP concentration of 0.8 mM. myr-NCδ caused 3.2-fold stimulation from 31.6 to 102 pmol of cyclic GMP min^{-1} (mg of protein)$^{-1}$ and ANF an additional 4.5-fold stimulation to 454 pmol of cyclic GMP min^{-1} (mg of protein)$^{-1}$, amounting to a total of 7.7-fold stimulation of ANF-RGC activity (Figure 2A). Thus, the results demonstrate that

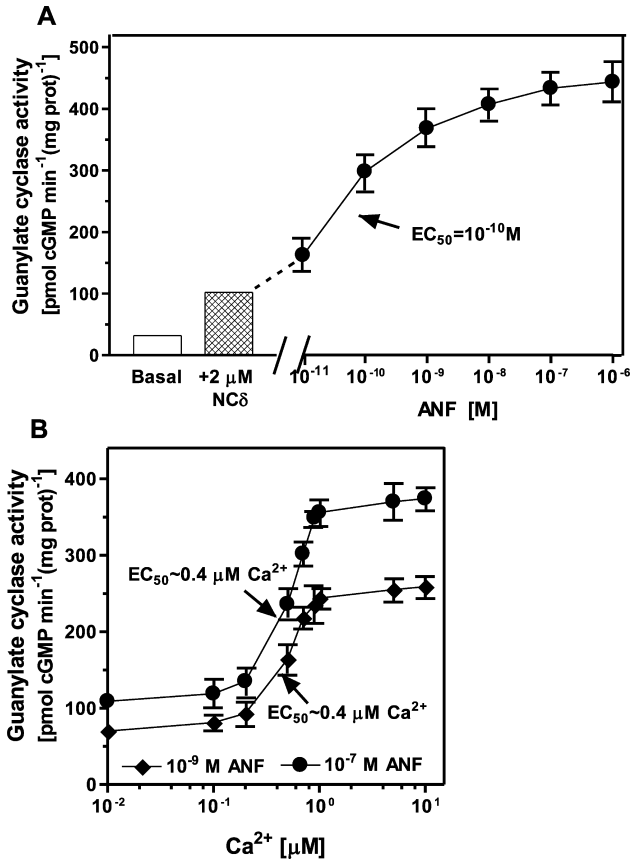


Figure 2. Stimulatory effects of ANF and Ca^{2+} on the activity of ANF-RGC are additive. (A) Membranes of COS cells expressing ANF-RGC were preincubated with 1 μM Ca^{2+} and 2 μM recombinant myristoylated neurocalcin δ (NCδ). This was followed by incubation with the indicated concentrations of ANF and 0.8 mM ATP, and the guanylate cyclase activity was assayed as described in Experimental Procedures. (B) Membranes of COS cells expressing ANF-RGC were incubated with 10^{-9} or 10^{-7} M ANF in the presence of 0.8 mM ATP, 2 μM NCδ, and increasing concentrations of Ca^{2+}. The guanylate cyclase activity was assessed. The experiments were repeated twice with different preparations of transfected cells. The results are from one experiment conducted in triplicate. The ANF EC_{50} values were determined graphically.

the effects of NCδ and ANF are additive. To further substantiate this conclusion, membranes of COS cells expressing ANF-RGC were exposed to 2 μM NCδ, 10^{-9} or 10^{-7} M ANF, 0.8 mM ATP, and increasing concentrations of Ca^{2+}, and the cyclase activity was assessed. At ~10 nM Ca^{2+}, the ANF-RGC activity was 70 ± 5 pmol of cyclic GMP min^{-1} (mg of protein)$^{-1}$ in the presence 10^{-9} M ANF and 109 ± 9 pmol of cyclic GMP min^{-1} (mg of protein)$^{-1}$ in the presence of 10^{-7} M ANF (Figure 2B). These activities are comparable to those achieved by ANF-RGC in the presence of only 10^{-9} or 10^{-7} M ANF (compare Figure 1A) and consistent with the fact that Ca^{2+}-free NCδ does not stimulate ANF-RGC (compare Figure 1B). Thus, the cyclase activity at low Ca^{2+} concentrations is regulated exclusively by ANF. Increasing the concentration of Ca^{2+} in the assay mixture resulted in an increased cyclase activity at both ANF concentrations. Because the NCδ concentration was constant, 2 μM, these results indicate that increasing the amount of Ca^{2+}-bound NCδ in the assay mixture resulted in increased ANF-RGC activity. The half-maximal ANF-RGC activation was achieved at ~0.4 μM Ca^{2+} and maximal at ~1 μM. On the basis of these results, we conclude that the two modes of ANF-RGC signaling, hormonal ANF and NCδ-mediated Ca^{2+}, are processed by individual mechanisms. Because deletion of the extracellular domain of ANF-RGC has no influence on NCδ-modulated Ca^{2+} signaling but is critical for ANF signaling,[20] we conclude that the two signaling modes are generated through different domains of ANF-RGC: ANF by the extracellular domain and Ca^{2+} by the intracellular domain.

The Myristoylated Form of NCδ Is Necessary for the Effective Ca^{2+} Signaling of ANF-RGC. NCδ belongs to the family of neuronal calcium sensor proteins (NCS). A characteristic feature of a majority, but not all, of these proteins is that they are myristoylated at their N-termini and the myristoylation is important for their cellular functions.

To determine if myristoylation is required for NCδ to transduce the Ca^{2+} signal for ANF-RGC activation, its myristoylated and nonmyristoylated forms were expressed, purified, and individually incubated in the presence of 1 μM Ca^{2+} with membranes of COS cells expressing recombinant ANF-RGC. The results are shown in Figure 3. The non-

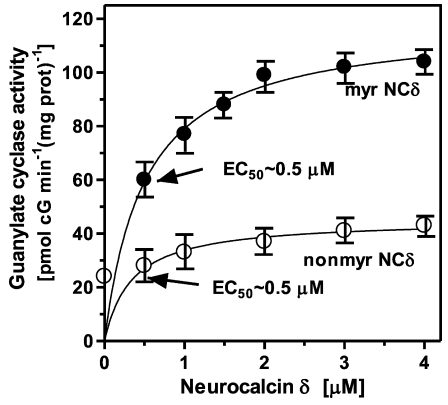


Figure 3. Myristoylated neurocalcin δ effectively transmits the Ca^{2+} signal to ANF-RGC. Membranes of COS cells expressing ANF-RGC were exposed to 1 μM Ca^{2+} and increasing concentrations of neurocalcin δ in its myristoylated (myr-NCδ) or nonmyristoylated (non-myr-NCδ) form. The experiment was conducted in triplicate and repeated four times with separate membrane or neurocalcin δ preparations. The results (means ± SD) are from one experiment.

myristoylated form caused only partial stimulation of ANF-RGC: from 24 to 43 pmol of cyclic GMP min^{-1} (mg of protein)$^{-1}$ [Figure 3 (○)]. In contrast, the myristoylated form robustly stimulated ANF-RGC: from 24 to 108 pmol of cyclic GMP min^{-1} (mg of protein)$^{-1}$ [Figure 3 (●)]. The EC_{50} values of both forms were identical, 0.5 μM, indicating their similar affinities for ANF-RGC. In the absence of Ca^{2+} (presence of 1 mM EGTA), both the myristoylated and nonmyristoylated forms caused no stimulation of ANF-RGC (not shown). The conclusion, therefore, is that the

dx.doi.org/10.1021/bi301176c | Biochemistry 2012, 51, 9394−9405

myristoylated form of NCδ is the transducer of the Ca^{2+} signal in the activation of ANF-RGC.

In addition to increasing the V_{max}, myristoylation of NCδ influences other enzymatic properties of ANF-RGC. It lowers its K_M for GTP from 0.86 to 0.37 mM and increases its enzymatic efficiency, k_{cat}, from 6.5 ± 0.3 to 41.4 ± 0.5 pmol of cyclic GMP/s.

The Core Catalytic Region of ANF-RGC, Amino Acids D^{849}–V^{866}, Binds Myristoylated NCδ. Guided by the information that the NCδ binding site on ROS-GC1, amino acids V^{837}–L^{858}, and on ONE-GC, amino acids M^{880}–L^{921}, resides on the core catalytic domain[16,28] and this domain is conserved in all members of the membrane guanylate cyclase family, we tested whether the corresponding domain in ANF-RGC is the target site of NCδ. Two approaches were used. In the first, the ANF-RGC fragment, amino acids 788–1029, encompassing the core catalytic domain, I^{820}–G^{1029}, was expressed as a soluble protein and purified to homogeneity. The expressed protein was biologically active and possessed an intrinsic guanylate cyclase activity of 18 ± 4 pmol of cyclic GMP min^{-1} (mg of protein)$^{-1}$. That the fragment is Ca^{2+} modulated via NCδ was verified by its dose-dependent response to varying concentrations of NCδ in the presence of 1 μM Ca^{2+}. myr-NCδ stimulated its guanylate cyclase activity in a dose-dependent fashion with an EC_{50} of 0.7 μM (Figure 4A), which is comparable to the value of 0.5 μM estimated for full-length ANF-RGC. These results demonstrated that the NCδ signaling site resides in this fragment, which contains the core catalytic domain of ANF-RGC.

In the second approach, the NCδ binding site was precisely mapped through peptide competition analysis. We took advantage of the knowledge that the experimentally validated sites on ROS-GC1 and ONE-GC are located within the V^{837}–L^{858} and V^{900}–L^{921} amino acid segments, respectively, and the segments have, among themselves, 100% sequence conservation. The corresponding site on ANF-RGC, ^{849}DIVGFTALSAESTPMQVVTLLMQ^{871}, has 70% sequence conservation in comparison with ROS-GC1 and ONE-GC. An ANF-RGC sequence-specific peptide, ^{849}DIVGFTALSAESTPMQVV^{866}, was synthesized and used in a functional interference experiment. A scrambled VDASAIVMFVGLPSQTET peptide was used as a control in this experiment.

COS cell membranes expressing ANF-RGC were incubated with 2 μM myr-NCδ, increasing concentrations of the peptide, and 1 μM Ca^{2+} (Figure 4B). The peptide caused almost complete inhibition of the NCδ-stimulated ANF-RGC activity at 200 μM with an IC_{50} value of 80 μM. Under the same conditions, the scrambled peptide did not exhibit any inhibitory effect. These results demonstrate that the ANF-RGC ^{849}DIVGFTALSAESTPMQVV^{866} region mediates NCδ-dependent Ca^{2+} stimulation of ANF-RGC activity. This region is a part of the core catalytic domain, and like the corresponding sites in other membrane guanylate cyclases,[28] it has a helix–loop–helix secondary structure and is acidic in nature with a pI of 3.37.

An Interactive NCδ Dimer with an ANF-RGC Dimer Constitutes the Functional Ca^{2+} Signal Transduction Unit. This concept based on the complementary biochemical and homology-based modeling studies indicates that the secondary structure of the functional form of all membrane guanylate cyclases is homodimeric.[28−30] The contact points for their homodimeric formation reside (1) in their extracellular domain[31] and in the intracellular domains of (2) the highly conserved dimerization domain[32] and (3) the catalytic core

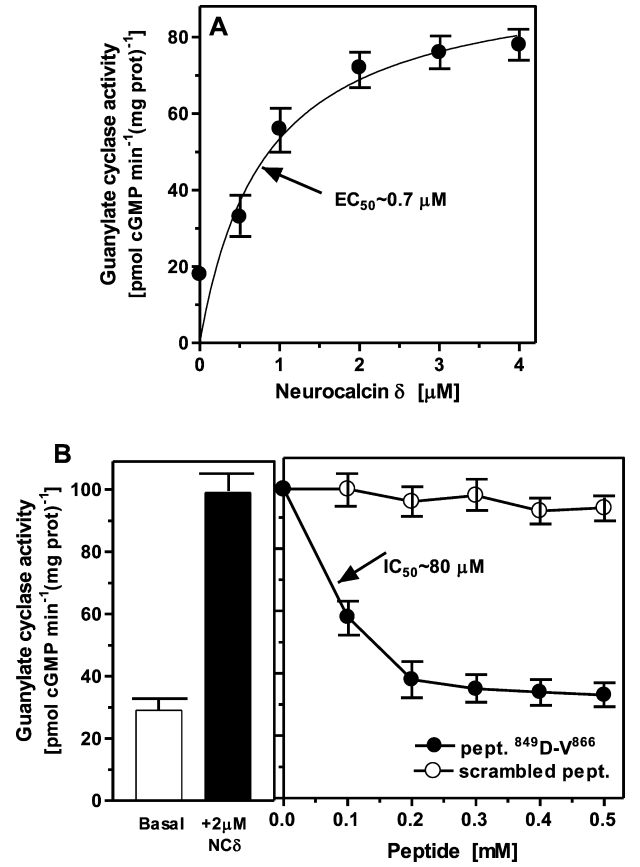


Figure 4. (A) Neurocalcin δ interacts with the catalytic domain of ANF-RGC. The ANF-RGC fragment of amino acids 788–1029 was expressed in Sf-9 cells and purified to homogeneity. The expressed protein was assayed by guanylate cyclase activity in the presence of 1 μM Ca^{2+} and the indicated concentrations of myristoylated neurocalcin δ. (B) Site of interaction of neurocalcin δ with ANF-RGC: functional interference. ANF-RGC expressed in COS cells was exposed first to 2 μM myristoylated neurocalcin δ and 1 μM Ca^{2+} and then to increasing concentrations (up to 0.5 mM) of peptide covering the ANF-RGC sequence of amino acids D^{849}–V^{866} or control scrambled peptide, which had the same D^{849}–V^{866} peptide amino acid composition but random sequence. The experiments were conducted in triplicate and repeated twice. The results are means ± SD of these experiments. The IC_{50} values were determined graphically.

domain.[28] The X-ray crystallographic studies have demonstrated that NCδ also exists as a dimer.[33] Thus, the theoretical prediction was that the Ca^{2+}-modulated functional unit is the interactive NCδ dimer and the ANF-RGC dimer.

To assess this prediction experimentally, monomeric and dimeric forms of myristoylated NCδ were separated by FPLC and were used for the reconstitution experiment with ANF-RGC. The dimeric form stimulated ANF-RGC 5-fold above the basal activity, from 24 to 120 pmol of cGMP min^{-1} (mg of protein)$^{-1}$ (Figure 5). Three separate experiments yielded calculated Hill coefficients for the stimulation of ANF-RGC of 1.08 ± 0.21, 0.91 ± 0.29, and 0.9 ± 0.23. The stimulation by the monomeric form was only marginal, from 24 to 38 pmol of cGMP min^{-1} (mg of protein)$^{-1}$ (Figure 5). These results, thus, demonstrate and validate the prediction that the functional Ca^{2+} signal transduction unit is composed of one NCδ dimer and one ANF-RGC dimer.

The Functional Ca^{2+} Signal Transduction Unit Is Present in the Mouse Adrenal Gland. To grasp the

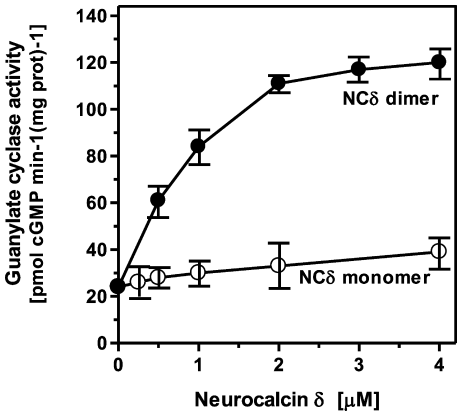


Figure 5. Dimer that is the functional entity of neurocalcin δ. Myristoylated neurocalcin δ was expressed and purified by FPLC as described in Experimental Procedures. The monomeric and dimeric fractions were collected. These were individually used to assess their stimulatory effect on ANF-RGC activity expressed in COS cells. The experiment was conducted in triplicate and repeated four times with different neurocalcin δ preparations and ANF-RGC expressions. The results shown are from one experiment.

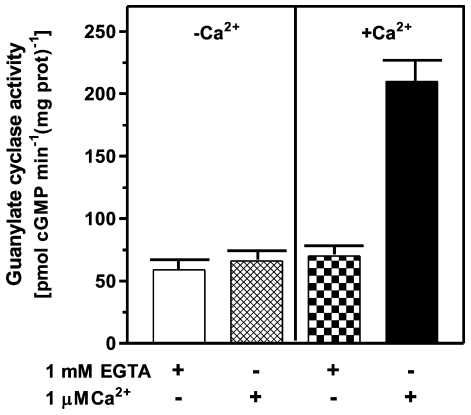


Figure 6. ANF-RGC activity in mouse adrenal gland that is Ca^{2+}-dependent. Particulate fractions of mouse adrenal gland were isolated in the presence of 1 mM EGTA ($-Ca^{2+}$ panel) or 10 μM Ca^{2+} ($+Ca^{2+}$ panel) as described in Experimental Procedures. The membranes were assayed for guanylate cyclase activity in the presence of 1 mM EGTA or 1 μM Ca^{2+}. The experiment was repeated three times with separate membrane preparations. The results are means ± SD of these experiments.

physiological relevance of the biochemical findings described above, these authors followed their preliminary study in which they observed first through Western blot analysis and then through immunohistochemistry the copresence of both elements of the functional transduction unit: NCδ and ANF-RGC in the glomerulosa cells of the mouse adrenal gland.[20]

The first question was whether the mouse adrenal gland contains Ca^{2+}-modulated membrane guanylate cyclase activity. The rationale was founded on the earlier studies, which had shown that two vital endocrine glands containing the functional ANF-RGC transduction system were adrenal and kidney glands.[7,34,35] Mouse adrenal gland was chosen for these studies. The gland was homogenized in the presence of 1 mM EGTA or 10 μM Ca^{2+}, and the particulate fractions were prepared. Each preparation was analyzed for guanylate cyclase activity in the presence of 1 mM EGTA or 1 μM Ca^{2+}. The membranes isolated in the absence of Ca^{2+} (1 mM EGTA) exhibited comparable cyclase activity regardless of the presence or absence of Ca^{2+} in the assay mixture. The activities were 59 ± 5 and 66 ± 5 pmol of cyclic GMP min^{-1} (mg of protein)$^{-1}$ in the absence and presence of Ca^{2+}, respectively (Figure 6, $-Ca^{2+}$ panel). However, the membranes isolated in the presence of Ca^{2+} exhibited Ca^{2+}-dependent activity; in the absence of Ca^{2+} from the assay mixture, it was 70 ± 5 pmol of cyclic GMP min^{-1} (mg of protein)$^{-1}$, an activity comparable to the activity in membranes isolated in the absence of Ca^{2+}, but in its presence, the activity was 210 ± 13 pmol of cyclic GMP min^{-1} (mg of protein)$^{-1}$ (Figure 6, $+Ca^{2+}$ panel). These results demonstrate that the adrenal gland houses functional Ca^{2+}-dependent ANF-RGC signal transduction machinery.

The second question was whether the Ca^{2+}-dependent ANF-RGC signal transduction machinery expressed in the mouse adrenocortical glomerulosa cells is NCδ-modulated. This problem was approached through immunocytochemistry. Because both antibodies at hand, against ANF-RGC and NCδ, were raised in rabbits, using them for co-immunostaining experiments was not possible. Hence, the goal was achieved indirectly. It has been established that the cyclic GMP-stimulated phosphodiesterase PDE2A is a specific marker for the glomerulosa cells.[36] Therefore, the copresence of ANF-RGC and PDE2A was determined first and, then, that of NCδ and PDE2A, and from them, the copresence of ANF-RGC, NCδ, and PDE2A was assessed.

The ANF-RGC panel of Figure 7A shows that the glomerulosa cells in this section exhibit an intense signal (green) generated with the ANF-RGC antibody. The PDE2A panel of Figure 7A shows the same section with the red signal generated with the PDE2A antibody. The merged image of the two signals generating yellow fluorescence shows that the immunostaining of ANF-RGC and PDE2A overlaps, demonstrating that these two proteins are copresent in the glomerulosa cells of the adrenal gland. Similarly, double staining with NCδ and PDE2A antibodies (Figure 7B) demonstrates that NCδ and PDE2A are also copresent in these cells. It is thus concluded that ANF-RGC and NCδ together with PDE2A are present in the same adrenocortical glomerulosa cells.

NCδ Is the Ca^{2+} Sensor Modulator of ANF-RGC in the Adrenocortical Glomerulosa Cells. To demonstrate that the ANF-RGC Ca^{2+} signal transduction unit present in the mouse adrenocortical glomerulosa cells is functional, two independent approaches were used. First, a mouse model with deletion of one copy of the NCδ gene, NC$\delta^{+/-}$, was constructed. It was reasoned that if NCδ is indeed the Ca^{2+} sensor modulator of ANF-RGC, in the adrenal glands of these mice the NCδ-modulated Ca^{2+} signaling pathway should be half as active as in those of wild-type mice (wt; NC$\delta^{+/+}$). To test this prediction, the particulate fractions of the adrenal glands from wild-type and NC$\delta^{+/-}$ mice were prepared in the presence and absence of Ca^{2+}, and the experiment was performed according to the protocol described earlier. Similar to the results presented in Figure 6, the guanylate cyclase activity in membranes isolated in the absence of Ca^{2+} was ~65 ± 8 pmol of cyclic GMP min^{-1} (mg of protein)$^{-1}$ for the wt and NC$\delta^{+/-}$ mice, and the activity was not affected by the presence or absence of Ca^{2+} in the assay mixture (Figure 8A). However, the cyclase activity in membranes isolated in the presence of Ca^{2+} was strongly dependent on the mice genotype and Ca^{2+} in the assay mixture. When assessed in the absence of Ca^{2+}, the activity was 70 ± 8 pmol of cyclic GMP min^{-1} (mg of protein)$^{-1}$ for the wt and

dx.doi.org/10.1021/bi301176c | Biochemistry 2012, 51, 9394−9405

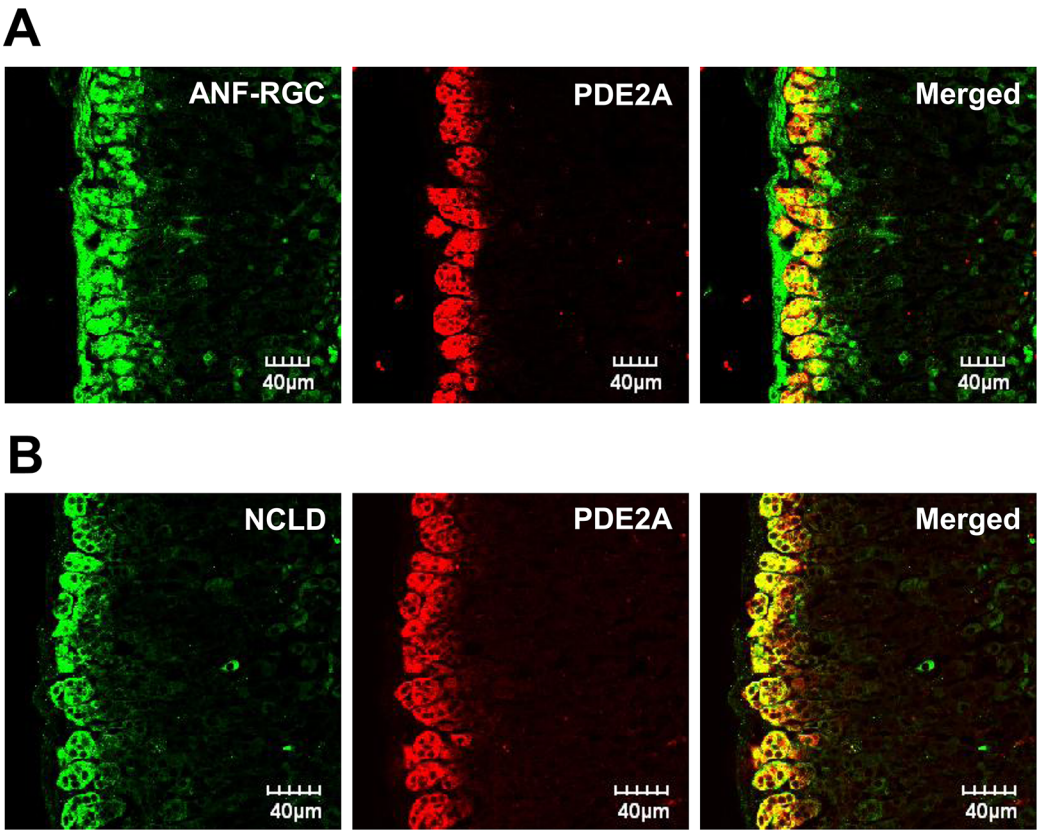


Figure 7. Neurocalcin δ is expressed in the same manner as ANF-RGC and PDE2A mouse adrenocortical zona glomerulosa cells. Cryosections of the mouse adrenal gland were immunostained with ANF-RGC and PDE2A antibodies (A) or neurocalcin δ and PDE2A antibodies (B) as described in Experimental Procedures. The right-hand panels (Merged) present the composite images of ANF-RGC and PDE2 or neurocalcin δ and PDE2 staining and document that both ANF-RGC and neurocalcin δ are coexpressed with PDE2A.

NC$\delta^{+/-}$ mice, but when assessed in the presence of 1 μM Ca^{2+}, the activity was 223 ± 20 pmol of cyclic GMP min^{-1} (mg of protein)$^{-1}$ for wt mice and 135 ± 10 pmol of cyclic GMP min^{-1} (mg of protein)$^{-1}$ for NC$\delta^{+/-}$ mice. These results, as predicted, demonstrate that the Ca^{2+}-dependent NCδ-modulated ANF-RGC signaling pathway in the mice with one copy of the NCδ gene deleted (NC$\delta^{+/-}$) is functionally half as active as in wt mice. To further validate that the decrease in the Ca^{2+}-dependent cyclase activity in the adrenal gland membranes of NC$\delta^{+/-}$ mice is the exclusive consequence of lower NCδ expression, 2 μM exogenous NCδ was added to the NC$\delta^{+/+}$ and NC$\delta^{+/-}$ membranes (isolated in the presence of Ca^{2+}) and the cyclase activity was assessed in the presence of 1 μM Ca^{2+}. The cyclase activity in the NC$\delta^{+/+}$ adrenal membranes increased only minimally, from 220 to 279 ± 21 pmol of cyclic GMP min^{-1} (mg of protein)$^{-1}$, but in the NC$\delta^{+/-}$ membranes, it increased from 133 to 284 ± 24 pmol of cyclic GMP min^{-1} (mg of protein)$^{-1}$ (Figure 8B). Thus, the activity achieved was practically the same for both types of membranes. These results demonstrate that addition of exogenous NCδ to the NC$\delta^{+/-}$ adrenal gland membranes restores the guanylate cyclase activity and brings it to the level of activity in the NC$\delta^{+/+}$ membranes. The slight activity increase in the NC$\delta^{+/+}$ membranes can be explained by a partial loss of the native NCδ during the membrane preparation procedure.

In the second approach, Ca^{2+}-dependent reconstitution of the transduction system was conducted using the heterologous system of COS cells. The cells were cotransfected with ANF-RGC and NCδ cDNAs. Their membranes were isolated in the presence and absence of Ca^{2+} and assayed for guanylate cyclase activity without and with Ca^{2+} in the assay mixture. The results are shown in Figure 9. The cyclase activity remained at the basal level in the membranes isolated without Ca^{2+} but increased 3.5-fold in membranes isolated and assayed in a buffer containing Ca^{2+}. Membranes of COS cells transfected with ANF-RGC cDNA alone exhibited a cyclase activity of 25 ± 4 pmol of cyclic GMP min^{-1} (mg of protein)$^{-1}$, and the activity was unaffected by the presence or absence of Ca^{2+} during their preparation or activity assay. Because the only difference between the cotransfected cells and those transfected with ANF-RGC cDNA only was the expression of NCδ in the cotransfected cells, these results demonstrate that in living cells NCδ exhibits Ca^{2+}-dependent association with the membranes and transmits the Ca^{2+} signal to ANF-RGC.

Together, the results of these two approaches establish that the Ca^{2+} modulation of ANF-RGC not only is an in vitro phenomenon but also occurs in vivo both in mouse adrenal gland and in the reconstituted system of transfected cells, and NCδ is the transducer of the Ca^{2+} signal.

The NCδ-Modulated Ca^{2+} Signaling ANF-RGC Transduction System Modulates Blood Pressure. The primary role of ANF-RGC in the adrenal gland is to offset the rennin–angiotensin system and inhibit aldosterone synthesis and, thus, to lower blood pressure.[37−39] It does so by responding to the two most hypotensive peptide hormones, ANF and BNP, and producing their second-messenger cyclic GMP. Cyclic GMP then elicits the natriuretic, diuretic, vasorelaxant, and antiproliferative effects programmed by the ANF and BNP

dx.doi.org/10.1021/bi301176c | *Biochemistry* 2012, 51, 9394−9405

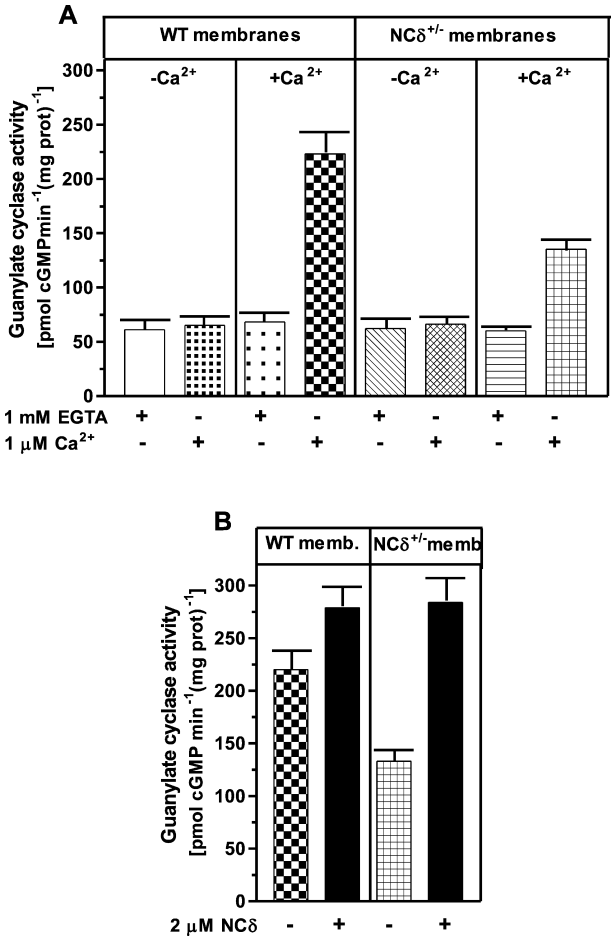


Figure 8. Neurocalcin δ modulates ANF-RGC activity in mouse adrenal glands. (A) Adrenal glands were removed from the wild-type (WT) and neurocalcin $\delta^{+/-}$ (NC$\delta^{+/-}$) mice, and their particulate fractions were isolated in the presence of 1 mM EGTA ($-Ca^{2+}$ panels in the WT membranes and NC$\delta^{+/-}$ membranes sections) or 10 μM Ca^{2+} ($+Ca^{2+}$ panels in both sections). These were assayed for guanylate cyclase activity in the presence of 1 mM EGTA or 1 μM Ca^{2+}. (B) myr-NCδ (2 μM) was added to the adrenal gland membranes isolated in the presence of 10 μM Ca^{2+} from WT (WT memb.) or NC$\delta^{+/-}$ (NC$\delta^{+/-}$ memb.) mice, and the guanylate cyclase activity was assessed in the presence of 1 μM Ca^{2+}. The experiments were repeated twice with separate membrane preparations. The results are means ± SD of these experiments.

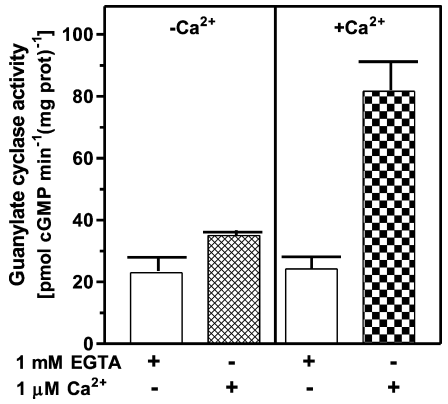


Figure 9. Ca^{2+}–neurocalcin δ modulation of ANF-RGC activity is reconstituted in cotransfected COS cells. COS cells were cotransfected with ANF-RGC and neurocalcin δ cDNAs. On the third day post-transfection, their membranes were isolated and assayed for guanylate cyclase activity as described in the legends of Figures 8 and 10. The experiment was repeated twice. The results presented (means ± SD) are from one experiment.

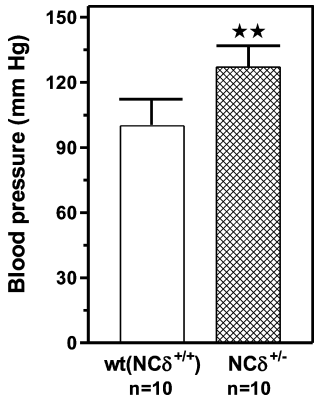


Figure 10. Systolic blood pressure in NCδ gene-targeted mice. The following mice were used: NCδ two-copy (+/+) wild-type allele (control) and NCδ one-copy (±) gene-disrupted heterozygous allele. The mice were fed a normal-salt diet. Systolic blood pressure was measured every day for 1 week by the noninvasive computerized tail-cuff method. An average blood pressure level of 10 sessions a day was calculated for analysis after 3 days of training. Bars indicate means ± SD for the representative genotypes. *n* describes the number of mice analyzed for each genotype. Stars indicate that the *P* value was <0.005.

signals. The obvious question, therefore, was whether the Ca^{2+}–neurocalcin δ–ANF-RGC transduction system is also involved in the regulation of blood pressure. To answer that question, the systolic blood pressure of the NC$\delta^{+/-}$ mice and of the isogenic control (wild type, NC$\delta^{+/+}$) was measured.

After 3 days of mock measurement sessions for training purposes, the systolic blood pressure was determined to be 92 ± 6 mmHg for the wild-type mice and 127 ± 9 mmHg for the NC$\delta^{+/-}$ mice (Figure 10). The increase was statistically highly significant ($P < 0.005$). These results clearly show that the deletion of one gene copy of neurocalcin δ leads to a significant increase in blood pressure. Thus, we conclude that the Ca^{2+}–neurocalcin δ–ANF-RGC transduction system is a physiological modulator of blood pressure.

DISCUSSION

Coinciding with time periods of observation that the hormone-dependent guanylate cyclase that is independent of the soluble form exists in the mammalian cells,[25,40,41] the discovery of the ANF was announced.[42] ANF regulated sodium excretion, water balance, and blood pressure.[43,44] A hint that there is a link between ANF actions and the particulate form of guanylate cyclase emerged with the findings that ANF stimulated membrane guanylate cyclase activities in the rat tissues.[45,46] The linkage was made firmer by the findings that ANF-RGC is the receptor of ANF and is also its signal transducer.[1−8] This marked the beginning of the membrane guanylate cyclase field and also its entry into cardiovascular research.

More than three decades of research involving ANF and ANF-RGC gene deletions and biochemical studies[8,47] has now established that abnormalities in the ANF-GC transduction pathway cause many cardiovascular complications, including

dx.doi.org/10.1021/bi301176c | Biochemistry 2012, 51, 9394−9405

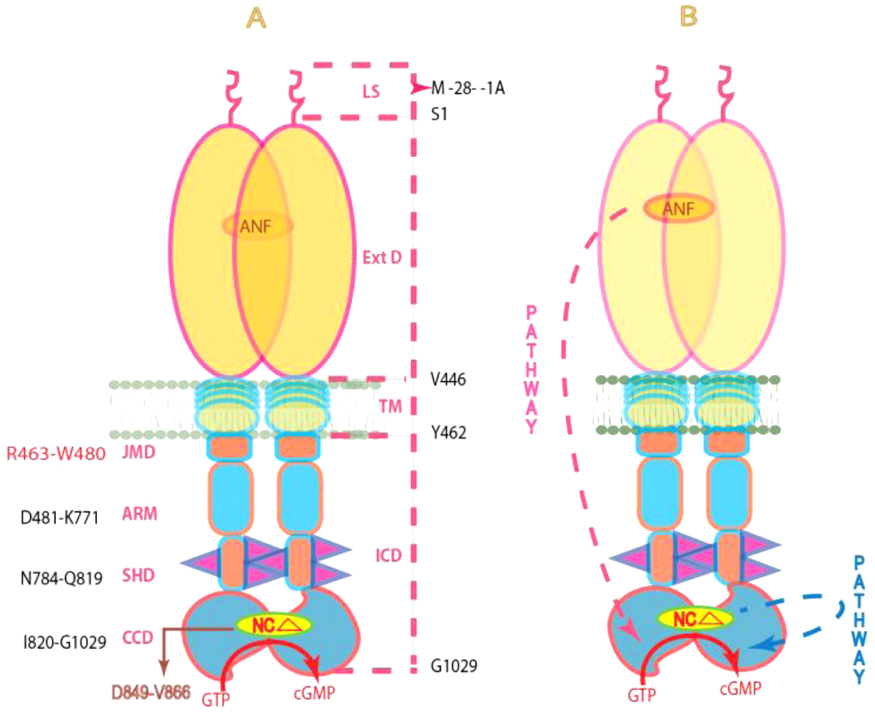


Figure 11. (A) Schematic representation of the structural topography of ANF-RGC. ANF-RGC is a single-transmembrane-spanning homodimer protein. The dashed lines at the right show the defined boundaries of its segments: LS, leader sequence; ExtD, extracellular domain; TM, transmembrane domain; ICD, intracellular domain. The functional domains housed in ICD, their designated names, and the amino acid residues constituting their boundaries are indicated at the left: JMD, juxtamembrane domain; ARM, ATP-regulated module; SHD, signaling helix domain; CCD, core catalytic domain. The site targeted by neurocalcin δ (NCΔ) (encircled) is located within CCD. (B) The signaling pathways of ANF and NCδ are independent. The trajectory of the ANF pathway is shown with a red dashed arrow. From its origin at the ExtD, it passes through the structural domains of TM, ARM, and SHD on its way to the CCD. In contrast, the trajectory of the NCδ pathway (shown as a blue dashed arrow) is within the CCD. The CCD exists as an antiparallel homodimer.

hypertension and heart failure. The ANF/ANF-RGC gene-deleted mice exhibit salt-sensitive and salt-insensitive hypertension. These defects in cardiovasculature occur because ANF-RGC does not properly transduce the ANF signal in the generation of cyclic GMP, the second messenger of ANF. Cyclic GMP is a major regulator of blood pressure. For these reasons, it is critical to understand the basic mechanism(s) by which the ANF-RGC signal transduction machinery operates. Many gaps remain, yet important strides have been made in this direction. The current, and (note) the only, model is that in which ANF-RGC signal transduction is meant to process only the hormonal signals of ANF and BNP, the signals generated in the domains in the extracellular region of ANF-RGC. These signals are then processed through an ATP-dependent two-step activation process.[48] In the model, the ANF signal originates by the binding of one molecule of ANF to the extracellular domain of ANF-RGC. The binding modifies the juxtamembrane region in which the disulfide ^{423}C−C^{432} structural motif is a key element in this modification. The signal twists the transmembrane domain, induces structural changes in the ARM domain, and prepares it for ATP activation. In step 1, binding of ATP to the ARM domain leads to a cascade of temporal and spatial changes. They involve (1) a shift in ATP binding pocket position by 3−4 Å and a rotation of its floor by 15° (G^{505} acts as a critical PIVOT for both the shift and the rotation), (2) movement by 2−7 Å but not the rotation of its β4 and β5 strands or its loop, and (3) movement of its αEF helix by 2−5 Å. This movement exposes its hydrophobic motif, ^{669}WTAPELL^{675}, which facilitates its direct (or indirect) interaction with the catalytic module, resulting in its partial, ∼50%, activation. In step 2, the six phosphorylation sites are brought from their buried state to the exposed state. Through ATP and a hypothetical protein kinase, they become phosphorylated, and the full activation (additional 50%) of ANF-RGC is achieved. Concomitantly, phosphorylation converts the ATP binding site from high to low affinity; ATP dissociates, and ANF-RGC returns to its ground state. A conspicuous feature of this model is that the trajectory of the pathway originating at the extracellular domain to the core catalytic domain, the site of signal translation into the production of cyclic GMP, is downstream (Figure 11).

Our ongoing studies provide physiological support to this model (unpublished studies). The mice lacking the ANF-RGC gene-encoded ^{669}WTAPELL^{675} motif are hypertensive. The systolic blood pressure of the ^{669}WTAPELL^{675} knockout mice is 147 ± 4 mmHg, whereas for the isogenic wt mice, it is 99 ± 9 mmHg.

This comprehensive study defines a new paradigm of ANF-RGC signal transduction. (1) ANF-RGC is the transducer of the Ca^{2+} signal. (2) This pathway is independent of ANF and BNP signal transduction and, thus, does not utilize the extracellular domain of ANF-RGC. (3) The trajectory of the pathway is not downstream. It originates and acts directly on the core catalytic domain (Figure 11), providing a novel mechanism of signal transduction like that of the sensory neuron-linked membrane guanylate cyclases: ROS-GC1 and ONE-GC. (4) Like the physiology of ANF and BNP signaling modes, the Ca^{2+} signaling mode is present in the glomerulosa cells of the adrenal cortex and importantly, like those modes,

dx.doi.org/10.1021/bi301176c | *Biochemistry* 2012, 51, 9394−9405

regulates blood pressure in mice. The absence of those modes leads to hypertension.

There are two functional and independent structural compartments of ANF-RGC. One compartment processes the signals of ANF and BNP and the other those of intracellularly generated Ca^{2+} (Figure 11). The transduction mechanisms of these two types of signals are fundamentally different, yet both modulate blood pressure; the absence of either of them causes hypertension in the genetically targeted mice.

Ca^{2+} Signal Transduction Model. On the basis of facts provided in this study, a preliminary stepwise Ca^{2+} signal transduction is envisioned. In the ground state, the dimer form of myr-NCδ is bound to a dimer of ANF-RGC. It constitutes the Ca^{2+} sensor element of ANF-RGC and binds to the ^{849}DIVGFTALSAESTPMQVV^{866} domain of ANF-RGC. The presence of the Ca^{2+} sensor element keeps the basic enzymatic efficiency of ANF-RGC at the threshold level in the production of cyclic GMP from GTP. In the activation and activated state, an increase in the Ca^{2+} concentration to the semimicromolar range is sensed by NCδ; Ca^{2+} with a $K_{1/2}$ of 0.5 μM binds myr-NCδ, which undergoes Ca^{2+}-dependent configurational changes. These, in turn, cause a structural change in the NCδ binding domain of ANF-RGC, which is also a part of the core catalytic domain. This increases the catalytic efficiency of ANF-RGC (k_{cat}) by more than 6-fold, from 6.5 to 41 pmol of cyclic GMP/s, and results in the accelerated production of cyclic GMP.

This model opens up important avenues of future cardiovascular research. We envision that in the adrenal glomerulosa cells, cyclic GMP formed through the Ca^{2+}-dependent mechanism acts as a second messenger, offsets, at least partially, the rennin−angiotensin system, inhibits aldosterone synthesis, and lowers blood pressure. Significantly, the Ca^{2+} concentration of 0.4−0.5 μM that, through NCδ, causes half-maximal activation of ANF-RGC (Figures 1A and 2B) is within the range of cytoplasmic Ca^{2+} concentrations in glomerulosa cells synthesizing aldosterone.[49] Whether, in these, the operation of the ANF-RGC−Ca^{2+}−NCδ system precedes the operation of the ANF/ANF-RGC system or is concomitant with it remains to be determined.

The model may also aid in explaining the contractile and relaxation properties of vascular smooth muscle cells (VSMC). The magic signaling molecule in these cells is Ca^{2+}. Volumes of documented evidence indicate that in VSMC the pulsated increase in the Ca^{2+} concentration causes their contraction and its decrease relaxation.[50−52] It is also well established that in these cells cyclic GMP generated through the ANF-RGC transduction system causes relaxation.[53] This study demonstrates, however, that Ca^{2+} directly through ANF-RGC can generate cyclic GMP and cause relaxation of VSMC. Thus, the intracellular increase in the Ca^{2+} concentration in VSMC can cause both their contraction and relaxation. In accordance with the presently proposed Ca^{2+} relaxation-related hypothesis, this study demonstrates that deletion of the Ca^{2+} sensor, NCδ, from the ANF-RGC transduction system causes hypertension in mice.

The Ca^{2+}- and NCδ-modulated ANF-RGC transduction pathway is a new regulator of mouse blood pressure. A future goal is to identify the source of Ca^{2+} that turns on the Ca^{2+} sensor element of the ANF-RGC transduction machinery.

AUTHOR INFORMATION

Corresponding Author

*T.D.: Research Divisions of Biochemistry and Molecular Biology, The Unit of Regulatory and Molecular Biology, Salus University, 8360 Old York Rd., Elkins Park, PA 19027; phone, (215) 780-3112; e-mail, tduda@salus.edu. R.K.S.: Research Divisions of Biochemistry and Molecular Biology, The Unit of Regulatory and Molecular Biology, Salus University, 8360 Old York Rd., Elkins Park, PA 19027; phone, (215) 780-3124; e-mail, rsharma@salus.edu.

Funding

This study was supported by National Heart, Lung and Blood Institute Grants HL084584 and S82701 and by the Pennsylvania Lions Sight Conservation and Eye Research Foundation.

Notes

The authors declare no competing financial interest.

ACKNOWLEDGMENTS

We acknowledge Guanylate Cyclase Innovative Biotechnologies (GCIB) for the support of neurocalcin $\delta^{+/-}$ mice.

ABBREVIATIONS

ANF, atrial natriuretic factor; BNP, B-type natriuretic peptide; CNP, C-type natriuretic peptide; ANF-RGC, atrial natriuretic factor receptor guanylate cyclase; ARM, ATP regulatory module; EGTA, ethylene glycol tetraacetic acid; GCAP, guanylate cyclase activating protein; ONE-GC, olfactory neuroepithelial guanylate cyclase; ROS-GC, rod outer segment guanylate cyclase; SD, standard deviation.

REFERENCES

(1) Paul, A. K. (1986) Particulate guanylate cyclase from adrenocortical carcinoma 494. Purification, biochemical and immunological characterization. Ph.D. Thesis, University of Tennessee, Knoxville, TN.

(2) Paul, A. K., Marala, R. B., Jaiswal, R. K., and Sharma, R. K. (1987) Coexistence of guanylate cyclase and atrial natriuretic factor receptor in a 180-kD protein. *Science 235*, 1224−1226.

(3) Kuno, T., Andresen, J. W., Kamisaki, Y., Waldman, S. A., Chang, L. Y., Saheki, S., Leitman, D. C., Nakane, M., and Murad, F. (1986) Co-purification of an atrial natriuretic factor receptor and particulate guanylate cyclase from rat lung. *J. Biol. Chem. 261*, 5817−5823.

(4) Meloche, S., McNicoll, N., Liu, B., Ong, H., and De Lean, A. (1988) Atrial natriuretic factor R1 receptor from bovine adrenal zona glomerulosa: Purification, characterization, and modulation by amiloride. *Biochemistry 27*, 8151−8158.

(5) Takayanagi, R., Inagami, T., Snajdar, R. M., Imada, T., Tamura, M., and Misono, K. S. (1987) Two distinct forms of receptors for atrial natriuretic factor in bovine adrenocortical cells. Purification, ligand binding, and peptide mapping. *J. Biol. Chem. 262*, 12104−12113.

(6) Chinkers, M., Garbers, D. L., Chang, M. S., Lowe, D. G., Chin, H. M., Goeddel, D. V., and Schulz, S. (1989) A membrane form of guanylate cyclase is an atrial natriuretic peptide receptor. *Nature 338*, 78−83.

(7) Duda, T., Goraczniak, R. M., and Sharma, R. K. (1991) Site-directed mutational analysis of a membrane guanylate cyclase cDNA reveals the atrial natriuretic factor signaling site. *Proc. Natl. Acad. Sci. U.S.A. 88*, 7882−7886.

(8) Sharma, R. K. (2010) Membrane guanylate cyclase is a beautiful signal transduction machine: Overview. *Mol. Cell. Biochem. 334*, 3−36.

(9) Duda, T. (2010) Atrial natriuretic factor-receptor guanylate cyclase signal transduction mechanism. *Mol. Cell. Biochem. 334*, 37−51.

(10) Schulz, S., Singh, S., Bellet, R. A., Singh, G., Tubb, D. J., Chin, H., and Garbers, D. L. (1989) The primary structure of a plasma

membrane guanylate cyclase demonstrates diversity within this new receptor family. *Cell 58*, 1155−1162.
(11) Duda, T., Goraczniak, R. M., Sitaramayya, A., and Sharma, R. K. (1993) Cloning and expression of an ATP-regulated human retina C-type natriuretic factor receptor guanylate cyclase. *Biochemistry 32*, 1391−1395.
(12) de Sauvage, F. J., Camerato, T. R., and Goeddel, D. V. (1991) Primary structure and functional expression of the human receptor for *Escherichia coli* heat-stable enterotoxin. *J. Biol. Chem. 266*, 17912−17918.
(13) Currie, M. G., Fok, K. F., Kato, J., Moore, R. J., Hamra, F. K., Duffin, K. L., and Smith, C. E. (1992) Guanylin: An endogenous activator of intestinal guanylate cyclase. *Proc. Natl. Acad. Sci. U.S.A. 89*, 947−951.
(14) Duda, T., Venkataraman, V., Krishnan, A., Nagele, R. G., and Sharma, R. K. (2001) Negatively calcium-modulated membrane guanylate cyclase signaling system in the rat olfactory bulb. *Biochemistry 40*, 4654−4662.
(15) Leinders-Zufall, T., Cockerham, R. E., Michalakis, S., Biel, M., Garbers, D. L., Reed, R. R., Zufall, F., and Munger, S. D. (2007) Contribution of the receptor guanylyl cyclase GC-D to chemosensory function in the olfactory epithelium. *Proc. Natl. Acad. Sci. U.S.A. 104*, 14507−14512.
(16) Duda, T., and Sharma, R. K. (2008) ONE-GC membrane guanylate cyclase, a trimodal odorant signal transducer. *Biochem. Biophys. Res. Commun. 367*, 440−445.
(17) Sun, L., Wang, H., Hu, J., Han, J., Matsunami, H., and Luo, M. (2009) Guanylyl cyclase-D in the olfactory CO_2 neurons is activated by bicarbonate. *Proc. Natl. Acad. Sci. U.S.A. 106*, 2041−2046.
(18) Duda, T., and Sharma, R. K. (2010) Distinct ONE-GC transduction modes and motifs of the odorants: Uroguanylin and CO_2. *Biochem. Biophys. Res. Commun. 391*, 1379−1384.
(19) Sharma, R. K., and Duda, T. (2012) Ca^{2+}-sensors and ROS-GC: Interlocked sensory transduction elements: A review. *Front. Mol. Neurosci. 5*, 42.
(20) Duda, T., Pertzev, A., Koch, K. W., and Sharma, R. K. (2012) Antithetical modes of and the Ca^{2+} sensors targeting in ANF-RGC and ROS-GC1 membrane guanylate cyclases. *Front. Mol. Neurosci. 5*, 44.
(21) Duda, T., Fik-Rymarkiewicz, E., Venkataraman, V., Krishnan, A., and Sharma, R. K. (2004) Calcium-modulated ciliary membrane guanylate cyclase transduction machinery: Constitution and operational principles. *Mol. Cell. Biochem. 267*, 107−122.
(22) Krishnan, A., Duda, T., Pertzev, A., Kobayashi, M., Takamatsu, K., and Sharma, R. K. (2009) Hippocalcin, new Ca^{2+} sensor of a ROS-GC subfamily member, ONE-GC, membrane guanylate cyclase transduction system. *Mol. Cell. Biochem. 325*, 1−14.
(23) Duda, T., and Sharma, R. K. (2009) Ca^{2+}-modulated ONE-GC odorant signal transduction. *FEBS Lett. 583*, 1327−1330.
(24) Sambrook, M. J., Fritsch, E. F., and Maniatis, T. (1989) *Molecular Cloning: A Laboratory Manual*, 2nd ed., Cold Spring Harbor Laboratory Press, Plainview, NY.
(25) Nambi, P., Aiyar, N. V., and Sharma, R. K. (1982) Adrenocorticotropin-dependent particulate guanylate cyclase in rat adrenal and adrenocortical carcinoma: Comparison of its properties with soluble guanylate cyclase and its relationship with ACTH-induced steroidogenesis. *Arch. Biochem. Biophys. 217*, 638−646.
(26) Krishnan, A., Venkataraman, V., Fik-Rymarkiewicz, E., Duda, T., and Sharma, R. K. (2004) Structural, biochemical, and functional characterization of the calcium sensor neurocalcin δ in the inner retinal neurons and its linkage with the rod outer segment membrane guanylate cyclase transduction system. *Biochemistry 43*, 2708−2723.
(27) Burczynska, B., Duda, T., and Sharma, R. K. (2007) ATP signaling site in the ARM domain of atrial natriuretic factor receptor guanylate cyclase. *Mol. Cell. Biochem. 301*, 93−107.
(28) Venkataraman, V., Duda, T., Ravichandran, S., and Sharma, R. K. (2008) Neurocalcin δ modulation of ROS-GC1, a new model of Ca^{2+} signaling. *Biochemistry 47*, 6590−6601.
(29) Rondeau, J. J., McNicoll, N., Gagnon, J., Bouchard, N., Ong, H., and De Léan, A. (1995) Stoichiometry of the atrial natriuretic factor-R1 receptor complex in the bovine zona glomerulosa. *Biochemistry 34*, 2130−2136.
(30) Liu, Y., Ruohom, A. E., Rao, V. D., and Hurley, J. H. (1997) Catalytic mechanism of the adenylyl and guanylyl cyclases: Modeling and mutational analysis. *Proc. Natl. Acad. Sci. U.S.A. 94*, 13414−13419.
(31) Misono, K. S., Philo, J. S., Arakawa, T., Ogata, C. M., Qiu, Y., Ogawa, H., and Young, H. S. (2011) Structure, signaling mechanism and regulation of the natriuretic peptide receptor guanylate cyclase. *FEBS J. 278*, 1818−1829.
(32) Wilson, E. M., and Chinkers, M. (1995) Identification of sequences mediating guanylyl cyclase dimerization. *Biochemistry 34*, 4696−4701.
(33) Vijay-Kumar, S., and Kumar, V. D. (1999) Crystal structure of recombinant bovine neurocalcin. *Nat. Struct. Biol. 6*, 80−88.
(34) Sharma, R. K., Marala, R. B., and Duda, T. (1989) Purification and characterization of the 180-kDa membrane guanylate cyclase containing atrial natriuretic factor receptor from rat adrenal gland and its regulation by protein kinase C. *Steroids 53*, 437−460.
(35) Ballermann, B. J., Marala, R. B., and Sharma, R. K. (1988) Characterization and regulation by protein kinase C of renal glomerular atrial natriuretic peptide receptor-coupled guanylate cyclase. *Biochem. Biophys. Res. Commun. 157*, 755−761.
(36) Stephenson, D. T., Coskran, T. M., Wilhelms, M. B., Adamowicz, W. O., O'Donnell, M. M., Muravnick, K. B., Menniti, F. S., Kleiman, R. J., and Morton, D. (2009) Immunohistochemical localization of phosphodiesterase 2A in multiple mammalian species. *J. Histochem. Cytochem. 57*, 933−949.
(37) Aoki, H., Richmond, M., Izumo, S., and Sadoshima, J. (2000) Specific role of the extracellular signal-regulated kinase pathway in angiotensin II-induced cardiac hypertrophy in vitro. *Biochem. J. 347*, 275−284.
(38) Burnett, J. C., Jr., Granger, J. P., and Opgenorth, T. J. (1984) Effect of synthetic atrial natriuretic factor on renal function and rennin release. *Am. J. Physiol. 247*, F863−F866.
(39) Shi, S.-J., Nguyen, H. T., Sharma, G. D., Navar, G., and Pandey, K. N. (2001) Genetic disruption of atrial natriuretic peptide receptor-A alters rennin and angiotensinII levels. *Am. J. Physiol. 281*, F665−F673.
(40) Nambi, P., and Sharma, R. K. (1981) Adrenocorticotropic hormone-responsive guanylate cyclase in the particulate fraction of rat adrenal glands. *Endocrinology 108*, 2025−2027.
(41) Sharma, R. K. (1985) Biochemical Control in Adrenocortical Carcinoma. In *Hormonally Responsive Tumors* (Hollander, V. P., Ed.) pp 185−213, Academic Press, New York.
(42) de Bold, A. J. (1982) Atrial natriuretic factor of the rat heart. Studies on isolation and properties. *Proc. Soc. Exp. Biol. Med. 170*, 133−138.
(43) Cantin, M., and Genest, J. (1985) The heart, an endocrine gland. *Ann. Endocrinol. 46*, 219−228.
(44) Atlas, S. A., and Laragh, J. H. (1986) Atrial natriuretic peptide: A new factor in hormonal control of blood pressure and electrolyte homeostasis. *Annu. Rev. Med. 37*, 397−414.
(45) Waldman, S. A., Rapoport, R. M., and Murad, F. (1984) Atrial natriuretic factor selectively activates particulate guanylate cyclase and elevates cyclic GMP in rat tissues. *J. Biol. Chem. 259*, 14332−14334.
(46) Hamet, P., Tremblay, J., Pang, S. C., Garcia, R., Thibault, G., Gutkowska, J., Cantin, M., and Genest, J. (1984) Effect of native and synthetic atrial natriuretic factor on cyclic GMP. *Biochem. Biophys. Res. Commun. 123*, 515−527.
(47) Pandey, K. N. (2011) Guanylyl cyclase/atrial natriuretic peptide receptor-A: Role in the pathophysiology of cardiovascular regulation. *Can. J. Physiol. Pharmacol. 89*, 557−573.
(48) Duda, T., Yadav, P., and Sharma, R. K. (2011) Allosteric modification, the primary ATP activation mechanism of atrial natriuretic factor receptor guanylate cyclase. *Biochemistry 50*, 1213−1225.
(49) Quinn, S. J., Williams, G. H., and Tillotson, D. L. (1988) Calcium oscillations in single adrenal glomerulosa cells stimulated by angiotensin II. *Proc. Natl. Acad. Sci. U.S.A. 85*, 5754−5758.

(50) Kamm, K. E., and Stull, J. T. (1985) The function of myosin and myosin light chain kinase phosphorylation in smooth muscle. *Annu. Rev. Pharmacol. Toxicol. 25*, 593−562.
(51) Somlyo, A. P., and Somlyo, A. V. (1994) Signal transduction and regulation in smooth muscle. *Nature 372*, 231−236.
(52) Somlyo, A. P., and Himpens, B. (1989) Cell calcium and its regulation in smooth muscle. *FASEB J. 3*, 2266−2276.
(53) Martel, G., Hamet, P., and Tremblay, J. (2010) Central role of guanylyl cyclase in natriuretic peptide signaling in hypertension and metabolic syndrome. *Mol. Cell. Biochem. 334*, 53−65.