BIOCHEMISTRY
including biophysical chemistry & molecular biology

Article

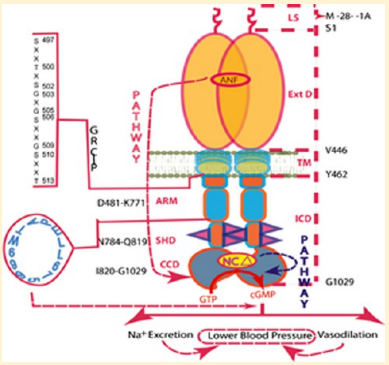
pubs.acs.org/biochemistry

The ANF-RGC Gene Motif ^{669}WTAPELL^{675} Is Vital for Blood Pressure Regulation: Biochemical Mechanism

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 membrane guanylate cyclase, both the receptor and the signal transducer of the hormones ANF and BNP. After binding them at the extracellular domain, it, at its intracellular domain, signals activation of the C-terminal catalytic module and accelerates production of the second messenger, cyclic GMP. This, in turn, controls the physiological processes of blood pressure, cardiovascular function, fluid secretion, and others: metabolic syndrome, obesity, and apoptosis. The biochemical mechanism by which this single molecule controls these diverse processes, explicitly blood pressure regulation, is the subject of this study. In line with the concept that the structural modules of ANF-RGC are designed to respond to more than one yet distinctive signals, the study demonstrates the construction of a novel ANF-RGC-In-gene-^{669}WTAPELL^{675} mouse model. Through this model, the study establishes that ^{669}WTAPELL^{675} is a vital ANF signal transducer motif of the guanylate cyclase. Its striking physiological features linked with their biochemistry are the following. (1) It controls the hormonally dependent cyclic GMP production in the kidney and the adrenal gland. Its deletion causes (2) hypertension and (3) cardiac hypertrophy. (4) These mice show higher levels of the plasma aldosterone. For the first time, a mere seven-amino acid-encoded motif of the mouse gene has been directly linked with the physiological control of blood pressure regulation, a detailed biochemistry of this linkage has been established, and a model for this linkage has been described.



Characterization of the first membrane guanylate cyclase ANF-RGC (atrial natriuretic factor receptor membrane guanylate cyclase) marked the dawn of a new era in the field of membrane guanylate cyclases.[1−3] The structural and functional uniqueness of ANF-RGC brought forth seminal principles of cellular signal transduction (reviewed in refs 4 and 5). First, it terminated the more than two-decade-long debate about its existence being independent of the soluble form. Second, unlike the three-signaling-component composition of its predecessors, adenylate cyclase and G-protein-coupled receptors, ANF-RGC was a single transduction component, being both a receptor and the signal transducer. Third, unlike the soluble form of adenylate cyclase, it was membrane-bound. Fourth, in contrast to the seven-transmembrane-spanning structure of G-proteins, it was a single-transmembrane-spanning protein. Fifth, being a physiological receptor of the two most hypotensive hormones, ANF and BNP, it was a potential regulator of the cardiovascular processes, including blood pressure, natriuresis, and fluid secretion. Indeed, through biochemical, physiological, and gene deletion studies, it is now established that the ANF-RGC signal transduction system is a central component of cardiovascular regulation. Depending upon the degree, its imbalance is a primary cause of ventricular heart failure and hypertension (reviewed in refs 6−9).

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] a three-member natriuretic peptide hormone surface receptor membrane guanylate cyclase family was established.

The sequential developments saw the addition of two additional branches to the family: Ca^{2+}-modulated, rod outer segment guanylate cyclase, ROS-GC, with two members, ROS-GC1 and ROS-GC2,[14] and the odorant (uroguanylin) surface receptor and Ca^{2+}-modulated subfamily with one member, olfactory neuroepithelial guanylate cyclase, ONE-GC.[15,16] The ROS-GC subfamily primarily exists in the vision-linked neurons; it is the central component of phototransduction (reviewed in refs 14, 17, and 18) and is also present in the mitral cells of the olfactory bulb neurons, but its physiological linkage with olfaction has not been established.[19] ONE-GC is expressed in the olfactory sensory neurons; it is the receptor of the odorant uroguanylin[15,20] and, indirectly, of atmospheric CO_2.[21,22]

The common structural trait of the family is that it represents a single-transmembrane-spanning protein. It is composed of modular blocks (reviewed in refs 4 and 5). Analyses of its members indicate that it is homodimeric. The transmembrane module in each monomeric subunit divides the protein into two roughly equal portions, extracellular and intracellular. The family contains a conserved core catalytic domain, which is a common signal translational site for the production of cyclic GMP. A striking topographical difference in the orientation of

Received: February 12, 2013
Revised: March 5, 2013
Published: March 6, 2013

   dx.doi.org/10.1021/bi400175d | *Biochemistry* 2013, 52, 2337−2347

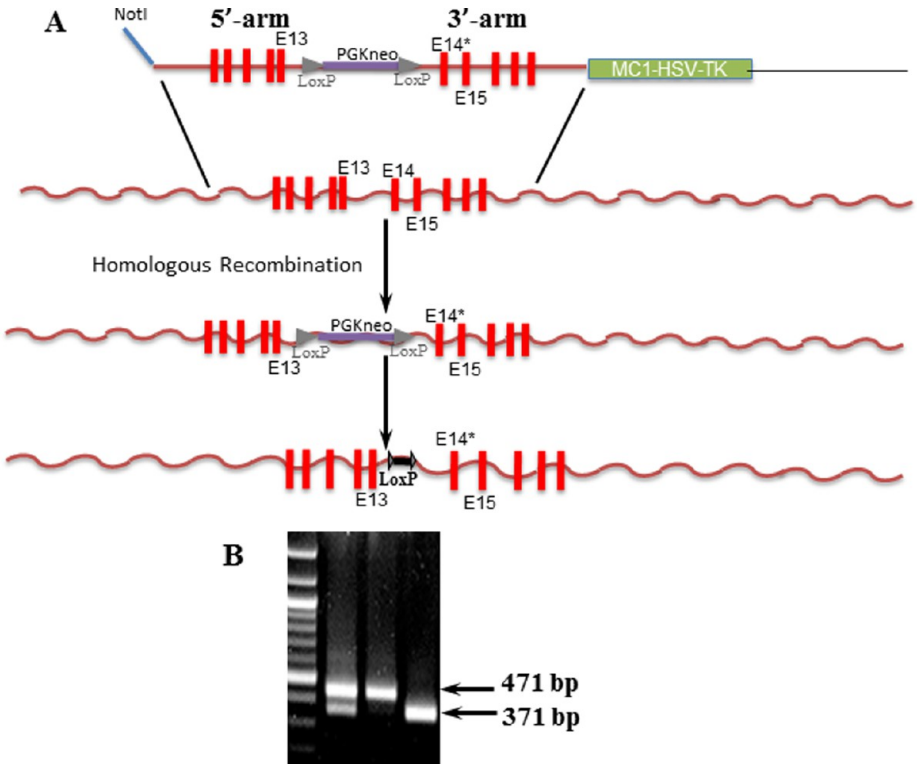


Figure 1. Schematic representation of the creation of the ANF-RGC ^{669}WTAPELL^{675} motif-targeted mice. (A) Two fragments (5′-arm, from intron 8 to 13; 3′-arm, from intron 13 to 18) of the ANF-RGC were subcloned into the multiple-cloning sites (separated by the PGK-neo cassette flanked by two LoxP sequences) of the HSV-TK vector. The ^{669}WTAPELL^{675} motif (located in exon 14 of the ANF-RGC gene) was deleted from the construct using site-directed mutagenesis. The 5′-arm-loxP-PGKneo-loxP-3′-arm fragment was excised from the mutated targeting vector and electroporated into mouse ES cells. G418- and ganciclovir (Ganc)-resistant ES cell clones were expanded and analyzed by long-range PCR using primers with sequences homologous to regions outside the arms to identify targeted ES clones. Clones that had undergone homologous recombination were processed as described in Experimental Procedures. (B) Agarose gel showing genotyping for ^{669}WTAPELL^{675} motif deletion. The retained LoxP sites are used for genotyping. Amplification of the wild-type allele yields a fragment of 371 bp and of the ^{669}WTAPELL^{675} deletion allele a fragment of 471 bp.

the core catalytic domain between the subfamily of ANF-RGC and those of ROS-GC and ONE-GC exists. This is caused by the presence of the C-terminal extension (CTE) segment in the latter two, which is absent in ANF-RGC and CNP-RGC. The CTE follows the core catalytic domain and contains selective Ca^{2+} sensor sites. In ROS-GC, the diverse Ca^{2+} signaling pathways run both downstream and upstream, yet they are translated at the common core catalytic domain.[23] In contrast, until recently, it was believed that the signaling pathways of the ANF-RGC receptor subfamily flow only downstream to be translated at the core catalytic domain.[5,23] In a surprising change of this paradigm, two recent findings of these authors demonstrate an additional model of ANF-RGC signal transduction.[24,25] In addition to being a transducer of the ANF and BNP hormonal signals, ANF-RGC is also the transducer of Ca^{2+} signals generated in the zona glomerulosa of the adrenal gland.[25] The Ca^{2+} signal is captured by its sensor myristoylated neurocalcin δ (NCδ), which is bound directly to the core catalytic domain. Thereby, the Ca^{2+} sensor element serves as a control switch of blood pressure regulation. Deletion of this switch in the mice results in hypertension.[25]

This information provides new insights into a mechanism and the function-related structure of the core catalytic module. The module, besides functioning as a central and common translational center of all the signals generated by the guanylate cyclases in the production of cyclic GMP, also functions as a regulatory center for the Ca^{2+} signal. The core catalytic module, therefore, is composed of two subtle structural compartments, one for regulating and the other for translating the Ca^{2+} signals; importantly, the translating compartment is common for both types, hormonal and Ca^{2+} signals. Thus, ANF-RGC is not only the traditional transducer of the extracellularly generated hormonal signals but also the transducer of the intracellular Ca^{2+} signal. Because the end results of these two types of signals are the same, generation of cyclic GMP, the net physiological response to both signals is the same; i.e., both contribute to the regulation of cardiovascular function in identical fashions. In support of this concept, the latest finding of the authors demonstrates that, indeed, deletion of the Ca^{2+} switch in mice causes hypertension.[25]

The ^{669}WTAPELL^{675} motif of ANF-RGC is conserved in the membrane guanylate cyclase family. In ANF-RGC, it controls the transmission of the hormonal signal to the catalytic domain.[26] In the photoreceptor ROS-GC1 guanylate cyclase, it controls the Ca^{2+}-modulated phototransduction machinery present in the sensory neurons of rods and cones.[27] Thus, in this evolving concept, the same motif is vital for the operation of two very different membrane guanylate cyclase machineries, and it performs very different functions. It programs the signaling functions according to the peculiarities and cellular location of that cyclase.

This concept is advanced in this study. In addition to its vital role in the regulation of the processes involved in blood pressure, cardiovascular function, and fluid secretion, ANF-

dx.doi.org/10.1021/bi400175d | *Biochemistry* 2013, 52, 2337−2347

RGC also is linked with other biological processes: metabolic syndrome, obesity, and apoptosis. How does this single receptor membrane guanylate cyclase control so many diverse processes? To begin to answer this question, through gene targeting, we deleted the seven-amino acid motif, ^{669}WTAPELL^{675}, from murine ANF-RGC. The mutated protein has been analyzed for its control of the physiology of blood pressure regulation; the regulation has been explained in biochemical terms, and a pictorial model for this mechanism is described.

EXPERIMENTAL PROCEDURES

ANF-RGC-In-gene-^{669}WTAPELL$^{675(-/-)}$ Mouse Model. Care of the experimental animals conformed to the protocols approved by the IACUC at Salus University and was in strict compliance with National Institutes of Health guidelines.

Construction of the Model. Two fragments (5′-arm, from intron 8 to 13; 3′-arm, from intron 13 to 18) of the ANF-RGC gene were amplified from the mouse genomic DNA. These were subcloned into the multiple cloning sites (separated by a PGK-neo cassette flanked by two LoxP sequences) of the HSV-TK vector. The ^{669}WTAPELL^{675} motif (located in exon 14 of the ANF-RGC gene) was deleted from the construct using site-directed mutagenesis. The 5′-arm-loxP-PGKneo-loxP-3′-arm fragment was excised from the mutated targeting vector and electroporated into mouse embryonic stem (ES) cells. G418- and ganciclovir (Ganc)-resistant ES cell clones were expanded and analyzed by long-range polymerase chain reaction (PCR) using primers with sequences homologous to regions outside the arms to identify targeted ES clones. Clones that had undergone homologous recombination were injected into C57BL/6 blastocytes. Male chimeras were bred with wild-type CD1 female mice to determine the germline transmission and then with Hprt-Cre mice to remove the PGK-neo cassette. Finally, the male mice were bred with C57BL/6 females to obtain ANF-RGC ^{669}WTAPELL$^{675(+/-)}$ heterozygous mutants and finally homozygous ^{669}WTAPELL$^{675(-/-)}$ mice. The targeting of the ANF-RGC ^{669}WTAPELL^{675} motif is schematically shown in Figure 1A. The retained LoxP sites are used for genotyping with the following primers: 5′-CGCAGCCTTCT-CAGATT (forward) and 5′-CAGTTTTTCTGTGGATCAG (reverse). Amplification of the wild-type allele yields a fragment of 371 bp and of the ^{669}WTAPELL^{675} deletion allele a fragment of 471 bp (Figure 1B).

With this strategy, the construction of the mouse model was contracted to the Gene Targeting and Transgenic Facility of the University of Connecticut (Storrs, CT).

Membrane Preparation. Tissues (heart, kidney, and adrenal glands) were removed from the wild-type (control), ANF-RGC ^{669}WTAPELL$^{675(+/-)}$, and ^{669}WTAPELL$^{675(-/-)}$ mice. The tissues were powdered under liquid nitrogen, homogenized in a buffer consisting of 250 mM sucrose, 10 mM Tris-HCl (pH 7.4), and protease inhibitor cocktail (Sigma), 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.

ANF-RGC ^{699}WTAPELL^{675} Deletion Mutant. This mutant was constructed as described previously.[26]

Expression in COS Cells. COS cells maintained in DMEM medium supplemented with 10% fetal bovine serum and penicillin/streptomycin antibiotics were transfected with the ANF-RGC cDNA expression construct using the calcium phosphate coprecipitation technique;[28] 64 h after transfection, 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.

Guanylate Cyclase Activity Assay. The membrane fraction was incubated on an ice bath with ANF and ATP in the assay system containing 10 mM theophylline, 15 mM phosphocreatine, 20 μg of creatine kinase, and 50 mM Tris-HCl (pH 7.5). 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) followed by heating on a boiling water bath for 3 min. The amount of cyclic GMP formed was determined by a radioimmunoassay.[29]

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 phosphate-buffered saline (PBS) and then with freshly prepared 4% paraformaldehyde in Tris-buffered saline (TBS). The kidneys and 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 mixture (TBST) for 1 h at room temperature, washed with TBST, incubated with the ANF-RGC antibody (50:1) in a blocking solution overnight at 4 °C, washed with TBST, incubated with DyLight 488-conjugated donkey anti-rabbit antibody (100:1) 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.

Antibodies. Antibodies against ANF-RGC were raised in rabbits. Their specificities were described previously.[24,25] The antibodies were affinity purified. Secondary antibodies conjugated to a fluorescent dye (DyLight 488) were purchased from Jackson ImmunoResearch Laboratories, Inc. (West Grove, PA).

Blood Pressure Measurements. The systolic and diastolic blood pressures of the ANF-RGC WTAPELL$^{+/+}$ mice (wild type, control) or the heterozygous ANF-RGC ^{669}WTAPELL$^{675(+/-)}$ and homozygous ^{669}WTAPELL$^{675(-/-)}$ mice 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 3 days of mice training. The mice were maintained on normal chow and drinking water available ad libitum.

The mean arterial pressure (MAP) was calculated from the values of systolic (S) and diastolic (D) pressure according to the equation MAP $\approx (2D + S)/3$.

Aldosterone Level. Aldosterone levels in the plasma of the ANF-RGC ^{669}WTAPELL$^{675(+/+)}$, ANF-RGC ^{669}WTAPELL$^{675(+/-)}$, or ANF-RGC ^{669}WTAPELL$^{675(-/-)}$ mice were determined using the RIA kit and the manufacturer's protocol (Siemens Medical Solutions Diagnostics).

Statistical Analysis. The results are presented as means ± the standard deviation. Differences among the three groups of mice studied, ANF-RGC ^{669}WTAPELL$^{675(+/+)}$, ANF-RGC

^{669}WTAPELL$^{675(+/-)}$, and ^{669}WTAPELL$^{675(-/-)}$, were compared using a Student's t test. A P value of <0.05 was considered significant.

RESULTS AND DISCUSSION

ANF-RGC ^{669}WTAPELL^{675} Motif-Targeted Mice. All biological effects of ANF are mediated by its second-messenger cyclic GMP produced by ANF-RGC. Studies of the recombinant system have shown that the ^{669}WTAPELL^{675} motif of ANF-RGC is critical for the transduction of the ANF signal into an increased level of synthesis of cyclic GMP.[26] To examine whether the same applies to ANF signaling in the biological systems, an in-ANF-RGC-gene ^{669}WTAPELL^{675} deletion mouse model was developed. To date, no ANF-RGC domain-specific genetically modified animal model is available. The only available is that with the entire ANF-RGC gene deleted.[30,31] It was hypothesized that absence of the ^{669}WTAPELL^{675} signaling motif from the ANF-RGC protein would result in a mouse phenotype where the mutant specifically lacks the ability to transmit the ANF-dependent ATP signal from the ARM (ATP-regulated module) to the ANF-RGC catalytic domain. It, then, cannot accelerate production of the ANF second-messenger cyclic GMP, and the mouse will be inflicted with the pathologies of hypertension and cardiac and renal hypertrophies.

Viable homozygous mice, with the ^{669}WTAPELL^{675} coding sequence deleted from both copies of the ANF-RGC gene [^{669}WTAPELL$^{675(-/-)}$], prove that the deletion is not immediately lethal and does not interfere with reproduction. The homo- and heterozygous mice do not differ in their appearance from their wild-type littermates.

What follows is the detailed physiological analysis and biochemistry behind the ^{669}WTAPELL^{675} deletion mutation phenotype in mice.

PHYSIOLOGY

The ^{669}WTAPELL^{675} Motif Is Critical for Cardiovascular Physiology. It is well established that elimination of either ANF-RGC or ANF expression leads to increased blood pressure, cardiac and renal hypertrophy, and fibrosis, to name some of the related pathological conditions in mice.[6,31−35] It is also established that the seven-amino acid ^{669}WTAPELL^{675} motif controls the signal transduction activity of ANF-RGC.[26] Does this motif control the physiological functions of ANF-RGC as well? As we will show, it does.

Blood Pressure. After 3 days of mock sessions for training purposes, the systolic blood pressure of the ^{669}WTAPELL$^{675(+/+)}$, ^{669}WTAPELL$^{675(+/-)}$, and ^{669}WTAPELL$^{675(-/-)}$ mice was measured. It was 102 ± 9 mmHg for the wild-type mice, 134 ± 17 mmHg for the heterozygous mice, and 159 ± 11 mmHg for the homozygous mice (Figure 2A). The increase was statistically highly significant (P < 0.005). Together with the systolic pressure, the diastolic pressure was recorded, and from their combined values, the mean arterial pressure was calculated. It was 78 ± 6 mmHg for the wild-type mice, 108 ± 11 mmHg for the heterozygous mice, and 127 ± 10 mmHg for the homozygous mice (Figure 2B). As with the systolic pressure, the differences between the wild-type and ^{669}WTAPELL$^{675(+/-)}$ heterozygous mice as well as between the heterozygous and ^{669}WTAPELL$^{675(-/-)}$ homozygous mice were statistically very significant (P < 0.005). These results show that the progressive elevation of blood pressure directly correlates with the number of ANF-RGC gene copies with the WTAPELL motif's coding sequence deleted, demonstrating that the motif is critical for ANF-RGC function in blood pressure regulation.

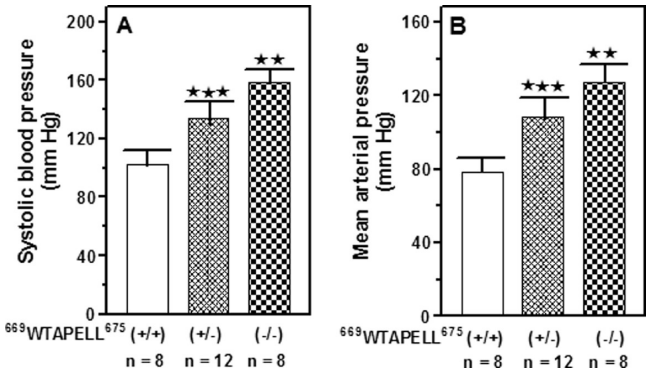


Figure 2. Systolic (A) and mean arterial (B) blood pressure in ANF-RGC ^{669}WTAPELL^{675} motif-targeted mice. The following mice were used: ^{669}WTAPELL$^{675(+/+)}$ wild-type allele, ^{669}WTAPELL$^{675(+/-)}$ heterozygous-type allele, and ^{669}WTAPELL$^{675(-/-)}$ homozygous-type allele. The mice were fed a normal-salt diet. The 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. The mean arterial blood pressure was calculated from the values of systolic and diastolic pressures as described in Experimental Procedures. Bars indicate means ± the standard deviation for the representative genotype. n describes the number of mice analyzed for each genotype. Two asterisks indicate that the P value was <0.005 and three asterisks that the P value was <0.001.

Plasma Aldosterone Level. Volume homeostasis and blood pressure are influenced by the steroid hormone aldosterone secreted by the cells of the zona glomerulosa of the adrenal gland. Aldosterone inhibits sodium secretion, and diuresis thus increases blood pressure. Its secretion is controlled by the renin angiotensin system (RAS). RAS is kept in balance through cyclic GMP generated by ANF-RGC.[36−38] In the adrenocortical zona glomerulosa, cyclic GMP inhibits aldosterone synthesis and prevents the elevation of blood pressure. The issue was whether the WTAPELL motif allows ANF-RGC to modulate blood pressure through this mechanism, e.g., by synthesizing cyclic GMP in a quantity sufficient to inhibit aldosterone synthesis. As we will show, it does.

Plasma aldosterone concentrations were measured in both types of genetically modified mice (hetero- and homozygous) and in their isogenic controls (wild type). The results are shown in Figure 3. In the plasma of wild-type mice, the aldosterone concentration was 147 ± 12 pg/mL; it increased to 204 ± 18 pg/mL in the heterozygous mice (P < 0.005) and, further, to 256 ± 22 pg/mL in the homozygous mice (P < 0.05 for the increase between hetero- and homozygous mice). It is therefore concluded that deletion of the ^{669}WTAPELL^{675} motif from ANF-RGC correlates with the increased aldosterone concentrations in the ^{669}WTAPELL^{675} motif-targeted mice in comparison with the wild-type animals.

Cardiac Hypertrophy. The final issue was whether the chronic pressure overload leads to cardiac hypertrophy. The results show that it does.

The ratio of the heart weight (in milligrams) to the whole body weight (in grams) of 10-week-old ^{669}WTAPELL$^{675(+/+)}$, ^{669}WTAPELL$^{675(+/-)}$, and ^{669}WTAPELL$^{675(-/-)}$ mice was determined, and the results are shown in Figure 4. The ratio

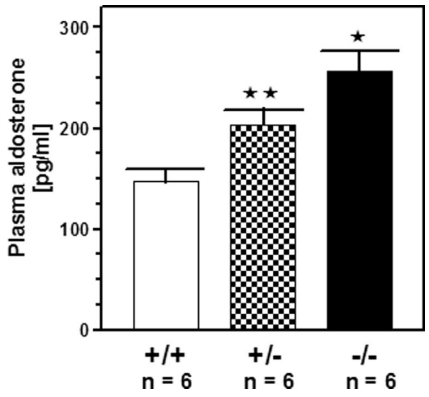


Figure 3. Plasma aldosterone levels in ANF-RGC ^{669}WTAPELL^{675} motif-targeted mice. The following mice were used: ^{669}WTAPELL$^{675(+/+)}$ wild-type allele, ^{669}WTAPELL$^{675(+/-)}$ heterozygous-type allele, and ^{669}WTAPELL$^{675(-/-)}$ homozygous-type allele. The mice were fed a normal-salt diet. Aldosterone concentrations were determined using Coat-A Count aldosterone RIA kit. Bars indicate means ± the standard deviation for the representative genotype. *n* describes the number of mice analyzed for each genotype. Two asterisks indicate that the *P* value was <0.005, and one asterisk indicates that the *P* value was <0.05.

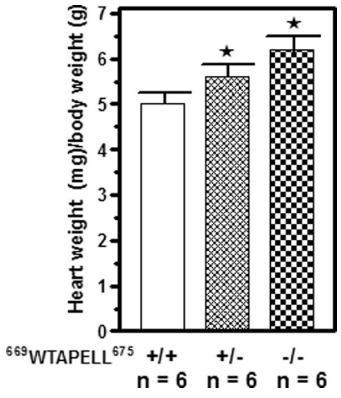


Figure 4. Absence of the ^{669}WTAPELL^{675} motif in ANF-RGC that causes cardiac hypertrophy. The ratio of the heart weight (in milligrams) to whole body weight (in grams) of 10-week-old ^{669}WTAPELL$^{675(+/+)}$ wild-type, ^{669}WTAPELL$^{675(+/-)}$ heterozygous, and ^{669}WTAPELL$^{675(-/-)}$ homozygous mice was determined. The mice were fed a normal-salt diet. Bars indicate means ± the standard deviation for the representative genotype. *n* describes the number of mice analyzed for each genotype. An asterisk indicates that the *P* value was <0.05.

was 5 ± 0.3, 5.6 ± 0.3, and 6.1 ± 0.5 for the ^{669}WTAPELL$^{675(+/+)}$, ^{669}WTAPELL$^{675(+/-)}$, and ^{669}WTAPELL$^{675(-/-)}$ mice, respectively. Thus, a progressive increase in cardiac hypertrophy correlates with the number of ANF-RGC gene copies with the deleted sequence encoding the ^{669}WTAPELL^{675} motif.

The results presented demonstrate that deletion of the ^{669}WTAPELL^{675} motif from ANF-RGC mimics the effects of the loss of ANF-RGC protein.[39] Thus, at the molecular level, the ^{669}WTAPELL^{675} motif of ANF-RGC is the regulator of the cyclase signal transduction, and its absence disables the cyclase's physiological functions and leads to cardiovascular pathologies.

BIOCHEMISTRY

What Is the Biochemical Explanation for the Inability of the Mutated ANF-RGC To Perform Its Biological Functions? Three issues were considered: level of expression of the mutated cyclase versus that of the wild type, its basal activity, and its response to the ANF signal.

The ^{669}WTAPELL^{675} Encoded Gene Motif Has No Role in ANF-RGC Expression in the Kidney and Adrenal Gland. After being excreted via the atrial stretch, ANF through the bloodstream is transported to two key organs, adrenal gland and kidney,[40] where it signals the catalytic activation of ANF-RGC.[41−45] These organs are critical in maintaining cardiovascular and renal homeostasis.[6,46,47]

The issue was whether the ^{669}WTAPELL^{675} encoded gene motif affects the ANF-RGC gene expression in these organs. The results show that it does not.

It was analyzed by immunocytochemistry. The kidneys and adrenal glands were removed from the wild-type [^{669}WTAPELL$^{675(+/+)}$], heterozygous [^{669}WTAPELL$^{675(+/-)}$], and homozygous [^{669}WTAPELL$^{675(-/-)}$] male mice, and the frozen sections of these tissues were stained with immunopurified anti ANF-RGC antibody.

Panels A and B of Figure 5 [^{669}WTAPELL$^{675(+/+)}$] show images generated in the kidney and the adrenal gland, respectively, of a wild-type mouse. Intense immunoreactivity (green color) was observed in the renal apical and basolateral aspects of the tubular elements (Figure 5A) and adrenocortical zona glomerulosa (Figure 5B). These results are in agreement with previous localizations of ANF-RGC in the kidney and adrenal gland.[24,25,45] An identical pattern of immunoreactivity was observed when the kidney and adrenal gland sections from the ^{669}WTAPELL$^{675(+/-)}$ and ^{669}WTAPELL$^{675(-/-)}$ mice were analyzed (Figure 5A,B, panels ^{669}WTAPELL$^{675(+/-)}$ and ^{669}WTAPELL$^{675(-/-)}$, respectively). Visual examination of the stained sections as well as of the respective differential interference contrast (DIC) images indicates that the deletion does also not affect the integrity of these tissues. These results demonstrate that deletion of the sequence encoding the ^{669}WTAPELL^{675} motif from either one or two ANF-RGC gene copies does not affect the cyclase tissue-specific expression.

The ^{669}WTAPELL^{675} Motif Does Not Control the Structure of the ANF-RGC Catalytic Site and Therefore Does Not Affect the Basal Cyclase Activity in the Kidney or Adrenal Gland. The next question was whether the apparent unchanged expression of the ANF-RGC mutant protein is accompanied by unchanged basal activity of the cyclase in the analyzed tissues. The answer is yes.

Membrane fractions of the kidney and the adrenal gland were isolated from the ^{669}WTAPELL$^{675(+/+)}$ mice, the heterozygous ^{669}WTAPELL$^{675(+/-)}$ mice, and the homozygous ^{669}WTAPELL$^{675(-/-)}$ mice, and they were assessed for basal guanylate cyclase activity. Membranes of COS cells expressing wild-type ANF-RGC and its ^{669}WTAPELL^{675} deletion mutant were assayed in parallel as controls. The activities of wild-type ANF-RGC and its ^{669}WTAPELL^{675} deletion mutant expressed in COS cells were virtually identical, ∼23 pmol of cyclic GMP min^{-1} (mg of protein)$^{-1}$. The particulate guanylate cyclase activities expressed in the kidneys and adrenal glands were also practically indistinguishable between the membranes isolated from the wild-type and the genetically modified mice. They were ∼41 and ∼78 pmol of cyclic GMP min^{-1} (mg of

dx.doi.org/10.1021/bi400175d | *Biochemistry* 2013, 52, 2337−2347

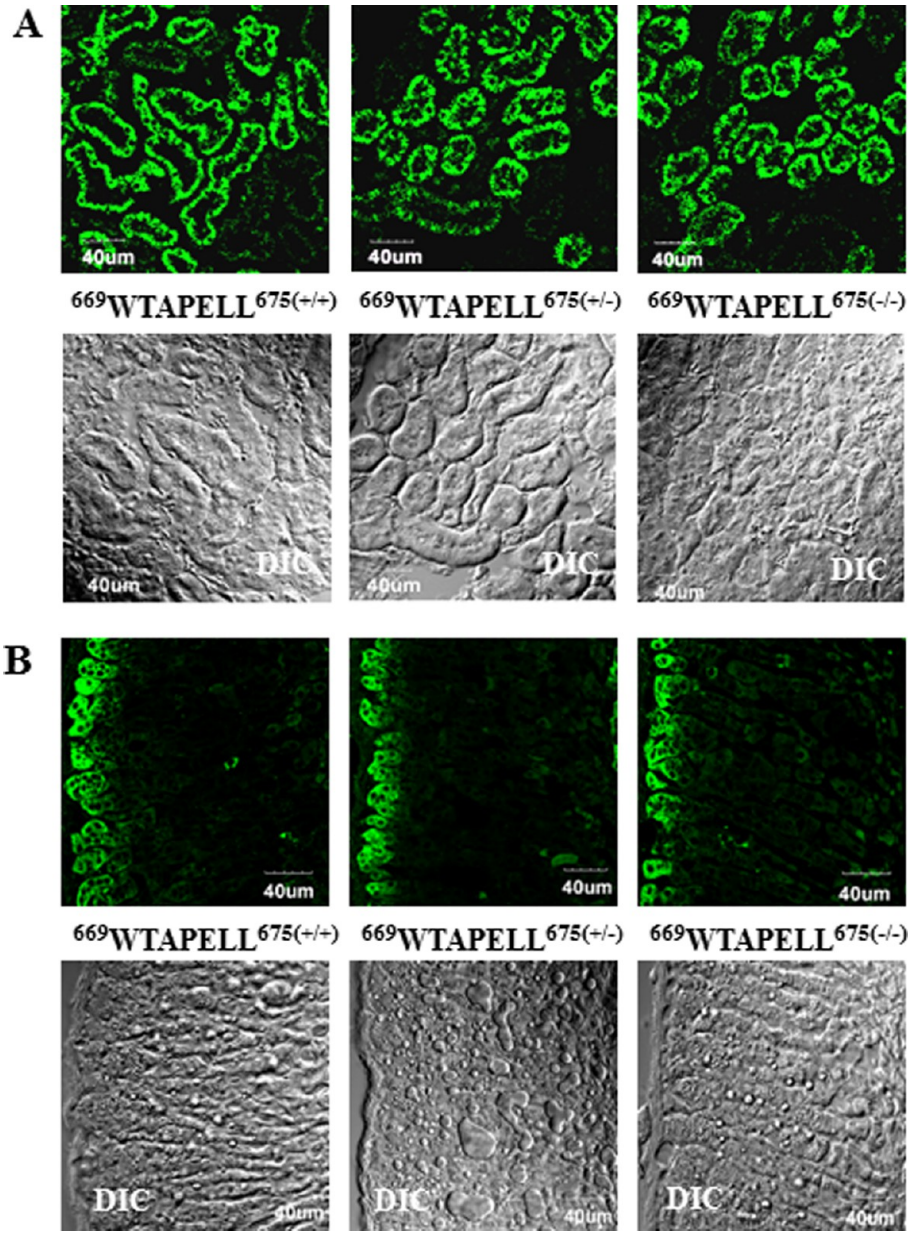


Figure 5. Deletion of the sequence encoding the ^{669}WTAPELL^{675} motif from the ANF-RGC gene does not affect expression of the mutated cyclase in the kidney (A) or adrenal gland (B). Cryosections of the kidney and adrenal gland from the wild-type [^{669}WTAPELL$^{675(+/+)}$], heterozygous [^{669}WTAPELL$^{675(+/-)}$], and homozygous [^{669}WTAPELL$^{675(-/-)}$] mice were immunostained with ANF-RGC antibodies. The DIC images showing the integrity of the sections are also shown ("DIC"). Intense staining was observed in the renal apical and basolateral aspects of the tubular elements and adrenocortical zona glomerulosa.

protein)$^{-1}$ for the kidney and adrenal gland, respectively (Table 1). These results demonstrate that in both the recombinant system and the in vivo system deletion of the ^{669}WTAPELL^{675} motif does not affect the basal activity of the catalytic domain of ANF-RGC. Therefore, the motif does not control the structure of the catalytic domain.

The ^{669}WTAPELL^{675} Motif Controls the ANF-Dependent Regulatory Activity of ANF-RGC. The membrane fractions of the kidneys and the adrenal glands isolated from the ^{669}WTAPELL$^{675(+/+)}$ mice, the heterozygous ^{669}WTAPELL$^{675(+/-)}$ mice, and the homozygous ^{669}WTAPELL$^{675(-/-)}$ mice were evaluated for guanylate cyclase activity in the presence of increasing concentrations of ANF and 0.8 mM ATP. Recombinant ANF-RGC and its ^{669}WTAPELL^{675} deletion mutant expressed individually in COS cells were treated identically as the control. The results are presented in Figure 6. In the wild-type mice, the guanylate cyclase activity was stimulated in an ANF dose-dependent fashion >9-fold in the kidney membranes and >5-fold in the adrenal gland membranes {form 41 ± 6 to 389 ± 40 pmol of cyclic GMP min^{-1} (mg of protein)$^{-1}$ for the kidney [Figure 6A (○)] and from 78 ± 13 to 413 ± 35 pmol of cyclic GMP min^{-1} (mg of protein)$^{-1}$ for the adrenal gland [Figure 6B (○)]}. For both types of membranes, the half-maximal stimulation was at ~10^{-9} M ANF. The guanylate cyclase activity in these membranes had an ANF dose-dependent profile comparable if not identical with the activity of COS cell membranes expressing wild-type ANF-RGC (compare the ○ curves in panels A and B of Figure 6 with that of panel C). Also, the calculated Hill coefficients for the dose−response curves were very similar: 0.91 ± 0.2 for the

dx.doi.org/10.1021/bi400175d | *Biochemistry* 2013, 52, 2337−2347

Table 1. Guanylate Cyclase Activity in the Membrane Fraction of the Kidney and Adrenal Gland of Wild-Type and ANF-RGC ^{669}WTAPELL^{675}-Modified Mice[a]

tissue or activity	wild type	^{669}WTAPELL$^{675(+/-)}$	^{669}WTAPELL$^{675(-/-)}$
kidney	42 ± 4	40 ± 6	41 ± 5
adrenal gland	78 ± 9	75 ± 10	81 ± 9
COS cells	23 ± 3		22 ± 3

[a]The membrane fractions were prepared as described in Experimental Procedures. Membranes of the COS cells expressing wild-type ANF-RGC and its ^{669}WTAPELL^{675} deletion mutant were assayed as the control. The experiment was conducted in triplicate and repeated three times with different membrane preparations. The results are means ± the standard deviation from these experiments. The activity is expressed as picomoles of cyclic GMP per minute per milligram of protein.

membranes isolated from kidney and adrenal gland and 0.95 ± 0.12 for recombinant wild-type ANF-RGC.

In the membranes isolated from the heterozygous mice [^{669}WTAPELL$^{675(+/-)}$], the cyclase remained responsive to ANF/ATP, the ANF EC_{50} value was again at ~10^{-9} M, but the saturated activity was only 47% of that achieved by the wild-type cyclase in kidney [Figure 6A (●)] and 61% in the adrenal gland [Figure 6B (●)]. These values, oscillating around 50% of the wild-type values, are indicative that in the heterozygous mice where the product of only one ANF-RGC gene copy is of the wild type and the other of the deletion-mutated cyclase, only wild-type ANF-RGC is responsive to ANF/ATP and the mutant is not.

The membranes of the homozygous mice [^{669}WTAPELL$^{675(-/-)}$] isolated either from the kidney [Figure 6A (◆)] or the adrenal gland [Figure 6B (◆)] were totally unresponsive to ANF/ATP; the cyclase activity at all concentrations of ANF tested was the same as in their absence. Importantly, the activity of the recombinant ^{669}WTAPELL^{675} deletion mutant expressed in COS cells was also indifferent to the tested concentrations of ANF and ATP [Figure 6C (◆)].

These findings establish that the ^{669}WTAPELL^{675} motif selectively controls the ANF-dependent regulatory activity of ANF-RGC; it has no control over its basal activity. These are striking properties of ANF-RGC that could not be observed in the earlier total ANF-RGC gene knockout mouse model studies.[30,31] This model has made it possible to link the mechanism of ANF/ATP signaling of ANF-RGC activation with hypertension. It is therefore concluded that in the ANF-dependent regulatory activity of ANF-RGC the ^{669}WTAPELL^{675} motif plays an essential role in the action of ANF in the regulation of blood pressure. Disruption of this motif results in hypertension.

The ANF/ATP- and Ca^{2+}-Modulated ANF-RGC Signaling Pathways Independently Control Blood Pressure. The recent ground breaking discovery of the authors has disclosed that ANF-RGC is a bimodal signal transduction switch.[24,25] In addition to being a signal transducer of the hormones, ANF and BNP, it is also a signal transducer of $[Ca^{2+}]_i$. The sensor of Ca^{2+} is neurocalcin δ (NCδ). Importantly, the Ca^{2+} signal transduction pathway also controls mouse blood pressure through the control of aldosterone synthesis in the adrenal glands.[25] The NC$\delta^{+}/^{-}$ mice are hypertensive.[25]

The question was whether the ^{669}WTAPELL^{675} motif influences the $[Ca^{2+}]_i$-dependent ANF-RGC catalytic activities in the kidney and adrenal glands of the mouse. The answer is that it does not.

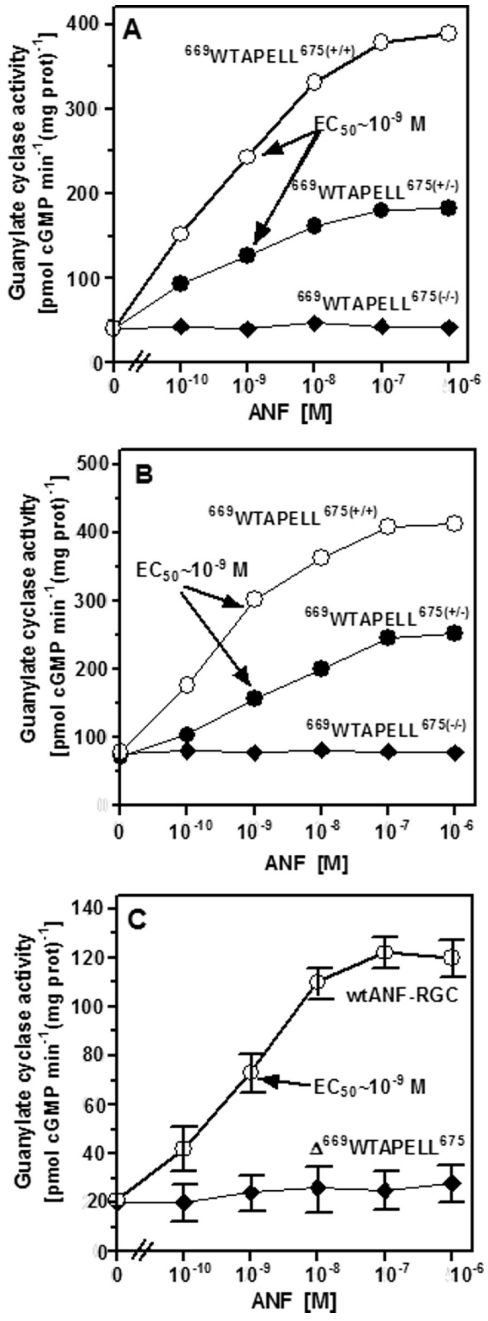


Figure 6. ANF/ATP-dependent ANF-RGC activity in the membranes of kidney (A) and adrenal gland (B) of the wild-type and ^{669}WTAPELL^{675} genetically modified mice and of COS cells transfected with wild-type ANF-RGC or its ^{669}WTAPELL^{675} deletion mutant (C). Membrane fractions of the kidneys and the adrenal glands were isolated from wild-type [^{669}WTAPELL$^{675(+/+)}$], heterozygous [^{669}WTAPELL$^{675(+/-)}$], and homozygous [^{669}WTAPELL$^{675(-/-)}$] mice and COS cells expressing wild-type ANF-RGC or its ^{669}WTAPELL^{675} deletion mutant. These were evaluated for guanylate cyclase activity in the presence of the indicated concentrations of ANF and 0.8 mM ATP, and the amount of cyclic GMP formed was measured by a radioimmunoassay. The experiment was conducted in triplicate and repeated three times with separate membrane preparations. The results presented (mean ± standard deviation) are from these experiments.

dx.doi.org/10.1021/bi400175d | *Biochemistry* 2013, 52, 2337−2347

Membranes isolated from the kidney and adrenal gland of the ^{669}WTAPELL^{675} targeted wild-type, heterozygous, and homozygous mice and the membranes of COS cells expressing recombinant ANF-RGC and its ^{669}WTAPELL^{675} deletion mutant were exposed to 4 μM NCδ and 1 μM Ca^{2+} (Figure 7). For all three mouse genotypes, the ANF-RGC activity was stimulated by 4.6-fold, from ~41 to 190 pmol of cyclic GMP min^{-1} (mg of protein)$^{-1}$ in the kidney membranes (Figure 7A), 3.2-fold, from ~78 to 247 pmol of cyclic GMP min^{-1} (mg of protein)$^{-1}$ in the adrenal gland membranes (Figure 7B), and approximately 5-fold, from 23 to 100 pmol of cyclic GMP min^{-1} (mg of protein)$^{-1}$, for both the wild-type and ^{669}WTAPELL^{675} motif-deleted ANF-RGC expressed in COS cell membranes. These results demonstrate that the ^{669}WTAPELL^{675} motif is not involved the Ca^{2+} modulation of ANF-RGC activity. Importantly, the two signaling pathways, the ANF-dependent pathway involving the ^{669}WTAPELL^{675} motif and the ANF-independent Ca^{2+}-modulated pathway in which the ^{669}WTAPELL^{675} motif is not involved, function through the generation of their second-messenger cyclic GMP, and through it, they both independently contribute to the regulation of blood pressure.

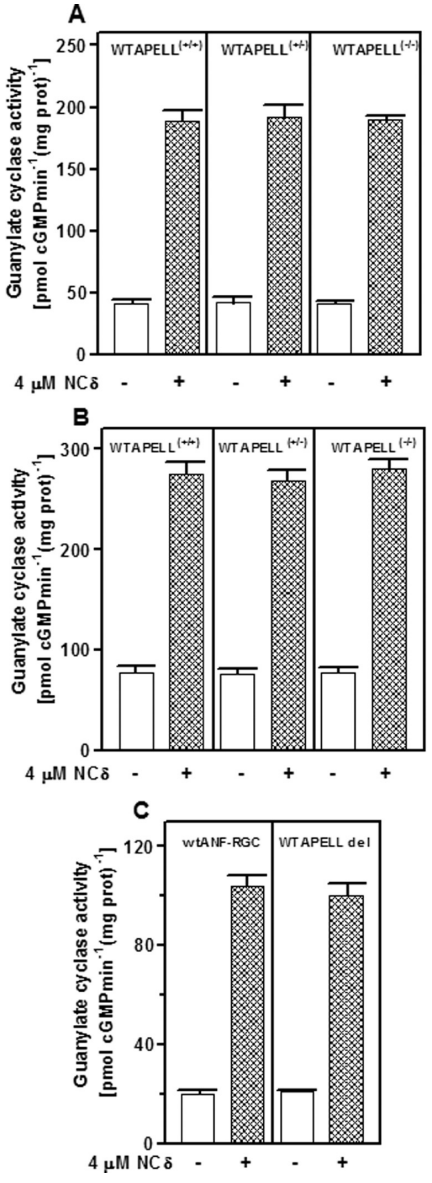


Figure 7. Motif ^{669}WTAPELL^{675} that is not involved in maintaining the integrity of the ANF-RGC catalytic domain. The particulate fractions of the kidney (A) and adrenal gland (B) from the wild-type ^{669}WTAPELL$^{675(+/+)}$, heterozygous ^{669}WTAPELL$^{675(+/-)}$, and homozygous ^{669}WTAPELL$^{675(-/-)}$ mice and from COS cells expressing wild-type ANF-RGC or its ^{669}WTAPELL^{675} deletion mutant were prepared and tested for their responsiveness to 4 μM neurocalcin δ in the presence of 1 μM Ca^{2+}. The experiment was conducted in triplicate and repeated twice. The results shown are means ± the standard deviation from these experiments.

CONCLUSION

Through a mouse molecular genetics approach involving a domain-specific targeting of ANF-RGC guanylate cyclase, this study directly links the biochemistry of the transduction events of the ^{669}WTAPELL^{675} motif with the physiological processes of blood pressure regulation. There are several striking features of this study. The first is the novelty of the approach; for the first time, a domain-based ANF-RGC mouse in-gene-deletion model has been used to decode the principles of ANF-RGC signal transduction. Second, the study demonstrates that a mere seven-amino acid motif, ^{669}WTAPELL^{675}, of ANF-RGC controls the ANF/ATP-dependent catalytic activity of the cyclase. Third, the motif controls only the hormone-dependent regulatory activity of ANF-RGC. Fourth, the absence of the motif in the ANF-RGC protein results in increased blood pressure. Fifth, the motif also is in command of the aldosterone plasma levels. Sixth, by causing chronic pressure overload, the absence of the motif inflicts the mouse with cardiac hypertrophy. Seventh, all these abnormalities are due to the dysfunctional ANF-dependent ANF-RGC signal transduction apparatus in the adrenal and kidney glands of the mouse.

We propose a model to explain these findings (Figure 8).

Model. *ANF Signaling of ANF-RGC, Cyclic GMP Production Events.* The signal originates by the binding of one molecule of ANF to the ECD of the ANF-RGC dimer.[48,49] The binding modifies the juxtamembrane region where the disulfide ^{423}C−C^{432} structural motif is a key element.[50,51] The signal twists the transmembrane domain,[52] induces a structural change in the ARM domain, and prepares it for ATP binding.[53] In step 1, the ARM domain binds ATP, which leads to a cascade of temporal and spatial changes.[54] They involve (1) a shift in the ATP binding pocket position by 3−4 Å and 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 and its loop, and (3) movement of its αEF helix by 2−5 Å. This movement exposes the hydrophobic motif, ^{669}WTAPELL^{675}, which facilitates its direct (or indirect) interaction with the catalytic module resulting in its partial, ~50%, activation.[26] In step 2, the six phosphorylation sites are brought from their buried state to their exposed state.[55] ATP, through a hypothetical protein kinase, phosphorylates the residues, and full activation (additional 50%) of ANF-RGC is achieved. ANF-dependent cyclic GMP is generated and functions as the second messenger of blood pressure regulation. Concomitantly, phosphorylation converts the ATP binding site from high to low affinity; ATP dissociates, and ANF-RGC returns to its ground state.[55]

Post-Cyclic GMP Production Events. The cyclic GMP that is generated suppresses the RAS-dependent secretion of aldosterone through yet a not fully understood mechanism. It is known, however, that it affects a number of effectors of aldosterone

dx.doi.org/10.1021/bi400175d | *Biochemistry* 2013, 52, 2337−2347

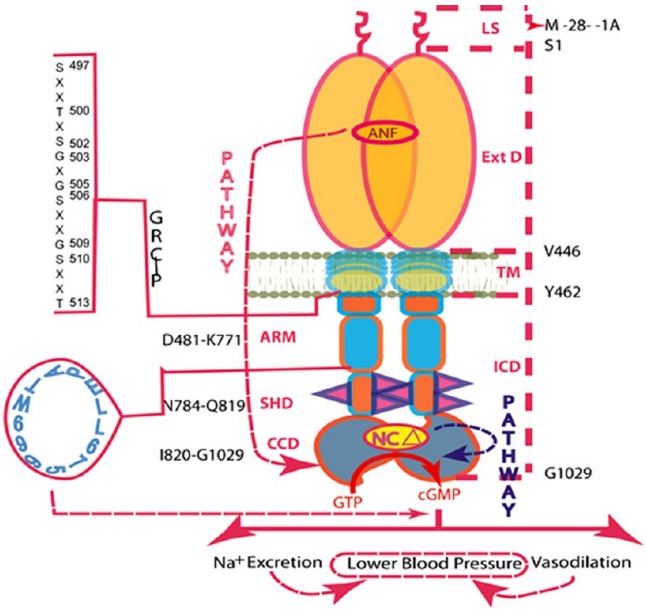


Figure 8. Model of ANF signaling of ANF-RGC activity linked with cardiovascular physiology. ANF-RGC is a single-transmembrane-spanning homodimer protein. The dashed lines on the right show the defined boundaries of its segments: LS, leader sequence; Ext D, extracellular domain; TM, transmembrane domain; ICD, intracellular domain. The functional domains housed in the intracellular domain (ICD), their designated names, and the amino acid residues constituting their boundaries are indicated at the left: ARM, ATP regulated module; SHD, signaling helix domain; CCD, core catalytic domain. The CCD exists as an antiparallel homodimer. The sequence of the ARM domain's region of the glycine rich cluster and the phosphorylation sites is shown on the left-hand side as GRC~P; the helical ^{669}WTAPELL^{675} motif is colored blue. The neurocalcin δ-targeted site within the catalytic domain is shown as NCΔ. The red dashed arrow indicates the physiological targets affected by deletion of the ^{669}WTAPELL^{675} motif of ANF-RGC.

synthesis. They include cyclic GMP-gated channels, cyclic GMP-dependent protein kinases, and cyclic GMP-regulated phosphodiesterases.[56−59] The lowered aldosterone level results in an increase in the level of sodium excretion and diuresis in the kidney and maintains physiological blood pressure.

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

The study was supported by National Heart, Lung and Blood Institute Grants HL084584 and S82701.

Notes

The authors declare no competing financial interest.

ACKNOWLEDGMENTS

We acknowledge Guanylate Cyclase Innovative Biotechnologies (GCIB) for the support of the ^{669}WTAPELL^{675}-deleted ANF-RGC gene mouse model.

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; GCAP, guanylate cyclase activating protein; NCδ, neurocalcin δ; ONE-GC, olfactory neuroepithelial guanylate cyclase; RAS, renin−angiotensin system; ROS-GC, rod outer segment guanylate cyclase.

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., Kamisak,i, 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) Sharma, R. K. (2002) Evolution of the membrane guanylate cyclase transduction system. *Mol. Cell. Biochem. 230*, 3−30.

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

(6) 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.

(7) 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.

(8) Gardner, D. G., Chen, S., Glenn, D. J., and Grigsby, C. L. (2007) Molecular biology of the natriuretic peptide system: Implications for physiology and hypertension. *Hypertension 49*, 419−426.

(9) Wedel, B. J., and Garbers, D. L. (1997) New insights on the functions of the guanylyl cyclase receptors. *FEBS Lett. 410*, 29−33.

(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) Koch, K. W., Duda, T., and Sharma, R. K. (2010) Ca^{2+}-modulated vision-linked ROS-GC guanylate cyclase transduction machinery. *Mol. Cell. Biochem. 334*, 105−115.

(15) Duda, T., and Sharma, R. K. (2008) ONE-GC membrane guanylate cyclase, a trimodal odorant signal transducer. *Biochem. Biophys. Res. Commun. 36*, 440−445.

(16) Sharma, R. K., and Duda, T. (2010) Odorant-linked ROS-GC subfamily membrane guanylate cyclase transduction system. *Mol. Cell. Biochem. 334*, 181−189.

(17) Koch, K. W., Duda, T., and Sharma, R. K. (2002) Photoreceptor specific guanylate cyclases in vertebrate phototransduction. *Mol. Cell. Biochem. 230*, 97−106.
(18) Pugh, E. N., Jr., Duda, T., Sitaramayya, A., and Sharma, R. K. (1997) Photoreceptor guanylate cyclases: A review. *Biosci. Rep. 1*, 429−473.
(19) 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.
(20) Leinders-Zufall, T., Cockerham, 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.
(21) 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. 10*, 2041−2046.
(22) 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.
(23) Sharma, R. K., and Duda, T. (2012) Ca^{2+}-sensors and ROS-GC: Interlocked sensory transduction elements: A review. *Front. Mol. Neurosci. 5*, 42.
(24) 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.
(25) Duda, T., Pertzev, A., and Sharma, R. K. (2012) Ca^{2+} Modulation of ANF-RGC: New Signaling Paradigm Interlocked with Blood Pressure Regulation. *Biochemistry 51*, 9394−9405.
(26) Duda, T., Bharill, S., Wojtas, I., Yadav, P., Gryczynski, I., Gryczynski, Z., and Sharma, R. K. (2009) Atrial natriuretic factor receptor guanylate cyclase signaling: New ATP-regulated transduction motif. *Mol. Cell. Biochem. 324*, 39−53.
(27) Duda, T., Pertzev, A., and Sharma, R. K. (2011) 657WTAPELL663 motif of the photoreceptor ROS-GC1: A general phototransduction switch. *Biochem. Biophys. Res. Commun. 408*, 236−241.
(28) Sambrook, M. J., Fritsch, E. F., and Maniatis, T. (1989) *Molecular Cloning: A Laboratory Manual*, 2nd ed., Cold Spring Harbor Laboratory Press, Plainview, NY.
(29) 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.
(30) Oliver, P. M., Fox, J. E., Kim, R., Rockman, H. A., Kim, H. S., Reddick, R. L., Pandey, K. N., Milgram, S. L., Smithies, O., and Maeda, N. (1997) Hypertension, cardiac hypertrophy, and sudden death in mice lacking natriuretic peptide receptor A. *Proc. Natl. Acad. Sci. U.S.A. 94*, 14730−14735.
(31) Lopez, M. J., Wong, S. K., Kishimoto, I., Dubois, S., Mach, V., Friesen, J., Garbers, D. L., and Beuve, A. (1995) Salt-resistant hypertension in mice lacking the guanylyl cyclase-A receptor for atrial natriuretic peptide. *Nature 378*, 65−68.
(32) Ellis, K. L., Newton-Cheh, C., Wang, T. J., Frampton, C. M., Doughty, R. N., Whalley, G. A., Ellis, C. J., Skelton, L., Davis, N., Yandle, T. G., Troughton, R. W., Richards, A. M., and Cameron, V. A. (2011) Association of genetic variation in the natriuretic peptide system with cardiovascular outcomes. *J. Mol. Cell. Cardiol. 50*, 695−701.
(33) Das, S., Au, E., Krazit, S. T., and Pandey, K. N. (2010) Targeted disruption of guanylyl cyclase-A/natriuretic peptide receptor-A gene provokes renal fibrosis and remodeling in null mutant mice: Role of proinflammatory cytokines. *Endocrinology 151*, 5841−5850.
(34) Ellmers, L. J., Scott, N. J., Piuhola, J., Maeda, N., Smithies, O., Frampton, C. M., Richards, A. M., and Cameron, V. A. (2007) Npr1-regulated gene pathways contributing to cardiac hypertrophy and fibrosis. *J. Mol. Endocrinol. 38*, 245−257.
(35) Wadei, H. M., and Textor, S. C. (2012) The role of the kidney in regulating arterial blood pressure. *Nat. Rev. Nephrol. 8*, 602−609.
(36) Anand-Srivastava, M. B., and Trachte, G. J. (1993) Atrial natriuretic factor receptors and signal transduction mechanisms. *Pharmacol. Rev. 45*, 455−497.
(37) Brenner, B. M., Ballermann, B. J., Gunning, M. E., and Zeidel, M. L. (1990) Diverse biological actions of atrial natriuretic peptide. *Physiol. Rev. 70*, 665−699.
(38) Ganguly, A. (1992) Atrial natriuretic peptide-induced inhibition of aldosterone secretion: A quest for mediator(s). *Am. J. Physiol. 263*, E181−E194.
(39) Zhao, D., Vellaichamy, E., Somanna, N. K., and Pandey, K. N. (2007) Guanylyl cyclase/natriuretic peptide receptor-A gene disruption causes increased adrenal angiotensin II and aldosterone levels. *Am. J. Physiol. 293*, F121−F127.
(40) de Bold, A. J. (1985) Atrial natriuretic factor: A hormone produced by the heart. *Science 230*, 767−770.
(41) Marala, R. B., and Sharma, R. K. (1991) Ubiquitous and bifunctional 180 kDa atrial natriuretic factor-dependent guanylate cyclase. *Mol. Cell. Biochem. 100*, 25−30.
(42) 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.
(43) De Léan, A., Gutkowska, J., McNicoll, N., Schiller, P. W., Cantin, M., and Genest, J. (1984) Characterization of specific receptors for atrial natriuretic factor in bovine adrenal zona glomerulosa. *Life Sci. 35*, 2311−2318.
(44) Lynch, D. R., Braas, K. M., and Snyder, S. H. (1986) Atrial natriuretic factor receptors in rat kidney, adrenal gland, and brain: Autoradiographic localization and fluid balance dependent changes. *Proc. Natl. Acad. Sci. U.S.A. 83*, 3557−3561.
(45) Chai, S. Y., Sexton, P. M., Allen, A. M., Figdor, R., and Mendelsohn, F. A. (1986) In vitro autoradiographic localization of ANP receptors in rat kidney and adrenal gland. *Am. J. Physiol. 250*, F753−F757.
(46) de Zeeuw, D., Janssen, W. M., and de Jong, P. E. (1992) Atrial natriuretic factor: its (patho)physiological significance in humans. *Kidney Int. 41*, 1115−1133.
(47) Misono, K. S. (2000) Atrial natriuretic factor binding to its receptor is dependent on chloride concentration: A possible feedback-control mechanism in renal salt regulation. *Circ. Res. 6*, 1135−1139.
(48) 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.
(49) van den Akker, F., Zhang, X., Miyagi, M., Huo, X., Misono, K. S., and Yee, V. C. (2000) Structure of the dimerized hormone-binding domain of a guanylyl-cyclase-coupled receptor. *Nature 406*, 101−104.
(50) Ogawa, H., Qiu, Y., Ogata, C. M., and Misono, K. S. (2004) Crystal structure of hormone-bound atrial natriuretic peptide receptor extracellular domain: Rotation mechanism for transmembrane signal transduction. *J. Biol. Chem. 279*, 28625−28631.
(51) Duda, T., and Sharma, R. K. (2005) Two membrane juxtaposed signaling modules in ANF-RGC are interlocked. *Biochem. Biophys. Res. Commun. 332*, 149−156.
(52) Joubert, S., Jossart, C., McNicoll, N., and De Léan, A. (2005) Atrial natriuretic peptide-dependent photolabeling of a regulatory ATP-binding site on the natriuretic peptide receptor-A. *FEBS J. 272*, 5572−5583.
(53) 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.
(54) Duda, T., Yadav, P., Jankowska, A., Venkataraman, V., and Sharma, R. K. (2001) Three dimensional atomic model and experimental validation for the ATP-regulated module (ARM) of the atrial natriuretic factor receptor guanylate cyclase. *Mol. Cell. Biochem. 217*, 165−172.

(55) 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.
(56) Lohmann, S. M., Vaandrager, A. B., Smolenski, A., Walter, U., and De Jonge, H. R. (1997) Distinct and specific functions of cGMP-dependent protein kinases. *Trends Biochem. Sci. 22*, 307−312.
(57) Pfeifer, A., Ruth, P., Dostmann, W., Sausbier, M., Klatt, P., and Hofmann, F. (1999) Structure and function of cGMP-dependent protein kinases. *Rev. Physiol., Biochem. Pharmacol. 135*, 105−149.
(58) MacFarland, R. T., Zelus, B. D., and Beavo, J. A. (1991) High concentrations of a cGMP-stimulated phosphodiesterase mediate ANP-induced decreases in cAMP and steroidogenesis in adrenal glomerulosa cells. *J. Biol. Chem. 266*, 136−142.
(59) Côté, M., Payet, M. D., Rousseau, E., Guillon, G., and Gallo-Payet, N. (1999) Comparative involvement of cyclic nucleotide phosphodiesterases and adenylyl cyclase on adrenocorticotropin-induced increase of cyclic adenosine monophosphate in rat and human glomerulosa cells. *Endocrinology 140*, 3594−3601.