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The glycine residue of ATP regulatory module in receptor guanylate cyclases that is essential in natriuretic factor signaling

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Atrial natriuretic factor (ANF) and C-type natriuretic peptide (CNP)-activated guanylate cyclases are single-chain transmembrane-spanning proteins, containing both ligand binding and catalytic activities. In both proteins, ligand binding to the extracellular receptor domain activates the cytosolic catalytic domain, generating the second messenger cyclic GMP. Obligatory in this activation process is an ATP-dependent step. ATP directly binds to a defined ATP-regulatory module (ARM) sequence motif in the cyclases and through ARM bridges the events of ligand binding and signal transduction. These ARM sequence motifs are respectively represented by Gly^{503}-Xa-Gly^{505}-Xa-Xa-Xa-Gly^{509} and Gly^{499}-Xa-Xa-Xa-Gly^{503} in the case of ANF receptor guanylate cyclase (ANF-RGC) and CNP receptor guanylate cyclase (CNP-RGC). Through genetic remodeling techniques, we now show that ARM-Gly^{505} in ANF-RGC and the corresponding ARM-Gly^{499} in CNP-RGC are critical for ANF and CNP signaling, and other ARM-Gly residues have minimal effect in the respective signaling processes.

Guanylate cyclase; ATP-regulatory module; Atrial natriuretic factor receptor; Type C natriuretic peptide receptor

1. INTRODUCTION

Atrial natriuretic factor (ANF) and brain natriuretic peptide (BNP) are the two members of the family of structurally related natriuretic peptides that regulate hemodynamics of the physiological processes of diuresis, water balance and blood pressure ([1]; reviewed in [2–4]). The third known member of this family is C-type natriuretic peptide (CNP), which in contrast to ANF and BNP, is far less effective in causing diuresis and lowering of blood pressure [5]. CNP is somewhat selectively predominant in the central nervous system [5,6], hence it is possible that it acts as a neurotransmitter and/or participates in the centrally-related fluid secretion and blood pressure regulation. One important second messenger of these hormones is cyclic GMP (reviewed in [2–4]). The receptors for ANF (ANF-RGC) [7–13] and CNP (CNP-RGC) [14,15] have been characterized; both are guanylate cyclases and are structurally similar. Topographical models of these receptor proteins indicate similarity in that both contain a single membrane-spanning helical domain which divides the protein into two roughly equal portions, the N-terminal extracellular and the C-terminal intracellular; the receptor domain lies in the extracellular portion while the intracellular portion contains two domains, the one adjacent to the transmembrane is termed 'kinase-like' domain due to its sequence similarity to the tyrosine kinase family, and the C-terminal region contains the catalytic domain [16]. This topographical arrangement for the ANF-RGC protein is supported by site-directed [10,17] and deletion-mutagenesis studies [16,17], in which the truncated-receptor cyclase showed no ANF-binding and ANF-dependent cyclase activities.

Studies with ANF-RGC and CNP-RGC show that the mere ligand binding to the receptor domain is not enough to maximally stimulate the cyclase activity [18,19]; obligatory in this activation process is an intervening step, which is regulated by ATP [20,21]. ATP binding causes an allosteric change in guanylate cyclase, bringing it to the activated-catalytic state. A defined ATP-regulatory module (ARM) of ANF-RGC with a sequence of Gly^{503}-Xa-Gly^{505}-Xa-Xa-Xa-Gly^{509} and a corresponding ARM sequence of CNP-RGC, Gly^{499}-Xa-Xa-Xa-Gly^{503}, are critical in ATP binding and amplification of the hormonal signal [22]. Sequence examination of these ARMs structural motifs reveals three interesting Gly-related patterns in ANF-RGC: (1) Gly-Xa-Gly; (2) Gly-Xa-Xa-Xa-Gly, and (3) Gly-Xa-Gly-Xa-Xa-Xa-Gly; and one pattern, Gly-Xa-Xa-Xa-Gly, in CNP-RGC, which is also shared by the ARM of ANF-RGC [22]. These structural features of ARM motifs raise a curious question regarding the role of individual glycine residues in the ATP-mediated event of ANF and CNP signal transduction processes.

The present study addresses this issue and shows that a single specific Gly-ARM residue suffices as being an ATP switch for both ANF and CNP signaling.

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2. MATERIALS AND METHODS

The 5-kDa CNP used in these studies was a 45 amino acid peptide, Ser-Gln-Asp-Ser-Ala-Phe-Arg-Ile-Gln-Glu-Arg-Leu-Arg-Asn-Ser-Lys-Met-Ala-His-Ser-Ser-Ser-Cys-Phe-Gly-Gln-Lys-Ile-Asp-Arg-Ile-Gly-Ala-Val-Ser-Arg-Leu-Gly-Cys-Asp-Gly-Leu-Arg-Leu-Phe; and ANF (rat, residues 8–33) was a 26 amino acid peptide, Arg-Arg-Ser-Ser-Cys-Phe-Gly-Gly-Arg-Ile-Asp-Arg-Ile-Gly-Ala-Gln-Ser-Gly-Leu-Gly-Cys-Asn-Ser-Phe-Arg-Tyr. These peptides were purchased from Peninsula Laboratories; GTP, cyclic GMP and bovine serum albumin were from Sigma; ATP was from Boehringer-Mannheim; [^{125}I]NaI was from ICN; cell culture media were from Gibco and restriction enzymes were purchased from USB and BioLabs.

CNP-RGC cDNA clone was isolated from the human retina cDNA library [15]; ANF-RGC (GCα-Dmut) was constructed from GCα cDNA [8]; ANF-RGCVal^{505}Asn^{506} was built as in [17], and ANF-RGCLeu^{503}Ser^{507} and CNP-RGCAla^{499} were prepared as in [22].

Other mutants corresponding to the ARM sequences of ANF-RGC and CNP-RGC were constructed as schematically represented in Fig. 1 and are described below: [M1] ANF-RGCΔTyr^{508} (Tyr^{508}-minus); [M2] ANF-RGCAla^{509} (Gly^{509}→Ala); [M3] ANF-RGCAla^{503}Ala^{509} (Gly^{503}→Ala and Gly^{509}→Ala); [M4] CNP-RGCAla^{503} (Gly^{503}→Ala).

Mutagenesis was performed on the 1.9 kb *SalI–XbaI* fragment of GCα cDNA or on the 1.85 kb *SalI–XbaI* fragment of CNP-RGC cDNA, subcloned individually into the pSelect-1 vector (Promega mutagenesis kit). The following primers, each corresponding to the indicated mutant, were used:

[M1] 5′-GGTGGTTAGCAGGGAGCCATTGGAGCCTCGCCCACT-3′
[M2] 5′-GGTTAGCAGGGAGGCATAATTGGAGCC-3′
[M3] 5′-AGCCGGCTGACCCTGAGTGCGCGAGGCTCCAA-3′
[M4] 5′-TGCGGGATCCAGTTACGCCTCGCTCATGAC-3′

The *Eco*RV–*XbaI* fragment excised from the pSelect-cDNA recombinants was used to replace the *Eco*RV–*XbaI* fragment of ANF-RGC or CNP-RGC cDNA, respectively.

All the mutated-recombinants were sequenced [23] to confirm their identities and correct ligations. The mutated-cDNAs were individually subcloned into the *XhoI–SmaI* or *XhoI–SacI* site of the pSVL vector to create pSVL-mutated cDNA expression constructs for ANF-RGC and CNP-RGC related mutants.

For expression studies, COS-7 cells (simian virus 40 (SV40)-transformed African Green Monkey kidney cells) were transfected with the expression vector by calcium phosphate technique [24]; 60 h after transfection, cells were washed with 50 mM Tris-HCl (pH 7.5)/10 mM $MgCl_2$ buffer, scraped into 2 ml of ice-cold buffer, homogenized, centrifuged for 15 min at 5,000 × g and washed with the same buffer. The pellet represented the crude membranes. In control experiments, the crude membranes prepared from cells transfected with the pSVL vector alone were used. The crude membranes were assayed for guanylate cyclase activity [12].

3. RESULTS AND DISCUSSION

Studies with ANF-RGC and CNP-RGC have shown that (1) each of these receptor cyclases contains an ARM sequence motif that is essential in the ATP-mediated occurrence that bridges the events of ligand binding and signal transduction; (2) this ARM sequence motif is represented by Gly^{503}-Xa-Gly^{505}-Xa-Xa-Xa-Gly^{509} in the case of ANF-RGC and by Xa^{497}-Xa-Gly^{499}-Xa-Xa-Xa-Gly^{503} in the case of CNP-RGC; (3) the core ARM sequence motif that is essential in both ANF and CNP signaling is Gly-Xa-Xa-Xa-Gly [22]. Analysis of these ARM sequence motifs reveals an intriguing glycine-related pattern Gly-Xa-Gly-Xa-Xa-Xa-Gly in ANF-RGC and Gly-Xa-Xa-Xa-Gly in CNP-RGC, raising a curious question regarding the individual role of these glycine residues in the ATP-regulated

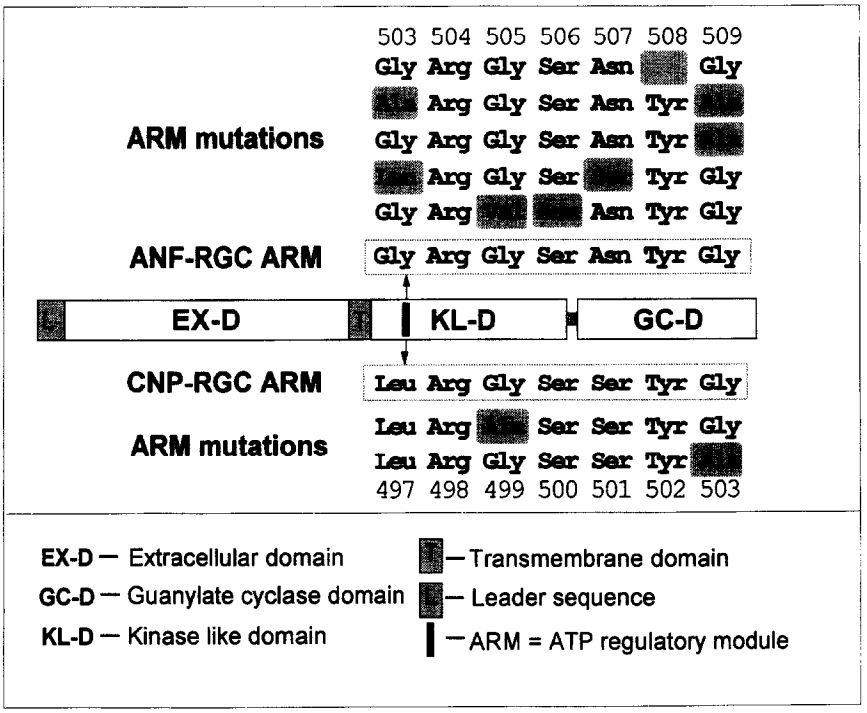

Fig. 1. Representation of ANF-RGC and CNP-RGC mutants. The mutated amino acid residues corresponding to the ARMs of ANF-RGC and CNP-RGC are indicated by shaded boxes. The appropriate changes in amino acid residues were created by site-directed mutagenesis as described in section 2.

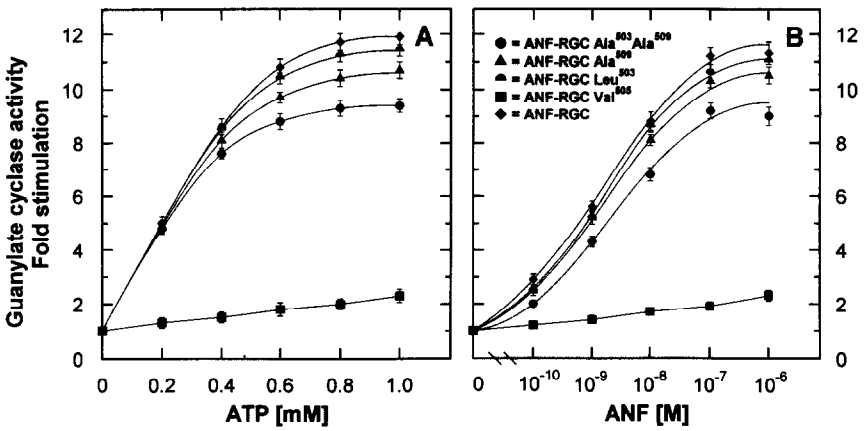

Fig. 2. Effect of ATP and ANF on particulate guanylate cyclase activity in membranes of COS-7 cells transfected with ANF-RGC cDNA or its mutants. Membranes, prepared as described in section 2, were assayed for guanylate cyclase activity using Mg^{2+} as a cofactor in the presence of (A) ANF (0.1 μM) with indicated additions of ATP and (B) in the presence of 0.8 mM ATP with varied additions of ANF. The experiments were done in triplicate and repeated three times. Results (mean ± S.D.) shown are from one typical experiment.

event of ANF and CNP signaling. This issue was addressed by using the combined tools of genetic remodeling and site-directed mutagenesis.

GCα is a wild type plasma membrane guanylate cyclase, that, with the exception of two amino acids, is structurally identical to ANF-RGC [8]. GCα cyclase activity is independent of ANF (and other natriuretic peptides), and GCα is not an ANF-receptor [8]. By oligonucleotide-directed mutagenesis, GCα was remodeled to create a mutant, GCα-Dmut, which is structurally and functionally identical to the cloned wild type ANF-RGC [8]. The individual glycine residues representing ARM of the genetically-constructed ANF-RGC were then changed through site-directed mutagenesis to the other indicated residues to create the following mutants: (1) **Leu**^{503}-Xa-Gly^{505}-Xa-Xa-Xa-Gly^{509} [22], (2) Gly^{503}-Xa-Gly^{505}-Xa-Xa-Xa-**Ala**^{509}, (3) **Ala**^{503}-Xa-Gly-Xa-Xa-Xa-**Ala**^{509}, and (4) Gly^{503}-Xa-**Val**^{505}-Xa-Xa-Xa-Gly^{509} [17]. The counterpart of the ANF-RGC ARM structural motif in CNP-RGC is Xa^{497}-Xa-Gly^{499}-Xa-Xa-Xa-Gly^{503}, indicating that there is a change in the sequence form – there being two glycine-surrounded domains (Gly-Xa-Gly-Xa-Xa-Xa-Gly) in ANF-RGC and only one (Gly^{499}-Xa-Xa-Xa-Gly^{503}) in CNP-RGC. To determine the role of Gly residues in CNP-RGC ARM sequence in CNP signaling, Gly^{503} and Gly^{499} residues were respectively substituted with alanine residues at equivalent positions to create the mutated structural motifs, Gly^{499}-Xa-Xa-Xa-**Ala**^{503} and **Ala**^{499}-Xa-Xa-Xa- Gly^{503}. The substituent residue alanine (or valine in the case of ANF-RGC ARM) is not charged and is very similar to glycine in its properties, it therefore should cause minimal change, if any, in the tertiary structure of the mutated-proteins.

The coding sequence of each of the mutated-proteins was introduced into an expression vector, pSVL, under the transcriptional control of the SV40 late promoter, which was then used to transfect COS-7 cells. The particulate fractions of these cells were appropriately treated and analyzed for the cyclase activities.

Earlier studies with ANF-RGC have indicated that maximal guanylate cyclase activity is attained with 0.1 μM ANF or CNP in the presence of 800 μM ATP [10,15,21], and neither ATP nor the hormones by themselves stimulate the guanylate cyclase activity [10,20,21]. In all experiments, therefore, the ATP-dependent cyclase activity was assessed in the presence of the 0.1 μM of ANF or CNP; and the hormone-dependent cyclase activity was measured in the presence of 800 μM ATP. These concentrations of the hormone and ATP are optimal in eliciting the maximal cyclase stimulation. The plasma membranes containing the mutated-proteins showed 30- to 50-fold higher basal activity than the membranes of control cells transfected with pSVL alone

Table I

Guanylate cyclase activity in membranes of transfected COS-7 cells

Transfection	Guanylate cyclase activity (pmol cyclic GMP/min/mg protein)
pSVL (control)	0.2
ANF-RGC	7.1 ± 1.0
ANF-RGCVal^{505}Asn^{506}	5.0 ± 0.4
ANF-RGCLeu^{503}Ser^{507}	8.1 ± 0.5
ANF-RGCAla^{509}	7.4 ± 0.6
ANF-RGCAla^{503}Asn^{509}	6.8 ± 0.3
ANF-RGCΔ Tyr^{508}	6.6 ± 0.4
CNP-RGC	10.1 ± 0.5
CNP-RGCAla^{499}	11.2 ± 0.6
CNP-RGCAla^{503}	8.2 ± 0.4

COS-7 cells were transfected with appropriate ANF-RGC or CNP-RGC mutant cDNAs in a pSVL expression vector. Membranes were prepared as described in section 2, and guanylate cyclase activity was determined.

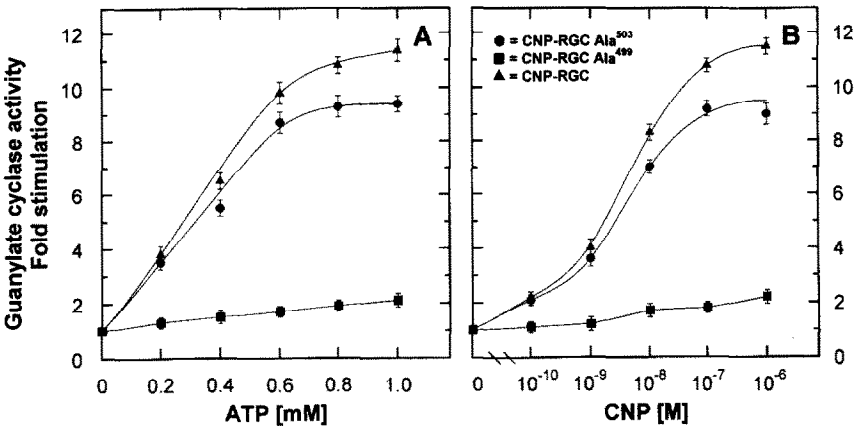

Fig. 3. Effect of ATP and CNP on particulate guanylate cyclase activity in membranes of COS-7 cells transfected with CNP-RGC cDNA or its mutants. Membranes, prepared as described in section 2, were assayed for guanylate cyclase activity using Mg^{2+} as a cofactor in the presence of (A) CNP (0.1 μM) with indicated additions of ATP and (B) in the presence of 0.8 mM ATP with varied additions of CNP. The experiments were done in triplicate and repeated three times. Results (mean ± S.D.) shown are from one typical experiment.

(Table I), indicating that the encoded protein is a guanylate cyclase.

Estimation of the role of each individual Gly residue comprising the ANF-RGC sequence motif Gly^{503}-Xa-Gly^{505}-Xa-Xa-Xa-Gly^{509} in the ATP-mediated ANF signaling was made by comparing the results of ANF-RGC with those of the corresponding mutants at their equivalent positions. In the presence of saturating amounts of ANF (0.1 μM), ATP in a dose-dependent fashion stimulated the cyclase activity of each of the mutants with the sequence motifs of Gly^{503}-Xa-Gly^{505}-Xa-Xa-Xa-Ala^{509}, Leu^{503}-Xa-Gly^{505}-Xa-Xa-Xa-Gly^{509} and Ala^{503}-Xa-Gly^{505}-Xa-Xa-Xa-Ala^{509}; the stimulation ranged between 9- and 11-fold at 800 μM ATP, and the concentration causing half-maximal response was between 300 to 400 μM (Fig. 2A). Likewise, the cyclase activity of these mutants in the presence of ATP was also stimulated by ANF in a dose-dependent manner (Fig. 2B); the EC_{50} concentration ranged between 1 and 4 nM; an excess of threefold stimulation occurring at ~0.1 nM ANF (Fig. 2B). These results indicate that the architectural integrity of the ARM sequence motifs – Gly^{503}- Xa-Gly^{505}-Xa-Xa-Xa-Gly^{509}, Gly^{503}-Xa-Gly^{505} and Gly^{505}-Xa-Xa-Xa-Gly^{509} – as such is nonessential in the ATP-mediated event of the ANF signaling; and they also reveal that similar is the case with the ARM-Gly residues positioned at 503 and 509. This interpretation was supported and extended by an expression study with the ANF-RGC mutant containing the site-directed mutation at Gly^{505}. This mutant with the ARM structural motif of Gly^{503}-Xa-Val^{505}-Xa-Xa-Xa-Gly^{509} did not respond to ATP in the presence of ANF (0.1 μM) nor to ANF in the presence of ATP (Fig. 2; [17]).

These results establish that the architectural pattern of the three glycine-related motifs of the ANF-RGC ARM sequence is not essential in the ATP-mediated event of ANF signaling; most significantly, they show that the most important signaling component that defines the ATP switch in ARM is the single Gly^{505} residue.

The counterpart of the ANF-RGC ARM-Gly^{505} residue in CNP-RGC ARM is Gly^{499}. To determine the role of this residue in the ATP-mediated event of CNP signaling, the comparative expression studies were conducted with the wild type CNP-RGC and its two mutants, Gly^{499}-Xa-Xa-Xa-Ala^{503} and Ala^{499}-Xa-Xa-Xa-Gly^{503}. CNP-RGC cyclase activity in the presence of saturating amounts of CNP (0.1 μM) was stimulated by ATP in a dose-dependent fashion with an EC_{50} of ~400 μM, and CNP in the presence of ATP (800 μM) caused the cyclase activation in a dose-dependent manner with an EC_{50} of 4 nM (Fig. 3); significant stimulation occurring below 0.1 nM CNP (Fig. 3B). A similar pattern of ATP and CNP stimulation of the cyclase activity was observed with the mutant Gly^{499}-Xa-Xa-Xa-Ala^{503}, but in complete contrast, the ATP-dependent hormone signaling was impaired in the mutant, Ala^{499}-Xa-Xa-Xa-Gly^{503}, indicating Gly^{499} is essential in CNP signaling.

It is thus concluded that the architectural pattern of the glycine-related CNP-RGC ARM sequence motif, like ANF-RGC sequence motifs, is also not essential in the ATP-mediated event of CNP signaling; and again in parallel to the results obtained with the ANF-RGC ARM, the critical ATP switching component of the CNP-RGC ARM is the single Gly^{499} residue.

A previous study has shown that the ANF-RGC ARM-Gly^{505} and the corresponding CNP-RGC ARM-Gly^{499} residues are critical in direct ATP binding [17,22]. In view of the present results indicating that the same Gly residues constitute the critical ATP signaling components of the ARM residues of ANF-RGC and CNP-RGC, it is concluded that these residues act as direct ATP switches.

These studies do not prove, however, that the ANF-RGC ARM-Gly residue 505 and CNP-RGC ARM-Gly

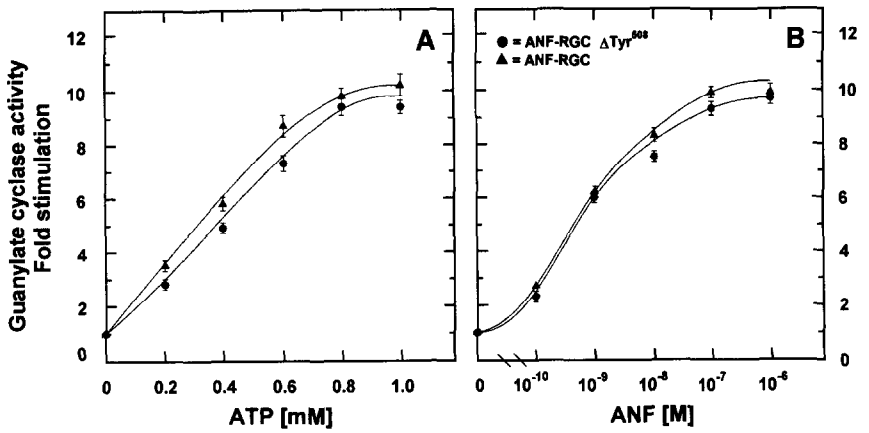

Fig. 4. Effect of ATP and ANF on particulate guanylate cyclase activity in membranes of COS-7 cells expressing ANF-RGC or ANF-RGC⊿Tyr^{508}. Membranes, prepared as described in section 2, were assayed for guanylate cyclase activity using Mg^{2+} as a cofactor in the presence of (A) ANF (0.1 μM) with indicated additions of ATP and (B) in the presence of 0.8 mM ATP with various concentrations of ANF. The experiments were done in triplicate and repeated twice. The experiments were done in triplicate and repeated three times. Results (mean ± S.D.) shown are from one typical experiment.

residue 499 are the direct ATP binding sites. But the ANF-RGC sequence motif – Gly^{503}-Xa-Gly^{505}-Xa-Xa-Xa-Gly^{509} – shows an interesting parallel with a conserved nucleotide binding sequence motif, Gly-Xa-Gly-Xa-Xa-Gly of protein kinases [25,26] and the GTP-binding protein, p21 [27]. The 'predicted nucleotide-binding protein model' indicates that the second glycine in the structural motif Gly-Xa-Gly-Xa-Xa-Gly is crucial in the direct GTP binding to the p21 protein [27], as also appears to be true in the case of ANF-RGC signaling. But this parallel is not total, because the first Gly-related structural motif (Gly-Xa-Gly) is absent in the CNP-RGC ARM motif Xa^{497}-Xa-Gly^{499}-Xa-Xa-Gly. In this case Gly^{499} is functionally equivalent to the middle glycine representing the structural motifs of ANF-RGC and the p21. Similarly, there does not appear to be a complete parallel in reference to the structural motif Gly-Xa-Xa-Xa-Gly comprising the ARMs of ANF-RGC and CNP-RGC with those of the protein kinases Gly-Xa-Xa-Gly, as is evident from the results obtained with the ANF-RGC mutant, Gly^{503}-Xa-Gly^{505}-Xa-Xa-Gly (ANF-RGC⊿Tyr^{508}): This mutant is responsive to the ATP-dependent hormone stimulation (Fig. 4). Thus, these structural parallels of protein kinases and GTP-binding proteins with those of the ATP-responsive membrane guanylate cyclases may merely be reflective of their evolutionary linkage rather than functional. In any event these ATP studies have started to show a unique regulatory signal transduction feature of the natriuretic factor receptor cyclases: the single ARM Gly residue defines the ATP switch in hormone signaling.

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