Distinct inhibitory ATP-regulated modulatory domain (ARM_i) in membrane guanylate cyclases

Teresa DUDA, Rafal GORACZNIK and Rameshwar K. SHARMA*

The Unit of Regulatory and Molecular Biology, Department of Cell Biology, University of Medicine and Dentistry of New Jersey, Stratford, NJ 08084, U.S.A.

Depending upon the cofactors Mg^{2+} or Mn^{2+} , ATP stimulates or inhibits the signal transduction activities of the natriuretic factor receptor guanylate cyclases, ANF-RGC and CNP-RGC: there is stimulation in the presence of Mg^{2+} and inhibition in the presence of Mn^{2+} . A defined core ATP-regulated modulatory (ARM) sequence motif within the intracellular 'kinase-like' domain of the cyclases is critical for stimulation, but the mechanism of the inhibitory transduction process is not known. In addition, ATP inhibits the basal cyclase activity of a rod outer segment membrane guanylate cyclase (ROS-GC). The mechanism of this inhibitory transduction process is also not known. These issues have been addressed in the present investigation through a program of deletion mutagenesis/expression studies of the cyclases. The study shows that the ATP-mediated inhibitory

transduction processes of the natriuretic factor receptor cyclases and of ROS-GC are identical. The ATP-regulated inhibitory domain of all these cyclases resides within the C-terminal segment of the cyclase. This domain is in a different location from the one representing the ATP-stimulatory ARM. The identification of the inhibitory domain in the C-terminal segment of the cyclase indicates that this segment is composed of two separate domains: one representing a catalytic cyclase domain and the other an ATP-regulated inhibitory (ARM_i) domain. These findings establish a novel ATP-mediated inhibitory transduction mechanism of the membrane guanylate cyclases which is distinct from that of its counterpart, the stimulatory ATP-mediated hormonal signal transduction mechanism. Thus, they define a new paradigm of guanylate cyclase-linked signal transduction pathways.

INTRODUCTION

Studies in the 1960s recognized two functional features that distinguished the guanylate cyclase enzyme from the adenylate cyclase. First, unlike adenylate cyclase activity, the preferred cofactor of which was Mg^{2+} , guanylate cyclase had an absolute dependence on Mn^{2+} . The enzyme activity was barely detectable when Mg^{2+} was substituted for Mn^{2+} [1–4]. Secondly, the guanylate cyclase activity was inhibited by physiological concentrations of ATP [1], the substrate of adenylate cyclase; but physiological concentrations of GTP, the counterpart of ATP, stimulated the adenylate cyclase activity. In these studies the cyclase response to ATP was observed in the presence of Mn^{2+} . At the time of these studies two separate classes of guanylate cyclase (membrane-bound and soluble) were not recognized, and it was not known that there are multiple forms of membrane guanylate cyclases. Studies in the 1980s provided kinetic evidence that clearly demarcated the particulate and soluble forms of guanylate cyclases [5,6] and showed that both forms were ATP-inhibited [7]. Again in these studies Mn^{2+} was used as a cofactor, and the identity of any pure membrane guanylate cyclase was not yet established. With the characterization of the first membrane guanylate cyclase (ANF-RGC) [8–13], which was also an atrial natriuretic factor (ANF) receptor, it was established that, depending upon the cofactors Mg^{2+} or Mn^{2+} , there is stimulation or inhibition of the hormone-dependent cyclase activity by ATP; stimulation in the presence of Mg^{2+} [14–16] and inhibition in the presence of Mn^{2+} [17,18]. With Mg^{2+} as cofactor, neither ATP nor ANF alone stimulated the cyclase activity. It was only when the two were together that the enzyme was activated [17,19–21]. Thus, ATP was obligatory for ANF signal transduction. These studies established three other important features of the cyclase. The first was that ATP bound ANF-RGC; hence, the ATP effect

was direct [17]. Secondly, in the presence of Mn^{2+} the basal cyclase activity was 10- to 30-fold higher than that in the presence of Mg^{2+} [7,17,22,23]. Thirdly, in the latter case the hormone-dependent cyclase activation was only minimal; however, it was ~2-fold compared with 3- to 10-fold in the presence of Mg^{2+} [24,25].

It is now known that ATP is also obligatory for the signal transduction activity of the other member of the natriuretic factor receptor guanylate cyclase subfamily, CNP-RGC [18,26]. This cyclase is also dually regulated by ATP [18]. The stimulatory ATP-mediated transduction mechanisms of both ANF-RGC and CNP-RGC are direct and identical [25,27,28]. A single amino acid residue in the defined ARM (ATP-regulated module) core sequence motif of the cyclases acts as a bimodal switch, critical to both the ligand binding and the signal transduction activities of the cyclases [29,30].

In contrast to the ATP-mediated stimulatory transduction process (see above), the mechanism of the ATP-mediated inhibitory transduction process is not known. Recent studies indicate that the inhibitory transduction mechanism is also operative in other membrane guanylate cyclase subfamilies, namely, enterotoxin receptor guanylate cyclase [31] and the photoreceptor guanylate cyclase, ROS-GC (rod outer segment guanylate cyclase) [32–34]. Unlike natriuretic factor receptor and enterotoxin/guanylin receptor guanylate cyclases, ROS-GC activity is not regulated by any known extracellular ligand [34]. Instead, it is indirectly modulated by both low [35,36] and high calcium levels [37,38]. Existence of the inhibitory transduction mechanism in ROS-GC, and in all known receptor guanylate cyclases (ANF-RGC, CNP-RGC and enterotoxin receptor guanylate cyclase) suggests a wider application of this transduction pathway in the membrane guanylate cyclase family. This transduction pathway may well be physiologically relevant. This

Abbreviations used: aa, amino acid; ANF, atrial natriuretic factor; ARM, ATP-regulated module; ROS-GC, rod outer segment guanylate cyclase.

* To whom correspondence and reprint requests should be addressed.

concept is supported by the observations that both forms of the cyclases, the surface receptor-regulated (enterotoxin receptor) [31] and the intracellularly regulated (ROS-GC) [39] form, are not only negatively regulated by ATP in the presence of the non-physiological ion, Mn^{2+} , but are also negatively regulated in the presence of the physiologically relevant ion, Mg^{2+} .

The present study shows that the ATP-regulated inhibitory transduction pathway in the natriuretic factor receptor cyclase family and in ROS-GC is identical. The ATP-regulated negative domain (ARM_1) is distinct from the stimulatory ARM domain (ARM_2). The ARM_1 domain is located within the C-terminal segment of the cyclase at a region separate from the catalytic domain. Thus, the C-terminal segment is composed of at least two functional domains: one representing the catalytic domain and the other the ARM_1 .

EXPERIMENTAL

Materials

ANF (rat, residues 8–33) and CNP-53 (porcine and rat) were purchased from Peninsula Laboratories; the mutagenesis kit and restriction enzymes were from Promega Corp.; GTP, cyclic GMP and BSA were purchased from Sigma; ATP was from Boehringer-Mannheim; $Na^{125}I$ was from ICN; cell culture media were from BioWittaker; ANF-RGC cDNA was constructed from GC α [24]; CNP-RGC cDNA was cloned from human retina [26] and ROS-GC cDNA from bovine retina [34].

Construction of mutants

The following segments of ANF-RGC, CNP-RGC and ROS-GC were truncated to construct their mutants: ext^- : ANF-RGC, amino acids (aa) 8–442; CNP-RGC, aa 8–430; ROS-GC, aa 8–408; kin^- : ANF-RGC, aa 473–745; CNP-RGC, aa 467–740; ROS-GC, aa 447–730. To construct the C-terminal ROS-GC mutant, C-324, the entire predicted ext (aa 8–408) and the predicted kin (aa 447–730) regions of the cyclase were deleted. The resulting mutant contained the N-terminal leader sequence, the predicted transmembrane domain and the C-terminal (aa 731–1054) segment. All these mutants are schematically represented in Figure 1.

To delete the ext and kin domains, two *HpaI* restriction sites were created in the cyclase DNAs. In the former case, the restriction sites were positioned at nucleotides 421 and 1721 in ANF-RGC, 370 and 1630 in CNP-RGC and 240 and 1443 in ROS-GC; and in the latter case, they were at the respective nucleotide positions 1819 and 2537, 1747 and 2570, and 1557 and 2412. The *HpaI* fragments were excised and the remaining cyclase parts were religated.

The C-324 mutant was constructed by creating the *BglII* restriction site at the nucleotide positions 1557 and 2408 in the ext^- mutant of ROS-GC. Excision of the 851 bp fragment and religation of the remaining cyclase parts resulted in deletion of the kinase-like domain, aa 447–730.

Identities and the ligations of the constructs were confirmed by sequence analysis. These constructs were then subcloned into pSVL or pcDNA3 expression vector.

Expression studies

COS-7 cells [simian virus 40 (SV40)-transformed African Green Monkey kidney cells] maintained in Dulbecco's modified Eagle's medium with penicillin/streptomycin and supplemented with 10% (v/v) fetal bovine serum (Sigma) were transfected with appropriate expression constructs using a calcium phosphate coprecipitation technique [40]. At 60 h post-transfection, cells were

Transfection	Domain Structure	Basal GC Activity (pmol cGMP/min/mg protein)
ANF-RGC		110 ± 8
ANF-RGC ext^-		107 ± 9
ANF-RGC kin^-		178 ± 14
CNP-RGC		105 ± 10
CNP-RGC ext^-		112 ± 11
CNP-RGC kin^-		170 ± 13
ROS-GC		230 ± 18
ROS-GC ext^-		230 ± 17
ROS-GC kin^-		350 ± 19
C324		132 ± 11
pcDNA3		<1

Figure 1 Schematic representation of ANF-RGC, CNP-RGC, ROS-GC, deletion mutants, and their activities in COS particulate fractions

The predicted domains are denoted by ext , extracellular domain; tm , transmembrane domain; kin , area with tyrosine kinase sequence similarity; and cat , catalytic domain. The right-hand column of the Figure depicts the cyclase activity of these expressed proteins as measured in crude membranes of COS cells transfected with the respective cDNAs.

washed twice with 50 mM Tris/HCl (pH 7.5)/10 mM $MgCl_2$ buffer, scraped into 1 ml of the same buffer, homogenized, centrifuged for 15 min at 5000 g and washed three times with the same buffer. The pellet represented the crude membranes.

Guanylate cyclase assay

The crude membranes (0.5–1 μ g of protein) were assayed for guanylate cyclase activity [9]. Briefly, membranes were pre-incubated on ice with or without ATP (0–1 mM) in the assay system containing 10 mM theophylline, 15 mM phosphocreatine, and 20 μ g creatine kinase in 50 mM Tris/HCl, pH 7.5. The total assay volume was 100 μ l. Reaction was initiated by addition of the substrate solution containing 4 mM $MnCl_2$ (or $MgCl_2$) and 1 mM GTP. Incubation (37 °C, 10 min) was terminated by the addition of 900 μ l of 50 mM sodium acetate buffer, pH 6.25, followed by heating in a boiling water-bath for 3 min. The amount of cyclic GMP formed was determined by RIA [41].

RESULTS AND DISCUSSION

Expression of the parent guanylate cyclases and their mutants in transfected COS cells

ANF-RGC, CNP-RGC, ROS-GC and their respective mutants (Figure 1), i.e. ext^- (deleted extracellular domain), kin^- (deleted kinase-like domain), and C-324 (consisting of the 324-residue C-terminal segment of ROS-GC), were transiently expressed in COS cells. In all mutants, the leader sequence and the sequence of the predicted transmembrane domain were retained to ensure proper translocation of the expressed protein. This leader sequence is composed of 28 residues in ANF-RGC [24], 22 residues in CNP-RGC [26], and 56 residues in ROS-GC [34], and the respective transmembrane regions are represented by the residues

Figure 2 ATP effect on basal and hormone-dependent guanylate cyclase activity of ANF-RGC (A) and CNP-RGC (B)

COS cells were transfected with ANF-RGC or CNP-RGC cDNA and their membranes were assayed for guanylate cyclase activity in the presence or absence of 10⁻⁷ M ANF or CNP and indicated concentrations of ATP, as described in the Experimental section. Experiments were done in triplicate and repeated three times for reproducibility. Results shown represent means $\pm$ S.E.M. from one typical experiment. Insets: 10⁻⁷ M ANF- or 10⁻⁷ M CNP-dependent stimulation of (A) ANF-RGC and (B) CNP-RGC guanylate cyclase activity at indicated concentrations of ATP. The cyclase activity in the absence of hormone is taken as unity for each concentration of ATP tested.

446–462, 434–456 and 412–436 [24,34]. The deleted fragments in ext⁻ mutants corresponded to the residues Val⁸ to Val⁴⁴² in ANF-RGC, Val⁸ to Pro⁴³⁰ in CNP-RGC and Leu⁸ to Gly⁴⁰⁹ in ROS-GC. In kin⁻ mutants, they corresponded to the fragments Ser⁴⁷³ to Gly⁷⁴⁵ in ANF-RGC, Ser⁴⁶⁷ to Val⁷⁴⁰ in CNP-RGC and Ser⁴⁴⁷ to Ile⁷³⁰ in ROS-GC. In the C-324 mutant, the entire ext and kin segments of ROS-GC were deleted. It is noted that in the kin⁻ mutants of ANF-RGC and CNP-RGC, the respective ARMs sequence motifs, i.e. aa 503–509 and aa 499–503, were also deleted. Thus, these mutants were ARM⁻kin⁻.

The mutants were transiently expressed in COS cells, and their functional configurational arrangements in the plasma membranes were confirmed by the 100- to 300-fold expression of their basal cyclase activity over the control cells transfected with vector only (Figure 1), and by their proper responsiveness to their appropriate stimuli.

ATP-dependent inhibition of the guanylate cyclase activity

The optimal membrane basal guanylate cyclase activity is observed at 3–5 mM Mn²⁺ with 1 mM GTP [5], and the hormone-

Figure 3 ATP effect on ROS-GC cyclase activity

COS cells were transfected with ROS-GC cDNA and their membranes were assayed for guanylate cyclase activity at various ATP concentrations in the presence of Mn²⁺ (○) or Mg²⁺ (●) as the cofactor. Experiments (in triplicate) were repeated three times, although data depicted are from one representative experiment.

dependent maximal cyclase activation of ANF-RGC and CNP-RGC is observed respectively at 10⁻⁷ M ANF and 10⁻⁷ M CNP [24,26]. To assess the effect of ATP on the basal and the hormone-dependent cyclase activity, all experiments were therefore conducted with the plasma membrane fractions of appropriately transfected COS cells containing 4 mM Mn²⁺ and 1 mM GTP in the presence, or absence, of 10⁻⁷ M ANF or 10⁻⁷ M CNP. Under these conditions, maximal hormone stimulation of approximately 2-fold above the basal level of the cyclases is achieved [17] (Figure 2). ATP apparently inhibited both the basal and the hormone-dependent cyclase activity of ANF-RGC and CNP-RGC in a dose-dependent manner, with a half-maximal concentration of $\sim$ 400 μ M (Figure 2). Similar patterns of basal and hormone-dependent cyclase activity inhibition were observed upon substitution of ATP with its non-dissociable analogue adenosine 5'-[γ -thio]triphosphate (ATP[S]) (results not shown). These results with recombinant cyclases are very similar to those obtained earlier with ANF in rat testicular plasma membranes [17] and with CNP in AT-t20 cell membranes [18]. The present studies also show, however, that when the basal cyclase-inhibited values are compared with those where the hormone was also added, there is no change in the fold stimulation caused by the hormone (Figure 2: insets), indicating that ATP selectively inhibits the basal cyclase activity without influencing the hormone-dependent cyclase activation. Thus, the mechanisms of ATP-mediated inhibition and stimulation of the guanylate cyclases differ in two important aspects: (1) in the inhibited mechanism, there is inhibition of the basal guanylate cyclase only, and ATP does not influence the ability of the cyclase to respond to the hormone; (2) in the stimulated-mechanism, there is only the hormone-dependent stimulation of the catalytic cyclase activity, and the basal guanylate cyclase activity is not affected by ATP [17,21,26]. This suggests that the inhibitory and the stimulatory domains of the cyclases are distinct and segregated from each other. To differentiate between these two domains, they are now being referred to as ARM_i and ARM_s.

Like the natriuretic factor receptor guanylate cyclases, ATP also inhibits the basal cyclase activity of ROS-GC (Figure 3). But in this case, the inhibition occurs regardless of the presence of either cofactor, Mg²⁺ or Mn²⁺ (Figure 3). It is noteworthy that

Figure 4 Kinetics of the ATP-dependent inhibition of ANF-RGC (A), CNP-RGC (B) and ROS-GC (C) guanylate cyclase activity

COS cell membranes expressing ANF-RGC, CNP-RGC, or ROS-GC were incubated with increasing concentrations of ATP (○, 0 mM ATP; ●, 0.25 mM; ▲, 0.5 mM; ★, 1 mM) and assayed for guanylate cyclase activity at indicated concentrations of GTP (0.25 mM, 0.5 mM, 0.75 mM and 1 mM) and 4 mM Mn^{2+} as a cofactor. Results were analysed using a linear regression algorithm and values for one typical experiment are presented as a Lineweaver–Burk plot. Error bars are within the size of the symbols. [S] = mM GTP; V = pmol of cGMP/mg of protein per min.

the stimulatory ARM_s sequence motif, present in ANF-RGC and CNP-RGC, is missing in ROS-GC. Thus, it is predicted that the ATP-mediated stimulatory signal transduction pathway will be absent in this cyclase. But the inhibitory transduction pathway might be similar in the two classes of cyclases. This hypothesis was supported by the following kinetic studies conducted with the recombinant cyclases, was reinforced by the deletion-mutant studies, and ultimately, was proven by the expression studies with the C-324 ROS-GC mutant. Barring its transmembrane domain, this mutant is exclusively composed of the C-terminal 324 residues of the cyclase.

Three incremental concentrations of ATP, i.e. 0.25 mM, 0.5 mM and 1 mM, were used to compete with the increasing concentrations of substrate (Mn^{2+} -GTP) and kinetics of the cyclase inhibition were analysed by Lineweaver–Burk plots (Figure 4). ATP inhibited catalytic activity of each of the cyclases (ANF-RGC, CNP-RGC and ROS-GC; Figures 2 and 3). In all cases, the mechanism of inhibition was non-competitive (Figure 4). These results support the earlier interpretation that modes of ATP inhibition of ROS-GC and of the natriuretic factor receptor cyclases are identical. The cyclase domains, comprising the catalytic and the ATP inhibitory domains, are distinct. Based on the kinetic evidence, a similar interpretation was made for these domains in the case of enterotoxin receptor guanylate cyclase [31]. This explanation was further supported by the expression

Figure 5 Kinetics of the ATP-dependent inhibition of ANF-RGC, CNP-RGC or ROS-GC deletion mutants (ext⁻ and kin⁻)

Membranes of COS cells expressing the indicated deletion mutants were incubated with incremental concentrations of ATP (○, 0 mM ATP; ●, 0.25 mM; ▲, 0.5 mM; ★, 1 mM) and assayed for guanylate cyclase activity using various concentrations of GTP (0.25 mM, 0.5 mM, 0.75 mM and 1 mM) and 4 mM Mn^{2+} . Results were analysed using a linear regression algorithm and values for one typical experiment are presented as a Lineweaver–Burk plot. Error bars are within the size of the symbols. [S] = mM GTP; V = pmol of cGMP/mg of protein per min.

studies of the cyclase mutants described below which reveal a distinct ATP_i domain at the C-terminal region of the ROS-GC.

Localization of the negatively modulated cyclase domain

To localize the negatively modulated ATP domain in ANF-RGC, CNP-RGC and ROS-GC, expression studies were conducted with the defined deletion mutants of these cyclases. To ensure that each of these mutants is properly translocated to the cell membrane and embodies the functional configuration, the construct retained the peptide leader sequence and the presumed transmembrane domain of the parent cyclase. Figure 1 shows that the basal guanylate cyclase activity of all the constructs was 100- to 300-fold higher than control cells transfected with vector only. Thus, all the cyclases were appropriately expressed.

The membrane fractions were appropriately treated, and the ATP-dependent kinetics of the cyclases were studied under identical conditions as described for their parent cyclases (see above): three incremental concentrations of ATP were used to compete with the increasing concentrations of the substrate (Mn^{2+} -GTP). Analysis of the enzyme kinetics by Lineweaver–Burk plots indicated that ATP inhibited the cyclase activity of each mutant by a non-competitive mechanism (Figure 5), similarly to their parent cyclases (Figure 4). These results indicated

Figure 6 Kinetics of the ATP-dependent inhibition of C-324 mutant

Membranes of COS cells expressing C-324 mutant (ROS-GCext⁻kin⁻) were assayed for guanylate cyclase activity at increasing concentrations of ATP (○, 0 mM ATP; ●, 0.25 mM; ▲, 0.5 mM; ★, 1 mM), various concentrations of GTP (0.25 mM, 0.5 mM, 0.75 mM and 1 mM) and in the presence of 4 mM Mn²⁺ (A) or 4 mM Mg²⁺ (B). Results were analysed using a linear regression algorithm and values for one typical experiment are presented as a Lineweaver-Burk plot. Error bars are within the size of the symbols. [S] = mM GTP; V = pmol of cGMP/mg of protein per min.

that the extracellular and kinase domains of these cyclases are not involved in the ATP-mediated inhibitory response. Direct results with the C-324 mutant confirmed this interpretation and further demonstrated that the inhibitory domain is localized in the C-terminal fragment of each of the cyclases (Figure 6).

Thus, the ATP-mediated inhibitory mechanism in ANF-RGC, CNP-RGC and ROS-GC is identical, and is mediated by the ARM_i domain located in the C-terminal of the cyclase. The ARM_i domain is distinct from the catalytic cyclase domain. Moreover, the ARM_s and the ARM_i sequence motifs of ANF-RGC and CNP-RGC are also distinct.

In conclusion, this study establishes the independent existence of an ARM_i domain that is involved with a novel ATP-mediated inhibitory transduction mechanism of membrane guanylate cyclases. Localization of this domain in the C-terminal region of the cyclases, furthermore, indicates that this C-terminal region is composed of at least two functional domains: one representing the ARM_i and the other being catalytic.

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