Vol. 209, No. 1, 1995 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
April 6, 1995 Pages 286-292

ATP BIMODAL SWITCH THAT REGULATES THE LIGAND BINDING AND SIGNAL TRANSDUCTION ACTIVITIES OF THE ATRIAL NATRIURETIC FACTOR RECEPTOR GUANYLATE CYCLASE

Teresa Duda and Rameshwar K. Sharma*

The Unit of Regulatory and Molecular Biology, Pennsylvania College of Optometry, 1200 West Godfrey Avenue, Philadelphia, PA 19141

Received March 2, 1995

SUMMARY: Atrial natriuretic factor (ANF)-dependent guanylate cyclase (ANF-RGC) is a single-chain transmembrane-spanning protein, containing both ANF binding and catalytic cyclase activity. ANF binding to the extracellular receptor domain activates the cytosolic catalytic domain, generating the second messenger cyclic GMP. Obligatory in this activation process is an intervening step regulated by the ATP binding to the cyclase. This is a signal transduction step that bridges the events of ligand binding and cyclase activation. A defined structural motif (Gly^{503}-Xa-Gly^{505}-Xa-Xa-Xa-Gly^{509}), termed ATP regulatory module (ARM), is critical for this step. The present study shows that the ARM-Gly^{505} residue acts as an ATP bimodal switch in regulating both the ligand binding and signal transduction activities of ANF-RGC, thus representing a critical site to turn the hormone signal on and off.

Atrial natriuretic factor (ANF) and the type C natriuretic peptide (CNP) 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). One important second messenger of these hormones is cyclic GMP whose generation takes place by the guanylate cyclases which are also the receptors for ANF and CNP (reviewed in: 5). These guanylate cyclases are respectively termed as ANF-RGC and CNP-RGC. The predicted 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 domain and the C-terminal intracellular domain; 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 (6,7).

*To whom correspondence should be addressed at The Unit of Regulatory and Molecular Biology, Pennsylvania College of Optometry, 1200 West Godfrey Avenue, Philadelphia, Pennsylvania 19141. Fax (215) 276-6081.

0006-291X/95 $5.00

This topographical arrangement for the ANF-RGC (8,9) and the CNP-RGC (10) proteins is supported by site-directed and deletion mutagenesis studies (9-12), which show that the ligand binding and catalytic cyclase activities occur at two opposite ends of the protein. The binding activity resides at the N-terminal extracellular region and the catalytic cyclase activity at the carboxyl end; and the ANF-RGC residue Leu^{364} residing in the extracellular region is critical for the ANF-binding activity (13), and the similarly located CNP-RGC residue Glu^{332} is critical for the CNP-binding activity (10).

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 (14,15); obligatory in this activation process is an intervening step, which is regulated by ATP (6,16). This step occurs by the direct binding of ATP to the guanylate cyclase (8,16,17). Within the "kinase-like" domain positioned at 40 amino acid residues distal to the last carboxy-terminal amino acid residue of the transmembrane domain is a defined ATP-regulatory module (ARM) of ANF-RGC with a sequence of Gly^{503}-Xa-Gly^{505}-Xa-Xa-Xa-Gly^{509} (9) and a corresponding ARM sequence of CNP-RGC, Gly^{499}-Xa-Xa-Xa-Gly^{503} (18), which are critical in the ATP binding and amplification of the hormonal signals. Thus, ARM is a critcal signal transducer site of the cyclases.

Analysis of the ANF-RGC and CNP-RGC ARMs by site-directed mutagenesis has further indicated that the single Gly^{505} residue of the ANF-RGC ARM and the Gly^{499} residue of the CNP-RGC ARM are most critical for the ATP-dependent signal transduction step, and the ANF-RGC ARM residues 503, 506, 507, 508, 509 have no effect on the ANF signaling (Duda et al., 1993b).

The present study shows that the ARM-Gly^{505} residue exhibits a dual control over the ATP-dependent regulation of ANF-RGC. It amplifies its signal transduction activity and inhibits its ligand binding activity. This raises the possibility that this residue acts as an ATP bimodal switch to turn the ANF signal on and off.

METHODS

Expression studies. COS-7 cells (simian virus 40-transformed African green monkey kidney cells), maintained in Dulbecco's modified Eagle's medium with penicillin, streptomycin, and 10% fetal bovine serum, were transfected with ANF-RGC or its ARM-505 mutant cDNA ligated into pSVL expression vector using the calcium phosphate coprecipitation technique (9,13). COS-7 cells transfected with pSVL vector alone were used as controls.

Plasma membrane fraction. Sixty hours after transfection, cells were washed twice with phosphate-buffered saline, pH 7.5, scraped into 50 mM Tris pH 7.5/10 mM $MgCl_2$ buffer, homogenized, centrifuged for 15 min at 5,000 x g. The pellet represented the crude membranes.

Binding assays. The crude membranes were suspended in the binding buffer consisting of 25 mM HEPES, pH 7.5, 2 mg/ml BSA, 150 mM NaCl, 4 mM $MgCl_2$ and 1 mM PMSF. The plasma membranes (10 - 20 μg) were incubated with [^{125}I]ANF (rat, residues 8-33; 150,000 - 200,000 cpm, specific activity 200 Ci/mmol) and ATP or other

nucleotides (10^{-6} - 10^{-3} M) for 90 min at 25°C. Incubation was terminated by diluting the incubation mixture with 3 ml of ice-cold washing buffer (25 mM HEPES, pH 7.5 and 150 mM NaCl) followed by immediate filtration through Whatman GF-C glass fiber filters (presoaked in 0.3% polyethyleneimine) which were washed with 3 x 3 ml of ice-cold washing buffer and counted for radioactivity. Nonspecific binding was measured in the presence of 10^{-11} - 10^{-6} M nonradioactive ANF. Specific binding was calculated by subtracting the nonspecific radioactivity from the total radioactivity bound to the filters. Experiments were conducted mostly in triplicates and sometimes in duplicates, and repeated three times for reproducibility. The results were expressed as the mean value of one typical experiment

Guanylate cyclase assay. Guanylate cyclase activity was assayed in crude membranes as described earlier (13). Briefly, the reaction mixture (100 µl) contained 50 mM Tris-HCl (pH 7.5), 10 mM theophylline, 15 mM creatine phosphate, 20 U/ml creatine phosphokinase (250 U/mg protein) and ~2 µg membrane protein. The reaction was started by the addition of 1 mM GTP and 4 mM $MgCl_2$ (substrate), incubated at 37°C, and terminated by the addition of 0.9 ml of ice-cold 50 mM sodium acetate buffer (pH 6.25), followed by heating on a boiling water bath for 3 min. To determine the effect of ANF and/or ATP on cyclase activity, the membrane fraction was preincubated with or without ANF and/or ATP at appropriate concentrations for 10 min on ice prior to the addition of the substrate. The cyclic GMP formed was estimated by radioimmunoassay (19).

RESULTS AND DISCUSSION

Studies with ANF-RGC show that (1) this cyclase contains an ARM motif that is essential in the ATP-mediated step of bridging the events of ligand binding and cyclase activation (9); (2) sequence of the ARM motif is represented by Gly^{503}-Xa-Gly^{505}-Xa-Xa-Xa-Gly^{509} (9); (3) the ARM-Gly^{505} residue is critical for amplification of the signal transduction step (20).

ATP not only amplifies the hormone-dependent cyclase activity of ANF-RGC, it also inhibits its ligand binding activity (17,21). To determine the possibility that both of these activities may be mediated by the ARM-Gly^{505} residue of ANF-RGC, the membrane fractions of the COS-7 cells expressing ANF-RGC and its ARM-Gly^{505}-mutant were appropriately treated and analyzed for these activities.

ATP in a dose-dependent fashion inhibited the binding of ANF to ANF-RGC (Fig. 1A). The maximum inhibition was ~45%. Similarly, ATPγS, the nonhydrolyzable analog of ATP, inhibited the ANF binding activity of ANF-RGC, this inhibition was ~50% (Fig. 1A); the Ki values for ATP and ATPγS were similar, ~8 µM (Fig. 1A); App(NH)p also inhibited the ANF binding activity, *albeit* at a lower level (Table 1).

ADPβS, the nonhydrolyzable analog of ADP, and GTPγS caused only a marginal (15 to 20%) inhibition of the ANF binding to ANF-RGC (Table 1), indicating ATP specificity on the ligand binding activity of the cyclase.

It is, therefore, concluded that ATP negatively regulates the ligand binding activity of ANF-RGC.

Estimation of the role of the ARM-Gly^{505} residue of ANF-RGC in the ATP-mediated ANF binding activity was made by comparing the results of ANF-RGC with that of its ARM-

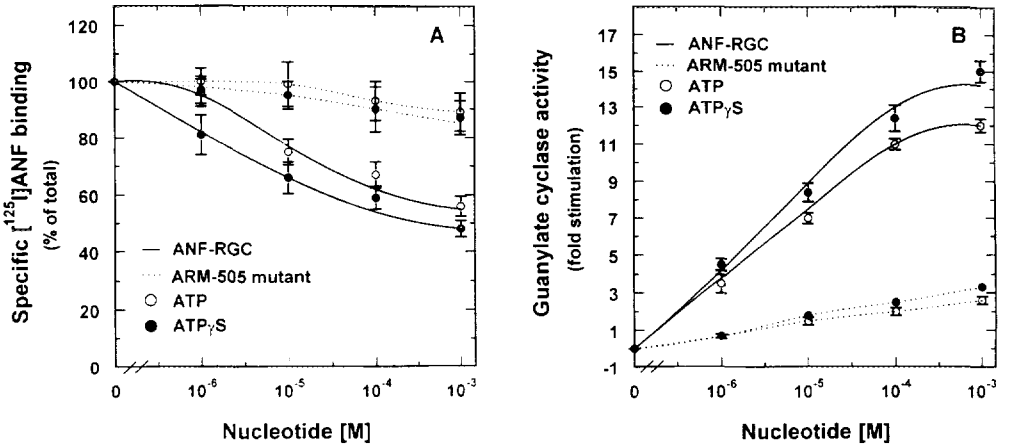

Fig. 1. ATP effect on ANF binding and ANF-dependent guanylate cyclase activity in membranes of COS cells expressing ANF-RGC or ARM-505 mutant. COS cells were transfected with ANF-RGC or its ARM-505 mutant cDNA in pSVL expression vector. Membranes were prepared as described under " Methods ". (A) membranes were incubated in a mixture containing [^{125}I]ANF (150,000 cpm, specific activity 200 Ci/mmol) and indicated concentrations of ATP or ATPγS. Nonspecific binding to the membranes was estimated in the presence of nonradioactive ANF (10^{-7} M). Specific binding was calculated by subtracting the nonspecific radioactivity from the total radioactivity bound to the filters. (B) membranes were assayed for guanylate cyclase activity as described under " Methods ". Experiments were done in triplicate and repeated three times. Data depicted ± SE are from one typical experiment.

Table 1

Effect of various nucleotides on the [^{125}I]ANF binding to the membranes of COS cells expressing ANF-RGC or its ARM-505 mutant.

Nucleotide (1 mM)	^{125}I-ANF bound (%)	
	ANF-RGC	ARM-505 mutant
none	100	100
ATP	57	90
ATPγS	50	82
App(NH)p	71	90
ADP	80	90
ADPβS	79	92
GTPγS	85	94

Membranes of COS cells transfected with ANF-RGC or its ARM-505 mutant cDNA were prepared as described under "**Methods**". The [^{125}I]ANF binding was measured as described in the legends to Figs. 1 and 2. Specific [^{125}I]ANF binding in the presence of the indicated nucleotides is expressed as percent of specific [^{125}I]ANF binding in the absence of the indicated nucleotides. Results shown are the mean of triplicate estimations from one typical experiment.

Gly^{505}-mutant. The ligand binding activity of this mutant, in contrast to its parent cyclase ANF-RGC (*vide supra*), was not significantly inhibited by ATP, or ATPγS (Fig. 1A).

It is thus concluded that the ARM-Gly^{505} residue of ANF-RGC is an essential ATP-modulatory component of the ligand binding activity.

To correlate the kinetics of the ligand binding and signal transduction steps, parallel experiments of signal transduction were conducted on the native cyclase and its mutant membranes, the membranes used in these experiments were the same as those used for the binding experiments described above. Earlier studies had indicated that maximal guanylate cyclase activity of ANF-RGC is attained with 0.1 μM of ANF in the presence of 800 μM ATP, and neither ATP nor ANF by itself stimulate the guanylate cyclase activity (8,16). In all signal transduction experiments, therefore, the ATP-dependent cyclase activity was assessed in the presence of the 0.1 μM of ANF.

ANF-RGC cyclase activity was stimulated by ATP or ATPγS in a dose-dependent fashion, the maximal cyclase response occurring at 800 μM ATP or ATPγS; ATPγS causing approximately 20% more stimulation than ATP: compare, 15-fold vs 12-fold (Fig. 1B). It is noteworthy that the EC_{50} value of ATP (or ATPγS) was approximately 6 μM (Fig. 1B), a value quite close to the one that causes half-maximal inhibition of the ligand-binding activity of ANF-RGC (Fig. 1A). This indicated that the ATP half-maximal concentration for the steps of signal transduction and receptor inactivation was roughly the same.

In complete contrast, the ATP-dependent signal transduction step was impaired in the ARM-Gly^{505}-mutant: the cyclase activity of the mutant was stimulated only 3-fold as compared to the 15-fold stimulation for the parent cyclase (Fig. 1B). This indicates that the Gly^{505} residue is essential in the ATP-regulated ANF signal transduction step, as established earlier (20).

Competitive binding studies indicated that the specific binding of [^{125}I]ANF to the ATP-treated and the untreated-cyclase was displaced by nonradioactive ANF in a dose-dependent fashion with an IC_{50} of ~0.4 nM in the absence of ATP; and with an IC_{50} of ~1 nM in the presence of ATP (Fig. 2A). This indicated that the ANF affinity for the ATP-treated cyclase was about 2.5-fold lower than that of the ATP-untreated cyclase, a conclusion supported by earlier studies (17,21). In contrast, the mutation in the ARM-505 residue eliminated the receptor transformation to the lower affinity form (Fig. 2B).

It is thus established that ATP converts the high affinity ANF receptor form to the low affinity form, and that ARM-505 residue plays a pivotal role in this transformation process.

These results demonstrate that the ARM-Gly^{505} residue is an essential residue for both ligand binding and signal transduction activities of the cyclase. Because the ARM-Gly^{505} residue is also critical in the direct ATP binding to ANF-RGC (20), the possibility

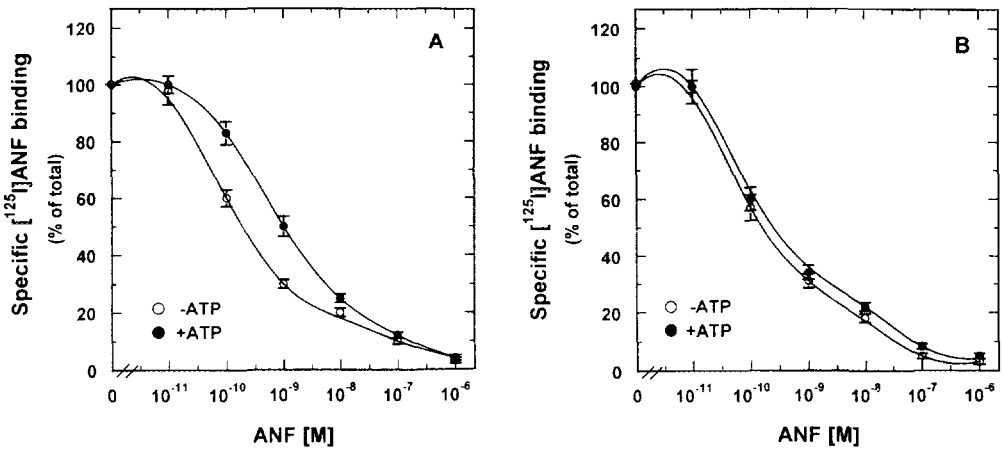

Fig. 2. Binding of [^{125}I]ANF to the membranes of COS cells expressing ANF-RGC (A) or its ARM-505 mutant (B) and specific displacement of bound [^{125}I]ANF by nonradioactive ANF. COS cells were transfected as described under "Methods", and their membranes were prepared as described in Fig. 1. Membranes were incubated with [^{125}I]ANF (150,000 cpm, specific activity 200 Ci/mmol) and indicated concentrations of nonradioactive ANF with or without 0.8 mM ATP and assayed for [^{125}I]ANF binding. Experiments were done in triplicate (or in duplicate in some cases) and repeated three times. Results from one typical experiment ± SE are shown.

arises that this residue acts as an ATP bimodal switch to turn the ANF signal on and off. Identification of this switch fills an important gap in the previously proposed "ANF-RGC multimodule signal transduction" model (5). This signaling model now envisions the following sequential events: (1) a signal is initiated by the binding of the hormone to the ligand binding module, Leu^{364} is a critical residue for this step (13); (2) there is a transmembrane migration of the binding signal; (3) upon binding to ANF-RGC ARM, ATP potentiates the signal (9); (4) the amplified signal is transduced at the catalytic site; and (5) the ATP-dependent transformation of the receptor to the low affinity form results in the dissociation of the hormone and termination of the signal. In both steps 3 and 5, ARM-Gly^{505} residue plays a pivotal role.

ACKNOWLEDGMENTS

This work was supported by grants from the National Institutes of Health (EY 10828, NS 23744) and the equipment grant from Pennsylvania Lions Eye Research Foundation.

REFERENCES

1. deBold, A. J., Bornstein, H. B., Veress, A. T., & Sonnenberg, H. (1981) *Life Sci* **28**, 89-94.
2. Needleman, P., Blaine, E. H., Greenwald, J. E., Michener, M. L., Saper, C. B., Stockmann, P. T., & Toulnay, H. E. (1989) *Annu. Rev. Pharm. Tox.* **29**, 23-54.

3. Brenner, B. M., Ballermann, B. J., Gunning, M. E., & Zeidel, M. L. (1990) *Physiological Rev.* ***70***, 665-699.
4. Rosenzweig, A., & Seidman, C. E. (1991) *Ann Rev Biochem* ***60***, 229-255.
5. Sharma, R. K., Duda, T., & Sitaramayya, A. (1994) *Amino Acids* ***7***, 117-127.
6. Chinkers, M., Garbers, D. L., Chang, M-S., Lowe, D. G., Chin, H., Goeddel, D. V., & Schulz, S. (1989) *Nature* ***338***, 78-83.
7. Chang, M-S., Lowe, D. G., Lewis, M., Hellmiss, R., Chen, E., & Goeddel, D. V. (1989) *Nature* ***341***, 68-72.
8. Marala, R., Duda, T., Goraczniak, R. M., & Sharma, R. K. (1992) *FEBS Lett* ***296,*** 254-258.
9. Goraczniak, R. M., Duda, T., & Sharma, R. K. (1992) *Biochem J* ***282***, 533-537.
10. Duda, T., Goraczniak, R. M., & Sharma, R. K. (1994) *Biochemistry* ***33***, 7430-7433.
11. Chinkers, M.,.& Garbers, D., L. (1989) *Science* ***245***, 1392-1394.
12. Thorpe, D. S., & Morkin, E. (1990) *J Biol Chem* ***265,*** 14717-14720.
13. Duda, T., Goraczniak, R. M., & Sharma, R. K. (1991) *Proc Natl Acad Sci USA* ***88,*** 7882-7886.
14. Kurose, H., Inagami, T., & Ui, M. (1987) *FEBS Lett* ***219,*** 375-379.
15. Chang, C. H., Kohse, K. P., Chang, B., Hirata, M., Jiang, B., Douglass, J. E., & Murad, F. (1990) *Biochim Biophys Acta* ***1052,*** 159-165.
16. Marala, R. B., Sitaramayya, A., & Sharma, R. K. (1991) *FEBS Lett* ***281,*** 73-76.
17. Larose, L., McNicoll, N., Ong, H., & DeLean, A. D. (1991) *Biochemistry* ***30,*** 8990-8995.
18. Duda, T., Goraczniak, R. M., & Sharma, R. K. (1993a) *FEBS Lett* ***315,*** 143-148.
19. Nambi, P., Aiyar, N.V., Roberts, A.N., Sharma, R.K. (1982) *Endocrinology* ***111***, 196-200.
20. Duda, T., Goraczniak, R. M., & Sharma, R. K. (1993b) *FEBS Lett* ***335***, 309-314.
21. Jewett, J. R. S., Koller, K. J., Goeddel, D. V., & Lowe, D. G. (1993) *EMBO* J ***12,*** 769-777.