Dual Role for Adenine Nucleotides in the Regulation of the Atrial Natriuretic Peptide Receptor, Guanylyl Cyclase-A

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The ability to both sensitize and desensitize a guanylyl cyclase receptor has not been previously accomplished in a broken cell or membrane preparation. The guanylyl cyclase-A (GC-A) receptor is known to require both atrial natriuretic peptide (ANP) and an adenine nucleotide for maximal cyclase activation. When membranes from NIH 3T3 cells stably overexpressing GC-A were incubated with ATP, AMPPNP, or ATPγS, only ATPγS dramatically potentiated ANP-dependent cyclase activity. When the membranes were incubated with ATPγS and then washed, GC-A now became sensitive to ANP/AMPPNP stimulation, suggestive that thio-phosphorylation had sensitized GC-A to ligand and adenine nucleotide binding. Consistent with this hypothesis, the ATPγS effects were both time- and concentration-dependent. Protein phosphatase stability of thio-phosphorylation (ATPγS) relative to phosphorylation (ATP) appeared to explain the differential effects of the two nucleotides since microcystin, β-glycerol phosphate, or okadaic acid coincident with ATP or ATPγS effectively sensitized GC-A to ligand stimulation over prolonged periods of time in either case. GC-A was phosphorylated in the presence of [γ32P]ATP, and the magnitude of the phosphorylation was increased by the addition of microcystin. Thus, the phosphorylation of GC-A correlates with the acquisition of ligand sensitivity. The establishment of an in vitro system to sensitize GC-A demonstrates that adenine nucleotides have a dual function in the regulation of GC-A through both phosphorylation of and binding to regulatory sites.

Atrial natriuretic peptide (ANP) is produced principally within the heart but is also synthesized in many other areas of the body (1). Major effects of ANP include the induction of natriuresis and diuresis in the kidney, the inhibition of adrenal gland aldosterone synthesis, and the relaxation of vascular smooth muscle (1). The two major binding proteins for ANP, the natriuretic peptide clearance receptor (NP-CR) and a guanylyl cyclase-linked receptor (GC-A), are found in the above target tissues as well as in many other regions of the body (1). Although NP-CR, a 65-kDa disulfide-linked dimer, has been suggested to function as a signaling molecule in addition to its role as a clearance receptor (2), GC-A appears to mediate a majority, if not all, of the known physiological effects of ANP. Mice lacking genes for either ANP or GC-A display an elevated form of blood pressure that has been suggested as salt-resistant (3, 4) or salt-sensitive (5, 6). In the GC-A deficient mouse infused ANP is unable to elicit a diuretic, natriuretic or smooth muscle relaxant effect (7).

Guanylyl cyclases, which catalyze the formation of cGMP, are divided into two classes: one is the family of soluble heterodimeric enzymes that are receptors for NO and possibly CO (8), and the other is the family of transmembrane receptor-linked enzymes, for which GC-A is a prototypical member (9). In mammals, 7 transmembrane forms of guanylyl cyclase (GC-A) are known to exist (10–14), whereas more than 25 putative guanylyl cyclases have been identified in Caenorhabditis elegans (15). These transmembrane proteins share several conserved features, including an amino-terminal extracellular domain separated by a single transmembrane segment from intracellular protein kinase-like and cyclase catalytic domains (10).

GC-A appears to exist as a higher ordered oligomer in the absence of ligand (16, 17), and it has been suggested that ANP binding induces a conformational change, perhaps resulting in relief of protein kinase homology domain inhibition of the cyclase catalytic domain (18). ANP binding to GC-A causes dramatic increases in intracellular cGMP levels, and the resultant homologous desensitization has been tightly correlated with GC-A dephosphorylation. Serine and threonine represent the phosphorylated amino acids in the basal state (19). Agents that activate protein kinase-C, such as certain pressor hormones and phorbol esters, also have been shown to desensitize GC-A (20–24). This heterologous desensitization also correlates with GC-A dephosphorylation (25). The tryptic phosphopeptide maps of GC-A appear different, dependent on whether desensitization is homologous or heterologous. ANP results in maps indistinguishable from untreated cells, although dephosphorylation occurs, while phorbol esters result in the disappearance of one major tryptic phosphopeptide (25). ANP, therefore, may cause complete dephosphorylation of a population of GC-A while phorbol esters result in selective dephosphorylation (25).

Possibly, ANP activates a protein phosphatase or induces a GC-A conformation conducive to dephosphorylation by a constitutively active protein phosphatase, whereas phorbol esters activate a protein phosphatase that selectively dephosphorylates due to the absence of ligand. Alternatively, the homolo-
Membranes were pre-assayed for guanylyl cyclase activity as described under "Experimental Procedures." Incubation mixtures (incubated for 9 min at 37 °C) contained 1 μM ANP and 1 mM of the indicated adenine nucleotides. The assay was linear with respect to protein concentration. Data are from one representative experiment, and error bars show the range of duplicate determinations.

We now demonstrate that adenine nucleotides play multiple roles in the activation of GC-A and for the first time demonstrate in vitro sensitization of GC-A to ligand. In a preparation of crude membranes, ATPγS, which can serve as a substrate for protein kinases for thiophosphorylation of proteins, sensitizes GC-A to stimulation with ANP and adenine nucleotides. After ATPγS treatment, even a nonhydrolyzable analog of ATP, AMPPNP, now facilitates stimulation of GC-A by ANP. How-ever, the activity of the enzyme rapidly declines, presumably recapitulating desensitization. Consistent with dephosphorylation of a site-regulating desensitization, not only does the protein phosphatase inhibitor microcystin block ligand-induced inactivation while potentiating ANP/ATPγS stimulation, but structurally different phosphatase inhibitors enhance ANP/adenine nucleotide signaling. Furthermore, phosphorylation of GC-A is observed under sensitization conditions, and phosphorylation is increased when microcystin is included in the reaction mixture.

EXPERIMENTAL PROCEDURES

Material—Rat ANP was from Peninsula Laboratories. Microcystin-LR and okadaic acid were from Life Technologies, Inc. Cyclosporin A was from Calbiochem. ATPγS and AMPPNP were from Boehringer Mannheim or Sigma, and ATP was from Sigma.

Cell Culture and Preparation of Particulate Fraction—NIH 3T3 cells stably expressing GC-A were prepared as described (25). 3T3GC-A cells were maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% heat-inactivated fetal bovine serum, 100 units/ml penicillin, 100 μg/ml streptomycin, 0.25 μg/ml amphotericin B, and 50 μg/ml biologically active G418. Particulate fractions were prepared by washing confluent 10- or 15-cm plates with ice-cold phosphate-buffered saline and scraping cells in 0.75–1 ml of homogenization buffer (HB) (50 mM Hepes, pH 7.4, 10% glycerol, 100 mM NaCl, 10 mM Hepes, 0.5% BSA, 5 mM MgCl2, 10 mM NaN3, 1 mM adenine nucleotide unless otherwise indicated, 1 μM ANP, 0.5 μCi [α32P]GTP (NEN Life Science Products, 3000 Ci/mmol), and membranes in a total volume of 100 μl for the indicated times. Phosphatase inhibitors also were included at the indicated concentrations. 3 mM MnCl2, and 0.1% Triton X-100 were included to determine maximal guanylyl cyclase activity. Assays were initiated by the addition of 20 μl (usually 20 μg of membrane protein) of membranes to the above mixture that had been pre-warmed at 37 °C for 20 s and terminated by the addition of zinc acetate/sodium carbonate. [α32P]cGMP was determined as described previously (32) and was produced in a linear manner as a function of protein concentration.

Primary Incubation and Washing Membranes—Membranes were incubated at 37 °C with the indicated variables in an assay buffer containing 25 mM Hepes, pH 7.4, 50 mM NaCl, 0.1 mM GTP, 0.25 mM IBMX, 0.1% BSA, 5 mM MgCl2, 10 mM NaNO3, 1 mM adenosine nucleotide unless otherwise indicated, 1 μM ANP, 1 μCi [α32P]GTP (NEN Life Science Products, 3000 Ci/mmol), and membranes in a total volume of 100 μl for the indicated times. Phosphatase inhibitors also were included at the indicated concentrations. 3 mM MnCl2, and 0.1% Triton X-100 were included to determine maximal guanylyl cyclase activity. Assays were initiated by the addition of 20 μl (usually 20 μg of membrane protein) of membranes to the above mixture that had been pre-warmed at 37 °C for 20 s and terminated by the addition of zinc acetate/sodium carbonate. [α32P]cGMP was determined as described previously (32) and was produced in a linear manner as a function of protein concentration.
of ice-cold HB (described above) containing 0.2 μM microcystin and centrifuged at 16,000 × g for 10 min at 4 °C. Pellets were washed with HB/microcystin and resuspended in HB with a 22-gauge needle. Membranes were used immediately for guanylyl cyclase assays as described above. The experiment in Fig. 2 contained 0.5 μCi [γ-32P]GTP (NEN Life Science Products, 3000 Ci/mmol) in the initial incubation, and the membranes were not washed before the addition of ligand(s). Membranes were washed twice with HB or HB containing 1 M NaCl to determine the effect of washing membranes on either the ability of ATPγS to sensitize GC-A, or microcystin to potentiate ANP/adenine nucleotide-dependent activity. Membranes were washed twice with HB or HB with 1 M NaCl and subsequently washed with HB and resuspended in HB by passing through a 22-gauge needle. The experiment in Fig. 2 contained 0.5 μCi [γ-32P]GTP (NEN Life Science Products, 3000 Ci/mmol) in the initial incubation, and the membranes were not washed before the addition of ligand(s). Membranes were washed twice with HB or HB containing 1 M NaCl to determine the effect of washing membranes on either the ability of ATPγS to sensitize GC-A, or microcystin to potentiate ANP/adenine nucleotide-dependent activity. Membranes were washed twice with HB or HB with 1 M NaCl and subsequently washed with HB and resuspended in HB by passing through a 22-gauge needle. The experiment in Fig. 2 contained 0.5 μCi [γ-32P]GTP (NEN Life Science Products, 3000 Ci/mmol) in the initial incubation, and the membranes were not washed before the addition of ligand(s). Membranes were washed twice with HB or HB containing 1 M NaCl to determine the effect of washing membranes on either the ability of ATPγS to sensitize GC-A, or microcystin to potentiate ANP/adenine nucleotide-dependent activity. Membranes were washed twice with HB or HB with 1 M NaCl and subsequently washed with HB and resuspended in HB by passing through a 22-gauge needle. When the ATPγS effect was examined, membranes were again washed after the incubation with ATPγS reaction mixture that included 25 mM Hepes, pH 7.4, 50 mM NaCl, 5 mM MgCl2, 10 mM NaF, and 1 mM ATPγS. Differently washed membranes were then subjected to the guanylyl cyclase assay as described.
Reversibility of Sensitization—Membranes were incubated with 1 mM ATP and 1 μM okadaic acid for 15 min at 37 °C followed by washing as described above. Membranes were then incubated at 37 °C for increasing times after which they were placed on ice. Some membranes were subsequently incubated with ATPγS reaction mixture that included 25 mM Hepes, pH 7.4, 50 mM NaCl, 10 mM NaN₃, 5 mM MgCl₂, and 1 mM ATPγS for 15 min at 37 °C followed by washing as described. Membranes were then subjected to the guanylyl cyclase assay as described.

Phosphorylation and Immunoprecipitation of GC-A—Membranes were incubated at 37 °C for 20 min in an assay buffer containing 25 mM Hepes, pH 7.4, 50 mM NaCl, 0.1 mM GTP, 0.25 mM IBMX, 0.1% BSA, 5 mM MgCl₂, 10 mM NaN₃, 10 μM ATP, and 200 μCi of [γ³²P]ATP (Amersham Pharmacia Biotech), in the presence or absence of 0.2 μM microcystin. Reactions were terminated by the addition of immunoprecipitation buffer (HB buffer containing 1% Triton X-100, 10 mM NaPO₄, pH 7.0, 0.1 M NaF, 1 mM Na₃VO₄, 0.1 μM okadaic acid, 80 μM β-glycerol phosphate, 1 μM microcystin-LR, and 10 mM EDTA). After rocking at 4 °C for 60 min, the detergent extract was cleared by centrifugation at 436,000 × g for 20 min at 4 °C. The extract was pre-cleared by initially incubating with a nonspecific antibody (Z660) raised against the carboxyl terminus of GC-C, the heat stable enterotoxin receptor, and protein A-agarose (Pierce). This antibody does not cross-react with GC-A. An antibody (A034) raised against the carboxyl-terminal 15 amino acids of GC-A was added to the cleared extract at a dilution of 1:200 and incubated at 4 °C for 60–120 min. Protein A-agarose was also included in this incubation to precipitate immune complexes. Immune complexes were washed with immunoprecipitation buffer supplemented with 0.1% SDS and 1% sodium deoxycholate, fractionated by SDS-polyacrylamide gel electrophoresis, blotted to Immobilon-P polyvinylidene fluoride membranes (Millipore), and GC-A visualized after autoradiography.³²P was quantitated using the ImageQuant software on a Molecular Dynamics PhosphorImager.

Immunoblot Analysis—Polyvinylidene fluoride membranes to which immunoprecipitated GC-A had been blotted were blocked for 15 min at room temperature with 5% non-fat dry milk in TBST (Tris-HCl, pH 7.5, 150 mM NaCl, and 0.1% Tween-20). Membranes were washed with TBST and incubated for 60 min at room temperature in 1:2500 dilution of antibody (A034) raised against the carboxyl-terminal 15 amino acids of GC-A. Membranes were washed and incubated with a 1:30,000 dilution of a horseradish-peroxidase-conjugated secondary antibody in TBST. After washing the membrane with TBST, protein bands were detected using the enhanced chemiluminescence detection method (ECL) (Amersham Pharmacia Biotech).

RESULTS AND DISCUSSION

Effect of Different Adenine Nucleotides on GC-A Activity—Various groups have demonstrated that adenine nucleotides are required in addition to ANP for maximal GC-A activity; it has been suggested that adenine nucleotide binding and not phosphoryl transfer is the regulatory event since a nonhydro-
lyzable ATP analog, AMPPNP, also facilitates slight ANP stimulation (27–29). The adenine nucleotide binding site has been suggested to be within the protein kinase homology domain of GC-A, but direct measurements of adenine nucleotide binding have not been reported. Although the current model of adenine nucleotide regulation of GC-A suggests that binding to a regulatory site is sufficient for transmission of ANP binding to activation of guanylyl cyclase, a positive correlation exists between the phosphorylation state of GC-A and its sensitivity to ligand (19, 25). The resensitization of GC-A in a broken cell preparation has not been achieved, and it therefore has remained unclear whether phosphorylation merely correlates with the sensitization/desensitization of the receptor or whether it is a primary regulatory event.

Crude membranes were obtained from cells stably expressing GC-A and different adenine nucleotides were tested for their ability to stimulate the cyclase in the presence of ANP. The effects of the adenine nucleotides were different, in that ATPγS was markedly more effective that ATP while AMPPNP was almost ineffective in yielding an ANP sensitive receptor (Fig. 1). These results suggested multiple adenine nucleotide requirements for ANP-dependent activation of GC-A, possibly as a substrate for transphosphorylation as well as for binding to a regulatory site.

Therefore, membranes were incubated with adenine nucleotides at concentrations that would not themselves yield an ANP-dependent activation of GC-A, and then ANP alone or ANP plus high concentrations of AMPPNP were added to determine whether such a prior incubation would sensitize the receptor. Low concentrations of ATPγS markedly sensitized GC-A to subsequent ANP/AMPPNP stimulation (Fig. 2). AMPPNP at millimolar concentrations in the absence of the first ATPγS incubation failed to significantly stimulate cyclase activity in the presence or absence of ANP. ATP also failed to yield an ANP/AMPPNP sensitive enzyme, but given that thiophosphorylated proteins are known as particularly poor substrates for protein phosphatases (33), the sensitization of GC-A by prior incubation with low concentrations of ATPγS but not ATP could be explained by the stability of thiophosphorylation. If a relatively stable thiophosphorylation explained the acquisition of a sensitive cyclase, then incubation with ATPγS followed by washing of the membrane preparation should now yield an ANP/AMPPNP-sensitive cyclase.

**ATPγS Sensitization of GC-A Is Stable**—Membranes were initially incubated with ATPγS, no adenine nucleotide, or AMPPNP and then washed, and the resulting membranes were assayed for ANP/AMPPNP-stimulated cyclase activity (Fig. 3A). Clearly, ATPγS but not AMPPNP sensitized GC-A to subsequent ANP/AMPPNP stimulation, consistent with thiophosphorylation of a regulatory site. When membranes were isolated and subsequently washed with buffers containing 1 M NaCl, the ability of ATPγS to sensitize GC-A was retained, suggestive that the proteins required for sensitization are tightly associated with the membrane (Fig. 3B).

**Time- and Concentration-dependence of ATPγS Sensitization**—When membranes were incubated with 1 mM ATPγS for increasing periods of time and washed, the resulting membranes displayed a time-dependent increase in ANP/AMPPNP-sensitive cyclase activity (Fig. 4A). The membranes incubated without ATPγS lost the slight ANP/AMPPNP responsiveness as a function of time. This was not due to a decreased capacity of the catalytic domain to synthesize cGMP since Mn2+/Triton X-100 activity (a treatment thought to maximally activate the cyclase independent of ligand) remained relatively constant throughout the incubation (Fig. 4A). The loss in ANP/AMPPNP responsiveness could be explained if a small amount of residual phosphoamino acid at the putative regulatory site was lost during the incubation. Increasing the concentration of ATPγS in the initial incubation also increased ANP/AMPPNP-stimu-
Incorporated into GC-A, where the amount of $^{32}$P found in the absence of ANP and AMPPNP was quantitated as described under "Experimental Procedures," immunoprecipitated, and visualized by autoradiography, or immunoblot. B, quantitation of GC-A phosphorylation. GC-A-associated $^{32}$P was quantitated as described under "Experimental Procedures" and is plotted as relative $^{32}$P incorporated into GC-A, where the amount of $^{32}$P found in the absence of microcystin is assigned a value of 1. Data are from one representative experiment, and error bars show the range of duplicate determinations. C, ATP and microcystin sensitize GC-A. 3T3GC-A membranes were initially incubated in a reaction mixture, including 1 mM ATP in the presence (filled bar) or absence (open bar) of 0.2 μM microcystin for 15 min at 37°C, and washed as described under "Experimental Procedures." Subsequent membranes were assayed for guanylyl cyclase activity in the presence of 1 μM ANP and 1 mM AMPPNP for 8 min at 37°C. Data are from one representative experiment, and error bars show the range of the duplicates.

Fig. 9. Microcystin increases the $^{32}$P content of GC-A. A, phosphorylation of GC-A. Membranes were incubated with [γ$^{32}$P]ATP under sensitizing conditions in the presence or absence of 0.2 μM microcystin as described under "Experimental Procedures," immunoprecipitated, and visualized by autoradiography, or immunoblot. B, quantitation of GC-A phosphorylation. GC-A-associated $^{32}$P was quantitated as described under "Experimental Procedures" and is plotted as relative $^{32}$P incorporated into GC-A, where the amount of $^{32}$P found in the absence of microcystin is assigned a value of 1. Data are from one representative experiment, and error bars show the range of duplicate determinations. C, ATP and microcystin sensitize GC-A. 3T3GC-A membranes were initially incubated in a reaction mixture, including 1 mM ATP in the presence (filled bar) or absence (open bar) of 0.2 μM microcystin for 15 min at 37°C, and washed as described under "Experimental Procedures." Subsequent membranes were assayed for guanylyl cyclase activity in the presence of 1 μM ANP and 1 mM AMPPNP for 8 min at 37°C. Data are from one representative experiment, and error bars show the range of the duplicates.
Fig. 10. **Model for the regulation of GC-A.** The model is described under “Results and Discussion.” Red arrows indicate fast steps relative to blue arrows. Red circle represents the putative regulatory phosphorylation site and may either be on GC-A or another protein. The green box represents adenine nucleotide binding and is labeled with AMPPPN. ATP may represent the endogenous regulator. The sensitization step is indicated by an “P” on the red circle, but this site may also be thio-phosphorylated when ATP/S is the sensitizing substrate. In the model, “inactive” GC-A does not contain the regulatory site in its phosphorylated state and is not stimulated by ANP/AMPNP. “Inactive and receptive” GC-A possesses a regulatory site that is phosphorylated but one that does not convey increased enzyme activity until both ANP and AMPPPN bind. Loss of the regulatory phosphorylation site results in GC-A desensitization or a return to the “inactive” state.

ently altering the phosphorylation state of unidentified regulatory phosphorylation sites.

**Phosphorylation of GC-A Correlates with Acquisition of Ligand Sensitivity**—Previous reports demonstrated that dephosphorylation of GC-A correlated with its loss of sensitivity to ANP (19, 25). However, a phosphorylation-induced sensitization of GC-A has not been accomplished. Given that conditions to sensitize GC-A have now been established here (Fig. 3) and phosphorylation of a regulatory site appears to represent a mechanism of sensitization, we examined phosphorylation of GC-A under sensitizing conditions. Phosphorylation of GC-A was observed, and clearly the GC-A-associated 32P was increased when microcystin was included in the reaction mixture (Fig. 9, A and B). The increase in 32P was not due to an increased amount of GC-A in the immunoprecipitate (Fig. 9A). Sensitization of GC-A by ATP and microcystin correlated well with GC-A phosphorylation (Fig. 9C), and thus for the first time, phosphorylation of GC-A is shown to correlate with GC-A sensitivity to stimulation by ANP/adenine nucleotides.

**Model for Regulation of GC-A Activation**—This is the first successful sensitization/desensitization of GC-A, a prototype of the other guanylyl cyclase receptors, in a broken cell preparation. Multiple roles for adenine nucleotides in regulation of the receptor are now clearly established based on this work. Phosphorylation of one or more regulatory sites as well as adenine nucleotide binding are required to generate a ligand-sensitive cyclase (Fig. 10). Although it is possible that phosphorylation of other sites participates in different aspects of GC-A regulation, such as ANP-independent activity, phosphorylation of these regulatory sites does not itself appear to alter the nonliganded activity of the receptor. The model now raises a number of important questions. The first is the site(s) of phosphorylation. These studies demonstrate that phosphorylation or thio-phosphorylation generates an ANP/AMPNP-sensitive receptor, however, they do not identify the localization of the regulatory sites. These could be within the cyclase itself or they could reside on associated proteins.

We have not established that adenine nucleotide binding is to the cyclase although the presence of the protein kinase homology domain is strongly suggestive that it represents the site of binding. Relatively high (millimolar) concentrations of ATP or AMPNP are required to facilitate ANP signaling, and thus it remains possible that ATP or AMPNP mimic the actions of a much more potent nucleotide regulator. The ability to obtain sensitized GC-A *in vitro* now allows searches for more potent regulators. Importantly, the ability to sensitize GC-A with ATP/S and prevent desensitization with phosphatase inhibitors is retained even after washing membranes with buffers with high salt concentrations, demonstrating that the components required for sensitization and desensitization of GC-A likely reside in the membrane itself (Fig. 3B and Fig. 6B).

The model also predicts several potential means by which to alter GC-A signaling. Other signaling systems could impinge on the sensitizing protein kinase or the putative desensitizing phosphatase, and given the dual role of adenine nucleotides in activation of GC-A, fluctuations in intracellular levels of ATP or of another regulatory nucleotide could also regulate its activity.

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