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Guanylyl Cyclases A and B Are Asymmetric Dimers That Are Allosterically Activated by ATP Binding to the Catalytic Domain

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Abstract

It is not known how natriuretic peptides and adenosine triphosphate (ATP) activate guanylyl cyclase A (GC-A) and GC-B, which generate the second messenger cyclic guanosine monophosphate. We determined that natriuretic peptides increased the maximum rate of these enzymes >10-fold in a positive cooperative manner in the absence of ATP. In the absence of natriuretic peptides, ATP shifted substrate-velocity profiles from cooperative to linear but did not increase the affinity of GCs for the substrate guanosine triphosphate (GTP) since the Michaelis constant was unchanged. However, in the presence of natriuretic peptides, ATP competed with GTP for binding to an allosteric site, which enhanced the activation of GCs by decreasing the Michaelis constant. Thus, natriuretic peptide binding was required for communication of the allosteric activation signal to the catalytic site. The ability of ATP to activate GCs decreased and enzyme potency (a measure of sensitivity to stimulation) increased with increasing GTP concentrations. Point mutations in the purine-binding site of the catalytic domain abolished GC activity but not allosteric activation. Coexpression of inactive mutants produced half the activity expected for symmetric catalytic dimers. 2'-Deoxy-ATP and 2'-deoxy-GTP were poor allosteric activators, but 2'-deoxy-GTP was an effective substrate, consistent with distinct binding requirements for the allosteric and catalytic sites. We conclude that membrane GC domains are asymmetric homodimers with distinct and reciprocally regulated catalytic and allosteric sites that bind to GTP and ATP, respectively. These data define a new membrane GC activation model and provide evidence of a previously unidentified GC drug interaction site.

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SUPPLEMENTARY MATERIALS

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Fig. S1. Detectable amounts of GC-A or GC-B protein are only found in HEK 293T cells specifically transfected with plasmids encoding each receptor.

Fig. S2. HEK 293T cells do not have detectable membrane GC activity.

Fig. S3. Kinetic values for substrate-velocity experiments presented in the indicated figures.

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INTRODUCTION

Guanylyl cyclases (GCs) catalyze the conversion of guanosine triphosphate (GTP) to cyclic guanosine monophosphate (cGMP) in organisms spanning the evolutionary spectrum. Single membrane-spanning GCs (mGCs) regulate blood pressure, bone growth, reproduction, intestinal hydration, CO₂ detection, phototransduction, feeding, and attention deficit disorder (1–6). The archetypal mammalian mGC is GC-A, a critical cardiovascular regulator that is stimulated by atrial natriuretic peptide (ANP) and B-type natriuretic peptide (BNP) (7, 8). GC-B has 78% sequence identity to GC-A at the intracellular amino acid level but is activated by C-type natriuretic peptide (CNP) (7, 9). In mice and humans, inactivating mutations in GC-A cause hypertension and cardiac hypertrophy (10, 11), whereas inactivating mutations in GC-B cause dwarfism and female infertility (12–15).

Both GC-A and GC-B are composed of a large extracellular ligand-binding domain, a single membrane span, and an intracellular segment consisting of a kinase homology domain (KHD), a dimerization domain, and a C-terminal catalytic domain. Unbound NP receptors are preformed, noncovalent homodimers (16). ANP binds to GC-A at a stoichiometry of 1:2 to induce a rotation of the juxtamembrane region that initiates enzyme activation by an unknown mechanism (17, 18). Structural modeling studies suggest that CNP binds to GC-B similarly (19, 20). Multiple phosphorylation sites have been identified in the intracellular juxtamembrane portions of GC-A and GC-B (21, 22). Phosphorylation is required for activation, and dephosphorylation is a mechanism of inactivation (23, 24).

Adenosine triphosphate (ATP) increases the activity of mGCs by binding to an unknown intracellular site (3). The most discussed activation model suggests that the binding of NP to the extracellular domain of an mGC enables ATP to bind to the KHD, which derepresses the catalytic domain and increases maximal velocity ($V_{\max}$; the maximum rate of product formation) (25–31). However, there are several inconsistencies with this model. First, mutation of the putative GXGXXXG ATP-binding site in the KHD failed to block the activation of GC-A by ATP (32, 33). Second, NPs activate GC-A and GC-B in the absence of ATP (32, 34). Third, an ATP analog that was chemically cross-linked to the KHD unexpectedly inhibited GC-A (31). Fourth, ATP reduced the Michaelis constant (K_m ; a measure of enzyme-substrate affinity) without increasing the maximal velocity of highly phosphorylated preparations of GC-A and GC-B (32, 34). A related, unexplained phenomenon is that mGCs demonstrate positive cooperativity through an allosteric site in the GC domain when assayed under synthetic conditions (in the presence of Mn²⁺ and nonionic detergent) but not under physiologic conditions (in the presence of Mg²⁺ and ATP) (32, 35, 36). Here, we connect these seemingly disparate observations into a new unified activation model that illuminates the activation mechanism of this important enzyme family. We demonstrated that the binding of NP to GC-A and GC-B enabled ATP to reduce the Hill coefficient and decreased the K_m by displacing GTP at an allosteric site in the catalytic domain. Hill coefficients greater or less than 1 indicate graded levels of positive or negative cooperativity, respectively, whereas a Hill coefficient of 1 indicates no cooperativity. In addition, we showed that ATP binding to the allosteric site was essential for the activation of GC-B under physiologic conditions.

RESULTS

ATP shifts product formation from cooperative to linear under basal conditions

To determine how NPs and adenine nucleotides activated GC-A and GC-B, we generated substrate-velocity curves from experiments with membranes from human embryonic kidney (HEK) 293T cells stably expressing GC-A or GC-B as the enzyme source. HEK 293T cells lack detectable endogenous mGCs, and both receptors were properly processed and regulated in these cells (figs. S1 and S2). Because highly purified preparations of GC-A are constitutively active and are not stimulated by ANP (37, 38), the HEK 293T system is the best system for studying the activation of NP receptors because the enzymes in these membranes are activated by NPs and ATP, and unlike endogenous preparations that contain multiple GCs, activity in the transfected HEK 293T cell membranes can be attributed to a single protein.

We generated substrate-velocity curves for GC-A and GC-B in the presence or absence of NPs or ATP (Fig. 1). A model is shown to the right of the graphs for each experiment to illustrate the proposed binding site for each regulator. In these models, the allosteric binding pocket is arbitrarily shown as the top site in the GC domain, whereas the catalytic binding pocket is arbitrarily shown as the bottom site. Under basal conditions (that is, in the absence of NP and ATP), maximal velocity was low, the Michaelis constant was high, and product formation was positive cooperative, as demonstrated by sigmoidal curves and Hill coefficients of 1.5 and 1.4 for GC-A and GC-B, respectively (Fig. 1, A to C; kinetic constants associated with substrate-velocity experiments are shown in fig. S3). This means that in the absence of NP and ATP, the enzyme has a low turnover rate, inefficient product formation, and binds to two molecules of GTP, one in the catalytic site and one in the allosteric site. In the absence of NPs, ATP shifted product formation from positive cooperative to linear, as demonstrated by reductions in the Hill coefficients from 1.4 or 1.5 to 1.1 or 1.0; however, ATP did not reduce the apparent K_m or increase the maximal velocities of the enzymes in the absence of NPs. Thus, although ATP disrupted cooperativity, it did not increase the affinities of the enzymes for GTP or their maximum activities.

NPs increase maximal velocity in the absence of ATP

In the next series of experiments, we investigated the effect of NPs on the activities of GC-A and GC-B in the absence of ATP (Fig. 1, D to F). ANP increased the maximal velocity of GC-A 23-fold and CNP increased the maximal velocity of GC-B 13-fold (Fig. 1, D and E). Both peptides reduced the K_m , but neither reduction was statistically significant; however, the Hill coefficient was not affected by the presence of NPs. These data are in direct contrast to previous reports of experiments with membranes prepared in the absence of phosphatase inhibitors, which showed that the receptors were not activated by NPs in the absence of ATP (27, 28).

The ATP-dependent reduction of the K_m requires the binding of NPs

In the third set of experiments, we examined the effect of ATP on NP-activated receptors (Fig. 1, G to I). In the absence of ATP, maximal velocity, K_m , and the Hill coefficients were

high. ATP shifted product formation from positive cooperative (as shown by the sigmoidal curve) to linear (as shown by the hyperbolic curve), and as demonstrated by a 0.4- or 0.5-U reduction in the Hill coefficient. ATP also reduced the K_m of both enzymes sevenfold to ~0.1 mM, which is in the range of cellular concentrations of GTP (39). Together, these data indicate that ATP binds to the receptors in the absence of NPs and that ATP is not required for NP-dependent increases in maximal velocity, as was previously reported for phosphorylated enzyme preparations (22, 23). However, the data also demonstrate that NP binding is required for the ATP-dependent reduction in the K_m . Thus, in a physiologic system, NP binding increases GC activity by two mechanisms: increasing V_{max} and reducing the K_m .

ATP competes with GTP for the allosteric site in a concentration-dependent manner

We hypothesized that ATP competed with GTP at an allosteric site in the catalytic domain. If this were true, then ATP should reduce the Hill coefficient in a concentration-dependent manner. We examined this possibility and found that increasing the concentration of ATP progressively reduced the Hill slopes of both enzymes (Fig. 2, A and B, and fig. S3). The ATP-dependent reduction in the Hill coefficient correlated with concomitant reductions in the K_m . These data are consistent with a model in which ATP competes with GTP for binding to an allosteric site, which then increases the affinity of the catalytic site for GTP.

The allosteric and catalytic sites are reciprocally regulated

If ATP increased enzyme activity solely by decreasing the K_m value, then stimulation by ATP should be greater at GTP concentrations below the K_m . To test this hypothesis, we performed concentration-response assays in the presence of four different concentrations of GTP (Fig. 2, C and D). At 0.1 mM GTP, which is at or below the K_m , ATP increased the activities of GC-A and GC-B 8- and 14-fold, respectively; however, at 5.4 mM GTP, which is substantially above the K_m , ATP increased the activities only 2.4-fold for GC-A and 1.4-fold for GC-B. We also tested whether intra-site communication occurred in the reverse direction by examining whether GTP increased the potency of ATP (Fig. 2, C and D). At 0.1 mM GTP, the concentration required to achieve half the maximal effect (EC_{50}) for ATP was 77 and 40 μ M for GC-A and GC-B, respectively; however, at 5.4 mM GTP, the EC_{50} decreased five- and twofold to 16 and 19 μ M for GC-A and GC-B, respectively. Thus, ATP binding to the allosteric site increased the extent of GTP binding to the catalytic site, whereas GTP binding to the catalytic site increased the extent of ATP binding to the allosteric site.

ATP and ADP, but not AMP or adenosine, allosterically activate GC-A and GC-B

We examined the structural requirements for allosteric regulation by adenine nucleotides by testing the abilities of adenosine, adenosine monophosphate (AMP), adenosine diphosphate (ADP), and ATP to activate GC-A in the presence of saturating concentrations of ANP (Fig. 3A). ADP and ATP increased activity six- and ninefold, respectively, whereas adenosine and AMP failed to increase activity. These data are consistent with the requirement for the β , but not the γ , phosphate of ATP for either binding to the allosteric site or transducing the effect of allosteric binding to the catalytic site.

Purines lacking ribosyl 2'-OH are poor allosteric activators of GC-A and GC-B

We also examined the effect of hydroxyl groups at the 2' and 3' positions of the ribose ring on the activities of GC-A and GC-B. In the absence of ATP, product formation by ANP-activated GC-A was cooperative, as demonstrated by a Hill coefficient of 1.5 (Fig. 3B). However, when this experiment was performed with 2'-deoxy-GTP as the substrate, product formation was linear and the Hill coefficient was 1. These data are consistent with the ribosyl 2'-OH group of GTP increasing binding to the allosteric site but not to the catalytic site.

Because we hypothesized that ATP competed with GTP at the same allosteric binding site, we tested whether ATP lacking a 2'-OH group was also a poor allosteric activator of GC-A. Consistent with our hypothesis, 2'-deoxy-ATP was a poorer activator of GC-A than was ATP in single-substrate concentration GC assays (Fig. 3C), consistent with the 2'-OH group increasing binding to or activation of the allosteric site. We also examined the effect of ATP molecules lacking either the 2'- or 3'-ribose-OH group on the activation of GC-B, and found that the 2'-OH group, but not the 3'-OH group, was required for maximal allosteric activation of GC-B (Fig. 3D). Furthermore, as would be expected if the 2'-OH group of the nucleotide increased binding to the allosteric site, 2'-deoxy-ATP reduced the Hill slope and K_m values for GC-A less than did ADP or ATP, both of which contain a ribosyl 2'-OH group (Fig. 3E). These data are consistent with the ribosyl 2'-OH, but not 3'-OH, group increasing binding to the allosteric site. We prepared models depicting the effects and differential binding capabilities of 2'-deoxy-GTP and 2'-deoxy-ATP to GC-A and GC-B (shown in Fig. 3F).

ATP binding to the allosteric site is required for the physiologic activation of GC-B

The physiologic relevance of ATP binding to the allosteric sites of GC-A and GC-B is unclear because some reports have indicated that ATP binding to the KHD is required for enzyme activation (27, 28, 30), whereas we previously showed that ATP was not required for maximal activation of GC-A and GC-B in enzyme assays conducted for very short periods and containing saturating concentrations (1 mM) of GTP (32, 34). Our goal was to determine how ATP activated GC-A and GC-B in cells, but because depleting cellular ATP concentrations below the EC_{50} for activation of the cyclases would kill the cells, we used an *in vitro* assay to address this question (Fig. 4). Here, we measured the activation of endogenous GC-B in NIH 3T3 cell membranes with increasing concentrations of CNP and physiologic (0.1 mM) concentrations of GTP in the presence or absence of physiologic (1 mM) concentrations of ATP. When assayed under enzyme conditions that closely reflected nucleotide concentrations measured in many cell types (39), ATP substantially increased the ability of CNP to activate GC-B (Fig. 4). For example, 125 nM CNP increased activity more than fourfold in the presence of ATP, but in the absence of ATP, no statistically significant increase in activity was observed. As shown earlier (Fig. 1H), the increased activity that resulted from the inclusion of ATP in the assay was solely a result of reductions in the K_m . ATP increased activity in previous assays because GTP concentrations were 10-fold higher and because the assays were conducted for much shorter periods (32, 34). Thus, ATP binding to the allosteric site in the catalytic domain of GC-B is essential for maximal CNP-dependent activation of the enzyme under biological conditions.

ATP activates mutants lacking a single GTP-binding site similarly to its activation of wild-type GC-A

Joubert *et al.* engineered two single purine-binding site mutations in individual complementary DNAs (cDNAs) encoding truncated GC-A molecules containing only the C-terminal dimerization and catalytic domains; these mutations were predicted to produce a catalytic dimer with only one nucleotide-binding site (35). One mutant contains a D849A substitution and the other mutant contains an N968S substitution. Either mutant GC-A expressed alone was inactive, but coexpression produced an active heterozygous (HET) enzyme that lacked cooperativity. On the basis of these and other data, Joubert *et al.* concluded that the truncated GC-A dimer is symmetric and contains two catalytic sites and that both sites are required for cooperativity.

We initially hypothesized that ATP reduced the K_m by displacing GTP at one of the two GTP-binding sites. Therefore, we predicted that ATP would not activate the HET mutant described by Joubert *et al.* (35). We generated D849A and N968S substitutions in individual full-length GC-A-encoding cDNAs, and we transfected HEK 293T cells with either construct alone, with both constructs together, or with a plasmid encoding wild-type GC-A to generate many possible dimer combinations (Fig. 5A). We measured GC activity in the presence of Mg^{2+} GTP alone (basal conditions) or with ANP in the presence or absence of ATP, and we also measured maximal GC activity under synthetic conditions by conducting the experiments in the presence of Mn^{2+} GTP and Triton X-100 (Fig. 5B). The individual mutants expressed alone were inactive under all assay conditions. However, ATP increased activity in membranes from cells containing mutant and wild-type receptors or two individual mutant receptors to make the HET receptor similar to that observed in membranes from cells expressing only the wild-type receptor. These data are inconsistent with the notion that ATP and GTP bind to the same site. Unexpectedly, activity measured in the presence of Triton X-100 and Mn^{2+} GTP was reduced in membranes from cells transfected with the mutant receptors, which likely results from the negative cooperativity of the HET receptor.

Full-length HET mutants of GC-A are negatively cooperative

To determine whether the full-length HET mutant lacked cooperativity, as was observed for the purified, truncated HET mutant (35), we generated substrate-velocity curves from experiments with membranes containing wild-type or HET receptors in the presence of Mn^{2+} and Triton X-100 (Fig. 5C). The double-reciprocal plot of the wild-type enzyme was concave upward, indicating positive cooperativity (Fig. 5D). However, the plot of the HET mutant was equally concave downward, indicating negative cooperativity, which is consistent with the reduced detergent-dependent activity observed for the mutant enzymes (Fig. 5B). These data suggest that ATP activated the HET enzyme because the mutations that abolished the catalytic site did not abolish the allosteric site. This finding is consistent with the different structural binding requirements for the catalytic and allosteric sites (Fig. 3). A better understanding of how binding to the allosteric site changes the conformation of the catalytic site is required to determine why the HET mutations caused negative cooperativity in the full-length, but not the truncated, enzyme.

GC activity measurements suggest the presence of asymmetric dimers

Finally, we investigated whether the catalytic domain of GC-A was a symmetric homodimer, as was suggested by Joubert and colleagues (35), or an asymmetric homodimer as envisioned for soluble GC (sGC) and adenylyl cyclase (AC) (35, 40, 41). On the basis of a symmetric homodimeric catalytic domain model, coexpression of mutant (D849A or N968S) and wild-type enzymes would be expected to produce 25% active homodimers of wild-type enzyme (Fig. 5A, i), 25% inactive homodimers of mutant enzymes (Fig. 5A, ii and iii) because each site contains an inactivating mutation, 25% active heterodimers with the mutation in the top purine-binding site (Fig. 5A, iv and vi), and 25% active heterodimers with the mutation in the bottom purine-binding site (Fig. 5A, v and vii), for a total of 75% of the activity of the wild-type enzyme. Asymmetric model expectations are similar, but only 50% of the activity of wild-type enzyme is expected because mutations in the catalytic site, which was arbitrarily designated as the bottom site, would be inactive. We observed $57 \pm 4\%$ and $59 \pm 2\%$ of the activity of wild-type enzyme when D849A and N968S mutants were coexpressed with wild-type GC-A, respectively, which is consistent with the asymmetric model (Fig. 5A, viii and ix).

The symmetric model also predicted that cells cotransfected with each mutant cDNA (HET) would produce 25% inactive homodimers of D849A, 25% inactive homodimers of N968S, 25% active heterodimers of N968S and D849A, in which the bottom site contains both mutations, and 25% active heterodimers of N968S and D849A, in which the upper site contains both mutations, for a total of 50% of the activity of the wild-type enzyme. However, only 25% of the activity of wild-type enzyme was anticipated for the asymmetric model because both mutations in the bottom catalytic domain would inactivate GC-A. We observed $33 \pm 8\%$ of the activity of wild-type enzyme in membranes from cells expressing both mutants (Fig. 5B). Thus, we conclude that GC-A forms an asymmetric catalytic dimer containing a single catalytic site.

DISCUSSION

ATP was first shown to increase the activity of GC-A in 1987 (42), but the mechanism of activation has been controversial. Early kinetic assays indicating that ATP increases maximal velocity together with the identification of a putative GXGXXXG ATP-binding motif in the KHD led to a model in which NP binding to the extracellular domain was proposed to stimulate the binding of ATP to the KHD (Fig. 6) (30, 42, 43). Two studies demonstrating cross-linking of azido-ATP analogs to the KHD of GC-A provide some support for this model, although the azido analog used in one study inhibited GC-A and the other study did not test the effects of the azido analog on enzyme activity (26, 31). In contrast, the discovery that both GC-A and GC-B are basally phosphorylated and require phosphorylation to be activated by NPs (23, 24, 44, 45) suggests that the maximal velocity effects were a result of increases in the extent of GC-A phosphorylation when ATP was included in the assay. This is also consistent with early observations showing that adenine analogs that cause prolonged phosphorylation, such as ATP γ S, stimulate the enzyme to a greater extent than do ATP analogs that are not kinase substrates, such as adenylyl-imidodiphosphate (27, 42). Subsequent experiments showed that thiophosphorylated ATP

analogs and phosphatase inhibitors increased the phosphate content and activity of GC-A and GC-B, confirming that the inclusion of ATP analogs in GC assays increased phosphorylation of the enzyme (34, 46, 47).

Our data generated from experiments with highly phosphorylated receptors have provided the foundation for a new model (Fig. 6). In enzyme assays lacking ATP, both GC-A and GC-B exhibited positive cooperativity because there was no molecule to compete with the binding of GTP to the allosteric site (Fig. 6). However, positive cooperativity was not observed in assays mimicking physiologic scenarios because the allosteric site was occupied by ATP and because cellular ATP concentrations are about 10-fold higher than those of GTP (39). This explains the conundrum of why positive cooperativity is only observed in assays conducted under synthetic, but not physiologic, assay conditions: Physiologic assays contain ATP.

Linear product formation in the presence of ATP, but in the absence of NPs, indicated that NP binding was not a prerequisite for ATP binding to the allosteric site (Fig. 6); however, because the K_m was high in the absence of NPs and low in the presence of NPs, ligand binding was required so that the K_m -reducing effects of ATP binding to the allosteric site were transduced to the catalytic site. Upon NP binding (Fig. 6), not only did maximal velocity increase substantially, the K_m also decreased markedly. The combination of increased V_{max} and decreased K_m ultimately led to a more than 100-fold activation of GC-A and GC-B upon NP binding (34).

Several observations and experiments support the idea that ATP binds to the allosteric site that resides in the catalytic domain of GC-A (35, 36). First, ATP and GTP are structurally similar. Second, both ATP and GTP reduce the K_m . Third, ATP is competitive with GTP for the allosteric regulation of the enzymes. Fourth, both nucleotides require the 2'-OH group of ribose for maximal allosteric activation. That the ribosyl 2'-OH group, but not the γ -phosphate, was required for maximum allosteric activation and that mutations that destroyed catalytic activity did not abolish allosterism clearly indicate that the binding requirements for the allosteric and catalytic sites are different.

It has been suggested that changes in cellular ATP concentrations regulate sGC in response to metabolic stress (48). However, because the EC_{50} for ATP activation of GC-A and GC-B is ~0.1 mM, whereas concentrations of ATP in living cells are >1 mM (39), it is unclear how changes in ATP concentrations could regulate the activation of these receptors if ATP concentrations are uniformly distributed throughout the cell. One possibility that could enable ATP to have a regulatory role is if ATP concentrations in the microenvironment containing the receptors are, under some circumstances, substantially lower than the concentrations measured in cellular extracts. Thus, at the moment, we can state that ATP is required for GC-A and GC-B activation under physiologic conditions, but whether changes in ATP concentrations are regulatory has yet to be demonstrated.

The requirement for ATP in the activation of mGCs is analogous to the requirement for GTP in the activation of ACs. Similar to the activation of GC-A and GC-B by ATP, GTP is required for the activation of AC by heterotrimeric guanine nucleotide-binding proteins (G

proteins); however, in both scenarios, changes in nucleotide concentrations have not been shown to affect the signaling of either enzyme, yet GTP binding to Gas is required for activation of AC, and ATP binding to the allosteric site in the catalytic domain is required for the activation of GC-A and GC-B.

Data from other studies are consistent with our findings. Thorpe *et al.* observed cooperative and linear kinetics for the recombinant GC-A catalytic domain with GTP and 2'-deoxy-GTP, respectively (49). Chang *et al.* observed that 2'-deoxy ATP, but not 3'-deoxy ATP, was a poor activator of GC-A compared to ATP (50). Similarly, Parkinson *et al.* reported that the 2'-ribosyl-substituted adenine analogs were poor activators of GC-C (51). Rauch *et al.* demonstrated that the catalytic domain of the mGC from cyanobacteria bound to ATP and GTP with similar affinity and was asymmetric, with only one active site in each dimer (52). Sinha *et al.* reported that a bacterial AC with similarity to mGCs has an asymmetric catalytic domain that contains "half the sites activity" (53), and the Waldman and Beuve groups reported that ATP inhibits sGCs by binding to the pseudo-symmetric site, which is analogous to the allosteric site in mGCs (40, 48). The Seifert group observed mixed-type inhibition of sGC by ATP and proposed a model similar to ours for GC-A and GC-B in which Mn^{2+} -GTP binds to an allosteric site in the catalytic domain of sGC (54). Why ATP activates GC-A and GC-B but inhibits sGC is not currently known. Finally, we note that ATP reduced the K_m for GC-A in experiments reported by other investigators, but for unknown reasons these authors did not comment on the K_m reductions (30, 43).

The binding of forskolin to the pseudosymmetric "P site" in AC led to the idea that an endogenous forskolin-like molecule may exist (41). A forskolin-like molecule was identified in human renal cysts, but this observation has yet to be corroborated by independent investigators (55). However, a number of stimulatory and inhibitory pharmacologic agents that target the diterpene site of AC have been identified (56). Analogous to AC and sGC, we showed that ATP allosterically regulated GC-A and GC-B by binding to a pseudosymmetric site in the catalytic domain. To our knowledge, this is the first example of an endogenous molecule that activates GCs by binding to the pseudosymmetric site.

In conclusion, we determined how adenine nucleotides activate GC-A and GC-B. ADP and ATP, but not AMP or adenosine, bind to an allosteric site in the catalytic domain that switches cGMP formation from having positively cooperative kinetics to linear kinetics and ultimately reduces the Michaelis constant for GTP. Because of the higher cellular concentrations of ATP and the similar EC_{50} values of ATP and ADP, we suggest that ATP, but not ADP, occupies the allosteric site under basal conditions, and that reductions in the Michaelis constant require activation of the receptors by NPs. Thus, we suggest that NPs increase the maximal velocity and reduce the Michaelis constants of GC-A and GC-B. In addition, we showed that an allosteric mechanism was required for the signaling of GC-B under physiologic conditions in which GTP concentrations are much lower than those of ATP. In contrast to studies conducted on a truncated mutant of GC-A, we found that the catalytic domain of the full-length receptor was an asymmetric dimer with reciprocally regulated allosteric and catalytic binding sites that have different binding preferences. Thus, in contrast to older models suggesting that heterodimeric and homodimeric GCs contain one and two catalytic domains, respectively (57), we postulate that all ACs and GCs contain a

single catalytic site. Finally, we suggest that this allosteric activation model may apply to other mGCs and that the allosteric site is a potential target for oral, non-peptide-based drugs.

MATERIALS AND METHODS

Reagents

¹²⁵I-cGMP radioimmunoassay kits were obtained from PerkinElmer Life Sciences. ANP, CNP, creatine kinase, and phosphocreatine were from Sigma-Aldrich. The protease inhibitor cocktail was from Roche.

Cells and transfections

HEK 293T, HEK 293T-GC-A, and HEK 293T-GC-B cells were maintained as previously described (58). HEK 293T cells do not contain detectable NP receptors (figs. S1 and S2); however, some HEK 293 cells contain small amounts of endogenous GC-A, which is consistent with GC-A being properly processed and regulated in these cells (45). NIH 3T3 cells were maintained as previously described (59). About 60% confluent HEK 293T cells grown on polylysine-coated 10-cm plates were transfected with 5 µg of plasmid DNA with the Hepes-buffered calcium phosphate precipitation method. Transfection efficiency was ~80% on the basis of green fluorescent protein abundance. The medium was replaced after 2 hours, and cellular membranes were prepared 48 hours after transfection.

Construction of GC-A mutants

Single amino acid substitutions of alanine for aspartate at position 849 and serine for asparagine at position 968 were performed by QuikChange mutagenesis of the pCMV3-GC-A plasmid according to the manufacturer's instructions, as previously described (45). Confirmation of the mutations and lack of additional mutations was done by nucleic acid sequencing.

Membrane preparation

Crude cellular membranes were prepared at 4°C in phosphatase inhibitor buffer, which consisted of 50 mM Hepes (pH 7.4), 50 mM NaCl, 20% glycerol, 50 mM NaF, 1 mM EDTA, 0.5 µM microcystin, and 1× Roche protease inhibitor cocktail.

GC assays

GC assays were performed at 37°C in a reaction mixture containing 25 mM Hepes (pH 7.4), 50 mM NaCl, 0.1% bovine serum albumin, 0.5 mM isobutylmethyl xanthine, 1 mM EDTA, 5 mM phosphocreatine, creatine kinase (0.1 mg/ml), 0.5 µM microcystin, and 5 mM MgCl₂. GTP concentrations were 0.1 mM for the experiments shown in Figs. 3 (A, C, and D) and 4. The incubation times for each reaction at 37°C are shown in the figure legends. In some assays, 1 mM ATP was included. GC activities in membranes from transfected cells were at least 50-fold higher than those in membranes from untransfected cells, which indicated that the GC activity was a result of the exogenously expressed enzyme (fig. S2). Reactions were initiated by adding 20 µl of crude membranes containing 10 to 18 µg of protein suspended in

phosphatase inhibitor buffer to 80 μ l of reaction mixture. Creatine kinase and phosphocreatine were not included in assays including AMP or ADP to prevent the conversion of these adenine nucleotides to ATP. Reactions were stopped with 0.4 ml of ice-cold 50 mM sodium acetate buffer containing 5 mM EDTA. If not indicated, NP concentrations were 1 μ M. Concentrations of cGMP were determined by radioimmunoassay as previously described (58). Because enzymatic activity was not completely linear with time, we qualified the kinetic parameters obtained under these conditions as “apparent.”

Statistical analysis

Substrate-velocity curves were analyzed by nonlinear regression with an allosteric sigmoidal model in Prism 5 to determine the $V_{\max}$, $S_{0.5}$, and Hill coefficients. A Michaelis-Menten model was used to determine $V_{\max}$ and K_m values in the presence of ATP. Statistically significant differences between nonlinear regression curves used to determine K_m and $V_{\max}$ values were evaluated with the extra sum of squares F test to generate P values with Prism software. $P < 0.05$ was considered statistically significant for all tests. Values are presented as means $\pm$ SEM. Vertical bars within the symbols represent the SEM. When not visible, error bars are contained within the symbol.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Fig. 1.

NPs alone increase the V_{max} of GCs, whereas ATP reduces the K_m in the presence of NPs. GC activity was measured in membranes from HEK 293T cells containing GC-A or GC-B with increasing concentrations of $Mg^{2+}GTP$. n_H , Hill coefficient. $n = 6$ experiments. (A to C) NPs increase maximal velocity in the absence of ATP. For GC-A, V_{max} values were 4 ± 0.9 and 104 ± 18.6 $\text{nmol mg}^{-1} (\text{9 min})^{-1}$ and the $S_{0.5}$ values were 1.1 ± 0.44 and 0.48 ± 0.211 mM in the absence and presence of ANP, respectively. For GC-B, V_{max} values were 4 ± 0.9 and 49 ± 3.7 $\text{nmol mg}^{-1} (\text{9 min})^{-1}$ and the $S_{0.5}$ values were 2.2 ± 0.84 and 1.0 ± 0.15 mM in the absence and presence of CNP, respectively. The NP-associated changes in V_{max} , but not K_m , were significantly different; $P < 0.05$. (D to F) Basal substrate-velocity curves are cooperative without ATP and linear with ATP. For GC-A, the V_{max} values were 3 ± 0.6 and 4 ± 0.8 $\text{nmol mg}^{-1} (\text{9 min})^{-1}$ and the $S_{0.5}$ values were 1.8 ± 0.62 and 1.6 ± 0.65 mM in

the absence and presence of ATP, respectively. For GC-B, the $V_{\max}$ values were 4 ± 0.2 and 4 ± 0.3 nmol mg⁻¹ (9 min)⁻¹ and the $S_{0.5}$ values were 1.0 ± 0.11 and 1.0 ± 0.15 mM in the absence and presence of ATP, respectively. Neither the ATP-associated changes in $V_{\max}$ nor those in K_m were significantly different; $P > 0.05$ for either receptor. (G to I) ATP decreases K_m only in the presence of NPs. For ANP-stimulated GC-A, the $V_{\max}$ values were 70 ± 4.6 and 84 ± 3.4 nmol mg⁻¹ (9 min)⁻¹ and the $S_{0.5}$ values were 0.7 ± 0.09 and 0.1 ± 0.1 mM in the absence and presence of ATP, respectively. For CNP-stimulated GC-B, the $V_{\max}$ values were 49 ± 7.0 and 41 ± 3.1 nmol mg⁻¹ (9 min)⁻¹ and the $S_{0.5}$ values were 0.6 ± 0.18 and 0.09 ± 0.02 mM in the absence and presence of ATP, respectively. The ATP-associated changes in K_m , but not $V_{\max}$, were significantly different; $P < 0.05$ for both receptors.

Fig. 2.

Reciprocal regulation of the catalytic and allosteric sites of GCs by ATP and GTP, respectively. NP-stimulated GC activity was measured for 9 min. $n = 4$ experiments. **(A)** ATP reduces cooperativity and K_m by competing for GTP in a concentration-dependent manner. Hill coefficients and Michaelis constants for GC-A in the presence of 0, 10, and 100 μM ATP were 1.4 ± 0.09 and 0.5 ± 0.03 mM, 1.2 ± 0.08 and 0.2 ± 0.02 mM, and 0.9 ± 0.07 and 0.08 ± 0.010 mM, respectively. **(B)** Hill coefficients and Michaelis constants for GC-B in the presence of 0, 10, and 750 μM ATP were 1.4 ± 0.39 and 0.9 ± 0.33 mM, 0.9 ± 0.13 and 0.4 ± 0.11 mM, and 0.9 ± 0.24 and 0.2 ± 0.18 mM, respectively. Fold reductions in the Michaelis constants were greater at lower GTP concentrations. **(C)** Maximum fold activation of GC-A by ATP was 8.0, 4.3, 3.4, and 2.4 at 0.1, 0.3, 0.9, and 5.4 mM GTP, respectively. **(D)** Maximum fold activation of GC-B by ATP was 14.0, 3.4, 2.1, and 1.4 at 0.1, 0.3, 0.9, and 5.4 mM GTP, respectively. ATP potency increased with increasing GTP. NP-stimulated GC activity was measured with the indicated concentrations of ATP and GTP, and EC_{50} values for ATP were determined by nonlinear curve fitting. **(C)** The EC_{50} values for ATP activation of GC-A were 77, 38, 38, and 16 μM when assayed with 0.1, 0.3, 0.9, and 5.4 mM GTP, respectively. **(D)** The EC_{50} values for ATP activation of GC-B were 40, 26, 16, and 19 μM when assayed with 0.1, 0.3, 0.9, and 5.4 mM GTP, respectively.

Fig. 3.

The purine ribosyl 2'-OH group, but not the 3'-OH group or γ -phosphate, is required for maximum allosteric regulation of GC-A and GC-B. (A) ANP-stimulated GC-A activity was measured with 0.1 mM GTP and increasing concentrations of the indicated adenine nucleotides in membranes from HEK 293T-GC-A cells. $n = 6$ experiments. (B) The Hill coefficient of ANP-stimulated GC-A activity measured with GTP was 1.5 ± 0.21 , but it was 1.0 ± 0.18 when measured with 2'-deoxy-GTP ($n = 4$ experiments). (C) Concentration-response curves for ANP-dependent GC-A activity determined in the presence of 0.1 mM GTP and the indicated concentrations of the individual adenine nucleotides ($n = 6$ experiments). (D) Concentration-response curves for CNP-dependent GC-B activity determined in the presence of 0.1 mM GTP and the indicated concentrations of the individual adenine nucleotides in membranes from HEK 293T-GC-B cells ($n = 4$ experiments). (E) Substrate-velocity curves were generated in membranes from HEK 293T-GC-A cells incubated for 9 min with 1 μ M ANP with or without the indicated nucleotides at concentrations of 1 mM ($n = 4$ experiments). Hill coefficients and K_m values were 1.4 ± 0.21 and 0.8 ± 0.14 mM for the control, 1.2 ± 0.24 and 0.4 ± 0.08 mM for 2'-deoxy-ATP, 1.1 ± 0.18 and 0.2 ± 0.04 mM for ADP, and 1.0 ± 0.15 and 0.1 ± 0.02 mM for ATP. (F) Model depicting allosteric regulation of NP-dependent mGC activity in the presence of GTP or 2'-deoxy-GTP (i and ii) and ATP or 2'-deoxy-ATP (iii and iv).

Fig. 4.

ATP is required for the activation of GC-B under physiologic conditions. GC activity was determined in crude membranes prepared from NIH 3T3 cells in the presence of 2 mM MgCl_2 and 0.1 mM GTP in the presence or absence of 1 mM ATP and the indicated concentrations of CNP. The bars within the symbols represent SEM. Where not visible, the bars are contained within the symbol. $n = 6$ experiments.

Fig. 5. Membrane GCs are asymmetric homodimers with distinct allosteric and catalytic sites. **(A)** Possible catalytic dimers are illustrated with wild-type (WT) or mutant residues shown in each purine-binding site. Residues that are mutated are either red or blue. In the asymmetric model, allosteric sites are triangles and catalytic sites are stars. **(B)** GC activity was determined in the presence of the indicated activators in membranes from HEK 293T cells transiently transfected with various combinations of plasmids encoding WT, D849A, or N968S GC-A isoforms ($n = 4$ experiments). **(C)** Substrate-velocity curves of GC activity determined in the presence of 3 mM Mn²⁺ and 1% Triton X-100 from membranes of HEK 293T cells transfected with plasmids encoding WT GC-A (left y axis) or both D849A and N968S GC-A (right y axis) ($n = 3$ experiments). **(D)** A double-reciprocal plot of the data from (C).

Fig. 6. Old and new activation models for GC-A and GC-B are shown. In the old “two-step” model, NP binding to the extracellular domain caused an intracellular conformational change that enabled ATP to bind to the KHD, which then enabled the two catalytic domains to come together to form two active sites. In the new “one-step” model, ATP is constitutively bound to an allosteric site in the catalytic domain. NP binding to the extracellular domain causes the catalytic domains to come together to increase the maximal velocity of the enzymes by forming a single active site, as well as enabling the effect of ATP binding to the allosteric site to be transduced to the catalytic site to increase affinity for GTP and decrease the K_m .