ACTIVATION AND INHIBITION MECHANISMS OF MEMBRANE GUANYLYL CYCLASES

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ABSTRACT

Guanylyl cyclase (GC)-A and GC-B are homologous enzymes that catalyze the formation of cyclic guanosine monophosphate and pyrophosphate from GTP. GC-A is activated by atrial natriuretic peptide and B-type natriuretic peptide and regulates the cardiovascular system. GC-B is activated by C-type natriuretic peptide and regulates the skeletal and female reproductive systems.

Activation of GC-A and GC-B was hypothesized to occur through two steps, binding of natriuretic peptide and subsequent binding of ATP to the kinase homology domain. However, our group reported that ATP binding does not increase maximal velocity but reduces the Michaelis constant. My work revealed an allosteric ATP binding site in the catalytic domain. Mutation and structure/function studies indicated that the allosteric and catalytic sites are different and that GC-A and GC-B are asymmetric homodimers, not symmetric homodimers as had been previously suggested. Interestingly, a constitutively active mutant of GC-B mimicked an ATP bound state.

ATP inhibits soluble guanylyl cyclases (sGC), and I demonstrated that physiological concentrations of ATP inhibit GC-A and GC-B. I went on to determine that the mechanism of inhibition was through binding the pyrophosphate-product site. Together, these data revealed how low and high concentrations of ATP activate and inhibit GC-A and GC-B, respectively.

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CHAPTER 1: INTRODUCTION TO ACTIVATION AND INHIBITION MECHANISMS OF MEMBRANE GUANYLYL CYCLASES

INTRODUCTION TO MEMBRANE GUANYLYL CYCLASES

Ashman and colleagues first identified cyclic guanosine monophosphate (cGMP) in rat urine in 1963 (Ashman et al. 1963). The enzymes that convert GTP into cGMP were purified six years later (Hardman and Sutherland 1969, Schultz et al. 1969, White and Aurbach 1969). They were originally called guanylate cyclases but were later renamed guanylyl cyclases. The Nomenclature Committee of the International Union of Biochemistry and Molecular Biology named them guanylate cyclase or GTP pyrophosphate-lyase EC 4.6.1.2. The reaction catalyzed requires at least one divalent metal ion bound to GTP and produces the products cGMP and pyrophosphate (PPi) (Hardman and Sutherland 1969). The physiologic metal is Mg^{2+}, though early studies used Mn^{2+} and non-ionic detergent to synthetically activate the enzymes because physiologic activators were not yet known (Hardman et al. 1971). By the mid-1970's, two major types of guanylyl cyclases were purified: soluble, heterodimeric-heme-binding forms that are activated by nitric oxide and particulate homodimeric forms that are activated by peptides (Chrisman et al. 1975, Garbers et al. 1975).

Molecular cloning techniques identified four soluble GCs (a_1, a_2, b_1, b_2) and seven particulate GCs (A-G) in rats. GC-D and -G appear to be regulated by carbon dioxide and are pseudo enzymes in humans (Manning et al. 2002). Soluble GCs (sGC) are heterodimers with a and b subunits, while transmembrane GCs (mGC) are homodimers, although guanylyl cyclase C migrated as a homotrimer when separated by gel electrophoresis (Vaandrager et al. 1994).

The first mammalian membrane GC cloned was GC-A, which is also known as NPR-A or NPR1 (Chinkers et al. 1989). Therefore, GC-A is the archetypal membrane

GC and is the best characterized family member. It is activated by the cardiac hormones atrial natriuretic peptide (ANP) or B-type natriuretic peptide (BNP). The main physiologic consequences of GC-A activation are reductions in blood pressure, blood volume, and heart size (Lopez et al. 1995, Kishimoto et al. 2001).

Guanylyl cyclase-B (GC-B) is also known as NPR-B or NPR2. It is homologous to GC-A but is activated by C-type natriuretic peptide. GC-B increases chondrocyte proliferation and hypertrophy, which results in long bone growth. Inactivating mutations in both alleles coding for GC-B cause acromesomelic dysplasia type Maroteaux (AMDM) dwarfism (Bartels et al. 2004). In contrast, a single residue, activating mutation in the catalytic domain of GC-B results in abnormally long and fragile bones (Fig. 1, V883M) (Miura et al. 2012). GC-B also prevents resumption of meiosis in oocytes during the majority of the female hormone cycle (Hubbard and Greenwald 1982). GC-B is expressed in endothelial cells, where it regulates vascular smooth muscle cell relaxation (Drewett et al. 1995) and proliferation in response to injury (Brown et al. 1997). Finally, GC-B is required for axonal bifurcation in the spinal cord (Schmidt et al. 2007). This chapter will primarily focus on GC-A and GC-B, but will relate relevant information to other GCs as is appropriate.

Guanylyl cyclase C (GC-C) is activated by the endogenous intestinal peptides guanylin and uroguanylin and is the target of pathogenic bacterial heat-stable enterotoxins (ST). GC-C stimulates fluid secretion into the intestine (Field et al. 1978, Hughes et al. 1978), and a single residue, activating mutation in the catalytic domain was linked to familial diarrhea in humans (Fig. 1, S840I). GC-C also is a negative regulator of intestinal epithelium proliferation (Steinbrecher et al. 2002). Recently, GC-

C was associated with attention deficit disorder and energy consumption and metabolism in mice (Gong et al. 2011, Valentino et al. 2011).

Guanylyl cyclase E (GC-E) and F (GC-F) are also known as Ret-GC1 and Ret-GC2, respectively. Both enzymes are found in the rods and cones of the retina, but GC-E is the primary regulator of the visual light and dark cycle. GC-E and GC-F are activated by two small calcium-binding proteins called guanylyl cyclase activator proteins (GCAP). In the presence of high intracellular calcium, guanylyl cyclase activator proteins inhibit GC-E and -F, but under low intracellular Ca^{2+} concentrations, GCAPs activate GC-E and -F.

Membrane Guanylyl Cyclase Structure

Topologically, mGCs have large extracellular and intracellular regions separated by a single membrane span. The extracellular domain of GC-A and GC-B is about 450 amino acids and contains three intra-molecular disulfide bonds. Given the diverse ligand specificities, the extracellular domain is the least similar region between family members, having 15-20% identity overall, with 35-40% identity within mGC subgroups (Potter 2005). GC-A, -B, and -C are glycosylated on asparagine residues (Bennett et al. 1991, Hasegawa et al. 1999), and GC-B is also glycosylated on serine residues (Muller et al. 2010). GC-E and -F are predicted to have one or no glycosylation sites, respectively, and enzymatic deglycosylation procedures do not affect activity.

The intracellular region is composed of a kinase homology-regulatory domain, a coiled-coiled-dimerization domain, and a C-terminal GC-catalytic domain. The kinase homology domain (KHD), so named because it shares identity with enzymes possessing known phosphotransferase activity, regulates the catalytic activity of GC-A and GC-B.

Deletion of the KHD from GC-A or GC-B results in a constitutively activated receptor that is insensitive to ligand, consistent with the KHD repressing the catalytic domain under basal conditions (Chinkers and Garbers 1989). Sustained exposure to ligand reduces the binding affinity of both GC-A and GC-C for ligand (Potter and Garbers 1992, Vieira et al. 2001). However, when the KHD is deleted from enzymes, this reduction in affinity does not occur. Thus, the KHD is required for communication between the extracellular and catalytic domains. The coiled-coiled domain adopts an amphipathic alpha helix that is necessary and sufficient for dimerization (Thompson and Garbers 1995, Wilson and Chinkers 1995). This domain has also been suggested to repress the activity of GC-C in the absence of ligand (Saha et al. 2009). The catalytic domain contains the active site that binds Mg^{2+}-GTP as well as a recently identified allosteric site that binds ATP (Robinson and Potter 2012). Whether the allosteric site binds Mg^{2+}-ATP or free ATP is not known. While no crystal structure is available for mammalian GC domains, crystal structures of dimeric guanylyl cyclase catalytic domains were determined for enzymes purified from cyanobacteria (Rauch et al. 2008) and green algae (Winger et al. 2008). The bacterial enzyme was active but the algal protein was not. The structures were similar to those determined for the adenylyl cyclase catalytic domain.

ACTIVATION OF MEMBRANE GUANYLYL CYCLASES

Most of what is known about nucleotide cyclizing enzymes comes from studies on adenylyl cyclases. Adenylyl cyclases (ACs) catalyze the formation of cAMP and

pyrophosphate from Mg-ATP. These enzymes contain twelve transmembrane spans and two similar but unique cytoplasmic catalytic domains (termed C1 and C2) that form an enzymatically active heterodimer. ACs are activated by heterotrimeric G-proteins but are also activated pharmacologically by forskolin (Tesmer et al. 1997). The catalytic domains of sGCs, mGCs, and ACs are homologous (Fig. 1) (Krupinski et al. 1989). The crystal structure of the catalytic domain of a heterodimeric type I AC is used as a model for the catalytic domains of GCs (Liu et al. 1997). The AC catalytic domain consists of a wreath like structure composed of alternating beta sheets within an alpha helical scaffolding. The heterodimer is composed of a head-to-tail arrangement between the two subunits that forms two small molecule-binding sites at the interface of the dimers. Like ACs, the catalytic domains of sGCs and mGCs are also head-to-tail dimers, with two purine binding sites formed at the dimer interface (Dessauer et al. 1997). Dimerization is necessary for enzyme activity because substrate-binding residues come from both monomers.

The catalytic site contains adenine-specific recognition residues for ACs and guanine-specific residues for GCs. The second purine-binding site known as the pseudosymmetric site binds forskolin in ACs and is thought to allosterically regulate the catalytic site; although whether an endogenous activator binds the pseudosymmetric site is currently unclear. Like forskolin activation of AC, the only molecules known to bind the pseudosymmetric site of sGC are drugs called YC-1 and BAY 41-2272 (Friebe et al. 1999, Friebe and Koesling 2003, Roy et al. 2008). Binding of activators to the pseudosymmetric site increases substrate affinity, while also increasing the potency of NO (Friebe and Koesling 1998, Friebe and Koesling 2003, Roy et al. 2008).

mGC catalytic domains exist as homodimers, and until recently, the most accepted model depicted a symmetric homodimer with two identical, catalytically active sites (Liu et al. 1997). mGCs were thought to be symmetric homodimers because they demonstrated positive cooperative substrate-velocity curves, which predicted two substrate-binding sites with binding to one site increasing the affinity of the second site for GTP. A truncated, heterodimeric mutant of GC-A containing single mutations in each monomer lacked cooperativity, as predicted by the symmetrical purine binding site model (Joubert et al. 2007). However, identical mutations in the full-length enzyme abolished catalytic activity but did not affect allosteric activity. These data indicated for the first time that GC-A exists as an asymmetric homodimer with unique catalytic and allosteric sites that bind GTP and ATP, respectively (Robinson and Potter 2012). We do not know why the effects of the catalytic mutations differed between the full length and truncated enzymes. Consistent with the asymmetric model, the crystal structure a homodimeric AC from *M. tuberculosis* indicated an asymmetric catalytic dimer with two quasi-symmetric purine-binding sites with only one site being catalytically active (Sinha et al. 2005). The noncatalytic site failed to bind forskolin but showed mild positive cooperativity, similar to mGCs (Sinha et al. 2005). Interestingly, the two-purine sites of mGCs demonstrate reciprocal regulation since ATP binding decreased the Km for GTP and GTP reduced the EC_{50} for ATP activation of GC-A and GC-B (Robinson and Potter 2011). Reciprocal regulation has not been reported for forskolin-dependent activation in ACs. YC-1 decreased the Km for sGCs similarly to how ATP decreased the Km for GC-A and GC-B. However, whether GTP affects the potency of YC-1 for sGC is not known.

Catalytic mechanism

A primary amino acid sequence alignment of the catalytic domains of human mGCs, sGC a1, sGC b1, and type I AC is shown in Fig. 1 to reveal the critical residues in these enzymes that are evolutionarily conserved. The sequence of Cya2 - a homodimeric mGC from cyanobacteria - is included because its crystal structure was determined (Rauch et al. 2008).

The catalytic mechanism of AC involves at least three steps. First, ATP binds in a conformation where the ribosyl 3'-OH group is close to the alpha phosphate. Carboxylic acid oxygens from a pair of aspartic acids coordinate two magnesium ions, and the magnesium ion nearest the alpha phosphate stabilizes the transition state. A carboxylic acid group from one of the aspartic acids involved with coordinating one of the magnesium ions (Fig. 1, cyan) deprotonates the ribosyl 3'-OH and the unpaired electrons from the oxygen attack the a phosphate, causing the cyclization of the alpha phosphate with the 3'-OH ribose to form a 3' – 5' cyclic bond. The pyrophosphate product is stabilized by a second Mg^{2+} ion. Mutational studies in GCs support a similar catalytic scheme (Yuen et al. 1994, Rudner et al. 1995, Thompson and Garbers 1995). For instance, mutation of Asp 893 to Ala in GC-A (Rudner et al. 1995, Thompson and Garbers 1995, Olson et al. 1998), which corresponds to the catalytic base (Asp 354) in AC (Hurley 1999), inactivated the enzyme. The same substitution also inactivated sGC (Yuen et al. 1994) (Fig. 1, cyan/red). Once the bond between the beta and gamma phosphates is broken and cGMP is formed, product release is random, meaning that cGMP and pyrophosphate are released independently. However, pyrophosphate release

was reported to be slow and partially rate-limiting for ACs, which may also be the case for GCs (Chang et al. 1990).

The substrate binding residues in GCs have been determined from modeling and amino acid substitution studies based on the crystal structures of AC. For example, the adenine 6-amino group interacts with an aspartic acid (Asp 1000), while the N1 interacts with a lysine (Lys 923) (Liu et al. 1997, Tesmer et al. 1997, Hurley 1999) (Fig. 1, magenta). Additionally, the lysine may help stabilize the conformation of the aspartic acid that interacts with the 6-amino group, thus forming a small hydrophobic pocket. Mutational data support the importance of the lysine residue, as mutation to an alanine increases the K_m 2-fold (Tang et al. 1995). Similarly, the catalytic site of GCs has specific guanine-recognizing residues (Fig. 1, magenta) (Liu et al. 1997). For human GC-A, the guanine O6 hydrogen bonds with the sulfhydryl group of Cys 965 and to the amine group of Arg 963. The N1 of guanine forms a hydrogen bond with the free amine group of Arg 963 (Fig. 2) (Liu et al. 1997, Rauch et al. 2008). Consistent with this scenario, mutation of the GC-specific glutamic acid and cysteine to the AC-specific lysine and aspartic acid converted GC-E to an AC (Tucker et al. 1998).

The 3-OH group on the ribose ring is the site of cyclization for GTP, and is activated by Asp 893 in GC-A (Fig. 2). A Mg^{2+} ion coordinates the alpha phosphate with help from Arg 972, and helps to direct electrons away from the phosphorous core as observed for DNA polymerases (Liu et al. 1997, Sawaya et al. 1997, Doublie et al. 1998). The carboxylic acid group of Asp 893 is hypothesized to deprotonate the 3-OH hydroxyl group of the ribose. The free electron pair of the deprotonated 3'-oxygen then initiates a nucleophilic attack on the a phosphate. Recent investigators suggest that there

are two molecules of Mg^{2+} present based on earlier kinetic studies and more recent GC crystal structures that were formed in the presence of Mg^{2+} (Garbers et al. 1975, Rauch et al. 2008, Winger et al. 2008). We observed maximum activity with a ratio of two Mg^{2+} ions per GTP, though we cannot distinguish whether both ions are binding in the active site (data not shown). We hypothesize that the second Mg^{2+} ion associates primarily with the beta and gamma phosphates and coordinates pyrophosphate binding and release. This binding scheme is supported by the recent crystal structures for a heterodimeric (Rauch et al. 2008) and homodimeric (Winger et al. 2008) GC, and is depicted in Figure 2 with the amino acids corresponding to human GC-A and GC-B (parentheses).

Peptide-dependent mGC activation

The stoichiometry for binding of ANP to GC-A is 1:2 (Ogawa et al. 2004). CNP is predicted to bind GC-B similarly (He et al. 2006, Yoder et al. 2008). ANP binds to the extracellular GC-A dimer in an asymmetric manner because different residues from each monomer bind ANP. Peptide binding causes a 24 degree rotation of the juxtamembrane region of GC-A (Ogawa et al. 2004). By an unknown mechanism, this results in an intracellular conformational change that activates the catalytic domain. In the absence of ATP, NP-stimulation of GC-A and GC-B results in a ten-fold or more increase in maximal velocity without affecting the Michaelis constant (Robinson and Potter 2012). However, in the presence of ATP, NP binding also reduces the Michaelis constant of GC-A and GC-B up to ten-fold by a process that requires ATP binding to the allosteric site in the catalytic domain (Antos and Potter 2007).

Guanylin (Currie et al. 1992), uroguanylin (Hamra et al. 1993) and heat stable enterotoxin (STa) from enterotoxigenic *E.coli* activate GC-C (Field et al. 1978, Hughes et al. 1978, Schulz et al. 1990). All three peptides act by increasing the maximal velocity of GC-C without affecting the Michaelis constant (Gazzano et al. 1991). Likewise, guanylyl cyclase activating proteins (GCAPs) increase maximal velocity of GC-E and GC-F without affecting the Km (Dizhoor et al. 1994, Gorczyca et al. 1994).

Phosphorylation-dependent GC regulation

Membrane GCs were first shown to be phosphorylated in sea urchins (Ward and Vacquier 1983). In 1992, the first mammalian mGC, GC-A, was shown to be phosphorylated on serine and threonine residues in the absence of NP (Potter and Garbers 1992). Prolonged incubation of GC-A or GC-B with NP reduced enzyme phosphate content and NP-dependent GC activity (Potter and Garbers 1992, Potter 1998). In vitro incubation of GC-A and GC-B with the catalytic domain from protein phosphatase 2A also resulted in losses in phosphate and NP-stimulated activity (Potter and Garbers 1992, Potter 1998).

The region preceding the KHD and the beginning part of the KHD of GC-A, -B, and -E is highly phosphorylated, while the C-terminal extension of GC-C is phosphorylated. Eight phosphorylation sites were identified on GC-A and seven sites have been identified on GC-B (Yoder et al. 2010, Yoder et al. 2012). Mutation of the phosphorylated residues to an alanine to mimic a constitutively dephosphorylated residue resulted in decreased NP-stimulated activity. Mutation of four or more phosphorylation sites in GC-A to alanine resulted in an enzyme that was not responsive to NP. In contrast, mutation of the same sites to a glutamate to mimic a constitutively

phosphorylated residue, resulted in an enzyme that was responsive to NP activation and was resistant to ANP-dependent inactivation in membranes and whole cells (Potter and Hunter 1999).

The KHD of GC-C is not phosphorylated, though reports suggest that activity is phosphorylation dependent (Wada et al. 1996). GC-C, -E, and -F have a C-terminal extension that is not present on GC-A or -B and it has been shown that GC-C is phosphorylated on a specific serine on the C-terminal tail in response to PMA-mediated PKC activation (Wada et al. 1996). GC-E has been reported to autophosphorylate residues just preceding the KHD, though this phosphorylation does not appear to be regulatory (Aparicio and Applebury 1996, Bereta et al. 2010).

ATP regulation of membrane guanylyl cyclases

Kurose *et al.* first reported that ATP potentiates the activation of ANP-stimulated GC activity in rat particulate tissue preparations in 1987 (Kurose et al. 1987). Importantly, most of this effect was phosphorylation-independent since the non-hydrolyzable ATP analogue AMPPNP and ADP activated almost as well as ATP. Foster and Garbers identified two distinct roles for ATP in regulation of GC-A activity: as a phosphate donor for protein kinases to phosphorylate GC-A, as well as a second, direct role as an allosteric activator of GC-A (Foster and Garbers 1998). They found that including ATPγS in the assay led to thiophosphorylated serine and threonine residues of GC-A, which made the enzyme resistant to dephosphorylation. This process sensitized the enzyme to further activation of GC-A by AMPPNP, an analog of ATP that is not a substrate for protein kinases. Chang *et al.* determined that ATP and ADP but not AMP

analogues activated GC-A and that the hydroxyl group at the 2 but not the 3 position of ribose is important for activation (Chang et al. 1990).

Two groups recently reported that ATP is required for ANP-dependent GC-A activation (Joubert et al. 2005, Burczynska et al. 2007). A two-step activation model was proposed where ANP binds the receptor and causes a conformational change that allows ATP to bind a putative GXGXXXG region the KHD, resulting in a conformational change that increased maximal activity. In contrast, we found that ATP is not required for maximal enzyme activation of GC-A in 293 or endogenously expressing cells, which is inconsistent with the two-step model (Antos et al. 2005, Antos and Potter 2007). Additionally, mutations known to block ATP binding to the similar GXGXXG site in protein kinases had no effect on the ability of ATP to activate GC-A (Koller et al. 1992, Antos and Potter 2007). We demonstrated that ATP is not required for mGC activation but rather functions to decrease the Michaelis constant for GTP in the presence of NP (Antos et al. 2005, Antos and Potter 2007, Robinson and Potter 2012). NPs alone maximally activate GC-A and GC-B, but the affinity of these enzymes for GTP as indicated by a high Michaelis constant was not affected by NPs in the absence of ATP. Surprisingly, including sub-millimolar concentrations of ATP in the assay reduced the Km from 1 mM to 0.1 mM GTP, which is within the range of cellular GTP concentrations (Traut 1994). In contrast to the allosteric activators for ACs or sGCs, ATP did not reduce the EC_{50} for NP activation or increase maximal velocity (Robinson and Potter 2012).

Similar to GC-A and GC-B, GC-C is also activated by adenine nucleotides (Gazzano et al. 1991, Vaandrager et al. 1993). GC-C activation is not dependent on

ATP, rather ATP was suggested to stabilize the active state of the enzyme (Vaandrager et al. 1994, Antos et al. 2005). In contrast to GC-A and -B, ATP was reported not to alter substrate affinity of GC-C (Vaandrager et al. 1993). GC-E was also shown to be activated by ATP (Gorczyca et al. 1994). Interestingly, ATP activates GC-E in the absence of GCAPs and the activation is independent of Ca^{2+} concentrations (Gorczyca et al. 1994, Aparicio and Applebury 1996, Yamazaki et al. 2003, Bereta et al. 2010).

INHIBITION OF MGCS

There are several ways that activated GCs are turned off. Homologous desensitization is enzyme inactivation resulting from prolonged exposure to the activating hormone. One mechanism for this process is enzyme dephosphorylation, which occurs over several minutes to hours (Potter and Garbers 1992, Koller et al. 1993, Potter 1998, Joubert et al. 2001). Heterologous desensitization is inactivation that results from the presence of a hormone that opposes the actions of the mGC, such as arginine vasopressin, angiotensin II, lysophosphatidic acid, sphingosine-1-phosphate, and platelet derived growth factor, all of which activate the phospholipase C/diacylglycerol/calcium/protein kinase C cascade (Potter and Garbers 1994). Inactivation associated with lysophosphatidic acid was correlated with GC-B dephosphorylation (Abbey and Potter 2003).

End-product inhibition

End product or feedback inhibition results when the products of the enzyme or degradation products of the products bind the enzyme and inhibit activity. End-product inhibition has been studied extensively for AC and sGC, and recent data from our group suggests both GC-A and GC-B are inhibited similarly. AC purine (P)-site inhibitors were first identified when cAMP and synthesized AMP derivatives were shown to inhibit AC activity at high concentrations (Fain et al. 1972, Desaubry et al. 1996, Desaubry et al. 1996, Johnson et al. 1997). Kinetic analysis revealed that adenosine analogs such as 2'd3'-AMP or 2'3'-ddAdo are noncompetitive inhibitors, while cAMP is a mixed-type inhibitor, (Johnson and Shoshani 1990) and Mg-pyrophosphate

enhanced the inhibitory effect of each purine inhibitor. Cyclic AMP competed with the substrate analog, AMPCPP, but only in the presence of pyrophosphate (Chang et al. 1990). The data are consistent with end products - such as linearized AMP - acting as dead-end inhibitors of pyrophosphate release by binding at the active site post catalysis.

P-site inhibitors were also studied for sGC (Makino et al. 2012). The end-product analog, 2'd3'-GMP, was a potent noncompetitive inhibitor of sGC. Interestingly, the pyrophosphate analog foscarnet enhanced affinity for 2'd3'-GMP. The data are consistent with an end-product inhibitory complex, in which GMP and pyrophosphate bind the active site and form a dead-end complex. The inhibitory effect was more pronounced with NO-binding, possibly as a result of the increased concentration of pyrophosphate resulting from the increased enzyme activity. P-site inhibition was also enhanced in the presence of the allosteric activator BAY 41-2272, likely due to increased product formation.

P-site inhibition of a truncated form of GC-A was also examined (Joubert et al. 2007). As with ACs and sGCs, the 2'd3'-GMP moiety was a potent inhibitor, and this inhibition was potentiated by pyrophosphate. It was concluded that pyrophosphate keeps the enzyme in a post-transitional state that has an enhanced affinity for GMP. Interestingly, a loss of cooperativity was observed when pyrophosphate and 2'd3'-GMP were bound, suggesting that binding of pyrophosphate and 2'd3'-GMP block binding to or transmission of the allosteric signal to the catalytic site. These observations are consistent with end products acting as feedback inhibitors that not only prevent substrate binding, but also reduce allosteric binding. Unfortunately, it is not known if P-site inhibitors affect the ability of ATP to bind to the allosteric site. It is also important to

note that these studies were conducted on a truncated from of the enzyme that we have shown does not behave like the full-length molecule.

Nucleotide triphosphate (NTP)-dependent inhibition of GCs

GTP and ATP have been shown to inhibit GC activity at higher concentrations. NTP-dependent inhibition of sGC has been studied extensively (Chang et al. 2005, Yazawa et al. 2006, Roy et al. 2008, Derbyshire et al. 2009). GTP, ATP, and ADP, as well as their respective 2' substituted analogs, inhibit sGC activity. In contrast, AMPS was not inhibitory, suggesting inhibition requires at least two phosphates. The inhibition was mixed, suggesting that the inhibitor does not interact with the substrate-binding site and binds in the presence and absence of GTP. The allosteric activators of sGC, YC-1 and BAY 41-2272, reduced binding of the ATP to the enzyme in a concentration-dependent manner, suggesting that the allosteric activators are competitive with the inhibitors. YC-1 and BAY 41-2272 were reported to bind the pseudosymmetric site and the authors concluded that the nucleoside inhibitors bind the pseudosymmetric site as well. Another group found a similar result, though they concluded that YC-1 did not bind the pseudosymmetric site, but rather YC-1 binds in the N-terminal region of the a subunit of sGCs (Stasch et al. 2001, Koglin and Behrends 2003). They concluded that ATP bound both the catalytic and pseudosymmetric site, resulting in mixed-type inhibition. In contrast to previous studies, we suggest that all phosphorylated compounds that inhibit GC-A and GC-B at millimolar concentrations do so by binding the pyrophosphate product site. Our hypothesis is consistent with previous observations in both sGC and mGCs. It has been shown that YC-1 and BAY 41-2272 binding increases maximal velocity of the enzyme while also decreasing the Km. As such, binding of YC-

1 or BAY 41-2272 could alter product inhibitor binding by reducing the affinity of the inhibitors for the active site, thereby reducing binding and increasing maximal velocity without directly binding the pseudosymmetric site. Recent data from our group shows that both pyrophosphate and ATP are mixed-type inhibitors of GC-A and GC-B, and their binding is mutually exclusive, demonstrating that PPi and ATP compete for binding the same product site (manuscript submitted).

CONCLUSION

At the beginning of my graduate studies, the prevailing dogma for GC-A and GC-B activation involved a two-step activation model where NP binding caused a conformational change in the KHD that allowed ATP to "derepress" the catalytic domain. This increased maximal velocity with no change in the Michaelis constant. However, Laura Antos demonstrated that GC-A and GC-B were maximally activated in the absence of ATP if substrate concentrations in the assay were high (Antos et al. 2005). Surprisingly, she found that ATP decreased the Michaelis constant of GC-A and GC-B (Antos and Potter 2007). Our work has refined the activation mechanism by demonstrating that ATP binds an unreported allosteric site within the catalytic domain under basal conditions and, in NP stimulated conditions, causes an increased affinity for substrate.

These studies also determined that the two-nucleotide binding sites in the enzyme were asymmetric. Previously, it was suggested that GC-A and GC-B exist as symmetric homodimers with two identical catalytic sites. In contrast, our work determined that GC-A and GC-B are asymmetric homodimers, with unique catalytic and

noncatalytic, allosteric sites that have different binding requirements. We also determined that the allosteric and catalytic sites are reciprocally regulated.

Finally, we demonstrated that ATP inhibits GC-A and GC-B by binding the pyrophosphate product site and prevents the recycling of GC-A and GC-B to a state that can bind and catalyze substrate. Finally, we demonstrated product inhibition for both GC-A and GC-B. This phosphorylation-independent inhibition was faster than the previously described dephosphorylation-dependent process and was reversible by dilution. In conclusion our work has advanced understanding of the structure, activation, and inactivation mechanisms of mGCs and has resolved decades-long discrepancies and controversies.

FIGURES

FIGURE 1. Sequence alignment of human mGCs (GC-A through F), human sGCa1, human sGCb1, the cyanobacterial mGC (Cya2), and human AC Type I domains C1 and C2.

Dark grey shading indicates identical amino acids. Light grey represents similar amino acids. Magenta amino acids determine substrate specificity. The conserved cyan aspartic acid is the catalytic base that the deprotonates the 3' hydroxyl group on the ribosyl moiety. Published inactivating mutations are shaded red, while activating mutations are shaded green. Specific GC-A, GC-B, or GC-C mutants are labeled above the respective amino acid abbreviation. Sequences were aligned using Clustal Omega alignment tool from the European Bioinformatics Institute (EBI).

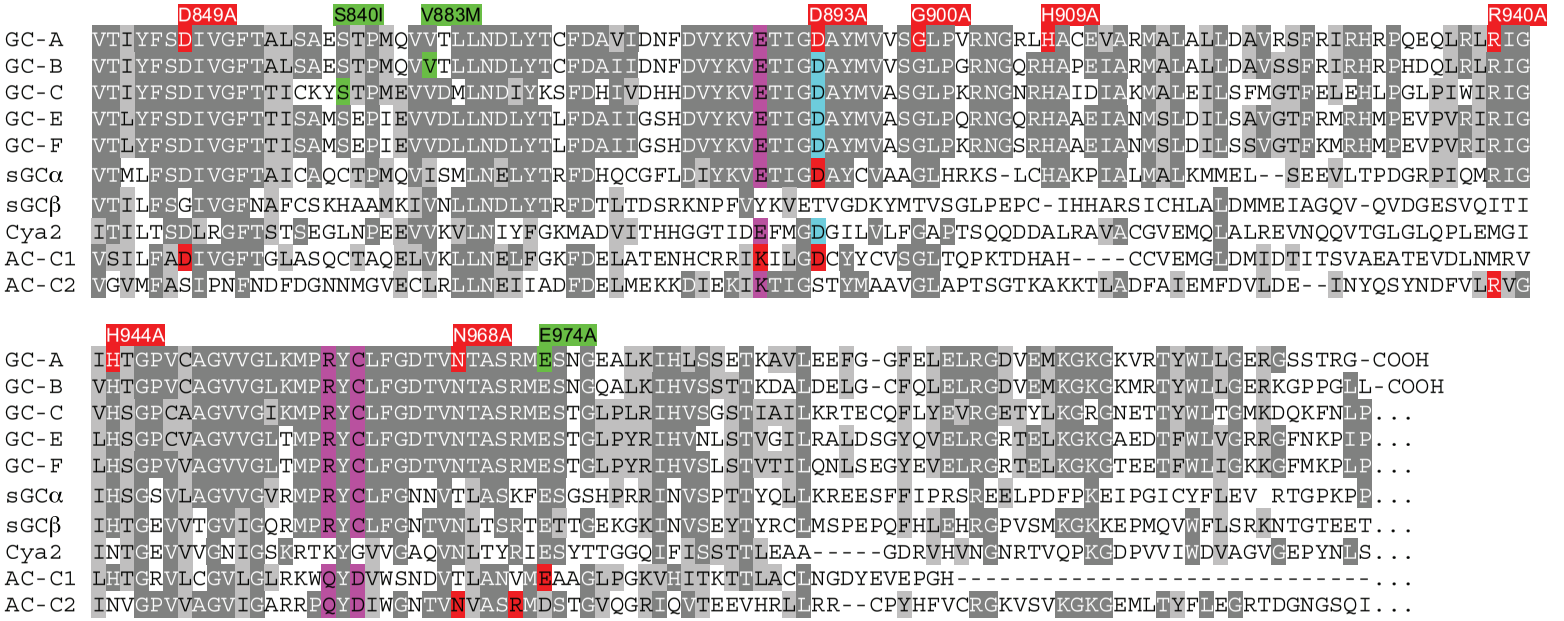
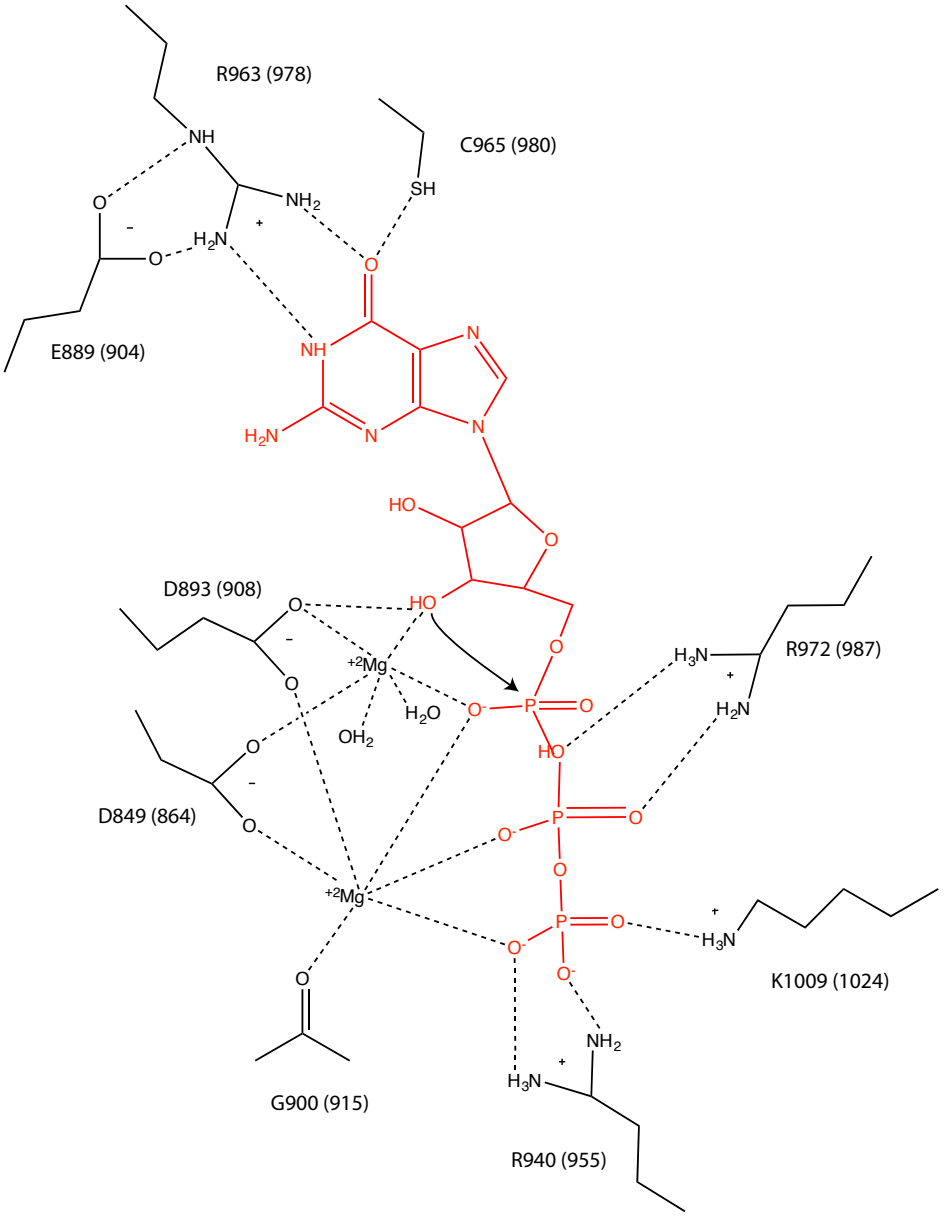

D849A
S840I
V883M
D893A
G900A
H909A
R940A
GC-A VTIYFSDIVGFTALSAESTPMQVVTLLNDLYTCFDAVIDNFDVYKVETIGDAYMVVSGLPVRNGRLHACEVARMALALLDAVRSFRIRHRPQEQLRLRIG
GC-B VTIYFSDIVGFTALSAESTPMQVVTLLNDLYTCFDAIIDNFDVYKVETIGDAYMVVSGLPGRNGQRHAPEIARMALALLDAVSSFRIRHRPHDQLRLRIG
GC-C VTIYFSDIVGFTTICKYSTPMEVVDMLNDIYKSFDHIVDHHDVYKVETIGDAYMVASGLPKRNGNRHAIDIAKMALEILSFMGTFELEHLPGLPIWIRIG
GC-E VTLYFSDIVGFTTISAMSEPIEVVDLLNDLYTLFDAIIGSHDVYKVETIGDAYMVASGLPQRNGQRHAAEIANMSLDILSAVGTFRMRHMPEVPVRIRIG
GC-F VTLYFSDIVGFTTISAMSEPIEVVDLLNDLYTLFDAIIGSHDVYKVETIGDAYMVASGLPKRNGSRHAAEIANMSLDILSSVGTFKMRHMPEVPVRIRIG
sGCα VTMLFSDIVGFTAICAQCTPMQVISMLNELYTRFDHQCGFLDIYKVETIGDAYCVAAGLHRKS-LCHAKPIALMALKMMEL--SEEVLTPDGRPIQMRIG
sGCβ VTILFSGIVGFNAFCSKHAAMKIVNLLNDLYTRFDTLTDSRKNPFVYKVETVGDKYMTVSGLPEPC-IHHARSICHLALDMMEIAGQV-QVDGESVQITI
Cya2 ITILTSDLRGFTSTSEGLNPEEVVKVLNIYFGKMADVITHHGGTIDEFMGDGILVLFGAPTSQQDDALRAVACGVEMQLALREVNQQVTGLGLQPLEMGI
AC-C1 VSILFADIVGFTGLASQCTAQELVKLLNELFGKFDELATENHCRRIKILGDCYYCVSGLTQPKTDHAH----CCVEMGLDMIDTITSVAEATEVDLNMRV
AC-C2 VGVMFASIPNFNDFDGNNMGVECLRLLNEIIADFDELMEKKDIEKIKTIGSTYMAAVGLAPTSGTKAKKTLADFAIEMFDVLDE--INYQSYNDFVLRVG
H944A
N968A
E974A
GC-A IHTGPVCAGVVGLKMPRYCLFGDTVNTASRMESNGEALKIHLSSETKAVLEEFG-GFELELRGDVEMKGKGKVRTYWLLGERGSSTRG-COOH
GC-B VHTGPVCAGVVGLKMPRYCLFGDTVNTASRMESNGQALKIHVSSTTKDALDELG-CFQLELRGDVEMKGKGKMRTYWLLGERKGPPGLL-COOH
GC-C VHSGPCAAGVVGIKMPRYCLFGDTVNTASRMESTGLPLRIHVSGSTIAILKRTECQFLYEVRGETYLKGRGNETTYWLTGMKDQKFNLP...
GC-E LHSGPCVAGVVGLTMPRYCLFGDTVNTASRMESTGLPYRIHVNLSTVGILRALDSGYQVELRGRTELKGKGAEDTFWLVGRRGFNKPIP...
GC-F LHSGPVVAGVVGLTMPRYCLFGDTVNTASRMESTGLPYRIHVSLSTVTILQNLSEGYEVELRGRTELKGKGTEETFWLIGKKGFMKPLP...
sGCα IHSGSVLAGVVGVRMPRYCLFGNNVTLASKFESGSHPRRINVSPTTYQLLKREESFFIPRSREELPDFPKEIPGICYFLEV RTGPKPP...
sGCβ IHTGEVVTGVIGQRMPRYCLFGNTVNLTSRTETTGEKGKINVSEYTYRCLMSPEPQFHLEHRGPVSMKGKKEPMQVWFLSRKNTGTEET...
Cya2 INTGEVVVGNIGSKRTKYGVVGAQVNLTYRIESYTTGGQIFISSTTLEAA-----GDRVHVNGNRTVQPKGDPVVIWDVAGVGEPYNLS...
AC-C1 LHTGRVLCGVLGLRKWQYDVWSNDVTLANVMEAACLPGKVHITKTTLACLNGDYEVEPGH----------...
AC-C2 INVGPVVAGVIGARRPQYDIWGNTVNVASRMDSTGVQGRIQVTEEVHRLLRR--CPYHFVCRGKVSVKGKGEMLTYFLEGRTDGNGSQI...

FIGURE 2. Catalytic mechanism for GC-A and GC-B.

Hypothetical model depicting GTP bound into the catalytic site of human GC-A or GC-B (parentheses). Residues E889, R963, and C965 mediate substrate recognition, while the carboxylate group of D893 is predicted to be the catalytic base that deprotonates the 3'-OH on the ribose ring. The top Mg2+ ion stabilizes the deprotonated 3'-O- and alpha phosphate. The amine group of R972 is thought to coordinate the alpha phosphate, which is subsequently attacked by the deprotonated ribosyl 3'-O-. A second (bottom) Mg2+ ion, along with several other residues on nearby amino acids, stabilizes the beta and gamma phosphates on GTP. The ribosyl 3'-O- forms a covalent bond with the alpha phosphate, resulting in the formation of 3' – 5' cyclic GMP and pyrophosphate.


R963 (978)
C965 (980)
E889 (904)
D893 (908)
D849 (864)
G900 (915)
R940 (955)
K1009 (1024)
R972 (987)

CHAPTER 2: THE INDOLOCARBAZOLE, GÖ6976, INHIBITS GUANYLYL CYCLASE-A AND -B

This chapter is a reprint of an original publication with minor alterations:
Robinson, Jerid W., Lou, Xiaoying, Potter, Lincoln R. (2011) The indolocarbazole, Go6976, inhibits guanylyl cyclase-A and -B. *British Journal of Pharmacology* 164(2b):499-506.

Jerid Robinson collected all data except for part of the top panel of Figure 7 and contributed to writing the manuscript.

Atrial natriuretic peptide (ANP) and B-type natriuretic peptide (BNP) decrease vascular volume and pressure by activating guanylyl cyclase-A (GC-A). C-type natriuretic peptide (CNP) activation of guanylyl cyclase-B (GC-B) stimulates long bone growth. This study investigated the inhibitory effects of the indolocarbazoles, Gö6976 and GF-109203X, on the guanylyl cyclase activity of GC-A and GC-B as a first step towards developing small molecule regulators of these enzymes. Gö6976, but not GF-109203X, blocked cellular CNP-dependent cGMP elevations in 293T-GC-B cells. The time required for Gö6976 to reduce CNP-dependent cGMP elevations to half maximal levels ($t_{1/2}$) was 7 seconds and the concentration required to reduce CNP-dependent cGMP elevations to half maximal levels (IC_{50}) was 380 nM. Gö6976 increased the EC_{50} for CNP 4.5 fold, but increasing the CNP concentration did not overcome the inhibition. Half of the inhibition was lost 1 h after removal of Gö6976 from the medium. Cellular exposure to Gö6976 reduced basal and natriuretic peptide-dependent, but not detergent-dependent, GC-A and GC-B cyclase activity. Inhibition was also observed when Gö6976 was added directly to the cyclase assay. A constitutively phosphorylated form of GC-B was inhibited like the wild type receptor. These data demonstrate that Gö6976 potently, rapidly and reversibly inhibits GC-A and GC-B via a process that does not require intact cell architecture, known phosphorylation sites or inactivation of all catalytic sites. This is the first report of an intracellular inhibitor of a transmembrane guanylyl cyclase and the first report of a non-kinase target for Gö6976.

INTRODUCTION

Natriuretic peptides are pleiotropic factors that regulate the cardiovascular and skeletal systems (Potter et al. 2009). Humans express three structurally related but genetically distinct natriuretic peptides known as atrial natriuretic peptide (ANP), B-type natriuretic peptide (BNP) and C-type natriuretic peptide (CNP). Three natriuretic peptide receptors have been identified as well. Guanylyl cyclase-A (GC-A) and guanylyl cyclase-B (GC-B) are composed of large extracellular ligand binding domains, single membrane-spanning regions and intracellular kinase homology, dimerization, and carboxyl-terminal guanylyl cyclase catalytic domains (Solis et al. 1994, Potter 2009, Potter et al. 2009). GC-A is activated by ANP and BNP, whereas GC-B is activated by CNP. Gene inactivation experiments in mice indicate that GC-A mediates the blood pressure and volume reducing effects of ANP and BNP, whereas GC-B mediates the skeletal growth effects of CNP (Lopez et al. 1995, Tamura et al. 2004).

Homozygous inactivating mutations in GC-B cause a severe form of human dwarfism called Acromesomelic Dysplasia, type Maroteaux (AMDM). Human chromosomal translocations that increase CNP concentrations are associated with Marfanoid-like skeletal overgrowth (Bocciardi et al. 2007, Moncla et al. 2007). Recently, CNP infusions were shown to increase long bone growth in a murine model of fibroblast growth factor receptor-3-dependent dwarfism (Yasoda et al. 2009). Together, these data indicate that GC-B inactivation causes dwarfism, that GC-B over-activation causes Marfanoid-like skeletal overgrowth, and that CNP rescues long bone growth in a murine model of the most common form of human dwarfism. Thus, identifying small

molecule inhibitors and activators of GC-B may lead to a new class of drugs for skeletal diseases.

In broken cell preparations, ATP increases the guanylyl cyclase activity of GC-A and GC-B when activated by natriuretic peptides but inhibits when activity is measured in the presence of manganese and nonionic detergent (Kurose et al. 1987, Gazzano et al. 1991). Whether ATP is required for initial natriuretic peptide activation of GC-A and GC-B is controversial. We found that initial activation does not require ATP (Antos et al. 2005) but that activities measured at longer time periods are increased in the presence of ATP due to reduction of the Michaelis constant for GTP (Antos and Potter 2007).

Both GC-A and GC-B are phosphorylated on multiple residues in resting cells (Yoder et al. 2010). Prolonged exposure to natriuretic peptide causes the dephosphorylation and desensitization of the receptors (Potter and Garbers 1992, Potter 1998). Conversion of known phosphorylation sites to alanine or glutamate yields hormonally unresponsive receptors or responsive receptors, respectively (Potter and Hunter 1999). Thus, phosphorylation is required for hormonal activation and dephosphorylation is a mechanism of desensitization.

Phorbol 12-myristate, 13-acetate (PMA) activation of protein kinase C (PKC) inhibits hormone-dependent activation of GC-A and GC-B in a manner that correlates with site-specific receptor dephosphorylation (Potter and Garbers 1994, Potter and Hunter 2000, Potthast et al. 2004). Guanylyl cyclase inhibition and dephosphorylation by PMA is blocked by the general PKC inhibitor GF-109203X, which competes for ATP binding to the active site of PKC (Potter and Garbers 1994, Abbey-Hosch et al.

2005). GF-109203X has no inhibitory effect on natriuretic peptide receptors in the absence of PKC activators. To identify the PKC isoform required for PMA-dependent inhibition of GC-B, cells were incubated with Gö6976, a compound that inhibits the conventional subgroup of the PKC family. However, an unexpected inhibitory effect of Gö6976 on GC-B activation was observed in the absence of PMA, which led to the studies described in this report. We found that Gö6976 potently, rapidly, and reversibly inhibits GC-B by a mechanism that does not require changes in known phosphorylation sites, cellular architecture or complete disruption of the active site.

EXPERIMENTAL PROCEDURES

Reagents. Cyclic GMP radioimmunoassay kits and ^{32}P-α GTP were from Perkin Elmer (Waltham, MA). Gö6976 was acquired from Sigma-Aldrich (Saint Louis, MO). GF-109203X, also known as Gö6850, was purchased from EMD chemicals (Gibbstown, NJ).

Cells. Human 293T cells stably expressing rat GC-A or GC-B were cultured as described (Potter and Hunter 2000, Fan et al. 2005). Mouse 3T3-L1 cells were cultured as described (Rodbell and Krishna 1974).

Whole cell stimulations. Cells were seeded on poly-D-lysine-coated 48-well plates and incubated >5 hr in serum-free media upon reaching 70% confluency. The medium was aspirated and replaced with 0.25 ml DMEM containing 1 mM 1-methyl-3-isobutylxanthine (IBMX) and 25 mM Hepes pH 7.4 for 10 min. Following pretreatment, the medium was aspirated and cells were incubated with the new medium with or without 1 μM CNP for 1 min. The medium was aspirated and the reaction was

stopped with 0.5 ml ice-cold 80% ethanol. The cGMP content of the extract was determined by radioimmunoassay as described (Abbey and Potter 2002).

Guanylyl cyclase assays. Crude membranes were prepared in phosphatase inhibitor buffer as previously described (Bryan and Potter 2002). Assays were performed at 37ºC for 5 min in a buffer containing 25 mM Hepes pH 7.4, 50 mM NaCl, 0.1% BSA, 0.5 mM IBMX, 1 mM GTP, 10-30 μCi of α-^{32}P-GTP, 1 mM EDTA, 0.5 μM microcystin, 1 mM ATP and 5 mM $MgCl_2$ in the presence (stimulated) or absence (basal) of the indicated natriuretic peptide. In some assays, Mn^{2+} was substituted for Mg^{2+} and Triton X-100 was added to a final concentration of 1% to artificially activate the enzyme. Synthesized ^{32}P-cGMP was purified and quantified as described (Bryan and Potter 2002).

Concentration response assays. Cells in 48 well plates were exposed to different concentrations of Gö6976 or an equivalent volume of DMSO for 1 hr at 37°C. The medium was aspirated and new medium with or without 1 μM CNP for was added. After 1 min, the medium was aspirated and the reaction was stopped with 0.5 ml ice-cold 80% ethanol and the cGMP content of the extract was determined.

Time course assays. Cells in 48-well plates were exposed to either 10 μM Gö6976 or an equivalent volume of DMSO for the periods of time indicated. The medium was then aspirated and replaced with new medium with or without 1 μM CNP. After 1 min, the medium was aspirated and the reaction was stopped with 0.5 ml ice-cold 80% ethanol and cGMP concentrations were determined.

Wash out experiments. 293T GC-B cells were incubated with medium containing 1 μM Gö6976, 10 μM Gö6976 or DMSO for 5 min. After 5 min the cells were washed twice with medium and then new medium containing 1 μM CNP was added for 1 min at the indicated post-wash time points. The medium was aspirated and the reaction was stopped with 0.5 ml ice-cold 80% ethanol and cellular cGMP concentrations of cell extracts determined.

Statistical analysis. All curves were fitted using GraphPad Prism 5.0 software. All p-values were obtained using a paired student's t-test in Microsoft Excel. Error bars represent the standard error of the mean, and when not visible, are contained within the symbol.

RESULTS

Gö6976 inhibits CNP-dependent intracellular cGMP elevations. Initial studies investigated the ability of PMA, Gö6976 and GF-109203X to modulate CNP-dependent cGMP elevations in 293T-GC-B cells. The structural similarity between Gö6976 and GF-109203X is shown in Fig. 1 (Top). One micromolar CNP elevated intracellular cGMP concentrations 48-fold, but prior PMA exposure reduced elevations more than 60% (Fig. 1, Bottom). Exposure to GF-109203X for 60 min prior to CNP stimulation yielded intracellular cGMP concentrations that were elevated 55-fold higher than those from control cells. PMA did not significantly reduce CNP-dependent cGMP concentrations in cells exposed to GF-109203X, consistent with PMA inhibiting GC-B through a PKC-dependent mechanism. Surprisingly, Gö6976 alone completely blocked CNP-dependent cGMP concentrations, and exposure to both Gö6976 and PMA reduced

CNP-dependent intracellular cGMP concentrations to levels that were below basal values.

Gö6976 inhibits the guanylyl cyclase activity of GC-B. To directly measure the effect of Gö6976 on the enzymatic activity of GC-B, intact 293T-GC-B cells were incubated with 10 μM Gö6976 for 1 h and guanylyl cyclase activities in crude membranes were determined under basal, CNP-stimulated or detergent-stimulated conditions (Fig. 2, Top). Ten micromolar Gö6976 exposure reduced basal and CNP-dependent guanylyl cyclase activities 51 and 49%, respectively, but failed to significantly reduce activity measured in the presence of Triton X-100 and Mn^{2+}GTP.

Gö6976 inhibits GC-A. Intact 293T cells stably expressing GC-A (293T-GC-A) were also incubated with 10 μM Gö6976 for 1 h and guanylyl cyclase activities in crude membranes were determined as described above except that ANP was substituted for CNP (Fig. 2, Bottom). Ten micromolar Gö6976 exposure reduced basal and ANP-dependent guanylyl cyclase activities 53 and 40%, respectively, but failed to significantly reduce activity measured in the presence of Triton X-100 and Mn^{2+}GTP.

Gö6976 is a potent inhibitor of GC-B. 293T-GC-B cells were incubated with increasing concentrations of Gö6976 and then CNP-dependent cGMP elevations were determined (Fig. 3, Top). The concentration required to inhibit half of the maximal CNP-dependent cGMP response (IC_{50}) was approximately 380 nM.

Gö6976 is a rapid inhibitor of GC-B. The time required for Gö6976 to reduce CNP-dependent concentrations to half of control values ($t_{1/2}$) was also determined. 293T-GC-B cells were incubated with 10 μM Gö6976 for the periods of time shown and then the cells were incubated with 1 μM CNP for 1 min and intracellular cGMP concentrations were determined. The $t_{½}$ was estimated to be 7 seconds (Fig. 3, Bottom).

Gö6976 modestly increases the EC_{50} but inhibition is maintained at saturating CNP concentrations. The ability of Gö6976 to competitively antagonize CNP activation of GC-B was investigated. Whole 293T-GC-B cells were incubated with or without 10 μM Gö6976 for 30 min and then exposed to various concentrations of CNP for 1 min and intracellular cGMP concentrations were determined (Fig. 4, Top). Absolute cGMP concentrations are shown in the top panel of Fig. 4 and the data are replotted with two Y-axes as shown in the bottom panel of Fig. 4. The calculated EC_{50}s for control (DMSO) and Gö6976 treated cells were 40 nM and 180 nM, respectively. A slight but significant 4.5-fold rightward shift in the dose-response curve in Gö6976 treated cells was observed (Fig. 4, Bottom). However, the inhibitory effect was not overcome at saturating CNP concentrations.

Gö6976 inhibition of GC-B is concentration-dependent and rapidly reversible. 293T-GC-B cells were incubated with 10 μM Gö6976 for 5 min, washed twice with DMEM, and incubated for the indicated periods of time before exposing the cells to CNP for 1 min to elevate intracellular cGMP concentrations (Fig. 5, Top). Approximately half of the activity was recovered 1 h after removal of Gö6976. When

the concentration of Gö6976 was reduced ten-fold to 1 μM, recovery was more rapid (Fig. 5, Bottom). The apparent half-time for recovery was 9 min and complete recovery was observed by 30 min in cells exposed to 1 μM Gö6976.

Gö6976 inhibition does not require changes in known GC-B phosphorylation sites. Since Gö6976 inhibits several protein kinases, and phosphorylation of GC-B is required for CNP-dependent activation (Potter 1998, Potter and Hunter 1998), we examined if Gö6976 reduces CNP-dependent activation by blocking GC-B phosphorylation (Fig. 6). A cell line stably expressing a version of GC-B containing glutamate substitutions for all six known GC-B phosphorylation sites (293-GC-B-6E) was used in these experiments (Yoder, Stone et al. 2010). Gö6976 inhibited GC-B-6E similarly to the wild type receptor, consistent with a mechanism that is independent of changes in the phosphorylation of known sites.

Gö6976 inhibits GC-B in broken cell preparations. In all previous experiments, measuring cGMP concentrations in whole cells or cyclase activity in crude membranes accessed the effect of whole cell Gö6976 exposure. Here, the ability of Gö6976 to inhibit GC-B activity when directly added to membranes was examined. As a positive control for Gö6976 inhibition, whole 293T-GC-B cells were incubated in the presence or absence of Gö6976 for 1 h and then crude membranes were prepared and assayed for basal, CNP or detergent-dependent guanylyl cyclase activity (Fig 7, Top (Whole Cell)). To test for direct inhibitory effects, crude membranes from 293T-GC-B cells that were not previously exposed to Gö6976 were incubated with Gö6976 or vehicle during the

cyclase assay (Fig. 7, Top (Membrane)). Addition of Gö6976 to whole cells or crude membranes inhibited CNP-dependent GC-B activity to a similar extent, consistent with Gö6976 acting in a manner that does not require intact intracellular architecture or cellular incubation. We also investigated whether Gö6976 inhibits GC-B in a separate cell line. Ten micromolar Gö6976 decreased CNP-dependent guanylyl cyclase activities at 5 and 10 min by 68 and 71%, respectively, in membranes from mouse 3T3-L1 cells. We have previously shown that 3T3 cells express GC-B, not GC-A (Abbey and Potter 2003).

DISCUSSION

Little is known about the inhibition of GC-A and GC-B. HS-142-1, a microbial polysaccharide, was shown to block CNP-dependent bone growth (Yasoda et al. 1998) and to inhibit CNP-dependent cGMP elevations in podocytes (Lewko et al. 2004), but no direct studies on GC-B guanylyl cyclase activities have been reported. Studies by Lewko and colleagues indicated an IC_{50} of HS-142-1 between 0.25 and 2.5 micromolar, which is similar to the IC_{50} of 380 nM determined for Gö6976. Both known GC-A antagonists, HS-142-1 and A71915, block ANP binding to the extracellular domain of GC-A (Delporte et al. 1992, Sano et al. 1992). In contrast, Gö6976 does not inhibit GC-B by only reducing CNP binding since the shift in the EC_{50} was relatively small and the inhibition was maintained at high CNP concentrations (Fig. 4, Top). Thus, Gö6976 uses a novel inhibitory mechanism compared to other antagonists of guanylyl cyclase-linked natriuretic peptide receptors.

The rapidity of the Gö6976-dependent inhibition of GC-B ($t_{1/2}$ = 7 sec) was striking. We have never seen anything inhibit GC-B remotely as fast as Gö6976. The fast kinetics of inhibition, relatively quick reversibility and ability to inhibit GC-B in broken cell preparations are consistent with a model where Gö6976 directly binds the receptor but this remains to be determined.

Gö6976 is structurally similar to ATP and competitively inhibits ATP binding to various PKCs (Martiny-Baron et al. 1993). Therefore, it is possible that Gö6976 inhibits GC-B by blocking the ability of ATP to activate the receptor. Two reports have identified putative ATP binding sites in the kinase homology domain (KHD) of GC-A (Joubert et al. 2005, Burczynska et al. 2007), but whether these sites are conserved in

GC-B and are required for ATP-dependent activation of GC-A is not known. An alternative hypothesis is that ATP binds one of the two putative nucleotide-binding sites in the catalytic domain of GC-B. Structural and functional data on a guanylyl cyclase isolated from cyanobacteria indicates two nucleotide-binding sites that have similar affinities for GTP and ATP (Rauch et al. 2008). When manganese is used as the divalent metal, GC-A, GC-B and the cyanobacteria guanylyl cyclase exhibit positive cooperative kinetics with respect to increasing GTP concentrations, consistent with two catalytic sites. However, under physiologic conditions where magnesium is the divalent cofactor, linear kinetics are observed for GC-A and GC-B, consistent with a single catalytic site. We found that ATP decreases the Michaelis constant for Mg^{2+}GTP, which is in agreement with a model where ATP increases the affinity of the catalytic site for GTP (Antos and Potter 2007). Thus, it is possible that Gö6976 inhibits GC-A and GC-B by blocking binding of ATP to a regulatory site in the catalytic domain. The fact that Gö6976 does not inhibit detergent dependent activity suggests that it does not directly inhibit the catalytic site, but we cannot rule out the possibility that the catalytic sites differ in the detergent-activated and natriuretic peptide-activated forms of the enzymes.

Recently Duda *et al.* reported that staurosporine activates GC-A similarly to AMP-PNP in a broken cell assay, which suggests that staurosporine mimics the ability of ATP to activate the receptor (Duda et al. 2010). However, we failed to observe activation of GC-B or GC-A by any of the indolocarbazoles that we tested in this report, including staurosporine (Robinson and Potter, 2011). Thus, in our hands, indolocarbazoles do not mimic the ability of ATP to activate receptor guanylyl cyclases.

In addition to the classic forms of PKC (Martiny-Baron et al. 1993), Gö6976 has been shown to inhibit Trk A, Trk B, JAK2 and FLT3 tyrosine kinases (Behrens et al. 1999, Grandage et al. 2006). However, GC-A and GC-B are the first reported non-kinase enzymatic targets of Gö6976. The IC_{50} of Gö6976 for whole cell inhibition of GC-B was similar to that reported for inhibition of JAK3 but higher than that reported for inhibition of the Trk A and Trk B receptors (Behrens et al. 1999, Grandage et al. 2006). Our data indicate that natriuretic peptide receptor guanylyl cyclase inhibition should be considered when interpreting the effects of Gö6976 on cell function.

In conclusion, we have shown for the first time that Gö6976 is a rapid, potent and reversible inhibitor of GC-A and GC-B that does not require changes in phosphorylation sites or modification of the active site. Future studies will investigate the mechanism of action of Gö6976 on GC-A and GC-B.

FIGURES

FIGURE 1. The PKC inhibitor Gö6976, but not GF-109203X, inhibits CNP-dependent activation of GC-B in whole cells.

Top. Comparison of the structure of GF-109203X and Gö6976 is shown. Bottom. 293T-GC-B cells were incubated with or without the indicated compounds for 30 min at 37oC and then cells were stimulated with 1 μM CNP for 1 min and intracellular cGMP concentrations were determined. n = 6 from two experiments. ** and *** indicate significance at p values of < 0.02 or 0.001, respectively, compared to cGMP concentrations measured in cells treated with CNP alone.

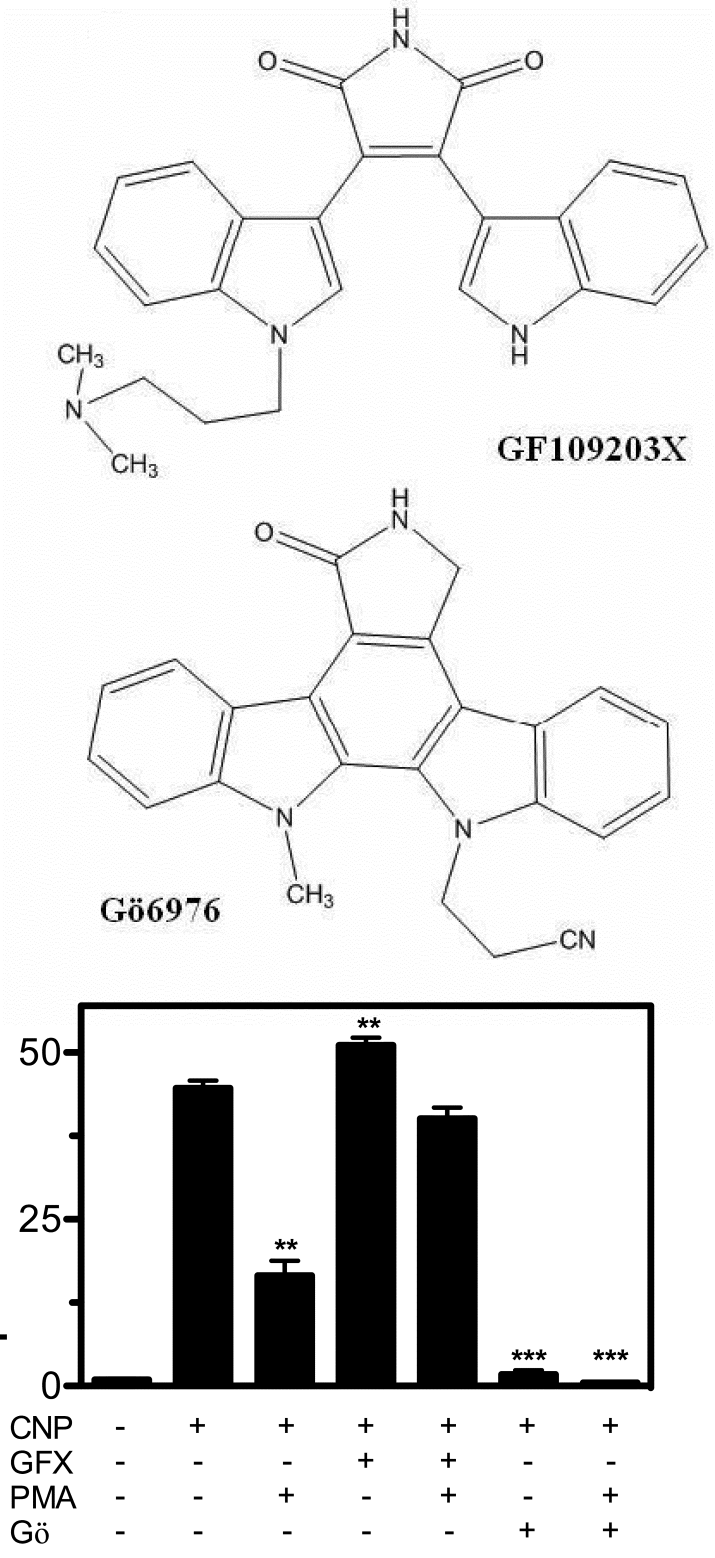

GF109203X
Gö6976
pmol cGMP well-1
50
25
0
**
**
***
***
CNP - + + + + + +
GFX - - - + + - -
PMA - - + - + - +
Gö - - - - - + +

FIGURE 2. Gö6976 inhibits basal and hormone- but not detergent-dependent guanylyl cyclase-A and B activity.

Top. 293T-GC-B cells were incubated with DMSO (vehicle) or 10 μM Gö6976 for 1 h at 37 ^{o}C and then crude membranes were prepared and guanylyl cyclase activities were measure under basal or CNP-stimulated conditions and presented as shown. Activities measured in the presence of Mn^{2+}GTP and 1% Triton X-100 were 112.7 ± 18.7 and 101.5 ± 13.2 for DMSO and Gö6976, respectively. ** indicates significance at a p value of <0.002, where n = 12 from six experiments. *Bottom*. 293T-GC-A cells were incubated with 10 μM Gö6976 and assay for guanylyl cyclase activity as described above. Activities measured in the presence of Mn^{2+}GTP and 1% Triton X-100 were 49.8 ± 8.5 and 45.2 ± 10.1 for DMSO and Gö6976, respectively * indicates significance at a p value of <0.02, where $n \geq 4$ from three experiments.

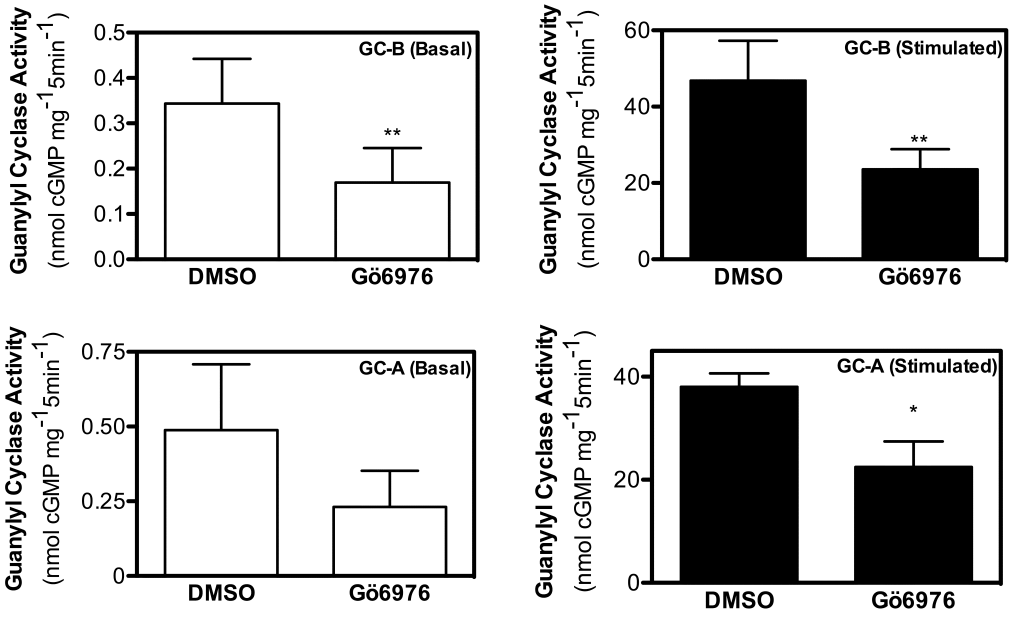
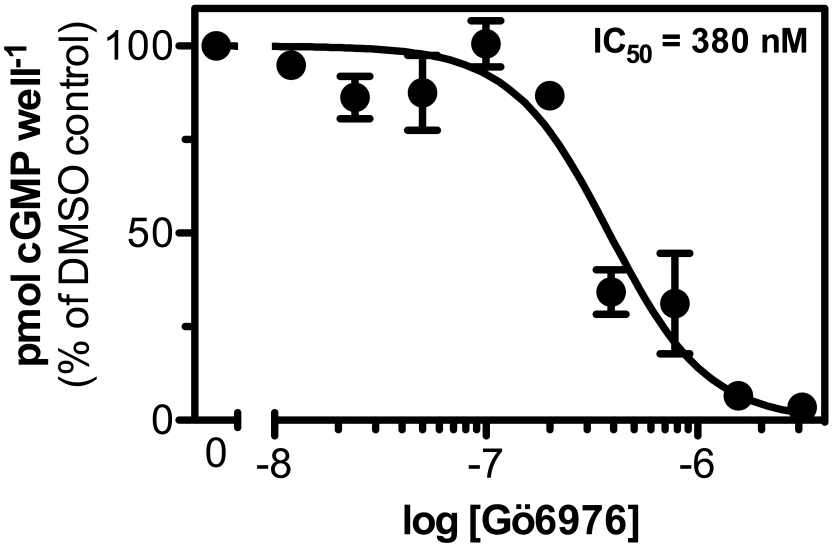

Guanylyl Cyclase Activity
(nmol cGMP mg-1 5min-1)
GC-B (Basal)
0.5
0.4
0.3
0.2
0.1
0.0
**
DMSO
Gö6976
Guanylyl Cyclase Activity
(nmol cGMP mg-1 5min-1)
GC-B (Stimulated)
60
40
20
0
**
DMSO
Gö6976
Guanylyl Cyclase Activity
(nmol cGMP mg-1 5min-1)
GC-A (Basal)
0.75
0.50
0.25
0
DMSO
Gö6976
Guanylyl Cyclase Activity
(nmol cGMP mg-1 5min-1)
GC-A (Stimulated)
40
20
0
*
DMSO
Gö6976

FIGURE 3. Gö6976 is a potent and rapid inhibitor of GC-B.

Top. 293T-GC-B cells were incubated with increasing concentrations of Gö6976 for 1 hr at 37°C and then the cells were stimulated with 1 μM CNP for 1 min and cGMP accumulations were determined and plotted as a function of the log of the Gö6976 concentration. IC_{50} = 607 nM, r^2 = 0.81, n = 6 from two experiments. *Bottom*. 293T-GCB cells were treated with 10 μM Gö6976 for the indicated periods of time and then stimulated with 1 μM CNP for 1 min. Cyclic GMP accumulations were measured and plotted as a function of time in the presence of Gö6976. $t_{½}$ = 7 sec, r^2 = 0.82, n ≥ 3 from two experiments. ^ indicates an n of 3, whereas all other points represent an n of 6.


pmol cGMP well-1
(% of DMSO control)
100
50
0
IC50 = 380 nM
0
-8
-7
-6
log [Gö6976]

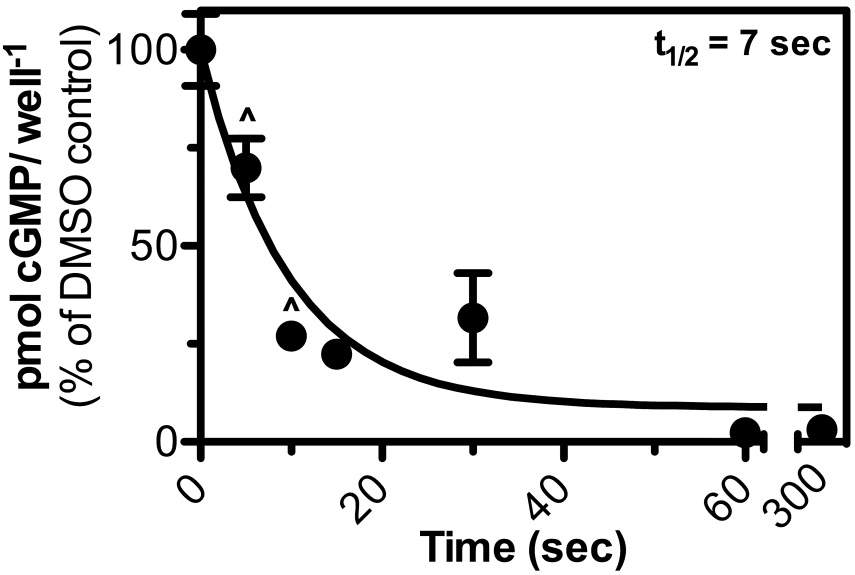
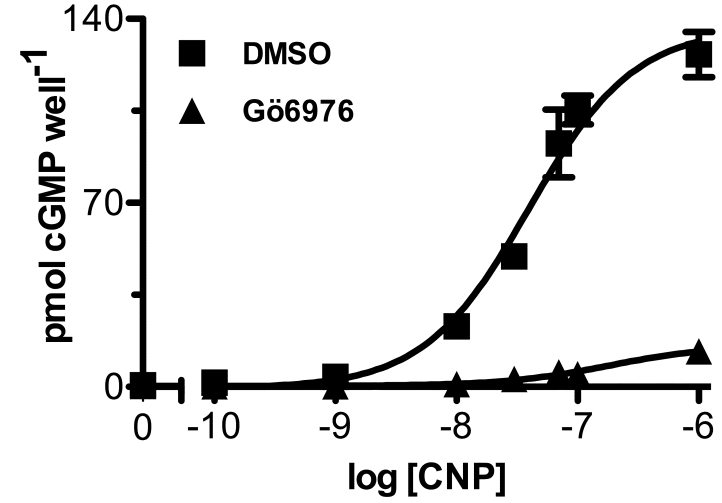

pmol cGMP/ well-1
(% of DMSO control)
100
50
0
t1/2 = 7 sec
0
20
40
60
300
Time (sec)

FIGURE 4. Gö6976 reduces maximum CNP-dependent cGMP elevations and increases the EC_{50} for CNP.

293T-GCB cells were treated with DMSO or 10 μM Gö6976 for 30 min and then stimulated with the indicated concentrations of CNP for 1 min and intracellular cGMP concentrations were determined. *Top*. Absolute values are plotted as pmol cGMP per well. *Bottom*. Data are replotted with two Y-axes to show the rightward shift in the CNP dose-response curve in presence of Gö6976. DMSO EC_{50} = 40 nM, r^2 = 0.91; Gö6976 EC_{50} = 181 nM, r^2 = 0.83, where n = 6 from two experiments.


140
70
0
pmol cGMP well-1
DMSO
Gö6976
0
-10
-9
-8
-7
-6
log [CNP]

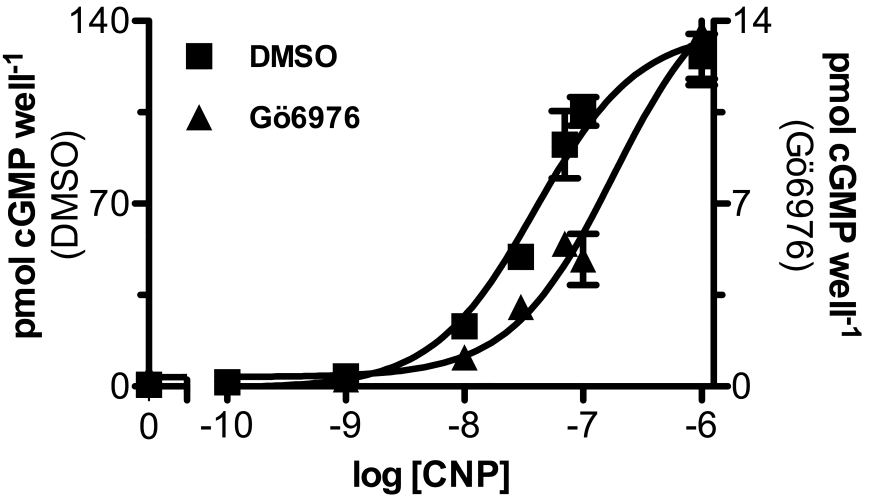
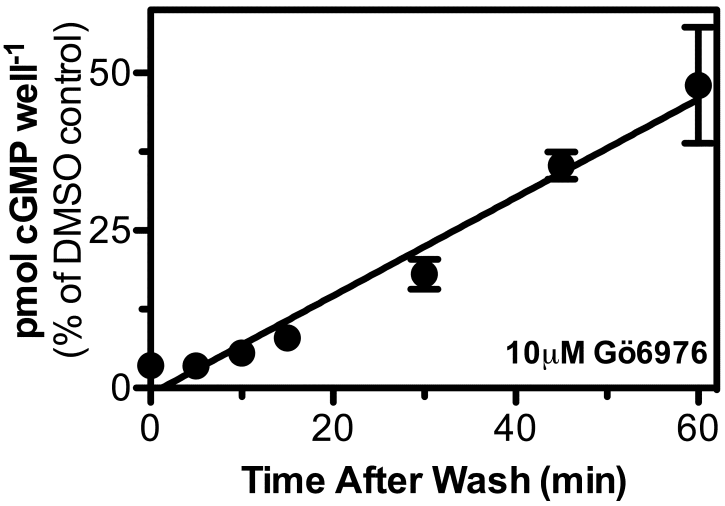

140
70
0
pmol cGMP well-1
(DMSO)
DMSO
Gö6976
14
7
0
pmol cGMP well-1
(Gö6976)
0
-10
-9
-8
-7
-6
log [CNP]

FIGURE 5. Inhibition of GC-B by Gö6976 is concentration-dependent and reversible.

Top. 293T-GC-B cells were treated with 10 μM Gö6976 for 5 min and then washed twice with DMEM. At the indicated times, cells were stimulated with 1 μM CNP for 1 min and cGMP accumulations were determined and plotted as a function of time after wash. Time required to recover half of the initial maximal activity was approximately 1 h, $r^2 = 0.93$, where n = 6 from two experiments. *Bottom*. 293T-GC-B cells were treated with 1 μM Gö6976 for 5 min and then washed twice with DMEM. At the indicated times, cells were stimulated with 1 μM CNP for 1 min and cGMP accumulations were determined and plotted as a function of time after wash. Time required to recover half of the initial maximal activity was approximately 9 min, $r^2 = 0.94$, where $n \geq 3$ from two experiments.


pmol cGMP well-1
(% of DMSO control)
50
25
0
10μM Gö6976
0
20
40
60
Time After Wash (min)

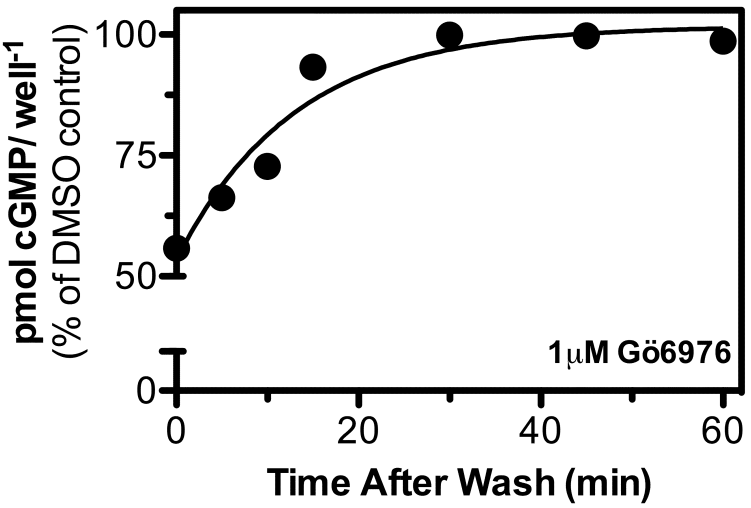

pmol cGMP/ well-1
(% of DMSO control)
100
75
50
0
1μM Gö6976
0
20
40
60
Time After Wash (min)

FIGURE 6. Gö6976 inhibition does not involve known GC-B phosphorylation sites. 293T-GC-B or 293T-GC-B 6E cells were incubated with DMSO or 10 μM Gö6976 for 1 h at 37 ^{o}C. Crude membranes were prepared and guanylyl cyclase assays were determined under basal or stimulated conditions. ** and *** indicate significance at p values of <0.01 and p<0.0005, respectively, where n = 6 from three experiments.

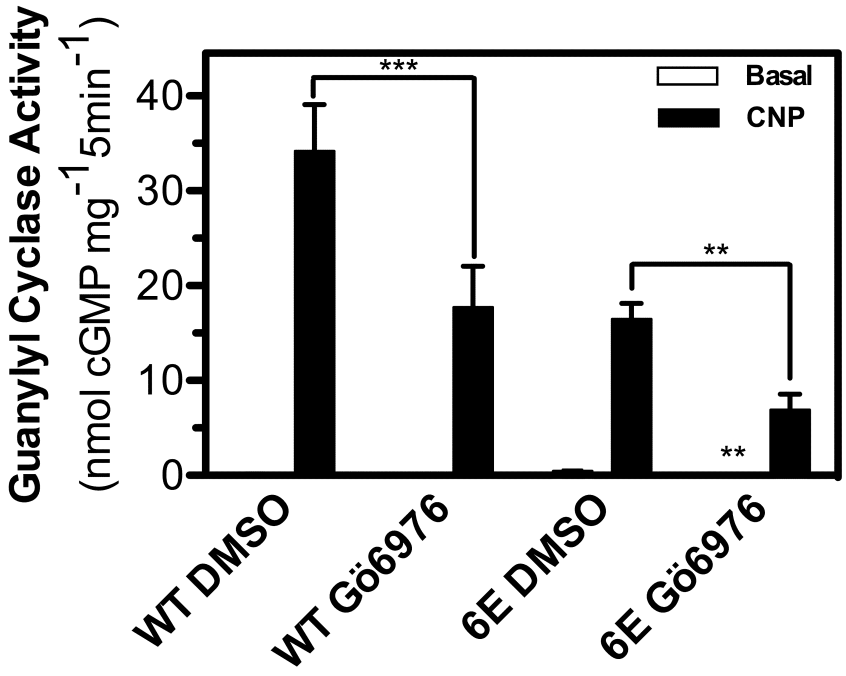
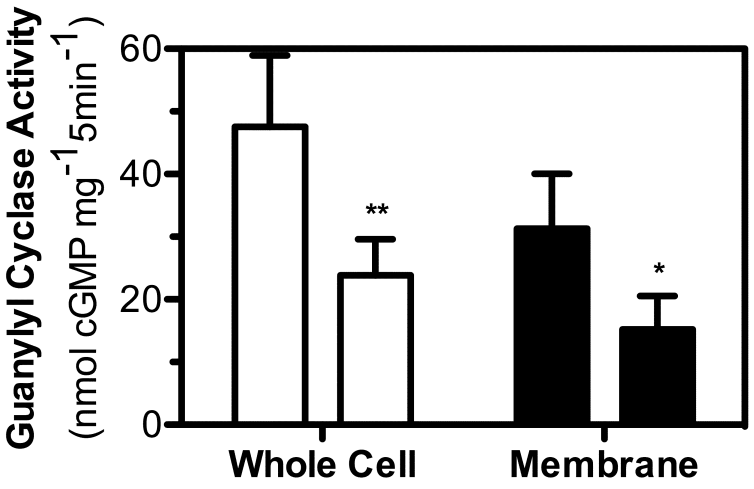
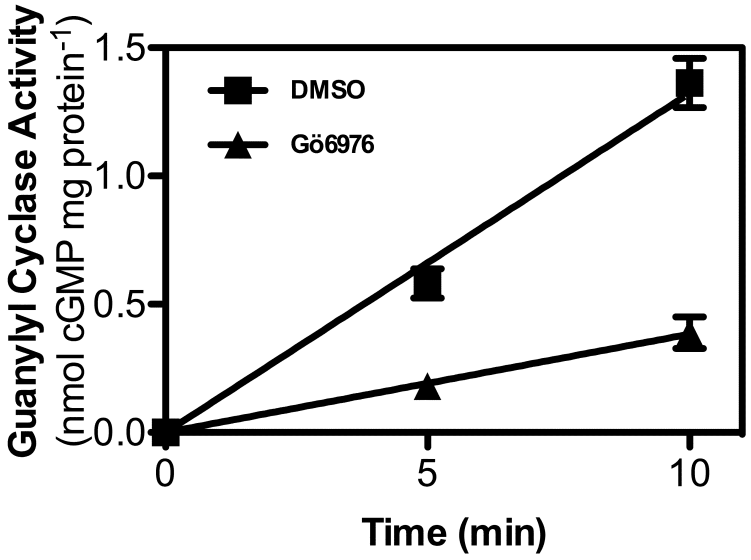

Guanylyl Cyclase Activity
(nmol cGMP mg-1 5min-1)
40
30
20
10
0
***
**
**
Basal
CNP
WT DMSO
WT Gö6976
6E DMSO
6E Gö6976

FIGURE 7. Gö6976 inhibits CNP-dependent activity in broken cell preparations. *Top*. Intact 293T-GC-B cells were incubated with 10 μM Gö6976 or DMSO for one hour at 37 ^{o}C and then membranes were prepared and assayed for guanylyl activity (Whole Cell). Alternatively, 10 μM Gö6976 or DMSO was directly added to membranes prepared from naive 293T-GC-B cells and guanylyl cyclase activity was measured under stimulated conditions (Membrane). * and ** indicate significance at p values of <0.05 and $p<0.004$, respectively, where n = 11 from six experiments (Whole Cell) or n = 4 from two experiments (Membrane). *Bottom*. 10 μM Gö6976 or DMSO was directly added to membranes prepared from 3T3-L1 cells and then guanylyl cyclase activity was measured in the presence of 1 mM CNP for the periods of time indicated. n = 10 from two experiments.


Guanylyl Cyclase Activity
(nmol cGMP mg-1 5min-1)
60
40
20
0
**
*
Whole Cell
Membrane


Guanylyl Cyclase Activity
(nmol cGMP mg protein-1)
1.5
1.0
0.5
0.0
DMSO
Gö6976
0
5
10
Time (min)

CHAPTER 3: ATP POTENTIATES COMPETITIVE INHIBITION OF GUANYLYL CYCLASE-B BY THE STAUROSPORINE ANALOG, GÖ6976: RECIPROCAL REGULATION OF ATP AND GTP BINDING

This chapter is a reprint of an original publication with minor alterations: Robinson, Jerid W. and Potter, Lincoln R. (2011) ATP potentiates competitive inhibition of guanylyl cyclase-B by the staurosporine analog, Go6976: Reciprocal regulation of ATP and GTP binding. *Journal of Biological Chemistry* 286(39):33841-4.

Jerid Robinson collected and analyzed all data and contributed to writing the manuscript.

Natriuretic peptides and ATP activate and Gö6976 inhibits guanylyl cyclase (GC)-A and GC-B. Here, the mechanism of inhibition was determined. Gö6976 progressively increased the K_m and decreased the Hill coefficient without reducing V_{max} of GC-A and GC-B. In the presence of 1 mM ATP, the K_i was 1 mM for both enzymes. Inhibition of GC-B was minimal in the absence of ATP and 1 mM ATP increased the inhibition 4-fold. In reciprocal manner, 10 μM Gö6976 increased the potency of ATP for GC-B 4-fold. In contrast to a recent report (Duda et al. 2010), neither staurosporine nor Gö6976 activated GC-A or GC-B. This is the first report to show that Gö6976 reduces GTP binding and the first demonstration of a competitive inhibitor of a receptor guanylyl cyclase. We conclude that Gö6976 reduces GTP binding to the catalytic site of GC-A and GC-B and that ATP increases the magnitude of the inhibition.

INTRODUCTION

Receptor guanylyl cyclases (GC) control a myriad of functions in organisms ranging from bacteria to humans by catalyzing the conversion of GTP to cGMP (Potter 2011). Guanylyl cyclase-A (GC-A) is the archetypical particulate GC that is activated by atrial natriuretic peptide (ANP) and B-type natriuretic peptide. The intracellular domain of guanylyl cyclase-B (GC-B) is 78% identical to GC-A but GC-B is activated by C-type natriuretic peptide (CNP) (Schulz et al. 1989). Both GC-A and GC-B are composed of a single polypeptide chain that contains a large extracellular domain, a single membrane span, and intracellular kinase homology, dimerization, and C-terminal guanylyl cyclase domains. ANP binds GC-A at a stoichiometry of 1:2 and induces a rotation of the juxta-membrane region (Rondeau et al. 1995, Ogawa et al. 2004). Structural modeling studies suggest that CNP binds GC-B similarly (He et al. 2006, Yoder et al. 2008). Natriuretic peptide binding is hypothesized to activate the receptors by relieving basal repression exerted by the KHDs on the guanylyl cyclase domains (Chinkers and Garbers 1989, Koller et al. 1992).

GC-A and GC-B are phosphorylated on multiple serines and threonines located slightly before and expanding into the N-terminal portion of the KHD (Yoder et al. 2010). Phosphorylation is required for peptide activation and prolonged natriuretic peptide exposure or acute exposure to phorbol esters or calcium elevating agents causes receptor dephosphorylation and inactivation (Dickey et al. 2010).

ATP increases the activity of both receptors in broken cell preparations by serving as a phosphate donor (Foster and Garbers 1998, Joubert et al. 2001, Abbey-Hosch et al. 2005). ATP is also an allosteric activator that reduces the Michaelis constant without

affecting maximal velocities (Antos and Potter 2007). In reciprocal manner, GTP decreases the EC_{50} for ATP. Thus, ATP increases GTP binding and GTP increases ATP binding.

We recently reported the staurosporine derivative, Gö6976, inhibits basal and natriuretic peptide-stimulated guanylyl cyclase activity of GC-A and GC-B, but does not inhibit activity measured in the presence of detergent with Mn^{2+}GTP as substrate (Robinson et al. 2011). Because Gö6976 inhibits ATP binding to protein kinases, we investigated whether Gö6976 inhibition requires changes in GC-B phosphorylation. However, Gö6976 inhibited a constitutively pseudo-phosphorylated form of GC-B similarly to the wild type receptor.

Here, we determined the mechanism of the inhibition. Gö6976 reduces the binding of GTP to the catalytic sites of GC-A and GC-B and ATP increases the magnitude of the inhibition by increasing affinity of the catalytic site for Gö6976.

EXPERIMENTAL PROCEDURES

Reagents. ^{125}I-cGMP radioimmunoassay kits were from Perkin Elmer (Waltham, MA). Gö6976, GF-109203X (Gö6850) and staurosporine were from EMD chemicals (Gibbstown, NJ) and were dissolved in DMSO.

Cells and Transfections.293T-GC-B cells were maintained as described (Fan et al. 2005).

Membrane preparation. Crude membranes were prepared in phosphatase inhibitor buffer as described (Flora and Potter 2010).

Guanylyl cyclase assays. All assays were performed at 37ºC in a cocktail containing 25 mM Hepes pH 7.4, 50 mM NaCl, 0.1% BSA, 0.5 mM IBMX, 1 mM EDTA, 0.5 μM microcystin and 5 mM $MgCl_2$. Unless otherwise indicated, 1 mM ATP and 1 mM GTP were included in the cocktail. Reactions were initiated by adding 20 μl of crude membranes containing 7 to 11 μg of protein to 80 μl of prewarmed reaction cocktail. Reactions were stopped with 0.4 ml of ice-cold 50 mM sodium acetate buffer containing 5 mM EDTA. Cyclic GMP concentrations were determined by radioimmunoassay as previously described (Abbey and Potter 2002). Concentration-response assays were conducted with the following Mg^{2+}GTP concentrations: 6000, 3000, 1500, 750, 375, 187.5, 93.8, 46.9, 23.4, 11.7, and 5.8 μM.

Statistical analysis. Data were analyzed using Prism 5 software. All experiments were conducted at least twice. p-values were obtained using student's paired t-test where $p \leq 0.05$ was considered significant. IC_{50} values were calculated using the following equation, where LOGIC50 equals the middle value of Y based on the nonlinear

regression curve fitting: Y=Bottom + (Top-Bottom)/(1+10^((X-LogIC50))). EC_{50} values were calculated based on the nonlinear curve fitting equation Y=Top*X/(EC_{50}+X). Apparent V_{max}, K_m and K_i values were determined using nonlinear regression analysis where least squares analysis determined the best model for inhibition.

RESULTS

Gö6976 is a competitive inhibitor of GC-A and GC-B. Substrate-velocity curves were generated using 293T-GC-A or 293T-GC-B membranes as the enzyme source in a reaction cocktail containing 1 μM natriuretic peptide, 1 mM ATP and increasing concentrations of Gö6976 and Mg^{2+}GTP to determine if the inhibition was competitive, uncompetitive or mixed. Increased K_m and unchanged V_{max} indicate competitive inhibition. Reduced K_m and V_{max} characterize uncompetitive inhibition, and reduced V_{max} and unchanged K_m are reflective of noncompetitive inhibition. Assays conducted with multiple concentrations of GTP and Gö6976 also allowed the K_i of inhibition to be determined.

Gö6976 markedly increased the K_m of GC-A but did not significantly reduce the V_{max} (Fig. 1). Similarly, Gö6976 increased the K_m of GC-B without reducing the V_{max} (Fig. 2). The K_i was 1.0 and 1.3 μM for GC-A and GC-B, respectively. Consistent with these values, when GC-B activity was determined in the presence of 1 mM ATP and 1 mM GTP and multiple concentrations of Gö6976, the IC_{50} was 2 μM (Fig. 3).

Unexpectedly, Gö6976 decreased the Hill coefficient for both enzymes. For GC-A, the Hill coefficient was 0.93, 0.91, 0.69 and 0.60 in the presence of 0, 1.7, 5 and 15 μM concentrations of Gö6976, respectively. For GC-B, the Hill coefficient was 1.19, 1.25, 1.02 and 0.87 in the presence of 0, 1.7, 5 and 15 μM concentrations of Gö6976, respectively. These data suggest that at the higher concentrations, Gö6976 may be inhibiting the allosteric activation of GC-A and GC-B.

ATP increases the magnitude of the Gö6976-dependent inhibition. Since Gö6976 competitively inhibits ATP binding to protein kinases (Martiny-Baron et al. 1993), we hypothesized that Gö6976 would compete for ATP binding to GC-B and that ATP would reduce the inhibitory effect of Gö6976. However, the opposite result was observed; ATP increased the magnitude of the inhibition in a concentration-dependent manner (Fig. 4). In the absence of ATP, 10 μM Gö6976 only reduced CNP-dependent GC-B activity 16% but in the presence of 1.5 mM ATP, activity was reduced 68%, a more than four-fold difference. The concentration of ATP required to elicit half the maximal inhibitory response was between 0.5 and 1 mM, which is below the cellular concentration of ATP.

Gö6976 increases the potency of ATP for GC-B. To determine if Gö6976 reciprocally increased ATP binding to GC-B, guanylyl cyclase activity was determined in the presence or absence of Gö6976 as a function of increasing ATP concentrations (Fig. 5). The EC_{50} for ATP activation of GC-B in the absence of Gö6976 was 11.2 μM, whereas the EC_{50} in the presence of Gö6976 was 2.7 μM. Thus, 10 μM Gö6976 reduced the EC_{50} for ATP more than four fold. Hence, like GTP, Gö6976 increases the potency of ATP for GC-B.

Staurosporine derivatives do not activate GC-A or GC-B. Two recent reports suggest that staurosporine effectively substitutes for ATP in the activation of GC-A (Duda et al. 2010, Duda et al. 2011). Therefore, we tested the ability of Gö6976 and the related compound, staurosporine, to activate GC-A and GC-B (Fig. 6). ATP markedly increased activity of both enzymes when assayed in the presence of their respective natriuretic peptide, but neither of the indolocarbazoles increased activity. In fact, both staurosporine

and Gö6976 inhibited GC-A and Gö6976 inhibited GC-B. The diminished inhibitory effect of Gö6976 was expected because ATP was not included in these assays. Thus, staurosporine and Gö6976 are inhibitors, not activators, of GC-A and GC-B.

DISCUSSION

As a result of these studies, the mechanism of Gö6976-mediated inhibition of GC-A and GC-B was determined. Substrate-velocity experiments indicated that Gö6976 is a competitive inhibitor of GTP binding to the catalytic site of both receptors. To our knowledge, this is the first report to show that a staurosporine derivative reduces GTP binding and the first report to describe a competitive inhibitor of a transmembrane guanylyl cyclase. Surprisingly, although Gö6976 blocks ATP binding to protein kinases, it did not block ATP-dependent activation of GC-A and GC-B. Instead, ATP potentiated the inhibitory effect by increasing the affinity of the catalytic domain for Gö6976, which ultimately increased the magnitude of the inhibition. Gö6976 also reduced the Hill coefficient of the receptors and increased the potency of ATP activation of GC-B, which is consistent with Gö6976 interacting with a second non-catalytic binding site. The ability of ATP to increase the potency of Gö6976 is also consistent with reduced IC_{50}s in whole cell experiments where intracellular ATP concentrations are higher and GTP concentrations are lower than those used in our assays (Robinson et al. 2011). The reciprocal regulation between the ATP and GTP sites in GC-A and GC-B is consistent with previous data showing that ATP decreases the K_m for GTP and that GTP decreases the EC_{50} for ATP (Antos and Potter 2007). Our data are also consistent with the work of Chang and colleagues showing an allosteric inhibitory role of ATP on soluble GC (Chang et al. 2005).

We do not know why our results are the opposite of those recently published by Duda et al (Duda et al. 2010, Duda et al. 2011). However, one major difference is that GTP concentrations were 1 mM in our assay but 0.1 mM in their assay. Other

differences are that we studied rat receptors stably expressed in human 293T cells whereas they studied rat GC-A transiently expressed in monkey COS cells and they used theophylline as a phosphodiesterase inhibitor whereas we used isobutylmethyl xanthine.

Based on the data reported here, we propose two possible inhibition models. In the direct competition model (Fig. 7, top), Gö6976 directly competes with GTP for binding to the catalytic site. If we make the assumption that the K_m (100 – 200 μM) and Ki (1-2 mM) for GTP and Gö6976, respectively, are reflective of the binding constants, then Gö6976 binds to the catalytic site about 100 fold tighter than GTP. Since the inhibitory affect of Gö6976 is increased in the presence of ATP, we conclude that ATP increases the affinity of the catalytic site for Gö6976 more than it increases the affinity for GTP. Because of the increased affinity for Gö6976 compared to GTP, the major effect of ATP binding is to accentuate the inhibitory effect of Gö6976 on the catalytic site. However, at higher concentrations, Gö6976 may bind a second site, which reduces the allosteric activation of the receptors as suggested by the decreased Hill coefficient.

In the allosteric competition model (Fig. 7, bottom), Gö6976 binds a separate, non-catalytic, site that allosterically reduces the affinity of the catalytic site for GTP. High concentrations of Gö6976 in this model may ultimately lead to a conformational change that not only inhibits GTP binding, but completely abolishes it, which would be congruent with the negative Hill slope observed at high Gö6976 concentrations. Future experiments will investigate the exact location of the Gö6976 binding site in these important signaling enzymes.

FIGURES

FIGURE 1. Gö6976 increases the K_m of GC-A.

Guanylyl cyclase activity was determined for 9 min in 293T-GC-A cell membranes containing 1 μM ANP, 1 mM ATP and the indicated concentrations of Gö6976 and GTP where n = 4 from two experiments. The K_i for Gö6976 was 1.0 μM.

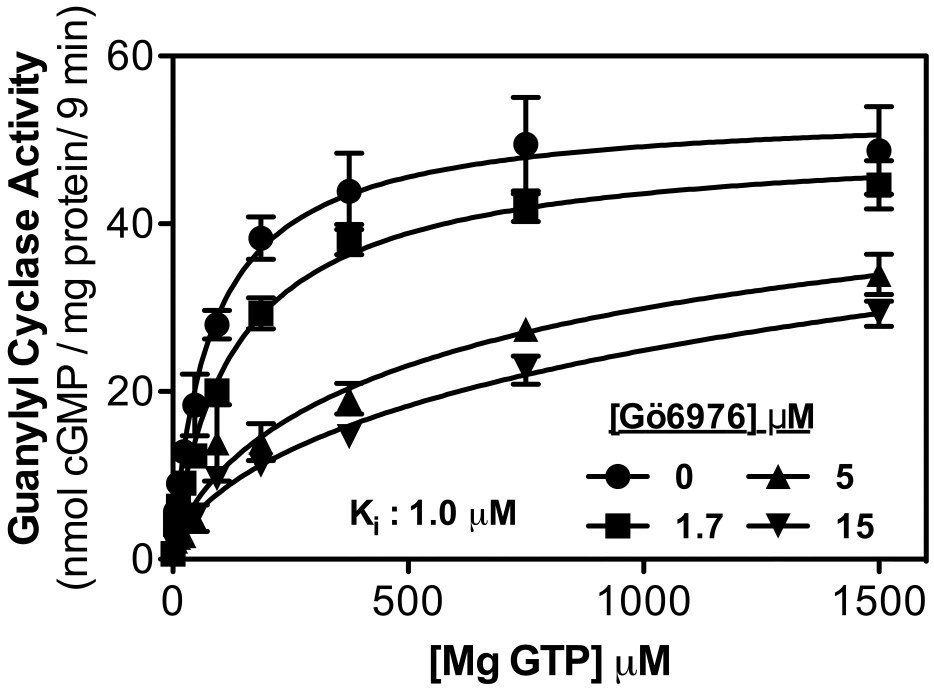

Guanylyl Cyclase Activity
(nmol cGMP / mg protein/ 9 min)
60
40
20
0
0
500
1000
1500
[Mg GTP] μM
[Gö6976] μM
0
5
1.7
15
K$_i$: 1.0 μM

FIGURE 2. Gö6976 increases the K_m of GC-B.

Guanylyl cyclase activity was determined for 9 min in 293T-GC-B cell membranes containing 1 μM CNP, 1 mM ATP and the indicated concentrations of Gö6976 and GTP where n = 4 from two experiments. The K_i for Gö6976 was 1.3 μM.

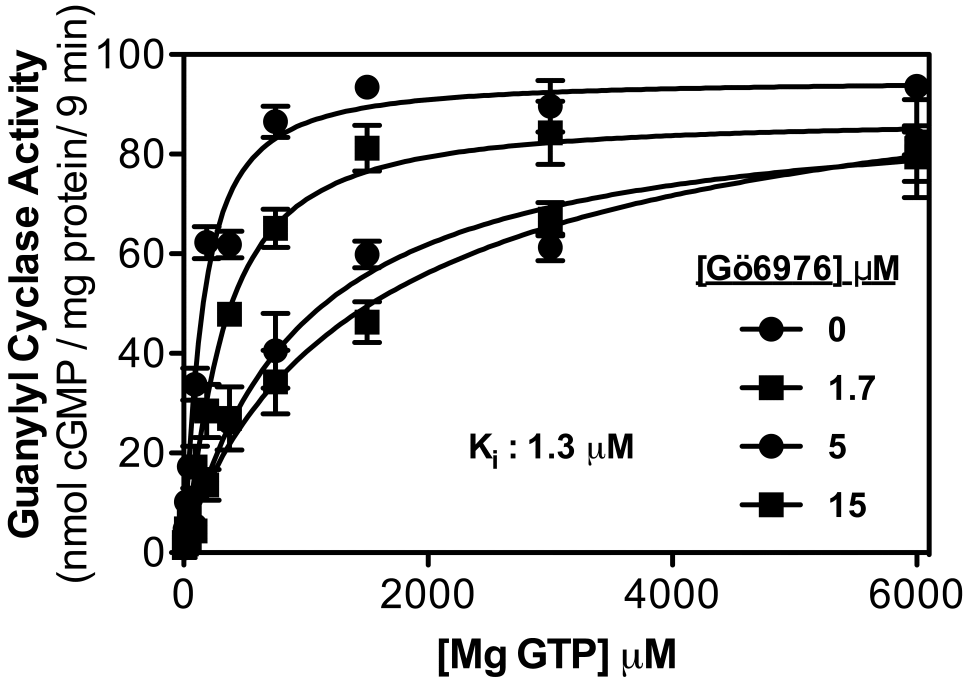

Guanylyl Cyclase Activity
(nmol cGMP / mg protein/ 9 min)
100
80
60
40
20
0
0
2000
4000
6000
[Mg GTP] μM
[Gö6976] μM
0
1.7
5
15
K$_i$: 1.3 μM

FIGURE 3. Gö6976 is a potent inhibitor of GC-B.

Guanylyl cyclase activity was determined in 293T GC-B membranes incubated with 1 μM CNP, 1 mM ATP and 1 mM GTP and the indicated concentrations of Gö6976 for 6 min where n = 12 from three experiments.

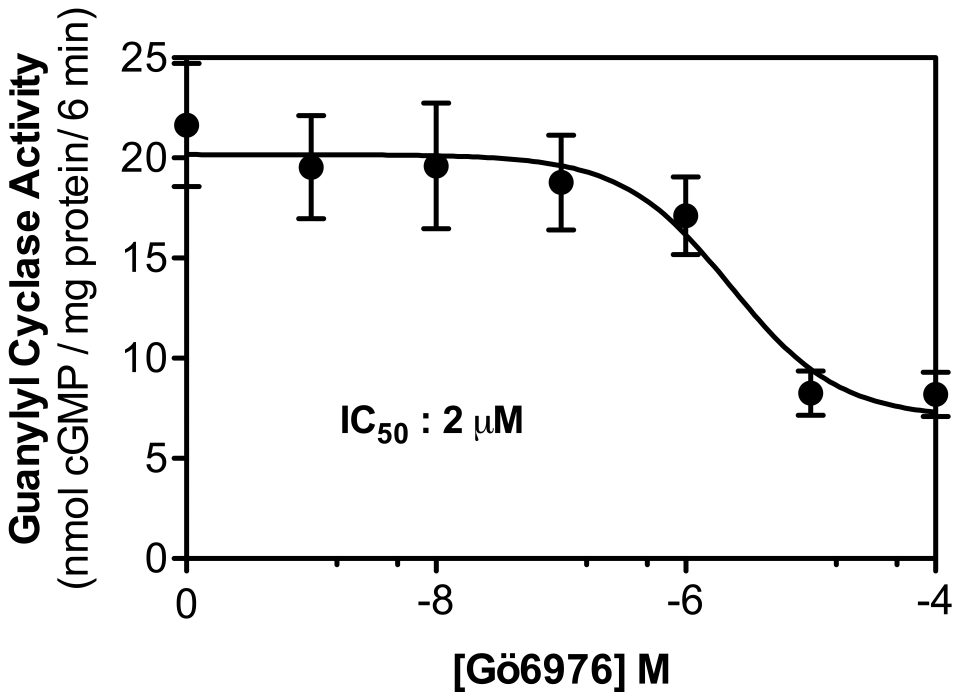

Guanylyl Cyclase Activity
(nmol cGMP / mg protein/ 6 min)
25
20
15
10
5
0
0
-8
-6
-4
IC50 : 2 μM
[Gö6976] M

FIGURE 4. ATP increases the magnitude of Gö6976-dependent inhibition of GC-B. Guanylyl cyclase activity determined in 293T-GC-B membranes containing 1 μM CNP, 1 mM GTP, 10 μM Gö6976 and the indicated ATP concentrations was divided by activities obtained under identical conditions except the samples lacked Gö6976 and the quotient was multiplied by 100. The resulting data were plotted as a function of the log of the ATP concentration where n = 6.

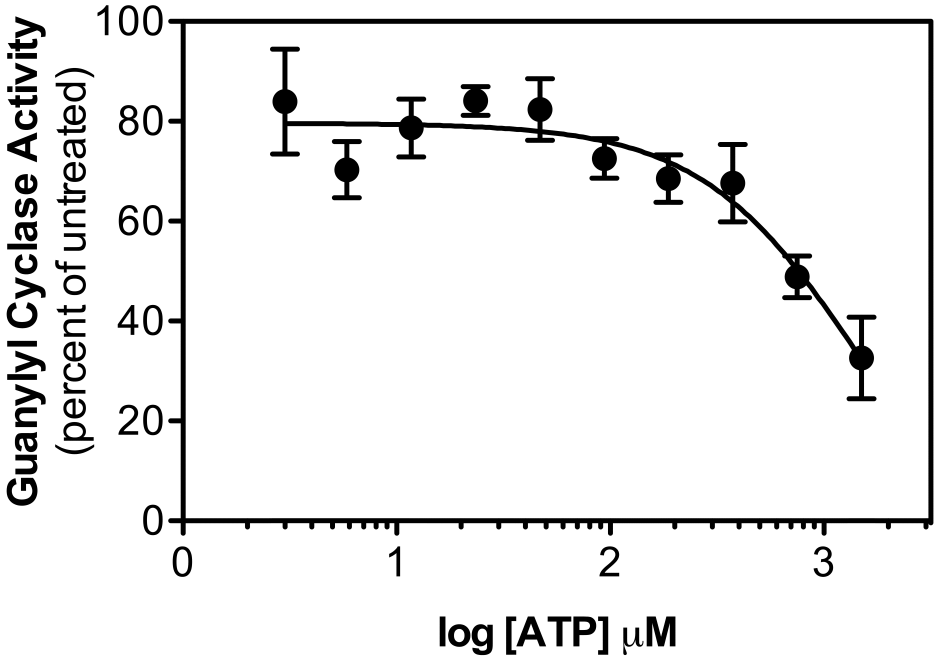

Guanylyl Cyclase Activity
(percent of untreated)
100
80
60
40
20
0
0
1
2
3
log [ATP] μM

FIGURE 5. Gö6976 increases the potency of ATP for GC-B.

Guanylyl cyclase activity was determined for 9 min in the presence of 10 μM Gö6976 or DMSO in 293T-GC-B membranes containing with 1 μM CNP, 1 mM GTP and the indicated ATP concentrations and plotted as percent of the maximum response where n = 6.

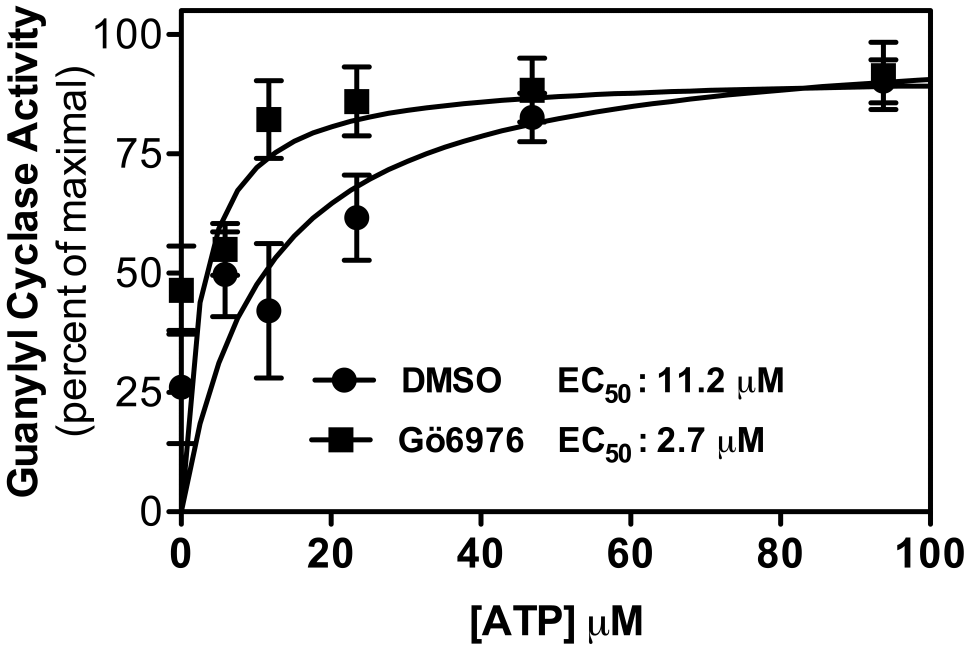
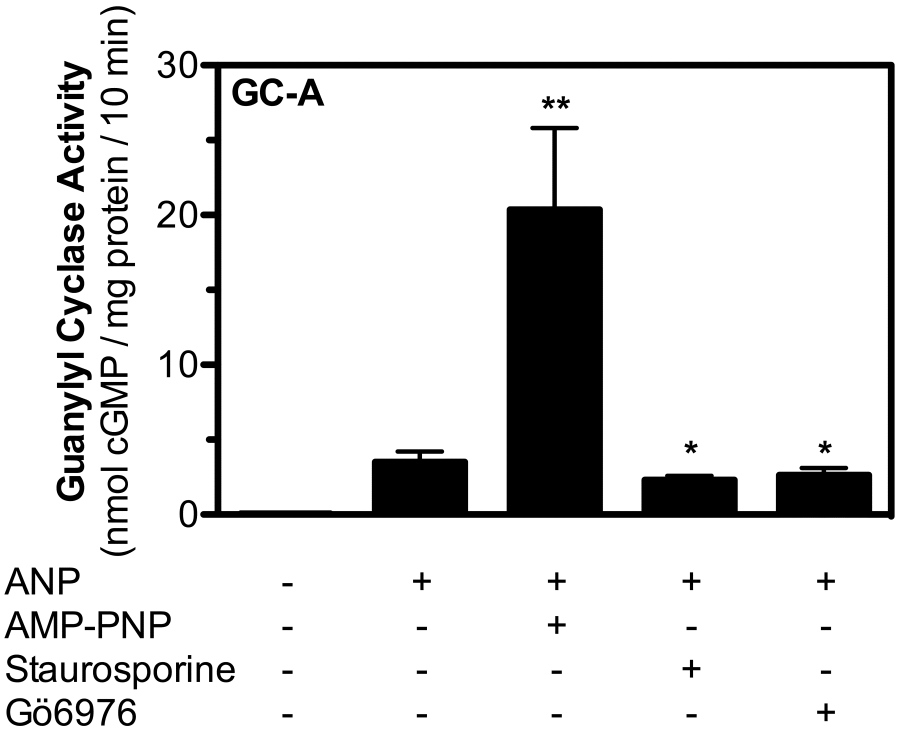

Guanylyl Cyclase Activity
(percent of maximal)
100
75
50
25
0
0
20
40
60
80
100
[ATP] μM
DMSO EC50 : 11.2 μM
Gö6976 EC50 : 2.7 μM

FIGURE 6. Staurosporine and related derivatives do not activate GC-A or GC-B. Guanylyl cyclase activity was measured in membranes from 293T-GC-A (top) or 293T-GC-B (bottom) cells for 10 min with 1 mM GTP in the absence or presence of 100 nM ANP or 100 nM CNP, 0.5 mM AMP-PNP or 10 μM staurosporine or Gö6976. * and ** indicate significance at p values of <0.05 and $p<0.01$, respectively, where n = 8 from four experiments.


GC-A
Guanylyl Cyclase Activity
(nmol cGMP / mg protein / 10 min)
30
20
10
0
**
*
*
ANP - + + + +
AMP-PNP - - + - -
Staurosporine - - - + -
Gö6976 - - - - +

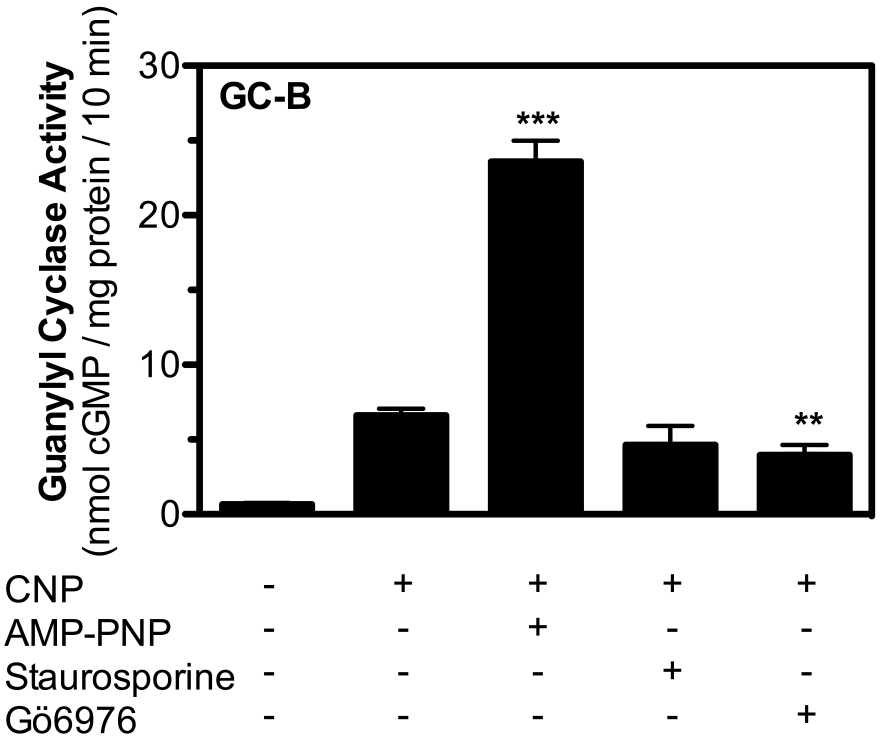
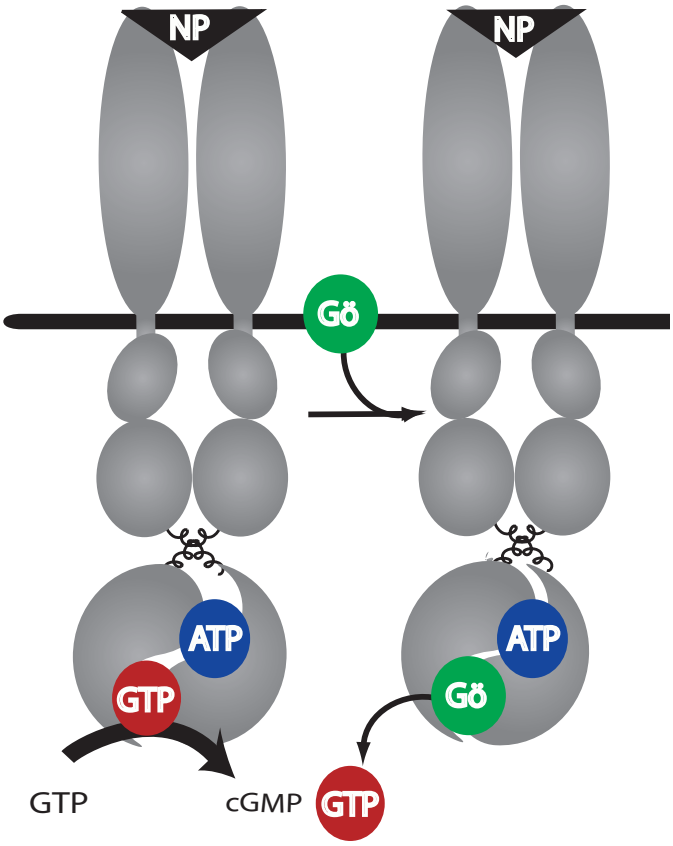

GC-B
Guanylyl Cyclase Activity
(nmol cGMP / mg protein / 10 min)
30
20
10
0
***
**
CNP - + + + +
AMP-PNP - - + - -
Staurosporine - - - + -
Gö6976 - - - - +

FIGURE 7. Two Models of Gö6976-dependent inhibition of GC-A and GC-B. Direct competition (upper panel). In this model, Gö6976 directly competes with GTP for binding to the catalytic site. Allosteric competition (lower panel). In this model, Gö6976 binds to a separate site that allosterically reduces the affinity of the catalytic site for GTP.


NP
NP
Gö
ATP
GTP
ATP
Gö
GTP
cGMP
NP
NP
Gö
ATP
GTP
Gö
ATP
GTP
cGMP

CHAPTER 4: LUTEINIZING HORMONE REDUCES THE ACTIVITY OF THE NPR2 GUANYLYL CYCLASE IN MOUSE OVARIAN FOLLICLES, CONTRIBUTING TO THE CYCLIC GMP DECREASE THAT PROMOTES RESUMPTION OF MEIOSIS IN OOCYTES

This chapter is a reprint of an original publication with minor alterations:
Robinson, Jerid W.; Zhang, Meijia; Shuhaibar, Leia C.; Norris, Rachael P.; Geerts, Andreas; Wunder, Frank; Eppig, John J.; Potter, Lincoln R.; and Jaffe, Laurinda A. (2012) Luteinizing hormone reduces the activity of the NPR2 guanylyl cyclase in mouse ovarian follicles, contributing to the cyclic GMP decrease that promotes resumption of meiosis in oocytes. *Developmental Biology* 366(2):308-16.

Jerid Robinson collected and analyzed all of the data for Figures 2 and 3 and contributed to the writing and preparation of the manuscript.

In preovulatory ovarian follicles of mice, meiotic prophase arrest in the oocyte is maintained by cyclic GMP from the surrounding granulosa cells that diffuses into the oocyte through gap junctions. The cGMP is synthesized in the granulosa cells by the transmembrane guanylyl cyclase natriuretic peptide receptor 2 (NPR2) in response to the agonist C-type natriuretic peptide (CNP). In response to luteinizing hormone (LH), cGMP in the granulosa cells decreases, and as a consequence, oocyte cGMP decreases and meiosis resumes. Here we report that within 20 minutes, LH treatment results in decreased guanylyl cyclase activity of NPR2, as determined in the presence of a maximally activating concentration of CNP. This occurs by a process that does not reduce the amount of NPR2 protein. We also show that by a slower process, first detected at 2 hours, LH decreases the amount of CNP available to bind to the receptor. Both of these LH actions contribute to decreasing cGMP in the follicle, thus signaling meiotic resumption in the oocyte.

INTRODUCTION

Mammalian oocytes are maintained in meiotic prophase for prolonged periods. During prophase arrest, the oocyte is located in a follicle in which it is surrounded by granulosa cells (Fig. 1A). As the follicle grows to its full size (~400-500 mm in mice), the oocyte acquires the ability to resume meiosis, but due to inhibitory signals from the granulosa cells, the oocyte remains in prophase (Jaffe 2010, Conti et al. 2012). Then during each reproductive cycle, luteinizing hormone (LH) from the pituitary acts on the granulosa cells of the fully grown follicle to cause the oocyte to mature into a fertilizable egg and be ovulated. This process begins with the transition from prophase to metaphase, marked by the breakdown of the nuclear envelope about 2 hours after LH exposure. However, other events of the prophase-to-metaphase transition occur before nuclear envelope breakdown: microtubule organizing centers assemble (Schuh and Ellenberg 2007), chromatin condenses (Racowsky and Baldwin 1989), and cell cycle regulatory proteins undergo changes in activity and localization (Solc et al. 2010).

Recent studies of the mouse ovary have shown that a key inhibitory substance for maintaining prophase arrest is cGMP, which diffuses from the granulosa cells into the oocyte through gap junctions (Norris et al. 2009, Vaccari et al. 2009). In the oocyte, cGMP inhibits the cAMP phosphodiesterase PDE3A, and thus prevents the degradation of cAMP. Elevated cAMP activates protein kinase A, which acts through a complex of mechanisms to inhibit the activity of the CDK1-cyclin B kinase and thus to inhibit the prophase-to-metaphase transition (Solc et al. 2010, Conti et al. 2012). If cGMP in a follicle-enclosed oocyte is experimentally decreased, by injection of a cGMP-specific

phosphodiesterase, cGMP is decreased, and as a consequence meiosis resumes (Norris et al. 2009).

The generation of the cGMP that maintains meiotic arrest requires the function in the granulosa cells of the transmembrane guanylyl cyclase natriuretic peptide receptor 2 (NPR2, also known as guanylyl cyclase-B) and its extracellular agonist C-type natriuretic peptide (CNP, also known as natriuretic peptide C, NPPC) (Zhang et al. 2010). In ovaries of mice carrying mutations in *Npr2* or *Nppc* genes, meiosis resumes precociously (Zhang et al. 2010). Although there is also evidence for expression of other guanylyl cyclases in granulosa cells (Sriraman et al. 2006) and some evidence that these may contribute to the maintenance of meiotic arrest (Tornell et al. 1990, Sela-Abramovich et al. 2008, Vaccari et al. 2009) and the response of the follicle to LH (Sriraman et al. 2006), CNP-dependent activation of NPR2 is fundamental for generating the inhibitory levels of cGMP.

CNP is synthesized by the outer (mural) granulosa cells, and binds to NPR2 throughout the follicle to stimulate cGMP production (Jankowski et al. 1997, Zhang et al. 2010). The connection of the cumulus cells to the mural granulosa cells is essential for maintaining meiotic arrest, since when this connection is broken, leaving the cumulus-oocyte complex free in the antral space, meiosis resumes (Racowsky and Baldwin 1989). This supports the concept that although *Npr2* mRNA is most concentrated in the cumulus cells (Zhang et al. 2010), cGMP generated by NPR2 in the mural layers also provides a critical part of the inhibitory cGMP to the oocyte.

Despite this knowledge of how CNP, NPR2, and cGMP function to maintain meiotic arrest, less is known about how signaling by LH reverses the arrest. LH acts on a

G-protein-linked receptor (LHCGR) (Rajagopalan-Gupta et al. 1998), which in rats and mice, is located in the mural granulosa cells, mostly within the outer several layers of cells, and is absent in the cumulus cells (Amsterdam et al. 1975, Eppig et al. 1997). In response to LH, the permeability of the gap junctions between the granulosa cells throughout the follicle is reduced, such that intercellular diffusion within the follicle of molecules of the size of cGMP is slowed (Sela-Abramovich et al. 2005, Norris et al. 2008). In parallel, cGMP levels in the follicle decrease (Hubbard 1986, Norris et al. 2009, Vaccari et al. 2009), from a basal level of ~3 mM, to ~0.5 mM at 20 minutes and ~0.1 mM at one hour after applying LH (Norris et al. 2010). CNP levels also decrease (Jankowski, Reis et al. 1997, Kawamura et al. 2011), but the earliest of these measurements were made at 4 hours after LH application, while the cGMP decrease occurs by 20 minutes, so their functional significance has not been certain. As cGMP in the follicle decreases, cGMP in the interconnected oocyte falls correspondingly, to a few percent of the basal level at one hour. As a consequence, the inhibition of PDE3A is relieved, cAMP decreases, and meiosis resumes (Norris et al. 2009, Vaccari et al. 2009).

The decrease in cGMP in the follicle could be caused by a decrease in cGMP synthesis, an increase in cGMP degradation, and/or an increase in cGMP efflux. Here we report that one mechanism by which LH signaling reduces cGMP is by reducing the activity of the guanylyl cyclase NPR2.

EXPERIMENTAL PROCEDURES

Mice and hormones. Ovaries were obtained from prepubertal B6SJLF1 mice (23-25 days old) from The Jackson Laboratory (Bar Harbor, ME); procedures were approved by the animal care committees of the University of Connecticut Health Center, China Agricultural University, and The Jackson Laboratory. For granulosa cell collection, cumulus-oocyte complex collection, CNP ELISA assays, and histological analysis, the mice were injected with 5 I.U. equine chorionic gonadotropin (eCG) 40-48 hours before use, to stimulate follicle growth and LH receptor expression. Mice for antral follicle isolation were not injected with eCG; instead the follicles were exposed to 10 ng/ml follicle stimulating hormone (FSH) in vitro.

Ovine LH, human LH, ovine FSH, and eCG, purified from biological sources, were obtained from A.F. Parlow (National Hormone and Peptide Program, Torrance, CA). Human recombinant LH was obtained from EMD Serono Research Institute, Inc. (Rockland, MA). Human chorionic gonadotropin (hCG) was purchased from Sigma-Aldrich (St. Louis, MO). Ovine LH was used for studies of isolated follicles (10 mg/ml). Because of their slower rate of degradation (Mock and Niswender, 1983), human LH or hCG was used for injection into mice (10 mg or 5 I.U., respectively).

Measurement of relative amounts of guanylyl cyclase mRNAs in granulosa cells. Mural granulosa cells were collected by puncturing antral follicles of isolated ovaries with 30 gauge needles. RNA was extracted using TRIzol reagent (Invitrogen Corporation, Carlsbad, CA). DNAse I digestion was performed to remove residual genomic DNA, and mRNAs were reverse transcribed using random hexamers.

Quantitative TaqMan analysis was performed using the Applied Biosystems PRISM 7900 sequence detection system, to determine the relative concentration of each guanylyl cyclase mRNA in granulosa cells. Differences in primer efficiency were determined by measuring the cycle threshold (C_t) values for each primer pair using 30 ng of genomic DNA. Only small differences were detected, and these were corrected for by use of the following formula:

C_t *(corrected)* = C_t *(measured)* + C_t *(mean of all probes, genomic DNA)* – C_t *(genomic DNA)*

Normalization was performed using the housekeeping gene *Rpl32* as a control. The resulting expression is given in arbitrary units.

Measurement of guanylyl cyclase activity in a crude membrane fraction of follicles. For each experiment, antral follicles from 4 mice were isolated and cultured for 24-30 hours in the presence of FSH to stimulate follicle growth and LH receptor expression (Norris et al. 2008, Norris et al. 2010). The follicles were divided into 2 equal groups, and half were exposed to LH for the indicated time. The 40-50 follicles in each group were washed in PBS and lysed in phosphatase inhibitor buffer (Dickey et al. 2007) in a 100 ml glass homogenizer. To obtain a crude membrane fraction, the homogenate (200 ml volume) was centrifuged at 10,000xg for 20 minutes; the pellet (~1 ml volume) was resuspended in 50 ml of phosphatase inhibitor buffer and sonicated briefly. Protein content was determined by solubilizing a 4 ml aliquot in 1% SDS and performing a BCA assay (Pierce, Thermo Fisher Scientific, Rockford, IL). The crude membrane fraction contained ~1 mg of protein per follicle. The samples were frozen in liquid N_2 and stored at -80ºC.

Guanylyl cyclase assays were conducted for each pair of follicle samples prepared as described above (one sample that had been treated with LH, one control sample without LH), using methods as previously described (Robinson and Potter 2011). Assays were performed at 37ºC using 1-2 mg of follicle protein per assay tube, in the presence or absence of 1 mM CNP (or ANP), which are maximally activating concentrations for their respective receptors (Abbey-Hosch, Smirnov et al. 2005, Dickey, Burnett et al. 2008) 0.5 mM isobutylmethylxanthine (IBMX) was included to inhibit cGMP phosphodiesterase activity. CNP (or ANP) dependent guanylyl cyclase activity refers to the activity measured in the presence of the natriuretic peptide minus the activity measured in the absence of the natriuretic peptide. Statistical significance of the data was tested using two-way repeated measures ANOVA with a Bonferroni post-test; control and LH-treated samples that had been prepared and assayed together were analyzed as pairs. The analysis was performed using Prism software (GraphPad Software, Inc., La Jolla, CA).

Measurement of cGMP in cumulus cells. Cumulus-oocyte complexes were isolated at various times after hCG injection, and cultured as previously described (Zhang et al. 2010), with or without 30 nM CNP for 1 hour. Cumulus cells were then separated for measurement of cGMP using an ELISA method as previously described (Zhang et al. 2010). Statistical significance of the data was tested using one-way ANOVA with a Dunnett multiple comparisons post-test.

Measurement of CNP in ovaries. CNP in ovaries was assayed by an ELISA method (#FEK-012-03, Phoenix Pharmaceuticals Inc., Burlingame, CA) with a primary antibody made against the 22 amino acid form of CNP. This antibody also recognizes the 53

amino acid form of CNP, and presumably the precursor forms, which include the same 22 amino acids at their C-termini (Wu et al. 2003, Potter et al. 2009).

Samples were prepared by a method modified from Jankowski *et al*. (Jankowski et al. 1997). Two ovaries in 70 ml of 1.0 M acetic acid were heated at 95ºC for 10 minutes, then lysed with a probe sonicator. 350 ml of MeOH was added to solubilize lipids, and the tube was centrifuged at 30,000 xg at 4ºC for 15 min. The supernatant ("ovary extract") contained ~250 mg of protein. ~50% of the CNP was recovered in this extract (determined by adding a known amount of CNP to the ovaries before extraction). For each sample, 10, 5, and 2 mg of the extract protein were lyophilized and assayed, following the manufacturer's instructions. Data were analyzed using Prism software. Statistical significance of the data was tested using one-way ANOVA with a Dunnett multiple comparisons post-test. The concentration of CNP in the ovary, if CNP was uniformly distributed, was estimated based on a volume per ovary of ~4 ml (~4 mg wet weight).

Histological analysis of nuclear envelope breakdown kinetics. Serial sections of mouse ovaries were prepared as previously described (Mehlmann et al. 2004). Follicles with a diameter of ≥350 mm in at least one dimension, as measured in the section containing the nucleolus or chromosomes, were analyzed for the presence of an intact nucleus (see Fig. 1A).

RESULTS AND DISCUSSION

In mural granulosa cells, mRNA encoding NPR2 is present at a higher concentration than mRNAs encoding other guanylyl cyclases. Although NPR2 is known to be present in mural granulosa cells and functionally important for maintaining meiotic arrest, there is also evidence that NPR1 and soluble guanylyl cyclase subunits could contribute to the control of meiotic arrest and progression (see Introduction). Because previous studies did not determine the relative expression levels of mRNA for NPR2 and other guanylyl cyclases in mouse granulosa cells, and because not all of the guanylyl cyclases were investigated, we quantitatively compared the amounts of mRNA in mural granulosa cells for each of the mouse guanylyl cyclase genes (Fig. 1B). The mouse genome contains 7 transmembrane and 4 soluble guanylyl cyclase genes (Potter 2011). We detected mRNA encoding two transmembrane guanylyl cyclases, NPR1 and NPR2, and two soluble guanylyl cyclase subunits, GUCY1A3 (soluble guanylyl cyclase alpha 1) and GUCY1B3 (soluble guanylyl cyclase beta 1). Among these, *Npr2* mRNA was expressed at a high level, ≥14 times higher than any of the other guanylyl cyclases. We also tested for mRNA encoding NPR3, which has sequence similarity to the extracellular domains of NPR1 and NPR2, but lacks the guanylyl cyclase domain and activity (Potter 2011). NPR3 is a clearance receptor for natriuretic peptides. Little or no *Npr3* mRNA was detected.

Although concentrations of mRNAs are not directly proportional to the amounts of the proteins they encode, these measurements further support the conclusion that NPR2 is the primary guanylyl cyclase that produces cGMP in the follicle.

LH signaling reduces NPR2 activity in the follicle. One way that LH activation of its receptors in the mural granulosa cells could decrease cGMP levels within the follicle is by reducing NPR2 activity. Two aspects of this question were considered: 1) whether LH signaling decreases NPR2 activity in the follicle as a whole, of which most of the volume is mural granulosa cells, and 2) whether LH signaling decreases NPR2 activity in the cumulus cells. A decrease in NPR2 activity in either or both of these regions could contribute to the measured decrease in cGMP in the oocyte. This section describes our studies of a crude membrane fraction from whole follicles, and a subsequent section describes our studies of cumulus cells.

Guanylyl cyclase activity was measured using the particulate fraction obtained by centrifuging a homogenate of follicles. When this crude membrane fraction was incubated without CNP, guanylyl cyclase activity was too low to measure accurately, but addition of 1 mM CNP increased the activity to 0.21 ± 0.02 nmole cGMP/mg protein/minute (n = 15 follicle preparations, see Fig. 2A, B). After a 20 minute exposure of follicles to LH, CNP-dependent guanylyl cyclase activity fell to 50% of the activity measured in the membrane fraction from follicles without LH exposure, and remained depressed for 2 hours after applying LH (Fig. 2 A, B).

The decrease in follicle cGMP that will result from a 50% decrease in NPR2 activity depends on the cGMP affinity of the phosphodiesterases present in the granulosa cells. If the affinity is higher (lower K_m), the cGMP concentration will fall to a lower level. Much of the cGMP phosphodiesterase activity in the follicle is sensitive to sildenafil and tadalafil, indicating an important PDE5 component (Vaccari et al. 2009).

Based on K_m values for PDE5, a 50% reduction in NPR2 activity could potentially account for the decrease in follicle cGMP from 3 mM before LH treatment to ~0.5 mM after 20 minutes (Norris et al. 2010).

Because a small amount of *Npr1* mRNA is also expressed in granulosa cells (Fig. 1B), we also evaluated the effect of LH on NPR1 activity, by measuring guanylyl cyclase activity in the presence of 1 mM atrial natriuretic peptide (ANP). Studies of human NPR1 and NPR2 have shown that 1 mM ANP activates NPR1, but has almost no effect on NPR2 (Dickey et al. 2008). In the crude membrane fraction from follicles, ANP-dependent guanylyl cyclase activity was 0.07 ± 0.03 nmole cGMP/mg protein/minute, or 33% of the CNP-dependent activity (n = 4). However, the ANP-dependent activity was unchanged by LH (Fig. 2C, D). Some of the ANP-dependent guanylyl cyclase activity that we measured might be due to NPR1 expressed in membranes from theca cells and blood vessels that were not completely removed from the follicle by microdissection (Fig. 1A). The lack of effect of LH on ANP-dependent cGMP accumulation serves as a control to indicate that the LH-induced decrease in CNP-dependent cGMP accumulation is not due to an LH effect on phosphodiesterase activity that could have been present in the crude membrane fraction despite the presence of IBMX.

The LH-induced decrease in NPR2 activity in the follicle occurs without a corresponding decrease in NPR2 protein. Previous studies have shown that other biological factors that rapidly decrease NPR2 activity in cultured cells do so in a manner that is independent of NPR2 protein levels (Abbey and Potter 2003, Abbey-Hosch et al. 2004). To test if LH decreased the amount of NPR2 protein in follicles, we first tried Western blotting, and immunoprecipitation followed by Western blotting. However, with the available antibodies, it was not possible to detect endogenous levels of the protein using these methods. So instead, we measured guanylyl cyclase activity in follicle membrane fractions after treatment with 1% Triton X-100 and 5 mM $MnCl_2$, a condition known to maximally activate NPR1 and NPR2 in the absence of natriuretic peptide and to be indicative of guanylyl cyclase protein levels (Abbey and Potter 2003). Guanylyl cyclase activity measured in the presence of Triton X-100 and $MnCl_2$ is independent of modification of the NPR2 protein by phosphorylation (Potter and Hunter 1998).

Detergent-dependent guanylyl cyclase was the same in samples from follicles with or without LH treatment for 20 minutes (Fig. 3). Since NPR1 and NPR2 are the only detectable membrane guanylyl cyclases in granulosa cells, and since NPR1 is a relatively minor component, these detergent measurements indicate that at 20 minutes, LH does not decrease the amount of NPR2 protein. A possible cause of the rapid LH-induced decrease in NPR2 activity is dephosphorylation, which can result from elevation of intracellular Ca^{2+} and/or activation of protein kinase C (Abbey-Hosch et al. 2005, Potter 2011).

In the cumulus cells, CNP-dependent cGMP production decreases in response to LH receptor stimulation, but more slowly than in the mural granulosa cells. Because of the direct connection between the cumulus cells and the oocyte, and because of the higher level of *Npr2* mRNA in the cumulus cells compared with the mural cells (Zhang et al. 2010), it was of particular interest to investigate whether LH signals that are initiated in the mural granulosa cells regulate NPR2 activity in the cumulus cells. As described above, LH receptors are not present in the cumulus cells, so such regulation would have to involve signaling between different regions of the follicle.

Due to the small amount of protein that could be obtained, we could not analyze guanylyl cyclase activity in a cumulus cell membrane fraction as we did for the more abundant material from whole follicles. Instead, we isolated cumulus-oocyte complexes from ovaries at various times after injection of mice with human chorionic gonadotropin (hCG) to stimulate the LH receptor, incubated the complexes in the presence or absence of 30 nM CNP for an additional hour, then isolated the cumulus cells and measured their cGMP content. hCG is often used instead of LH, since both hormones act on the same receptor. 30 nM CNP was used because this is approximately the minimum concentration needed to prevent spontaneous meiotic resumption in isolated cumulus-oocyte complexes (Zhang et al. 2010). Under these experimental conditions, measurements of a change in cellular cGMP content in response to LH receptor stimulation could indicate a change in guanylyl cyclase activity, or a change in cGMP phosphodiesterase activity, or a change in cGMP efflux. However, by measuring the effect of LH receptor stimulation on cGMP content in the presence and absence of CNP, we were able to distinguish between these possibilities.

Without injection of the mice with hCG, addition of 30 nM CNP to cumulus-oocyte complexes elevated the cGMP content of the cumulus cells by 4.1 ± 0.9 times (n = 6). When cumulus-oocyte complexes were isolated from mice at 1 hour after hormone injection, and then incubated in the presence of CNP for an additional hour, the cGMP content of the cumulus cells was the same as that in cumulus cells from mice without hormone injection (Fig. 4). However, when the cumulus-oocyte complexes were isolated at 2 hours after hormone injection, and incubated with CNP until 3 hours, cGMP had decreased to 70% of values obtained without hormone treatment (Fig. 4). With isolation of the complexes at 3 hours, followed by a CNP incubation and measurement at 4 hours, cGMP had decreased to 42% (Fig. 4).

In the absence of CNP, the cGMP content of the cumulus cells was low, as expected for this in vitro condition in which NPR2 would not be activated. Under this condition, the cGMP content was not decreased by LH receptor stimulation (Fig. 4), indicating that LH receptor signaling does not increase cGMP phosphodiesterase activity in these cells, or cause an increase in cGMP efflux. Thus the LH receptor-induced decrease in cumulus cell cGMP seen in the presence of CNP can be attributed to a decrease in cGMP production. These findings indicate that LH signaling decreases NPR2 activity in the cumulus cells, but only after 2-3 hours, versus 20 minutes in the mural cells. This delay is likely to be a consequence of the localization of the LH receptors in separate cells (the mural granulosa). As will be discussed below, this intercellular signaling is most likely mediated by the release of EGF-like growth factors from the mural granulosa cells.

The decrease in cGMP production in the cumulus cells could result from a decrease in the amount of NPR2 protein, or from a modification of the NPR2 protein such as dephosphorylation. In support of the first possibility, the amount of *Npr2* mRNA in the cumulus cells decreases to ~50% of the basal level at 3 hours after LH receptor stimulation (Zhang et al. 2011). However, it is unknown how rapidly NPR2 protein would decrease as a consequence. Protein turnover rates for NPR2 have not been investigated, but turnover of the closely related protein NPR1 in cultured cells is a slow process, with a half-time of ≥8 hours (Flora and Potter 2010).

The amount of CNP in the ovary decreases in response to LH, preceding nuclear envelope breakdown. Another factor that could contribute to the LH-induced reduction in cGMP levels in the follicle is a decrease in CNP. CNP decreases have been reported previously for rat, mouse, and human (Jankowski et al. 1997, Kawamura et al. 2011), but the earliest of these measurements were made at 4 hours after LH receptor stimulation, so it was unclear if the CNP decrease occurred early enough to contribute to causing nuclear envelope breakdown and the events that precede it, vs later events leading to ovulation. To investigate the time course of the decrease in CNP, we injected mice with LH, and at various times afterwards, collected their ovaries for analysis of CNP content using an ELISA based on an antibody that should recognize all forms of CNP and its precursor NPPC.

Without LH injection, there were ~150 fmoles of CNP per mg of ovary extract protein (Fig. 5A), corresponding to an overall concentration of ~10 nM. However, the immunoreactive material detected by the ELISA contains both extracellular peptide and intracellular precursor protein, and only the peptide that has been secreted into the

extracellular space can activate NPR2. Thus the concentration of peptide that could function to regulate NPR2 is unknown.

No change from the pre-LH level of CNP was seen at 1 hour after injection of LH, but by 2 hours, the amount of CNP had decreased to 37% (Fig. 5A). The decrease in CNP at 2 hours corresponds to the time at which nuclear envelope breakdown in the oocyte is beginning, as determined from histological sections of ovaries of similarly treated mice (Fig. 5B). Thus, the CNP decrease occurs well after cGMP decreases in the follicle detected at 20 minutes after LH (Norris et al., 2010), but early enough to potentially contribute to stimulating nuclear envelope breakdown. After nuclear envelope breakdown, CNP continued to decrease, reaching 15% at 4 hours after LH, and 7% at 8 hours (Fig. 5A).

A likely cause of the CNP decrease is that *Nppc* mRNA decreases to about half of the basal level by 2 hours after LH receptor stimulation (Kawamura et al. 2011). Thus LH signaling might reduce *Nppc* mRNA synthesis or increase its degradation. Because the turnover of CNP is very rapid, with a half-life of about 3 minutes in plasma (Hunt et al. 1994), a decrease in *Nppc* mRNA could rapidly decrease the amount of CNP. Other possible factors that could contribute to the decrease in CNP are an increase in the natriuretic peptide clearance receptor NPR3, and an increase in the activity of proteases that degrade CNP (Potter 2011).

The amount of CNP in the ovary increases as follicles develop to the preovulatory stage. We also examined the effect of equine chorionic gonadotropin (eCG, also called PMSG) on CNP levels. Unlike human chorionic gonadotropin (hCG), which binds to the LH receptor, eCG binds to the follicle stimulating hormone receptor, which stimulates

antral follicle growth and LH receptor expression. eCG is often used experimentally to cause follicles to grow and to progress to the preovulatory stage; it was used for this purpose for the CNP measurements described above.

We found that eCG injection of the mice, 44 hours before collecting the ovaries, increased their CNP content (Fig. 6). The increase in CNP in response to eCG is consistent with findings that mRNA encoding the CNP precursor (NPPC) increases in mouse ovaries in response to eCG (Kawamura et al. 2011), and that CNP and cGMP increase between the days of diestrus and proestrus, in rats and hamsters (Hubbard and Greenwald 1982, Jankowski et al. 1997). Thus our findings add to the accumulating evidence that during follicle growth to the preovulatory stage, CNP and cGMP content of the ovary increases. Prior to this stage, cyclic nucleotide regulation is not needed to maintain meiotic arrest (Erickson and Sorensen 1974). Then with follicle growth, as the oocyte accumulates more CDK1 and other factors that result in meiotic competence (Chesnel and Eppig 1995), CNP is synthesized in order to prevent premature meiotic progression.

Pathways by which LH signaling in the mural granulosa cells causes cGMP to decrease in the oocyte. The connections between LH-induced activation of G-proteins in the outer layers of the mural granulosa cells and the ensuing events in the follicle that lead to the decrease in cGMP and resumption of meiosis in the oocyte are only partially understood (Fig. 7) (Vaccari et al. 2009, Norris et al. 2010, Hsieh et al. 2011, Conti et al. 2012). These connections include not only the pathways leading to a decrease in granulosa cell guanylyl cyclase activity, but also pathways that reduce gap junction permeability through MAP kinase-dependent phosphorylation of connexin 43 (Sela-

Abramovich et al. 2005, Norris et al. 2008), thus reducing diffusion of cGMP into the oocyte. Both the decrease in gap junction permeability and the decrease in granulosa cell guanylyl cyclase activity contribute to the decrease in oocyte cGMP.

At the level of the mural granulosa cells, LH receptor signaling activates G_s and adenylyl cyclase (Rajagopalan-Gupta et al. 1998), thus elevating cAMP. LH receptor signaling also activates G_i, G_q, and phospholipase Cb (Rajagopalan-Gupta et al. 1998, Kuhn and Gudermann 1999), thus elevating calcium (via IP_3) in granulosa cells in culture (Davis et al. 1986, Flores et al. 1998). However, activation of protein kinase C by the diacylglycerol that is generated by phospholipase C has, to our knowledge, not been detected so far (Salvador et al. 2002). Further studies of these signaling events using intact follicles will be informative, because both calcium elevation and protein kinase C activation can lead to dephosphorylation and inactivation of NPR2 (Abbey-Hosch et al. 2005). Calcium elevation could also increase the activity of the PDE1 family of cGMP phosphodiesterases (Francis et al. 2011).

Since the mRNA encoding the precursor protein of CNP is expressed in the same cells as the LH receptor (Zhang et al. 2010), exposure to LH could result in a reduction in the amount of CNP by signaling within the same cells. Likewise, since the LH receptor is expressed in the outer several layers of the mural granulosa cells (Amsterdam et al. 1975), the LH-induced decrease in NPR2 activity in these cells could result from signaling within the same cell. However, the LH-induced decrease in NPR2 activity in the cumulus cells, or in the inner layers of the mural cell epithelium, both of which regions lack LH receptors (Amsterdam et al. 1975, Eppig et al. 1997), must involve signaling between cells.

Based on evidence that the cGMP decrease in the follicle is partially dependent on EGF receptor signaling (Vaccari et al. 2009, Norris et al. 2010, Hsieh et al. 2011, Conti et al. 2012), EGF-like growth factors released from the outer layers of mural granulosa cells in response to LH are mediators of the paracrine signals, and could also contribute to autocrine signaling in the outer layers of the mural granulosa cells. EGF receptor signaling is essential for LH-induced nuclear envelope breakdown (Park et al. 2004), and EGF receptor activation, as indicated by increased phosphorylation of the receptor protein, occurs as early as 30 minutes after LH treatment (Panigone et al. 2008). However, it remains unknown how LH receptor signaling triggers the synthesis and/or release of the EGF-like growth factors epiregulin and amphiregulin. RNA encoding precursors of these growth factors increases by 2 hours after LH receptor stimulation (Park et al. 2004), but in addition, LH signaling might activate the proteases that release epiregulin and amphiregulin from pre-existing precursors (Blobel et al. 2009, Killock and Ivetic 2010).

EGF receptor signaling is required for much of the increase in MAP kinase activity in response to LH (Panigone et al. 2008), and thus contributes to phosphorylation of connexin 43 and the resulting decrease in gap junction permeability (Norris et al. 2008, Conti et al. 2012). EGF receptor signaling also activates phospholipase Cγ (Chattopadhyay et al. 1999), and could thus elevate calcium and protein kinase C activity, amplifying the LH receptor signaling that may occur through through phospholipase Cβ. As discussed above, these signaling events could decrease NPR2 activity, and possibly increase PDE1 activity, thus lowering cGMP in the granulosa cells and oocyte.

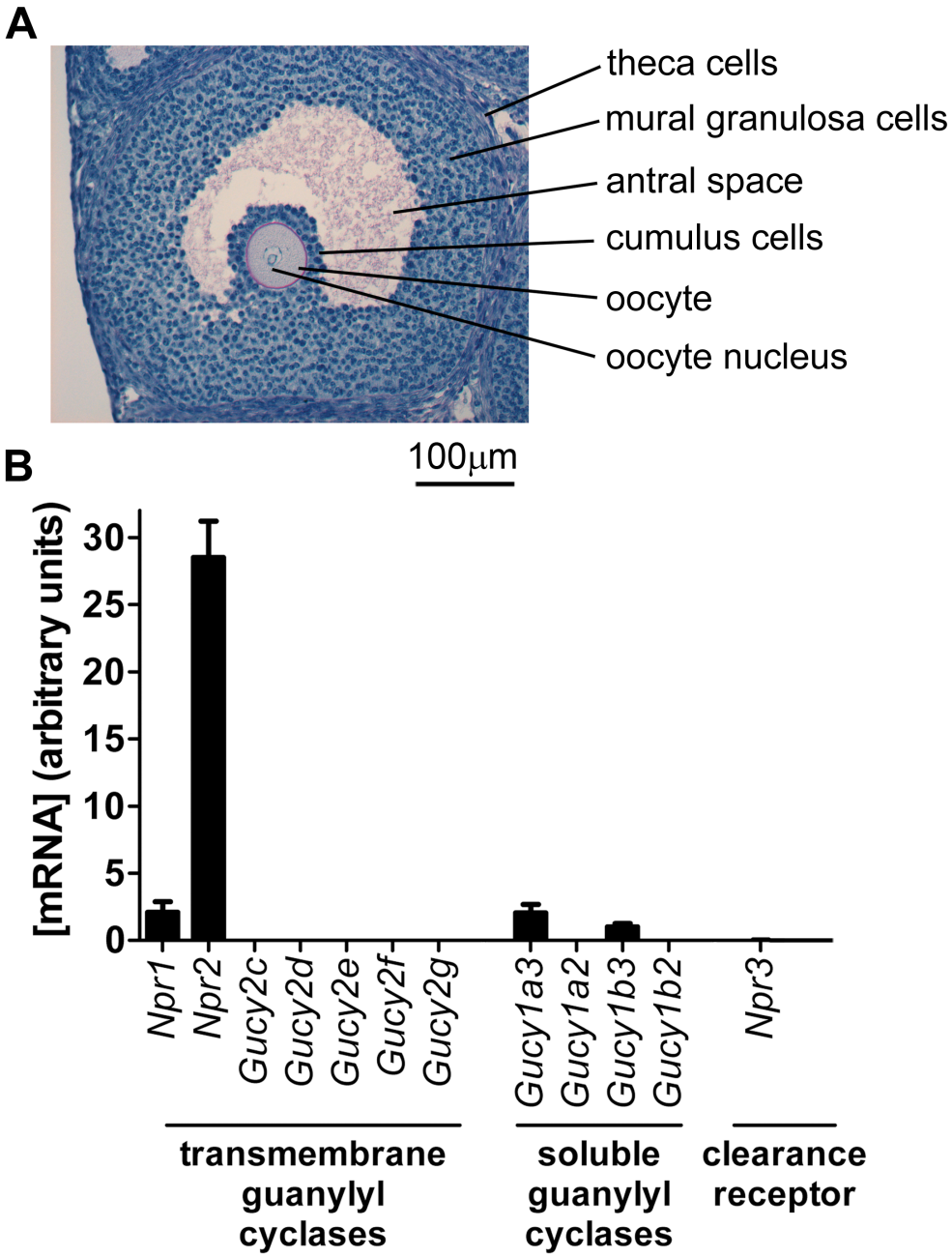
CONCLUSIONS

By 20 minutes after applying LH to ovarian follicles, the guanylyl cyclase activity of NPR2 elicited by a saturating concentration of CNP is decreased by half. This correlates with a similarly rapid decrease in follicle cGMP. There is then a slower decrease in NPR2 responsiveness to CNP in the cumulus cells, first seen at 2-3 hours. By 2 hours, LH signaling also induces a decrease in the amount of CNP in the ovary. Together, these 3 factors that decrease guanylyl cyclase activity contribute to the decrease in cGMP in the follicle. Because the mural granulosa cells, cumulus cells, and oocyte are connected by gap junctions to form a syncitium with respect to cGMP, cGMP in the oocyte equilibrates with that in the surrounding somatic cell compartment, and the resulting decrease in oocyte cGMP promotes meiotic resumption.

FIGURES

FIGURE 1. In mural granulosa cells, *Npr2* mRNA is present at a higher concentration than mRNAs for other guanylyl cyclases.

(**A**) Histological section of a mouse ovary, showing an antral follicle, and indicating the mural granulosa cells collected for analysis, as well as other cell types and structures in and around the follicle. (**B**) Relative concentrations of each guanylyl cyclase mRNA in isolated mural granulosa cells. Results for the natriuretic peptide clearance receptor, *Npr3*, are also shown. Where no bars are visible, concentrations of mRNAs were <0.1% of *Npr2*. The results show the mean ± s.e.m. for 3 RNA preparations.


A
theca cells
mural granulosa cells
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oocyte
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30
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Gucy2c
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Gucy2f
Gucy2g
Gucy1a3
Gucy1a2
Gucy1b3
Gucy1b2
Npr3
transmembrane guanylyl cyclases
soluble guanylyl cyclases
clearance receptor

FIGURE 2. LH signaling reduces NPR2 activity in the follicle.

(**A**) Guanylyl cyclase activity of crude membrane fractions prepared from follicles treated with or without LH for 20 minutes was measured with or without 1 μM CNP. Values indicate the mean ± range of duplicate measurements for each condition, using one follicle preparation made after LH treatment, and another preparation made in parallel but without LH treatment. (**B**) Combined data from 15 experiments like that in A, showing CNP-dependent guanylyl cyclase activity of crude membrane fractions from follicles treated with LH for the indicated times. CNP-dependent activity values were determined by subtracting the basal values measured in the absence of CNP. Activities are expressed as the mean ± s.e.m. for n follicle preparations. Activities for follicles treated with LH for 20, 60, or 120 minutes differed significantly from the activity for follicles without LH treatment (*, $p < 0.05$; **, $p < 0.01$). (**C, D**) Guanylyl cyclase activity of crude membrane fractions prepared from follicles treated with or without LH for 60 minutes was measured with or without 1 μM ANP. Data are presented as described for A and B. LH did not decrease the ANP-dependent guanylyl cyclase activity.

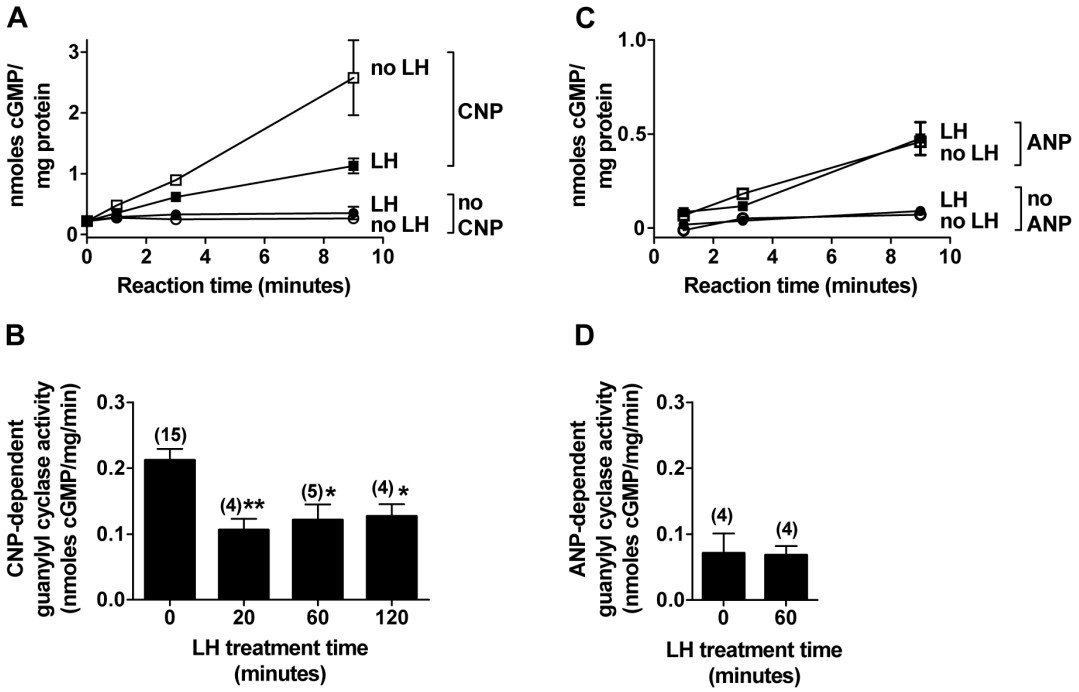

A
nmoles cGMP/
mg protein
3
2
1
0
0
2
4
6
8
10
Reaction time (minutes)
no LH
LH
LH
no LH
CNP
no
CNP
C
nmoles cGMP/
mg protein
1.0
0.5
0
0
2
4
6
8
10
Reaction time (minutes)
LH
no LH
ANP
LH
no LH
no
ANP
B
CNP-dependent
guanylyl cyclase activity
(nmoles cGMP/mg/min)
0.3
0.2
0.1
0.0
(15)
(4)**
(5)*
(4) *
0
20
60
120
LH treatment time
(minutes)
D
ANP-dependent
guanylyl cyclase activity
(nmoles cGMP/mg/min)
0.3
0.2
0.1
0.0
(4)
(4)
0
60
LH treatment time
(minutes)

FIGURE 3. The LH-induced decrease in NPR2 activity occurs without a corresponding decrease in NPR2 protein.

(**A**) Guanylyl cyclase activity of crude membrane fractions prepared from follicles treated with or without LH for 20 minutes was measured with or without 1% Triton X-100 and 5 mM $MnCl_2$, to maximally activate guanylyl cyclase. Values indicate the mean ± range of duplicate measurements for each condition, using one follicle preparation made after LH treatment, and another preparation made in parallel but without LH treatment. (**B**) Combined data from 3 experiments like that in A, showing Mn/triton-dependent guanylyl cyclase activity of crude membrane fractions from follicles treated with or without LH for 20 minutes (mean ± s.e.m.). LH did not decrease the Mn/triton-dependent guanylyl cyclase activity.

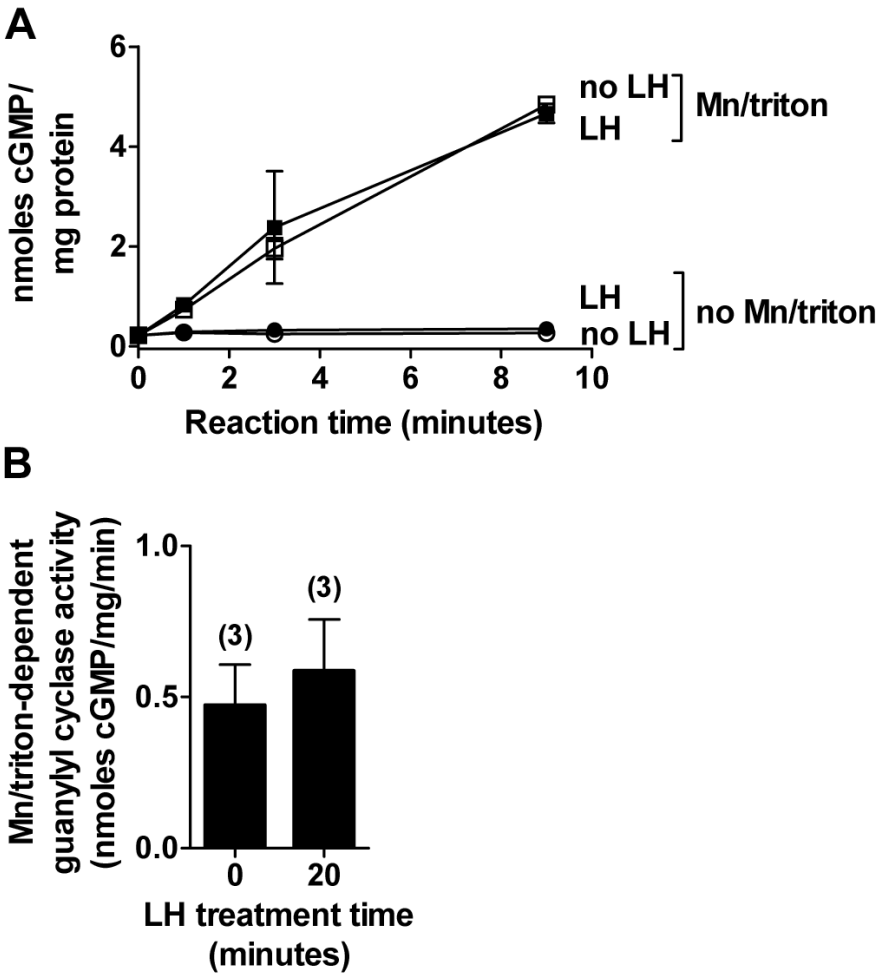

A
nmoles cGMP/
mg protein
6
4
2
0
0
2
4
6
8
10
Reaction time (minutes)
no LH
LH
Mn/triton
LH
no LH
no Mn/triton
B
Mn/triton-dependent
guanylyl cyclase activity
(nmoles cGMP/mg/min)
1.0
0.5
0.0
(3)
(3)
0
20
LH treatment time
(minutes)

FIGURE 4. In the cumulus cells, CNP-dependent cGMP production decreases in response to LH receptor stimulation, but more slowly than in the mural granulosa cells.

Cumulus-oocyte complexes were collected at various times after hCG injection into mice and incubated for one hour with or without 30 nM CNP. The cumulus cells were separated and cGMP content was measured. Values indicate the mean ± s.e.m. for the number of experiments shown in parentheses. Values for 2-3 and 3-4 hours post hCG were significantly different from the no hCG value (**, $p < 0.01$).

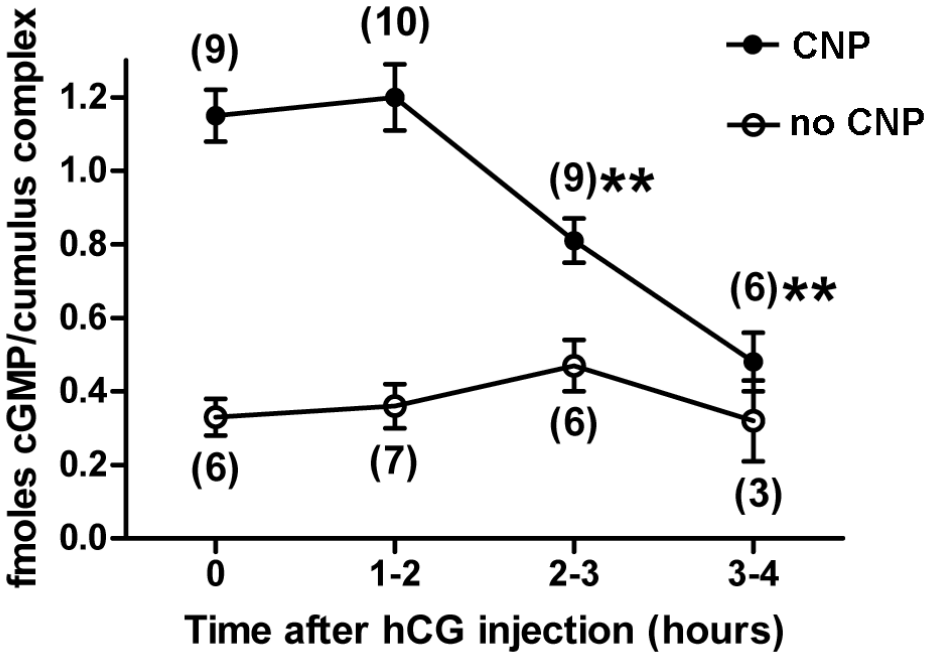

fmoles cGMP/cumulus complex
1.2
1.0
0.8
0.6
0.4
0.2
0.0
(9)
(10)
(9)**
(6)**
(6)
(7)
(6)
(3)
CNP
no CNP
0
1-2
2-3
3-4
Time after hCG injection (hours)

FIGURE 5. The amount of CNP in the ovary decreases in response to LH, preceding nuclear envelope breakdown.

(**A**) Time course of the decrease in the CNP content of ovary extracts, following LH injection into mice. Values indicate the mean ± s.e.m. for the number of mice shown in parentheses. Values for 2, 4, and 8 hour LH treatments were significantly different from the no LH value (*, $p < 0.05$; ** $p < 0.01$). (**B**) Time course of nuclear envelope breakdown, following LH injection into mice. Values indicate the percentage of fully grown follicles (≥350 μm in diameter) in which the oocyte contained a prophase-arrested nucleus; the number of follicles and the number of mice counted are indicated.

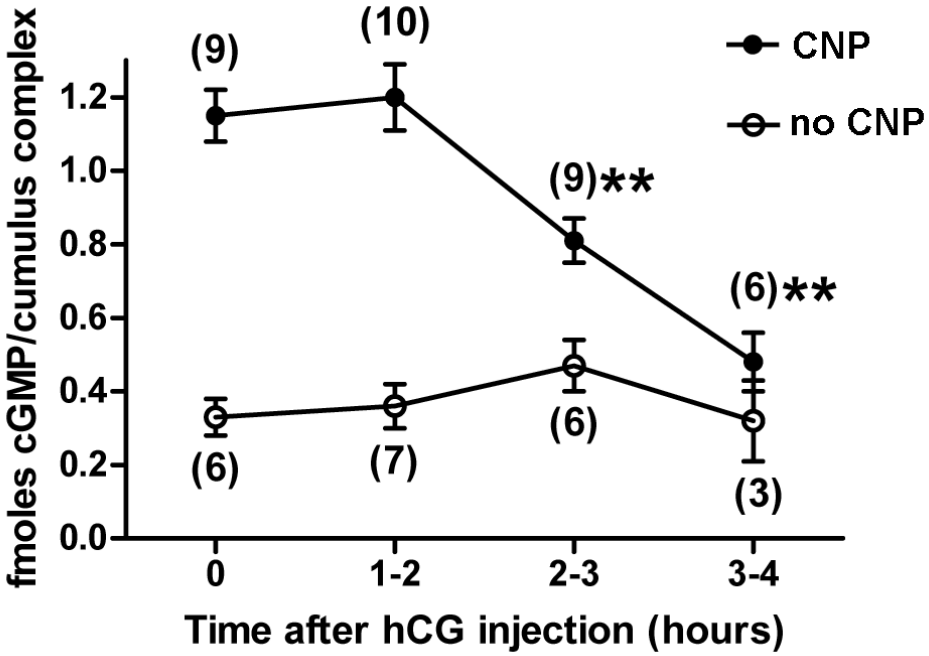

fmoles cGMP/cumulus complex
1.2
1.0
0.8
0.6
0.4
0.2
0.0
(9)
(10)
(9)**
(6)**
(6)
(7)
(6)
(3)
CNP
no CNP
0
1-2
2-3
3-4
Time after hCG injection (hours)

FIGURE 6. The amount of CNP in the ovary increases in response to activation of follicle stimulating hormone receptors.

The mice to be used for the CNP measurements shown in Fig. 5A were injected 44 hours previously with eCG to stimulate follicle growth. In response to eCG, the amount of CNP per mg of ovary protein increased ~4 times. The +eCG data shown in Fig. 6 are the same as the no LH (0 hour) data shown in Fig. 5A, so the statistical significance of these data was tested together. Values for ovaries from mice with or without eCG treatment were significantly different.

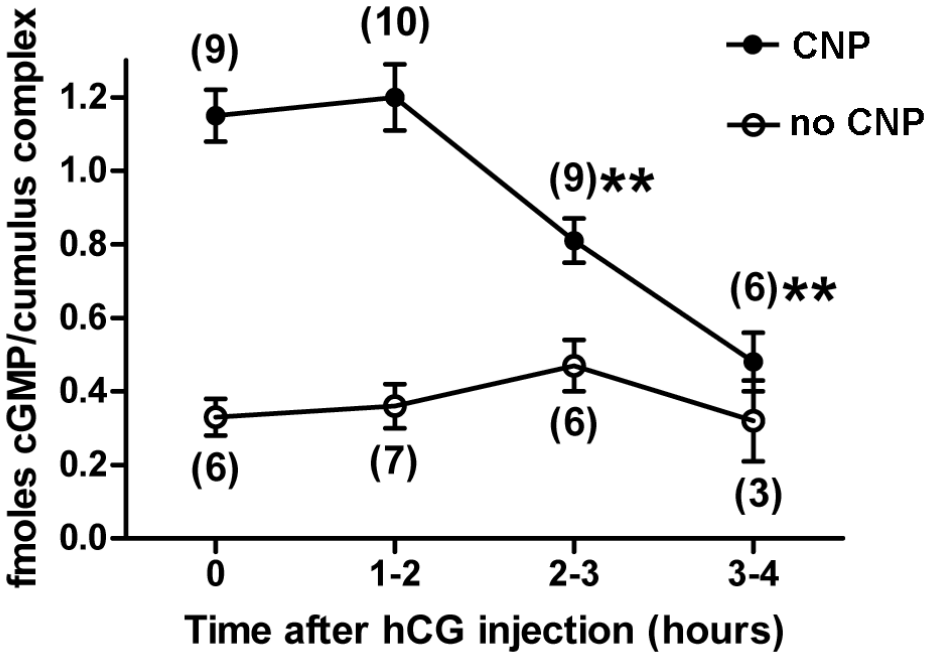
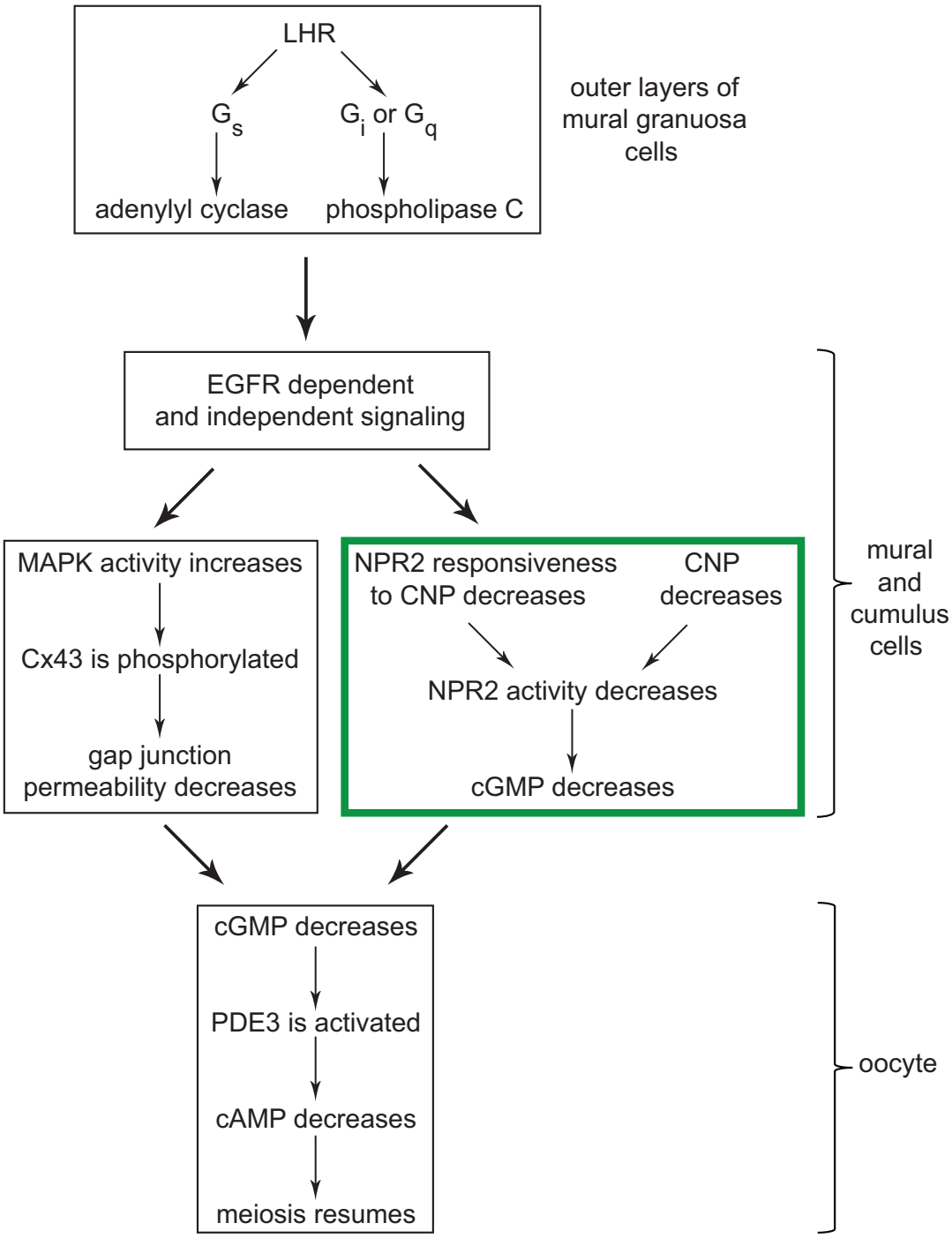

fmoles cGMP/cumulus complex
1.2
1.0
0.8
0.6
0.4
0.2
0.0
(9)
(10)
(9)**
(6)**
(6)
(7)
(6)
(3)
CNP
no CNP
0
1-2
2-3
3-4
Time after hCG injection (hours)

FIGURE 7. Signaling pathways connecting LH binding to its receptors in the outer layers of the mural granulosa cells to resumption of meiosis in a mammalian oocyte.

The green box indicates the findings of this study in the context of other aspects of the signaling network.


LHR
G_s
G_i or G_q
adenylyl cyclase
phospholipase C
outer layers of mural granuosa cells
EGFR dependent and independent signaling
MAPK activity increases
Cx43 is phosphorylated
gap junction permeability decreases
NPR2 responsiveness to CNP decreases
CNP decreases
NPR2 activity decreases
cGMP decreases
mural and cumulus cells
cGMP decreases
PDE3 is activated
cAMP decreases
meiosis resumes
oocyte

CHAPTER 5: GUANYLYL CYCLASE A AND B ARE ASYMMETRIC DIMERS THAT ARE ALLOSTERICALLY REGULATED BY ATP BINDING TO THE CATALYTIC DOMAIN

This chapter is a reprint of an original publication with minor alterations: Robinson, Jerid W and Potter, Lincoln R. (2012) Guanylyl cyclase A and B are asymmetric dimers that are allosterically regulated by ATP binding to the catalytic domain. *Science Signaling* 5(240):ra65.

Jerid Robinson collected and analyzed all data, created all figures and also contributed significantly to the writing of the manuscript and interpretation of the data.

How natriuretic peptides (NPs) and ATP activate guanylyl cyclase (GC)-A and GC-B is not known. We report that NPs increased Vmax > 10 fold in a positive cooperative manner in the absence of ATP. Without NPs, ATP shifted substrate-velocity profiles from cooperative to linear but did not reduce the Km. In the presence of NPs, ATP or ADP, but not AMP, reduced the Hill coefficient 0.5 units and reduced the Km 7-fold. ATP activation decreased and potency increased with increasing GTP concentrations. Single purine-binding site mutations in the catalytic domain abolished GC activity but did not block allosteric activation. Co-expression of inactive mutants produced half the activity expected for symmetric catalytic dimers. 2'deoxy-ATP and -GTP were poor allosteric activators, but 2'deoxy-GTP was an effective substrate. We conclude that membrane GC domains are asymmetric homodimers with unique and reciprocally regulated catalytic and allosteric sites that bind GTP and ATP, respectively.

INTRODUCTION

Guanylyl cyclases (GCs) catalyze the conversion of GTP to cGMP in organisms spanning the evolutionary spectrum. Single membrane-spanning GCs (mGC) regulate blood pressure, bone growth, reproduction, intestinal hydration, CO_2 detection, phototransduction, feeding and attention deficit disorder (Friebe et al. 1998, Kuhn 2003, Gong et al. 2011, Misono et al. 2011, Potter 2011, Valentino et al. 2011). The archetypal mammalian mGC is guanylyl cyclase-A (GC-A), a critical cardiovascular regulator that is stimulated by atrial natriuretic peptide (ANP) and B-type natriuretic peptide (BNP) (Koller et al. 1991, Suga et al. 1992). Guanylyl cyclase-B (GC-B) is 78% identical to GC-A at the intracellular amino acid level but is activated by C-type natriuretic peptide (CNP) (Schulz et al. 1989, Koller et al. 1991). In mice and/or humans, inactivating mutations in GC-A cause hypertension and cardiac hypertrophy (Lopez et al. 1995, Knowles et al. 2001), whereas inactivating mutations in GC-B cause dwarfism and infertility (Salomon et al. 1974, Bartels et al. 2004, Tamura et al. 2004).

Both GC-A and GC-B are composed of a large extracellular ligand-binding domain, a single membrane span and intracellular region consisting of a kinase homology domain (KHD), a dimerization domain and C-terminal catalytic domain. Unbound natriuretic peptide receptors are preformed, non-covalent homodimers (Chinkers and Wilson 1992). ANP binds GC-A at a stoichiometry of 1:2 to induce a rotation of the juxta-membrane region that initiates enzyme activation by an unknown mechanism (van den Akker et al. 2000, Ogawa et al. 2004). Structural modeling studies suggest that CNP binds GC-B similarly (He et al. 2006, Yoder et al. 2008). Multiple phosphorylation sites have been identified in the juxtamembrane portion of the intracellular region of GC-A and GC-B (Yoder et al. 2010, Yoder et al. 2012).

Phosphorylation is required for activation and dephosphorylation is an inactivation mechanism of the enzymes (Potter and Garbers 1992, Potter 1998).

ATP increases the activity of mGCs by binding an unknown intracellular site (Friebe et al. 1998). The most referenced activation model suggests that natriuretic peptide binding to the extracellular domain allows ATP to bind the KHD, which derepresses the catalytic domain and increases maximal velocity (Fig. 5, Old model) (Chinkers et al. 1991, Duda et al. 1993, Friebert et al. 1998, Bhandari et al. 2001, Joubert et al. 2005, Burczynska et al. 2007, Duda et al. 2010). However, there are several inconsistencies with this model. First, mutation of the putative GXGXXXG ATP binding site in the KHD failed to block activation of GC-A by ATP (Koller et al. 1993, Antos and Potter 2007). Second, natriuretic peptides were shown to activate GC-A and GC-B in the absence of ATP (Antos et al. 2005, Antos and Potter 2007). Third, an ATP analog that was chemically cross-linked to the KHD unexpectedly inhibited GC-A (Joubert et al. 2005). Fourth, ATP reduced the Michaelis constant without increasing the maximal velocity of highly phosphorylated preparations of GC-A and GC-B (Antos et al. 2005, Antos and Potter 2007). A related, unexplained phenomenon is that mGCs demonstrate positive cooperativity via an allosteric site in the GC domain when assayed under synthetic (manganese and nonionic detergent) but not physiologic (magnesium and ATP) conditions (Antos and Potter 2007, Joubert et al. 2007, Pattanaik et al. 2009). Here, we connect these seemingly disparate observations into a new unified activation model that illuminates the activation mechanism of this important enzyme family. We show that natriuretic peptide binding to GC-A and GC-B allows ATP to reduce the Hill coefficient and Km by displacing GTP at the allosteric site in the catalytic domain. Furthermore, we show that ATP binding to the allosteric site is essential for activation of GC-B under physiologic conditions.

RESULTS

To determine how NPs and adenine nucleotides activate GC-A and GC-B, we generated substrate-velocity curves using membranes from 293T cells stably expressing GC-A or GC-B as the enzyme source. 293T cells lack detectable endogenous mGCs and both receptors are properly processed and regulated in these cells. Since highly purified preparations of GC-A are constitutively active and are not further stimulated by ANP (Kuno et al. 1986, Takayanagi et al. 1987), this is the best system for studying natriuretic peptide receptor activation because the enzymes in these membranes are activated by both natriuretic peptides and ATP, and unlike endogenous preparations that express multiple GCs, activity in the transfected 293T cell membranes can be attributed to a single enzyme.

ATP shifts product formation from cooperative to linear under basal conditions. To understand how natriuretic peptides and adenine nucleotides regulate the enzymatic activity of GC-A and GC-B, we generated substrate-velocity curves for GC-A and GC-B in the presence and absence of NPs and 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 and the catalytic binding pocket is arbitrarily shown as the bottom site.

Under basal conditions (no NP or 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. 1A-C). In the absence of natriuretic peptides, ATP shifted product formation from positive cooperative to linear as demonstrated by a reduction in the Hill coefficients to 1. However,

ATP did not reduce the apparent $S_{0.5}$ or increase the maximal velocities of the enzymes in the absence of natriuretic peptides.

Natriuretic peptides increase maximal velocity in the absence of ATP. The next series of experiments investigated the effect of natriuretic peptides on the activity of GC-A and GC-B in the absence of ATP (Fig. 1D-F). ANP increased the maximal velocity of GC-A 23-fold and CNP increased the maximal velocity of GC-B 13-fold but neither peptide reduced the Hill coefficient or apparent $S_{0.5}$. These data are in direct contrast to previous reports generated on membranes prepared in the absence of phosphatase inhibitors that were not activated by natriuretic peptides in the absence of ATP (Chinkers et al. 1991, Duda et al. 1993).

The ATP-dependent reduction in the Km requires natriuretic peptide binding. In the third set of experiments, the effect of ATP on natriuretic peptide-activated receptors was examined (Fig. 1G-I). In absence of ATP, maximal velocity was high, the Km was high and the Hill coefficient was high. Strikingly, ATP shifted product formation from positive cooperative to linear as demonstrated by a 0.5 unit reduction in the Hill coefficient and reduced the $S_{0.5}$/Km of both enzymes seven-fold to around 0.1 mM, which is in the range of cellular GTP concentrations (Traut 1994). Together, these data indicate that ATP binds the receptors in the absence of natriuretic peptides and that ATP is not required for natriuretic peptide-dependent increases in maximal velocity as previously reported for dephosphorylated enzyme preparations (Chinkers et al. 1991, Duda et al. 1993). However, they also demonstrate for the first time that natriuretic peptide binding is required for the ATP-dependent reduction in the Km. Thus, in a physiologic system, natriuretic peptide binding increases GC activity by increasing Vmax and reducing the Km.

ATP competes with GTP for the allosteric site in a concentration-dependent manner. We hypothesized that ATP competes for GTP at an allosteric site in the catalytic domain. If this is 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 slope of both receptors (Fig. 2A and B). Importantly, the ATP-dependent reduction in the Hill coefficient correlated with concomitant reductions in the Km. These data are consistent with a model where ATP competes with GTP for binding to an allosteric site that increases the affinity of the catalytic site for GTP.

The allosteric and catalytic sites are reciprocally regulated. If ATP increases activity solely by decreasing the $S_{0.5}$/Km value, then stimulation must be greater at GTP concentrations below the Km. To test this hypothesis, we performed concentration-response assays in the presence of four different concentrations of GTP (Fig. 2C and D). At 0.1 mM GTP, which is at or below the Km, ATP increased the activities of GC-A and GC-B 8-fold and 14-fold, respectively; but at 5.4 mM GTP, which is above the Km, ATP only increased activity 2.4 fold for GC-A and 1.4 fold for GC-B.

We also tested if intra-site communication occurred in the reverse direction by examining if GTP increased the potency of ATP (Fig. 2C and D). At 0.1 mM GTP, the EC_{50} for ATP was 77 and 40 μM for GC-A and GC-B; but at 5.4 mM, the EC_{50} decreased 5 - and 2 - fold to 16 and 19 μM for GC-A and GC-B, respectively. Thus, ATP binding to the allosteric site increases GTP binding to the catalytic site, and GTP binding to the catalytic site increases ATP binding to the allosteric site.

ATP and ADP but not AMP allosterically activate GC-A. The structural requirements for allosteric regulation by adenine nucleotides were examined by testing the ability of adenosine, AMP, ADP and ATP to activate GC-A in the presence of saturating concentrations of ANP (Fig. 3A). ADP and ATP increased activity 6- and 9-fold, respectively, whereas adenosine and AMP failed to increase activity. These data indicate that the beta but not the gamma phosphate of ATP is required for binding the allosteric site.

Purines lacking ribosyl 2', but not 3', OH groups are poor allosteric activators of GC-A. To further investigate the structural requirements of the allosteric regulator, we examined the effect of the purine ribosyl 2' OH on GC-A activity. 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 repeated with 2'-deoxyGTP as substrate, product formation was linear and the Hill coefficient was 1. These data are consistent with the ribosyl 2' OH of GTP increasing binding to the allosteric but not to the catalytic site (Fig. 3C).

Since we hypothesized that ATP competes with GTP at the same allosteric binding site, we tested whether ATP lacking a 2' OH was also a poor allosteric activator of GC-A. Consistent with our hypothesis, compared to ATP, 2'-deoxyATP was poor activator of GC-A in single substrate concentration GC assays (Fig. 3D). Furthermore, as would be expected if the 2' OH group of the nucleotide increases binding to the allosteric site, 2'-deoxyATP reduced the Hill slope and $S_{0.5}$/Km values less than ADP or ATP, both of which contain a ribosyl 2' OH group (Fig. 3E). Models depicting the effects and differential binding capabilities of 2'-deoxyGTP and 2'-deoxyATP are shown in Figures 3C and 3F, respectively.

ATP binding 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 groups have indicated that ATP binding to the KHD is required for enzyme activation (Chinkers et al. 1991, Duda et al. 1993, Friebert et al. 1998), whereas we have shown that ATP is not required for maximal activation of GC-A and GC-B in enzyme assays containing saturating concentrations (1 mM) of the substrate GTP (Antos et al. 2005, Antos and Potter 2007). Our goal is to determine how ATP activates GC-A and GC-B in cells, but since depleting cellular ATP concentrations below the EC_{50} for activation of the cyclases would kill the cells, we used an *in vitro* assay (Fig. 4). Here, we measured the activation of endogenous GC-B in 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 reflect nucleotide concentrations measured in many cells (Traut 1994), ATP dramatically increased the ability of CNP to activate GC-B (Fig. 4). For example, 125 nM CNP increased activity 13 fold in the presence of ATP but in the absence of ATP, no significant increase in activity was observed. As shown in Fig. 1H, the increased activity resulting from the inclusion of ATP was solely due to reductions in the Km. The reason that ATP did not increase activity in previous assays is because GTP concentrations were ten-fold higher and the assay were 1/20 as long. Thus, ATP binding to the allosteric site in the catalytic domain of GC-B is essential for CNP activation of the enzyme under biological conditions.

Heterozygous receptors lacking a single purine-binding site have been studied. Joubert *et al.* engineered two single purine-binding site mutations in individual GC-A cDNAs encoding truncated enzymes containing only the C-terminal dimerization and catalytic domains; these mutations were predicted to produce a catalytic dimer with only one nucleotide binding site (Joubert et al. 2007). One mutant contained a D849A substitution and the other mutant contained a N968S substitution. Expression of either mutant alone was inactive but co-expression produced an active heterozygous (HET) enzyme that lacked cooperativity. Based on these and other data, Joubert *et al.* concluded that the truncated GC-A dimer is symmetrical, contains two catalytic sites, and that both sites are required for cooperativity.

ATP activates mutants lacking a single GTP binding site similarly to WT-GC-A. We initially hypothesized that ATP reduces the Km 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. (Joubert et al. 2007). D849A or N968S substitutions were generated in individual full-length GC-A cDNAs and transfected separately, together or with WT-GC-A into 293 cells. Please see Fig. 5A for graphical descriptions of the possible dimer combinations. GC activity was measured in the presence of Mg^{2+}GTP (basal), Mg^{2+}GTP and ANP (ANP), Mg^{2+}GTP, ANP and ATP (ANP + ATP) and Mn^{2+}GTP and Triton X-100 (Mn/Triton) (Fig. 5B). The individual mutants were inactive under all assay conditions when expressed alone. However, ATP increased activity in membranes from cells expressing mutant and WT receptors or two individual mutant receptors to make the HET receptor like it increased activity in membranes from cells only expressing the WT receptor. These data were inconsistent with the notion that ATP and GTP bind 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.

Full-length HET mutants of GC-A are negative cooperative. To determine if the full-length HET mutant lacked cooperativity as was observed for the purified, truncated HET mutant (Joubert et al. 2007), substrate-velocity curves were generated in the presence of Mn^{2+} and Triton X-100 on membranes expressing WT or HET receptors (Fig. 5C). The double reciprocal plot of the WT enzyme was concave upward, indicating positive cooperativity (Fig. 5D). However, the plot of the HET mutant was equally concave downward, indicating negative cooperativity, which explains why detergent-dependent activity was reduced for the mutant enzymes shown in Fig. 5B. These data suggest that the reason ATP activated the HET enzyme is because the mutations that abolish the catalytic site do not abolish the allosteric site. This is consistent with the different structural binding requirements for the catalytic and allosteric sites shown in 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 with the full-length but not with the truncated enzyme.

Guanylyl cyclase activity measurements are consistent with asymmetric dimers. Finally, we investigated whether the catalytic domain of GC-A is a symmetric homodimer as suggested by Joubert and colleagues (Joubert et al. 2007) or an asymmetric homodimer as envisioned for soluble GC and adenylyl cyclase (Gean et al. 1990, Hunt et al. 1994, Joubert et al. 2007). Based on a symmetric homodimeric catalytic domain model, coexpression of mutant (D849A or N968S) and WT enzymes is expected to produce 25% WT/WT active homodimers (cartoon A in Fig. 5A), 25% inactive mutant/mutant homodimers because each site contains an inactivating mutation (B or C), 25% active heterodimers with the mutation in

the top purine binding site (D or F) and 25% active heterodimers with the mutation in the bottom purine binding site (E or G) for a total of 75% of WT activity. Asymmetric model expectations are the same but only 50% of WT activity is expected because mutations in the catalytic site, which was arbitrarily designated as the star-shaped, bottom site, would be inactive. We observed 57% ± 4 and 59% ± 2% of WT activity when D849A and N968S mutants were coexpressed with WT-GC-A, respectively, consistent with the asymmetric model (Fig. 5B).

The symmetric model also predicts that cells cotransfected with each mutant cDNA (HET) will produce 25% inactive D849A/D849A homodimers (B), 25% inactive N968S/N968S homodimers (C), 25% active N968S/D849A heterodimers where the bottom site contains both mutations (H), and 25% active N968S/D849A heterodimers where the upper site contains both mutations (I) for a total of 50% of WT activity. However, only 25% of WT activity is anticipated for the asymmetric model because both mutations in the bottom catalytic domain (H) would inactivate GC-A. We observed 33% ± 8% of WT activity in membranes from cells cotransfected with 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 25 years ago (Kurose et al. 1987), but the mechanism of activation has been controversial from the beginning. 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 where natriuretic peptide binding to the extracellular domain stimulates ATP binding to the KHD (Fig. 6, Old model). (Kurose et al. 1987, Wong et al. 1995, Friebert et al. 1998). Two relatively recent papers demonstrating cross-linking of azido-ATP analogs to the KHD of GC-A provide some support for this model, although the azido analog used by one group inhibited GC-A and effects on enzyme activity were not tested by the other group (Joubert et al. 2005, Burczynska et al. 2007). In contrast, the discovery that both GC-A and GC-B are basally phosphorylated and require phosphorylation for natriuretic peptide activation (Potter and Garbers 1992, Potter 1998, Potter and Hunter 1998) suggests that the maximal velocity effects were due to increases in GC-A phosphorylation. This is also consistent with early observations showing that adenine analogs that cause prolonged phosphorylation like ATPγS lead to greater levels of enzyme stimulation than ATP analogs that are not kinase substrates (Kurose et al. 1987, Chinkers et al. 1991). Subsequent experiments confirmed that the inclusion of ATP analogs in the GC assays increased enzyme phosphorylation by showing that thiophosphorylated ATP analogs and phosphatase inhibitors increased the phosphate content and activity of GC-A and GC-B (Foster and Garbers 1998, Abbey-Hosch et al. 2005, Antos et al. 2005).

Data generated with highly phosphorylated receptors have provided the foundation for a new model (Fig. 6, New model). In enzyme assays lacking ATP, both GC-A and GC-B are positive cooperative because there is no molecule to compete with GTP binding to the

allosteric site (first figure in new model of Fig. 5). However, positive cooperativity is not observed in assays mimicking physiologic scenarios because the allosteric site is occupied by ATP due to ten-fold higher cellular ATP concentrations compared to GTP concentrations (Traut 1994). This explains the conundrum of why positive cooperativity is only observed in assays conducted under synthetic but not physiologic assay conditions since physiologic assays historically contain ATP.

Linear product formation in the presence of ATP but in the absence of natriuretic peptides indicates that natriuretic peptide binding is not a prerequisite for ATP binding to the allosteric site (middle figure in new model). However, since the Km is high in the absence of natriuretic peptides and low in the presence of natriuretic peptides, ligand binding is required to transduce the Km reducing effects of ATP binding to the catalytic site. Upon natriuretic peptide binding (last step in new model), not only does maximal velocity increase dramatically, but the Km also falls precipitously. The combination of increased Vmax and decreased Km ultimately leads to more than a one hundred fold activation of GC-A and GC-B upon natriuretic peptide binding (Antos et al. 2005).

Several observations and experiments support the idea that ATP binds the allosteric site that was previously shown to reside in the catalytic domain of GC-A (Joubert et al. 2007, Pattanaik et al. 2009). Firstly, ATP and GTP are structurally similar. Secondly, both ATP and GTP reduce the $Km/S_{0.5}$. Thirdly, ATP is competitive with GTP, and fourthly, both nucleotides require the 2' OH group of ribose for maximal allosteric activation. The observations that the ribosyl 2'OH but not the gamma phosphate is required for maximum allosteric activation and mutations that destroy catalytic activity do 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 soluble GC in response to metabolic stress (Ruiz-Stewart et al. 2004). However, in our opinion, it is unlikely that ATP would drop below the EC_{50} (~0.1 mM) for GC-A and GC-B activation in living cells. Thus, although ATP is absolutely required for these enzymes to signal under physiologic conditions, changes in cellular ATP concentrations are unlikely to be regulatory with respect to allosteric activation. This is analogous to the activation of adenylyl cyclase by G-proteins. Like ATP activation of GC-A and GC-B, GTP is required for the activation of adenylyl cyclase by heterotrimeric GTP binding proteins. However in both scenarios, changes in nucleotide concentrations are unlikely to affect the signaling of either enzyme, yet GTP binding to Gsα is required for activation of adenylyl cyclase and ATP binding to the catalytic allosteric site is required for the activation of GC-A and GC-B.

The convergence of data from our group with the data from independent groups increases the validity of our conclusions. Thorpe et al. observed cooperative and linear kinetics for the recombinant GC-A catalytic domain with GTP and 2'-deoxyGTP, respectively (Thorpe et al. 1996). Chang et al. observed that 2'-deoxy ATP was a poor activator of GC-A compared to ATP (Chang et al. 1990). Similarly, Parkinson et al. reported the 2' ribosyl-substituted adenine analogs were poor activators of GC-C (Parkinson et al. 1994). Rauch *et al.* demonstrated that the catalytic domain of the mGC from cyanobacteria bound ATP and GTP with similar affinity and was asymmetric with only one active site per dimer (Rauch et al. 2008). Sinha *et al.* reported that a bacterial adenylyl cyclase with similarity to mGCs possesses an asymmetric catalytic domain that contains "half the sites activity" (Sinha et al. 2005), and the Waldman and Beuve groups reported that ATP inhibits soluble GCs by binding the pseudo-symmetric site, which is analogous to the allosteric site in mGCs (Hunt et al. 1994,

Ruiz-Stewart et al. 2004). The Marletta group found that ATP is a mixed type inhibitor of purified full-length and truncated sGC and concluded that ATP binds to both the catalytic and allosteric sites. The Seifert group also observed mixed type inhibition of sGC by ATP and proposed a model similar to ours for GC-A and GC-B where Mn^{2+}GTP binds an allosteric site in the catalytic domain of sGC (Beste et al. 2012). Why ATP inhibits GC-A and GC-B but inhibits soluble GC is not currently known. Finally, we note that ATP clearly reduced the Km for GC-A in experiments reported by other investigators, but for unknown reasons the authors did not comment on the Km reductions in the manuscripts describing these experiments (Wong et al. 1995, Friebert et al. 1998).

Forskolin binding to the pseudo-symmetric "p site" in adenylyl cyclase led to the idea that an endogenous forskolin-like molecule may exist (Gean et al. 1990). A forskolin-like molecule was identified in human renal cysts, but this observation has yet to be corroborated by independent investigators (Putnam et al. 2007). A number of stimulatory and inhibitory pharmacologic agents that target the adenylyl cyclase diterpene site have also been identified (Seifert et al. 2012). Analogously, we show that ATP allosteric regulates GC-A and GC-B by binding a pseudo-symmetric site in the catalytic domain. To our knowledge, this is the first example of an endogenous molecule that activates guanylyl cyclases by binding the pseudo-symmetric site.

In conclusion, we determined how adenine nucleotides activate GC-A and GC-B. ADP and ATP but not AMP or adenosine bind an allosteric site in the catalytic domain that switches the kinetics of cGMP formation from positive cooperative to linear and reduces the Michaelis constant for GTP. Due to higher cellular concentrations and similar EC_{50} values, we suggest that ATP, not ADP, occupies the allosteric site under basal conditions, and that

reductions in the Michaelis constant require natriuretic peptide activation of the receptors. Thus, natriuretic peptides increase maximal velocity and reduce the Michaelis constants of GC-A and GC-B. Importantly, we showed that ATP is required for the activation of GC-B under physiologic conditions. In contrast to studies conducted on a truncated mutant of GC-A, we found that the catalytic domain of the full-length receptor is an asymmetric dimer with reciprocally regulated allosteric and catalytic binding sites that have different binding preferences. 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.

EXPERIMENTAL PROCEDURES

Reagents. ^{125}I-cGMP radioimmunoassay kits were from Perkin Elmer Life Sciences (Waltham, MA). ANP, CNP, creatine kinase and phosphocreatine were from Sigma-Aldrich (St. Louis, MO). The protease inhibitor cocktail was from Roche (Indianapolis, IN).

Cells. 293T, 293T-GC-A and 293T-GC-B cells were maintained as previously described (Robinson and Potter 2011). 293T cells do not express detectable natriuretic peptide receptors. However, some 293 cells do endogenously express low levels of GC-A, which is consistent with GC-A being properly processed and regulated in these cells (Potter and Hunter 1998).

Cells and Transfections. Approximately 60% confluent 293T cells grown on polylysine coated 10 cm plates were transfected with 5 μg of plasmid DNA using the HEPES-buffered calcium phosphate precipitation method. Transfection efficiency was ~80% based on GFP expression. The medium was replaced after 2 h and membranes were harvested 48 hours after transfection. 3T3 cells were cultured as previously described (Potthast et al. 2004).

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 Quik change mutagenesis on the pCMV3-GC-A plasmid per manufacturer's instructions as previously described (Potter and Hunter 1998). Confirmation of the mutations and lack of additional mutations was determined by nucleic acid sequencing.

Membrane preparation. Crude membranes were prepared at 4^{o}C in phosphatase inhibitor buffer which consisted of 50 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid - pH

7.4, 50 mM NaCl, 20% glycerol, 50 mM NaF, 1 mM EDTA, 0.5 μM microcystin and 1X Roche protease inhibitor cocktail.

Guanylyl Cyclase Assays. GC assays were performed at 37ºC in a reaction mixture containing 25 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid - pH 7.4, 50 mM NaCl, 0.1% BSA, 0.5 mM isobutylmethyl xanthine, 1 mM EDTA, 5 mM phosphocreatine, 0.1 mg/ml creatine kinase, 0.5 μM microcystin, 5 mM $MgCl_2$. GTP concentrations were 0.1 mM in figures 3A, 3D and 4B. The time each reaction was incubated at 37^{o}C is 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 activities in membranes from untransfected cells, which indicated that the GC activity was due to the transfected enzyme. Reactions were initiated by adding 20 ml of crude membranes containing 10 to 18 μg of protein suspended in phosphatase inhibitor buffer to 80 ml of reaction mixture. Creatine kinase and phosphocreatine were not included in assays including AMP or ADP to prevent 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. Cyclic GMP concentrations were determined by radioimmunoassay as previously described (Robinson and Potter 2011). Because enzymatic activity was not completely linear with time, we qualify the kinetic parameters obtained under these conditions as “apparent.”

Statistical Analysis. Substrate-velocity curves were analyzed by non-linear regression using an allosteric sigmoidal model in Prism 5 to determine the Vmax, $S_{0.5}$ and Hill coefficients. A Michaelis-Menten model was used to determine Vmax and Km in the presence of ATP. Significant differences between nonlinear regression curves used to determine Km and Vmax values were evaluated using the extra sum of squares F test to generate p-values using Prism software. $p \leq 0.05$ was considered significant for all tests. Values were presented as mean ± SEM. Vertical bars within the symbols represent SEM. When not visible, error bars are contained within the symbol.

FIGURES

FIGURE 1. Natriuretic peptides (NPs) alone increase Vmax, and ATP reduces Km in the presence of NPs.

GC activity was measured in membranes from 293T cells expressing GC-A or GC-B with increasing concentrations of Mg^{2+}GTP where n_H = Hill coefficient and n = 6. (A-C) Basal substrate-velocity curves are cooperative without ATP and linear with ATP. For GC-A, the Vmax was 3 ± 0.6 nmol/mg/9 min and the $S_{0.5}$ was 1.8 ± 0.62 mM in the absence of ATP and 4 ± 0.8 nmol/mg/9 min and 1.6 ± 0.65 mM in the presence of ATP. For GC-B, the Vmax was 4 ± 0.2 nmol/mg/9 min and the $S_{0.5}$ was 1.0 ± 0.11 mM in the absence of ATP and 4 ± 0.3 nmol/mg/9 min and 1.0 ± 0.15 mM in the presence of ATP. Neither the ATP-associated changes in Vmax nor Km were significantly different at $p \leq 0.05$ for either receptor. (D-F) NPs increase maximal velocity in the absence of ATP. For GC-A, Vmax was 4 ± 0.9 nmol/mg/9 min and the $S_{0.5}$ was 1.1 ± 0.44 mM in the absence of ANP and 104 ± 18.6 nmol/mg/9 min and 0.48 ± 0.211 mM in the presence of ANP. For GC-B, Vmax was 4 ± 0.9 nmol/mg/9 min and the $S_{0.5}$ was 2.2 ± 0.84 mM in the absence of CNP and 49 ± 3.7 nmol/mg/9 min and 1.0 ± 0.15 mM in the presence of CNP. The NP-associated changes in Vmax but not Km were significantly different at $p \leq 0.05$. (G-I) ATP only decreases Km in the presence of NPs. For ANP-stimulated GC-A, the Vmax was 70 ± 4.6 nmol/mg/9 min and the $S_{0.5}$ was 0.7 ± 0.09 mM without ATP and 84 ± 3.4 nmol/mg/9 min and 0.1 ± 0.1 mM with ATP. For CNP-stimulated GC-B, the Vmax was 49 ± 7.0 nmol/mg/9 min and the $S_{0.5}$ was 0.6 ± 0.18 mM without ATP and 41 ± 3.1 nmol/mg/9 min and 0.09 ± 0.02 mM with ATP. The ATP-associated

changes in Km but not Vmax were significantly different at $p \leq 0.05$ for both receptors.

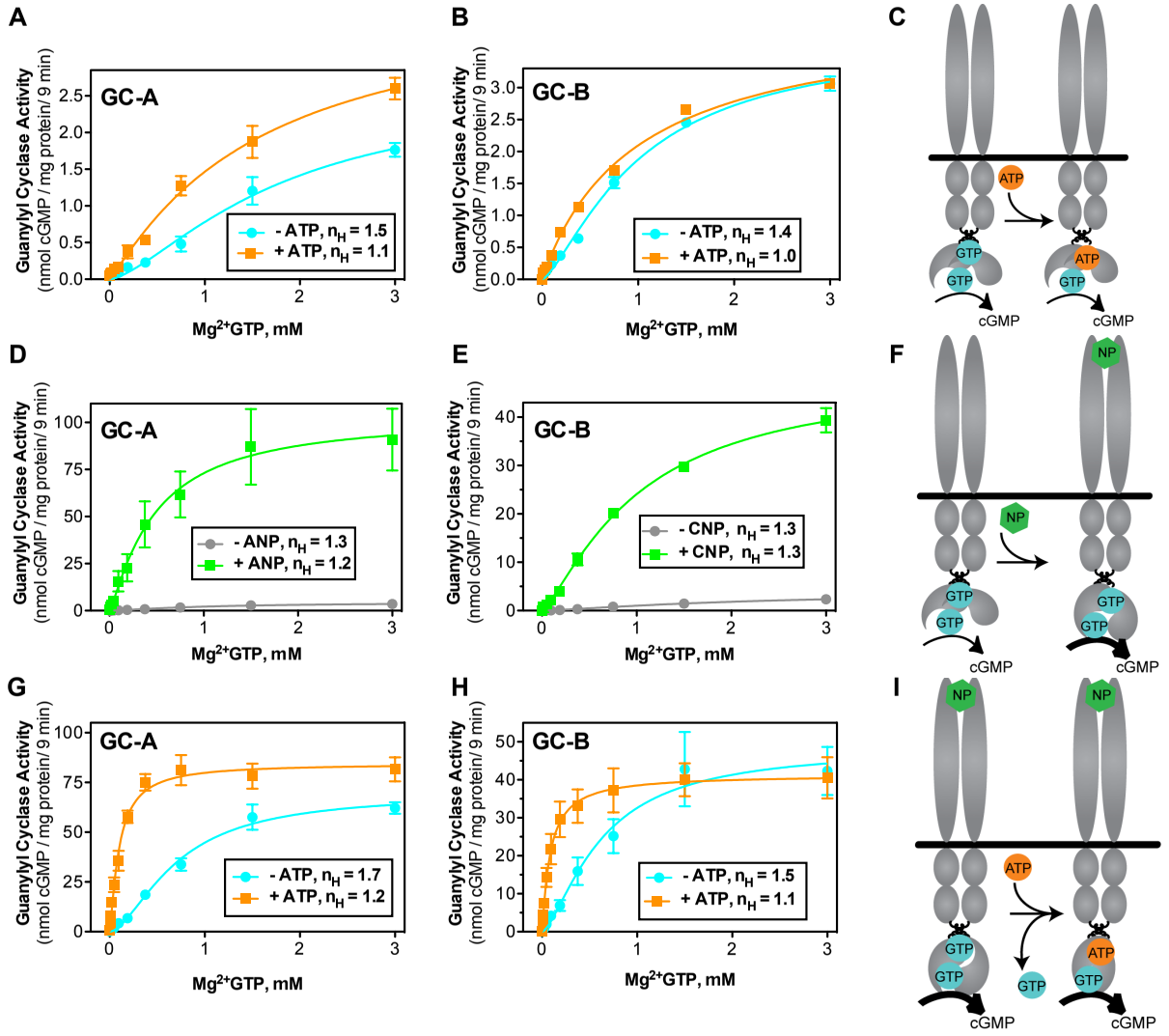

A
GC-A
Guanylyl Cyclase Activity
(nmol cGMP / mg protein/ 9 min)
- ATP, nH = 1.5
+ ATP, nH = 1.1
Mg2+GTP, mM
B
GC-B
- ATP, nH = 1.4
+ ATP, nH = 1.0
C
ATP
GTP
cGMP
D
GC-A
- ANP, nH = 1.3
+ ANP, nH = 1.2
E
GC-B
- CNP, nH = 1.3
+ CNP, nH = 1.3
F
NP
G
GC-A
- ATP, nH = 1.7
+ ATP, nH = 1.2
H
GC-B
- ATP, nH = 1.5
+ ATP, nH = 1.1
I

FIGURE 2. Reciprocal regulation of the catalytic and allosteric sites by ATP and GTP, respectively.

NP-stimulated GC activity was measured for 9 min where n = 4. (A) ATP reduces cooperativity and Km 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, 0.5 ± 0.03 mM; 1.2 ± 0.08, 0.2 ± 0.02 mM; 0.9 ± 0.07, 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, 0.9 ± 0.33 mM; 0.9 ± 0.13, 0.4 ± 0.11 mM; 0.9 ± 0.24, 0.2 ± 0.18 mM, respectively. Fold reductions in the Michaelis constants are 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 increases with increasing GTP. NP-stimulated GC activity was measured with the indicated concentrations of ATP and GTP and EC_{50}s for ATP were determined by non-linear curve fitting. (C) The EC_{50} for ATP activation of GC-A was 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} for ATP activation of GC-B was 40, 26, 16 and 19 μM when assayed with 0.1, 0.3, 0.9 and 5.4 mM GTP, respectively.

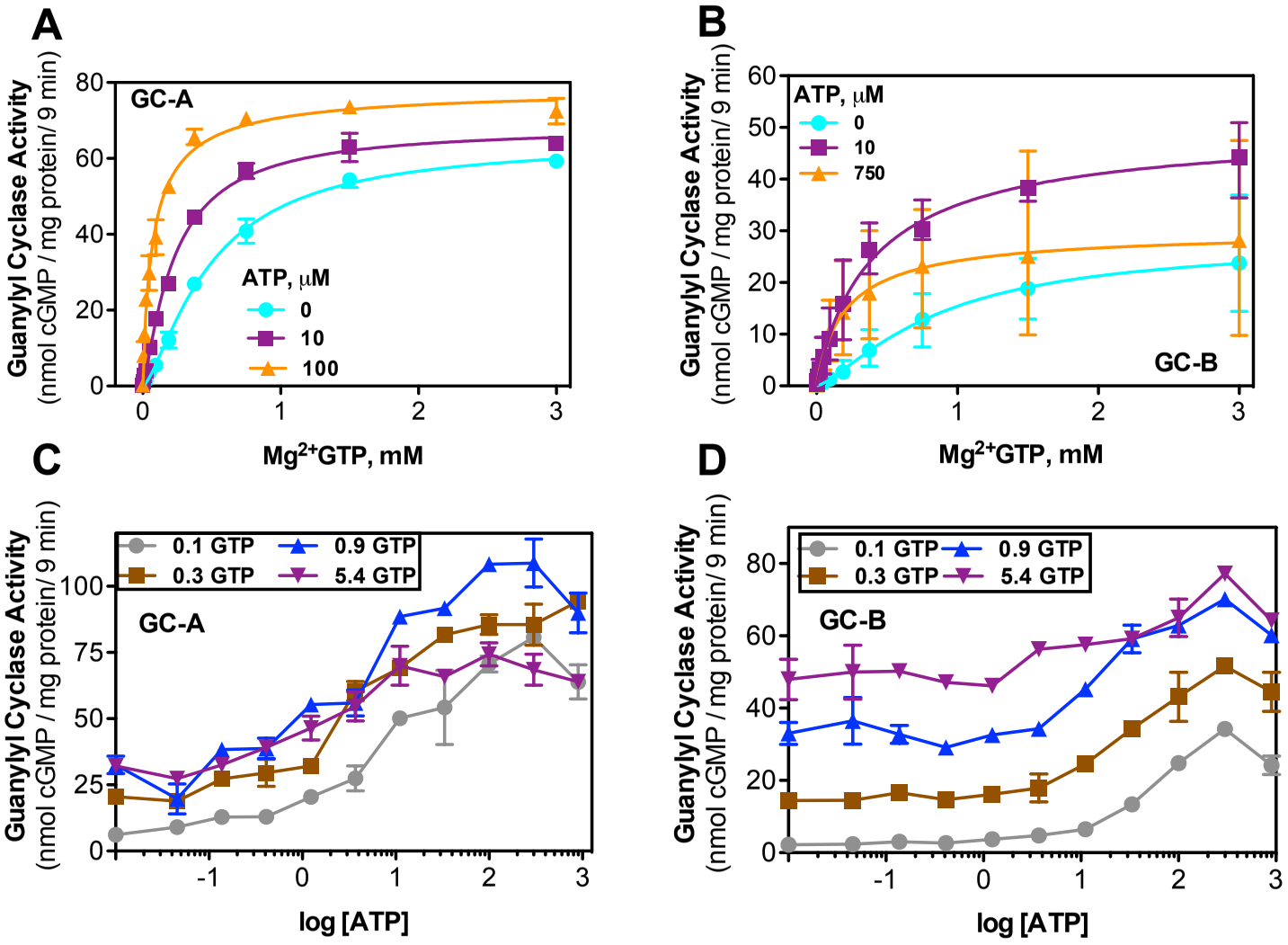

A
GC-A
Guanylyl Cyclase Activity
(nmol cGMP / mg protein/ 9 min)
ATP, μM
0
10
100
Mg2+GTP, mM
B
GC-B
ATP, μM
0
10
750
Mg2+GTP, mM
C
GC-A
0.1 GTP
0.3 GTP
0.9 GTP
5.4 GTP
log [ATP]
D
GC-B
0.1 GTP
0.3 GTP
0.9 GTP
5.4 GTP
log [ATP]

FIGURE 3. The purine ribosyl 2' OH but not gamma phosphate is required for maximum allosteric regulation of GC-A.

(A) ANP-stimulated GC-A activity was measured with 0.1 mM GTP and increasing concentrations of the indicated adenine nucleotides where n = 6. (B) The Hill coefficient of ANP-stimulated GC-A activity measured with 0.1 mM GTP was 1.5 ± 0.21 but was 1.0 ± 0.18 when measured with 0.1 mM 2-deoxyGTP, n = 4. (C) Model depicting ANP-dependent GC-A activation in the presence of GTP or 2-deoxyGTP. (D) Concentration-response curves for ANP-dependent GC-A activity determined in the presence of 0.1 mM GTP and the indicated concentrations of adenine nucleotide where n = 6. (E) Substrate-velocity curves were generated in membranes from 293T-GC-A cells for 9 min with 1 μM ANP ± 1 mM concentrations of the indicated nucleotides where n = 4. Hill coefficient and Km values were 1.4 ± 0.21, 1.0 ± 0.14 mM for control, 1.2 ± 0.24, 0.4 ± 0.08 mM for 2-deoxyATP; 1.1 ± 0.18, 0.2 ± 0.04 mM ADP and 1.0 ± 0.15, 0.1 ± 0.02 mM for ATP. (F) Model depicting allosteric regulation of ANP-dependent GC-A activity in the presence of ATP or 2-deoxyATP.

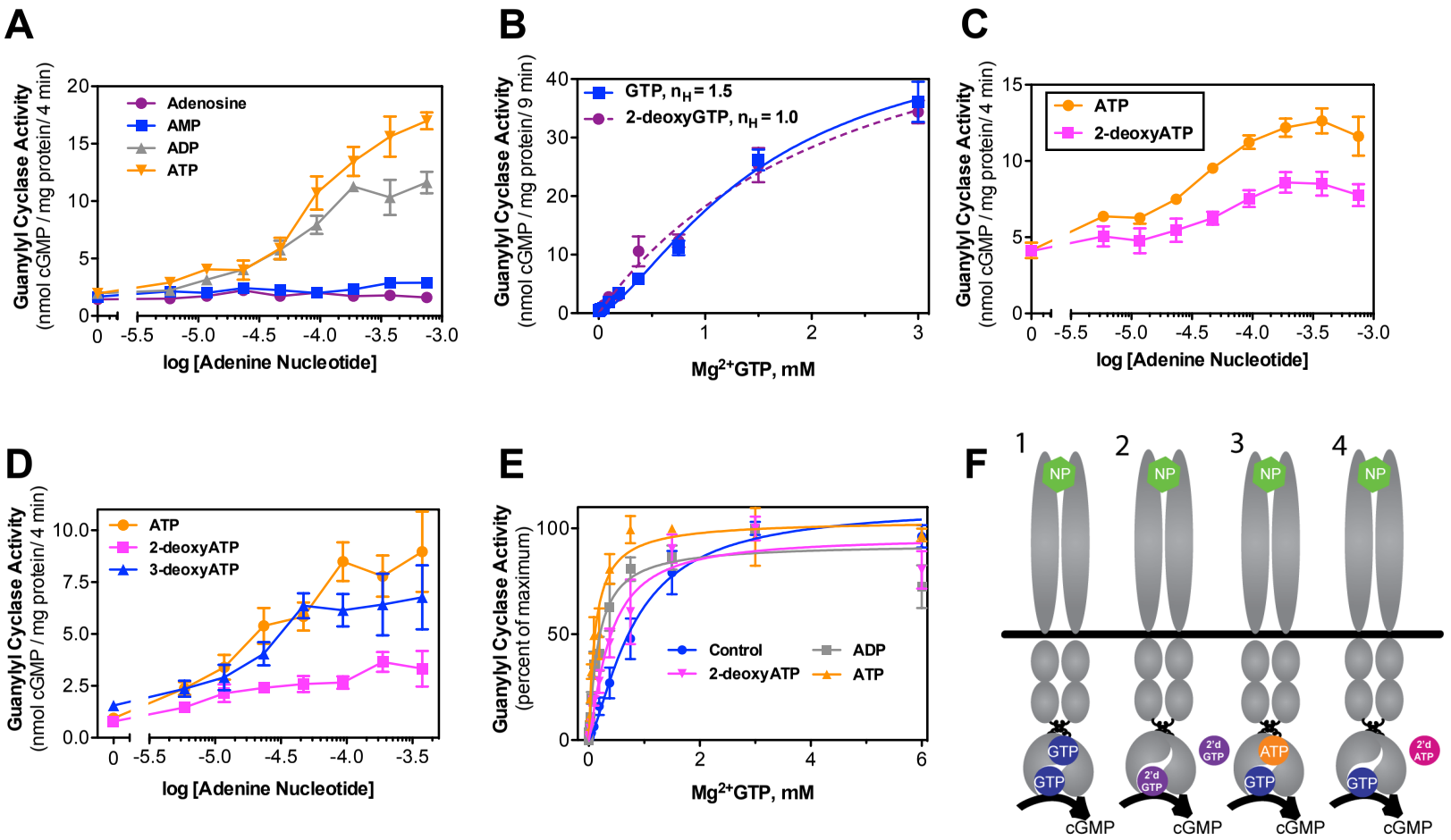

A
Guanylyl Cyclase Activity
(nmol cGMP / mg protein/ 4 min)
Adenosine
AMP
ADP
ATP
log [Adenine Nucleotide]
B
Guanylyl Cyclase Activity
(nmol cGMP / mg protein/ 9 min)
GTP, n_H = 1.5
2-deoxyGTP, n_H = 1.0
Mg2+GTP, mM
C
Guanylyl Cyclase Activity
(nmol cGMP / mg protein/ 4 min)
ATP
2-deoxyATP
log [Adenine Nucleotide]
D
Guanylyl Cyclase Activity
(nmol cGMP / mg protein/ 4 min)
ATP
2-deoxyATP
3-deoxyATP
log [Adenine Nucleotide]
E
Guanylyl Cyclase Activity
(percent of maximum)
Control
2-deoxyATP
ADP
ATP
Mg2+GTP, mM
F
1
2
3
4
NP
GTP
2'd GTP
ATP
2'd ATP
cGMP

FIGURE 4. ATP is required for the activation of GC-B under physiologic conditions.

GC activity was determined in crude membranes prepared from NIH3T3 cells in the presence of 5 mM $Mg^{2+}Cl_2$ and 0.1 mM GTP in the presence or absence of 1 mM ATP and the indicated concentrations of CNP. Each point represents 4 independent determinations. The bars within the symbols represent SEM. Where not visible the bars are contained within the symbol. The shaded area indicates the increased activity due to ATP binding the allosteric site in the GC-B catalytic domain at each individual CNP concentration.

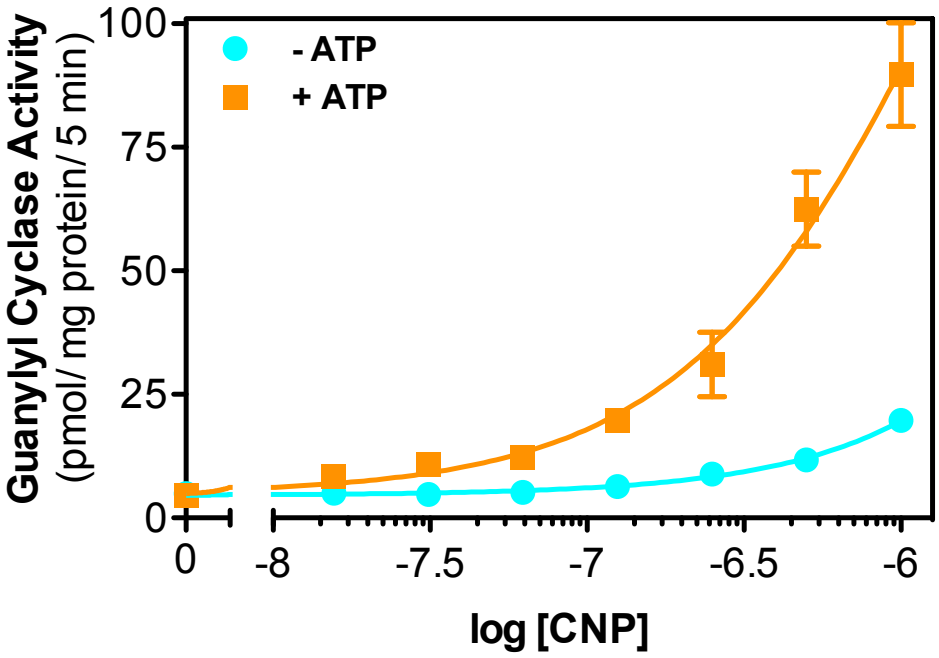

Guanylyl Cyclase Activity
(pmol/ mg protein/ 5 min)
100
75
50
25
0
- ATP
+ ATP
0
-8
-7.5
-7
-6.5
-6
log [CNP]

FIGURE 5. Membrane GCs are asymmetric homodimers with distinct allosteric and catalytic sites.

(A) Possible catalytic dimers are illustrated with WT or mutant residues shown in each purine-binding site. When the residue is mutated it is 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 293 cells transiently transfected with various combinations of WT, D849A or N968S GC-A receptors where n = 4. (C) Substrate-velocity curves of GC activity determined in the presence of 3 mM Mn^{2+} and 1% Triton X-100 from membranes of cells transfected with WT-GC-A (left Y-axis) or both D849A and N968S GC-A (right Y-axis) where n = 3. (D) A double reciprocal plot of data from C is shown.

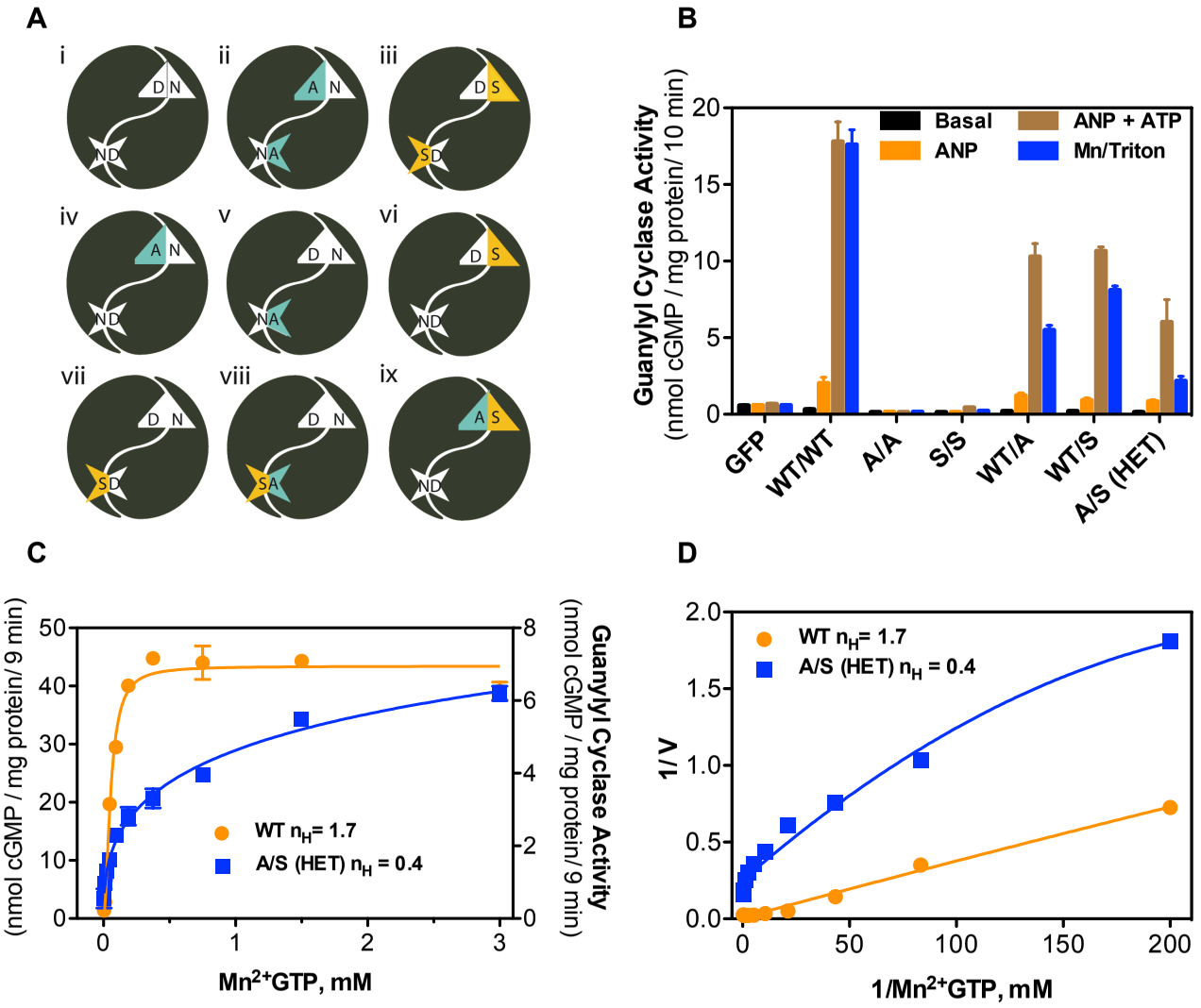
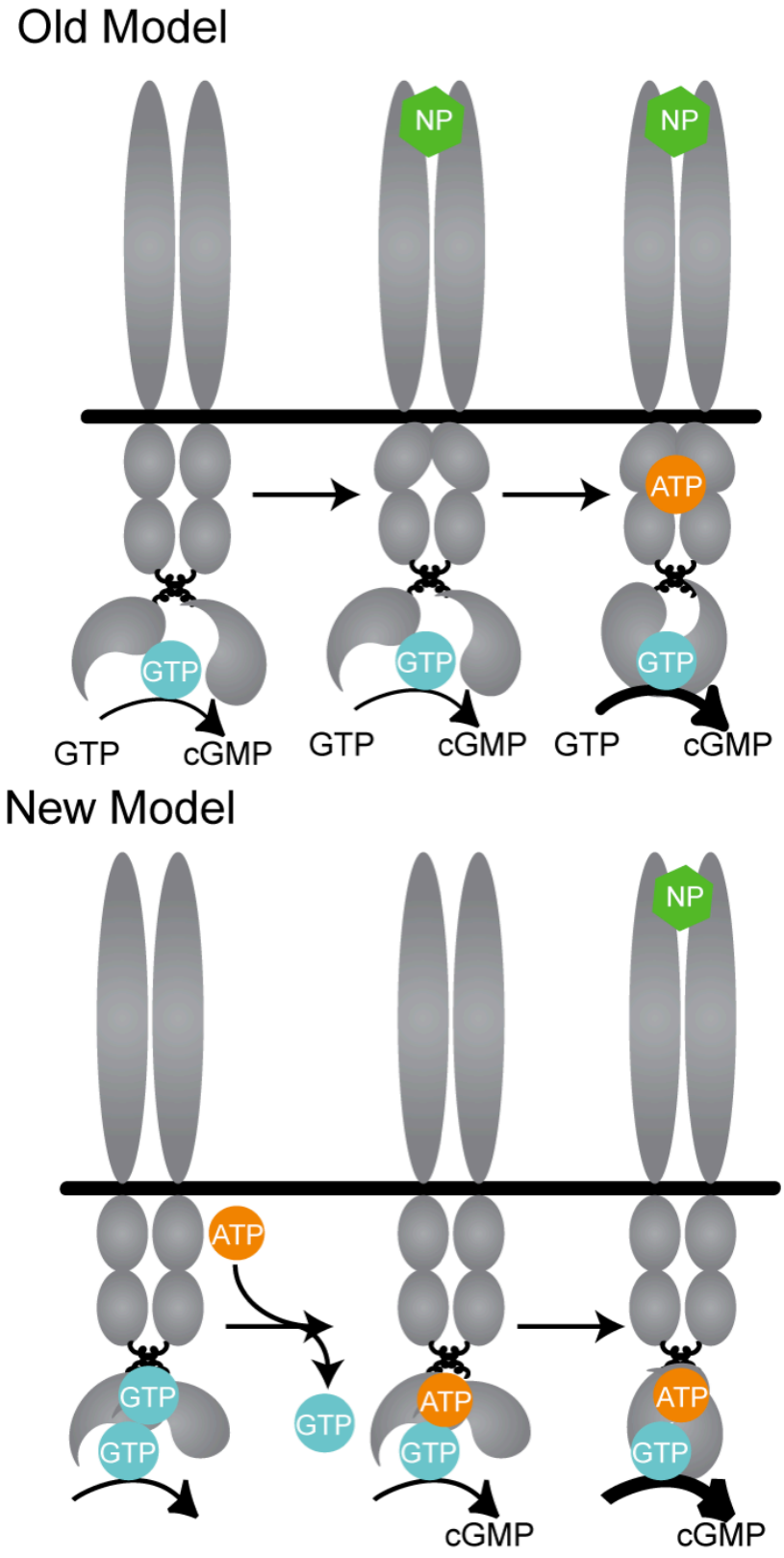

A
i
ii
iii
iv
v
vi
vii
viii
ix
B
Guanylyl Cyclase Activity
(nmol cGMP / mg protein/ 10 min)
Basal
ANP
ANP + ATP
Mn/Triton
GFP
WT/WT
A/A
S/S
WT/A
WT/S
A/S (HET)
C
Guanylyl Cyclase Activity
(nmol cGMP / mg protein/ 9 min)
WT n_H= 1.7
A/S (HET) n_H = 0.4
Mn2+GTP, mM
D
1/ V
WT n_H= 1.7
A/S (HET) n_H = 0.4
1/Mn2+GTP, mM

FIGURE 6. Old and New activation models for GC-A and GC-B are shown.

Details of each model are described under “Discussion.”


Old Model
NP
NP
ATP
GTP
GTP
GTP
GTP
cGMP
GTP
cGMP
GTP
cGMP
New Model
NP
ATP
GTP
GTP
GTP
ATP
GTP
ATP
GTP
cGMP
cGMP

CHAPTER 6: A HUMAN SKELETAL OVERGROWTH MUTATION INCREASES MAXIMAL VELOCITY AND BLOCKS DESENSITIZATION OF GUANYLYL CYCLASE-B

This chapter is a reprint of a submitted publication with minor alterations:

Robinson, Jerid W.; Dickey, Deborah M.; Miura, K.; Michigami, T.; Ozono, K.; and Potter, Lincoln R. (2013) A human skeletal overgrowth mutation increases maximal velocity and blocks desensitization of guanylyl cyclase-B.

Jerid Robinson collected the data for Figure 3, created Figure 6 and also contributed significantly to the writing of the manuscript and interpretation of the data.

C-type natriuretic peptide (CNP) increases long bone growth by stimulating guanylyl cyclase (GC)-B/NPR-B/NPR2. Recently, a single missense mutation in catalytic domain of GC-B (V883M) was identified in humans with increased blood cGMP that causes abnormally long and fragile bones. Here, we determined how this mutation increases GC-B activity. Compared to wild-type (WT)-GC-B, fully processed V883M-GC-B protein was reduced. However, basal cGMP levels in cells expressing V883M-GC-B were elevated 21-fold, and CNP only increased cGMP levels 2-fold. Kinetic analysis indicated that basal maximal velocity of V883M-GC-B was increased 15-fold compared to WT-GC-B but the Michaelis constant (Km) was unchanged. CNP decreased the Km of V883M-GC-B 10-fold in a concentration-dependent manner without increasing maximal velocity. However, unlike WT-GC-B, the Km reduction of V883M-GC-B did not require ATP. Unexpectedly, V883M-GC-B but not WT-GC-B failed to inactivate with time. Phosphorylation increased but was not required for the activity increase associated with the mutation because a GC-B mutant lacking all known phosphorylation sites was activated 17-fold by the substitution. We conclude that the single V883M mutation increases maximal velocity in the absence of CNP, eliminates the requirement for ATP in the CNP-dependent Km reduction, and disrupts the normal inactivation process.

INTRODUCTION

C-type natriuretic peptide (CNP) stimulates long bone growth and inhibits meiotic resumption in oocytes by activating the enzyme known as guanylyl cyclase (GC)-B, NPR2 or NPR-B, which catalyzes the synthesis of the intracellular signaling molecule, cGMP (Salomon et al. 1974, Tamura et al. 2004, Schmidt et al. 2007). GC-B is a homodimer containing an extracellular ligand-binding domain, a single membrane-spanning region, and intracellular highly phosphorylated kinase homology domain, dimerization domain and C-terminal GC catalytic domain (Potter and Hunter 2001). Phosphorylation of the kinase homology domain is required for maximal CNP-dependent activation of GC-B, and dephosphorylation results in enzyme inactivation (Potter 1998, Potthast et al. 2004, Abbey-Hosch et al. 2005, Yoder et al. 2010, Yoder, Robinson et al. 2012). ATP increases GC-B activity by serving as an allosteric activator and as a kinase substrate (Duda et al. 1993, Potter 1998). We recently demonstrated that ATP reduces the Hill coefficient of GC-B in the absence or presence of CNP by binding an allosteric site in the catalytic domain and that CNP reduces the Michaelis constant (Km) only in the presence of ATP (Antos and Potter 2007, Robinson and Potter 2012).

GC-B was identified in rat chondrocytes in 1994 (Hagiwara et al. 1994), but the ability of natriuretic peptides to stimulate skeletal growth was first observed in transgenic mice overexpressing BNP in 1998 (Suda et al. 1998). Subsequent bone culture studies indicated that CNP, not BNP, increased the proliferative and hypertrophic zones of the murine growth plate, which increases the length of long bones (Suda et al. 1998). CNP also increases the earliest stage of endochondral bone

development - the condensation of mesenchymal precursor cells - as well as stimulates glycosaminoglycan synthesis and extracellular matrix production (Krejci et al. 2005, Woods et al. 2007). Consistent with the requirement of CNP and GC-B in normal long bone growth in mammals, mice lacking either CNP or GC-B were dwarfs (Chusho et al. 2001, Tamura and Garbers 2003) and mice lacking the natriuretic peptide clearance receptor (NPR-C) that degrades CNP exhibited skeletal hyperplasia (Jaubert et al. 1999, Matsukawa et al. 1999). Importantly, infusion of CNP or CNP analogs was recently shown to increase long bone growth in murine models of achondroplasia (Kake et al. 2009, Yasoda et al. 2009, Lorget et al. 2012).

Homozygous inactivating mutations in both alleles of GC-B have been identified in humans with Acromesomelic Dysplasia, Type Maroteaux (AMDM) dwarfism (Bartels et al. 2004, Hachiya et al. 2007, Khan et al. 2012), and heterozygous mutations in GC-B were associated with non-pathological reductions in human stature (Olney et al. 2006). Conversely, mutations associated with CNP overexpression were identified in patients with skeletal overgrowth (Moncla et al. 2007, Bocciardi and Ravazzolo 2009), and a genome-wide association study identified correlations between genetic mutations that regulate CNP or NPR-C expression and height in Northwestern European populations (Estrada et al. 2009).

In 2012, Miura *et al.* identified a conserved valine to methionine missense mutation at position 883 in the catalytic domain of human GC-B (V883M-GC-B) in three generations of a Japanese family with skeletal overgrowth, fragile bones and elevated blood cGMP concentrations (Miura et al. 2012). Importantly, how this mutation increases GC-B activity was not determined. Here, we show that this single residue substitution in the catalytic domain of GC-B increases the maximal velocity in the absence of CNP and that CNP reduces the Km of V883M-GC-B an order of magnitude without ATP or without increasing maximal velocity. Finally, we show that the V883M substitution blocks the normal GC-B inactivation process.

EXPERIMENTAL PROCEDURES

Reagents. ^{125}I-cGMP radioimmunoassay kits and ^{32}P-α-GTP were from Perkin Elmer (Waltham, MA). CNP-22 was purchased from Sigma (St. Louis, MO). The plasmids encoding the N-terminally HA-tagged form of WT human GC-B (HA-WT-GC-B) (Hachiya et al. 2007) and HA-V883M-GC-B plasmid (Miura et al. 2012) have been described. The plasmids expressing rat GC-B-7A and GC-B-7E were also previously described (Potter and Hunter 1998, Yoder, Robinson et al. 2012). The ATDC5 chondrocytes were from ATCC (www.atcc.org).

Cells and transfections. 293 neo cells were maintained and transiently transfected by the HEPES-calcium-phosphate precipitation method as previously reported (Yoder, Robinson et al. 2012).

Whole cell cGMP elevation assays. Cyclic GMP concentrations were measured by radioimmunoassay in ethanol extracts of transiently transfected 293 cells that were incubated with increasing concentrations of CNP as previously described (Dickey et al. 2008).

Guanylyl cyclase assays. Crude membranes were prepared at 4^{o}C in phosphatase inhibitor buffer consisting of 50 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid - pH 7.4, 50 mM NaCl, 20% glycerol, 50 mM NaF, 1 mM EDTA, 0.5 μM microcystin and 1X Roche protease inhibitor cocktail. All assays were performed at 37ºC in a cocktail containing 25 mM Hepes pH 7.4, 50 mM NaCl, 0.1% BSA, 0.5 mM isobutylmethyl xanthine, 1 mM EDTA, 0.5 μM microcystin, 5 mM phosphocreatine, 0.1 mg/ml creatine kinase and 5 mM $MgCl_2$.

The single substrate concentration GC assays were performed using ^{32}P-GTP as substrate in the presence of 1 mM ATP and 1 mM GTP at 37^{o}C for 3 min as previously described (Dickey et al. 2008). For the desensitization assays, the reaction was performed using a pool of crude membranes. The reaction was initiated by the addition of pre-warmed cocktail. At the designated times, 0.1 ml aliquots were removed and added to ice-cold tubes containing 0.5 ml zinc acetate to stop the reaction. Alumina column chromatography purified the ^{32}P-cGMP, which was quantified by Cerenkov counting (Bryan and Potter 2002).

Substrate-velocity assays were performed for the indicated times with the indicated GTP concentrations. The resulting cGMP concentrations were determined by radioimmunoassay as described (Robinson and Potter 2011). When included, free manganese concentrations in the assays were 2 mM. Because enzymatic activity was not completely linear with time, the kinetic parameters obtained under these conditions are considered "apparent."

Western blotting. 293T cells were transfected with the indicated constructs, immunoprecipitated, fractionated by reducing SDS-PAGE and blotted to a Immobilon membrane for immune-detection as previously described (Abbey-Hosch et al. 2005). The blot was blocked and probed with at 1/2,500 dilution of rabbit serum 6328 followed by incubation with a 1/20,000 dilution of goat anti-rabbit IRDye 680 conjugated antibody and visualized on a LI-COR instrument as previously described (Flora and Potter 2010).

Statistical analysis. Statistics and graphs were generated with Prism 5 software. Student's paired t-test determined p values where $p \leq 0.05$ was considered significant. The vertical bars within the symbols represent the SEM. Where not visible the bars are contained within the symbol. EC_{50} values were calculated based on the nonlinear curve fitting equation $Y=Top*X/(EC_{50}+X)$. Substrate-velocity curves were analyzed using an allosteric sigmoidal model to generate Hill coefficients.

RESULTS

Cyclic GMP is dramatically elevated in cells expressing GC-B-V883M. HEK293 cells were transiently transfected with human isoforms of HA-WT-GC-B or HA-V883M-GC-B. Two days later, the cells were incubated in the presence of increasing concentrations of CNP for 3 min and intracellular cGMP concentrations were determined (Fig. 1A). Basal (no CNP) cGMP concentrations were elevated 21-fold in cells expressing HA-V883M-GC-B compared to cells expressing HA-WT-GC-B. Maximal concentrations of CNP increased cGMP concentrations 29-fold in HA-WT-GC-B expressing cells but only 2-fold in cells expressing HA-V883M-GC-B. The EC_{50} for CNP activation was not significantly different between the WT and mutant enzymes, consistent with the mutation not affecting the affinity of CNP for GC-B.

Plasmids expressing WT and GC-B-V883M were also transiently transfected into ATDC5 mouse chondrocytic cells that endogenously express GC-B. Overexpression of WT-GC-B slightly elevated cyclic GMP concentrations in the ATDC5 cells, but overexpression of the GC-B-V883M mutant resulted in cGMP levels that were more than four-fold higher than those observed in cells transfected with the WT enzyme (Fig. 1B). These data indicate that the increased basal activity associated with the V883M mutation occurs in a natural cellular environment for GC-B and is consistent with increased plasma cGMP concentrations measured in patients expressing V883M-GC-B (Miura et al. 2012).

Basal enzymatic activity of V883M-GC-B is elevated but expression is reduced. GC activity was measured in crude membranes from 293 cells expressing green fluorescent protein (GFP) as a control, WT-GC-B, HA-WT-GC-B or HA-V883M-GC-B under basal (1 mM Mg^{2+}GTP), hormone-stimulated (1 mM Mg^{2+}GTP, 1 mM ATP and 1 mM CNP), or detergent-stimulated (1 mM Mn^{2+}GTP and 1% Triton X-100) conditions (Fig. 2). Kinetic determinations were performed in the 293 cells because they do not express detectable endogenous GC activity (Robinson and Potter 2012), which allows definitive interpretation of the data because most tissues and cell lines express more than one GC.

GC activity measured in crude membranes from GFP transfected cells was insignificant under all conditions. Consistent with the whole cell cGMP analysis describe in Fig. 1, basal GC activity was low for WT-GC-B and HA-WT-GC-B but was elevated 28-fold over WT levels for HA-V883M-GC-B. Saturating concentrations of CNP and ATP stimulated WT-GC-B and HA-WT-GC-B similarly (>50-fold). However, GC activity of HA-WT-GC-B was almost double that of the WT enzyme lacking the HA tag, consistent with higher expression of the HA-tagged receptor. As in whole cells, CNP and ATP activated HA-V883M-GC-B about two-fold in enzyme assays. GC activity of HA-V883M-GC-B measured in the presence of detergent was lower than that observed for HA-WT-GC-B, which is consistent with reduced expression of HA-V883M-GC-B compared to HA-WT-GC-B. Western analysis of SDS-PAGE fractionated immunoprecipitated enzymes confirmed that the more slowly migrating species (upper band) was expressed at lower levels than the fully processed forms of the tagged or untagged WT versions of GC-B (Fig. 2, inset). We previously demonstrated

that only the upper band of GC-B is phosphorylated and that phosphorylation is required for CNP-dependent activation of GC-B (Potter 1998, Potter and Hunter 1998).

Maximal velocity of HA-GC-B-V883M is elevated. To determine how the mutation increased the enzymatic activity of GC-B, substrate-velocity curves were generated for HA-WT-GC-B and HA-V883M-GC-B with or without 1 mM CNP in the absence of ATP (Fig. 3A). Basal activity of the WT enzyme was low and CNP increased Vmax 12-fold without decreasing the Km. Consistent with previous observations (Robinson and Potter 2012), WT-GC-B was positive cooperative as indicated by a Hill slope of 1.3. In contrast, basal maximal velocity of the mutant enzyme was elevated 15-fold compared to WT-GC-B and the Km was unchanged. The Hill coefficient was 0.9, suggesting slight negative cooperativity. CNP failed to increase the maximal velocity of HA-V883M-GC-B, but reduced the Hill slope 0.4 units and the Km 10-fold. Thus, the V883M mutation increases basal maximal velocity, reduces the Hill coefficient and allows CNP to reduce the Km in the absence of ATP. In contrast, the reduction in Hill coefficient and Km for the WT enzyme was previously shown to be complete dependent on the presence of ATP (Antos and Potter 2007). These data are consistent with the V883M mutation producing a structural change in GC-B that locks it into a conformation that mimics that of the ATP-bound state. They also indicate for the first time that the CNP-dependent changes in the Vmax and Km of GC-B are separate but related processes.

CNP reduces the Hill coefficient and Km of HA-V883M-GC-B in a concentration-dependent manner in the absence of ATP. Substrate-velocity curves were generated for HA-V883M-GC-B in the presence of increasing concentrations of CNP to evaluate the concentration-dependence of CNP on reductions in the Hill coefficient and Michaelis constant (Fig. 3B). ATP was not included in these experiments. In the absence of CNP, no cooperativity was observed, but increasing concentrations of CNP progressively increased the amount of negative cooperativity while concomitantly decreasing the Km. These data indicate that CNP converts HA-V883M-GC-B to a strongly negative cooperative enzyme. Similarly, in the absence of CNP, the Km of the mutant enzyme was high; but in the presence of increasing concentrations of CNP, the Km dropped progressively while maximal velocity was unaffected.

ATP does not allosterically activate V883M-GC-B. We recently determined that CNP reduces the Hill coefficient and Km of WT-GC-B by a process that requires ATP binding to an allosteric site in the catalytic domain (Robinson and Potter 2012). Therefore, we investigated whether the V883M mutation affected these processes as well. Substrate-velocity curves were generated for HA-WT-GC-B and HA-V883M-GC-B in the presence of 1 mM CNP with or without 0.1 mM ATP. With the WT enzyme, ATP reduced the Km 6-fold and the Hill coefficient 0.3 units without affecting the Vmax (Fig. 3C). However, ATP failed to reduce the Km or Hill coefficient or increase the Vmax of HA-V883M-GC-B. These data are consistent with a scenario where the V883M mutation causes a conformational change in GC-B that abolishes the need for ATP in the CNP-dependent reduction in Hill coefficient and Km.

HA-V883M-GC-B is slightly negative cooperative in the presence of manganese. Substrate velocity curves were also generated on membranes expressing HA-WT-GC-B or HA-V883M-GC-B under non-physiologic, detergent conditions using manganese-GTP as substrate (Fig. 3D). Substrate-velocity curves generated under these conditions were previously shown to be positive cooperative for GC-A (Ishii et al. 1990). Vmax was lower when measured under these conditions but the $Km/S_{0.5}$ was much lower compared to physiologic activation. Maximal velocity was not different between the WT and mutant GC-B enzymes. The substrate-velocity curve for HA-WT-GC-B was positive cooperative as demonstrated by concave upward reciprocal plots and a Hill coefficient of 1.3 (Fig. 3D, inset). To our knowledge, this is the first demonstration of positive cooperativity for GC-B when assayed under detergent-stimulated conditions. In contrast to HA-WT-GC-B, HA-V883M-GC-B was weakly negative cooperative as indicated by a slightly concave downward curve and a Hill coefficient of 0.9 (Fig. 3D, inset), which is consistent with the slight negative cooperativity observed for V883-GC-B when assayed under basal conditions.

HA-V883M-GC-B is resistant to desensitization. Cyclic GMP concentrations in cells expressing V883M-GC-B were highly elevated two days after transfection (Fig. 1), which suggest that the mutant enzyme was not completely desensitized or down-regulated. In contrast, CNP activated WT-GC-B was shown to desensitize in less than one hour (Potter 1998). Therefore, we examined whether the V883M mutation disrupted the inactivation of GC-B.

GC activity was measured on membranes from cells expressing HA-WT-GC-B or HA-V883M-GC-B for up to 2 hours to evaluate the effect of the V883M mutation on the inactivation of GC-B as a function of time (Fig. 4, top panel). WT GC activity was determined in the presence of CNP, whereas mutant activity was determined in the presence and absence of CNP. The GC activity of the WT enzyme declined with time and was inactive after 60 min. In contrast, the GC activity of the mutant receptor was linear for the duration of the assay regardless of whether CNP was included in the assay.

We also examined the inactivation of the WT and mutant enzymes under whole cell conditions. In this experiment, intact cells were treated with 1 mM CNP for 0, 30 or 90 min then membranes were prepared from the cells and assayed for GC activity for 3 min (Fig. 4, bottom panel). The WT enzyme demonstrated a time-dependent inactivation similar to that previously reported for GC-B expressed in 3T3 cells (Potter 1998). However, exposure of the V883M-GC-B to saturating concentrations of CNP failed to inactivate the enzyme after 30 or 90 minutes. Together, these data indicate that the Val substitution at position 883 not only increases the maximal velocity of the enzyme, it also disrupts the normal desensitization process.

Activation of GC-B by the V883M substitution does not require phosphorylation. CNP only activated a GC-B construct containing alanine substitutions for the first six phosphorylation sites identified in GC-B (S513, T516, S518, S523, S526, T529) two-fold as opposed to greater than 30-fold for WT-GC-B (Potter and Hunter 1998), whereas the analogous substitutions left GC-A completely unresponsive to NP stimulation (Potter and Hunter 1998). These observations led to the idea that phosphorylation is required for

NP-dependent activation of GC-A and GC-B. Here, we asked whether phosphorylation is also required for the V883M mutation to increase GC-B activity.

To do this, we mutated Val-883 to Met in the rat GC-B-7A construct that contains alanine substituted for the first six identified sites plus Ser-522, which is not phosphorylated (Potter and Hunter 1998). We also created a constitutively phosphorylated mimetic version of rat GC-B-V883M by mutating Val-883 to Met in GC-B-7E. Rat GC-B-7E contains glutamate substitutions for the first six identified sites as well as Ser-489, a newly identified putative site that reduces the Km of GC-B when phosphorylated (Yoder, Robinson et al. 2012).

Introducing the Val-883-Met mutation into WT-GC-B increased basal activity 39-fold, whereas the same mutation in the dephosphorylated form of the enzyme (GC-B-7A) increased activity 17-fold (Fig. 5). However, introduction of the V883M mutation into the GC-B-7E construct increased activity 68-fold. Thus, phosphorylation is not required for the elevated basal activity associated with the V883M mutation, but results in greater activation since the WT and phosphorylation mimetic enzyme (GC-B-7E) were activated to a greater degree than the non-phosphorylated enzyme (GC-B-7A).

DISCUSSION

Characterization of the missense mutant revealed several important changes in GC-B that occurred as a result of this single amino acid substitution. First, maximal velocity was dramatically increased. Second, the CNP-dependent reduction in Km was rendered independent of ATP, and thirdly, the normal desensitization process was inactivated. Another point worthy of discussion is that the V883M-GC-B mutant is the first example of a GC where ligand binding reduces the Km without increasing maximal velocity. Thus, the kinetic analysis of this mutant allows the separation of the maximal velocity increasing effects of ligand binding from the Km reducing effects of ligand binding for the first time. Furthermore, this mutant enzyme provides unequivocal support for the new GC-B activation model where CNP binding increases activity by reducing the Km as well as increasing maximal velocity (Robinson and Potter 2012).

Early studies indicated that product formation by membrane GCs in the presence of detergent is positive cooperative (Kimura and Murad 1974, Chrisman et al. 1975). We found that GC-B is positive cooperative under basal conditions as well as when assayed in the presence of Mn^{2+}GTP and Triton X-100. However, the single V883M mutation converts the enzyme from positive cooperative to slightly negative cooperative when assayed under both physiologic and detergent-activated conditions. Interestingly, CNP increased the degree of negative cooperativity of V883M-GC-B in a concentration-dependent manner.

The reduction in Km and increase in negative cooperativity appear paradoxical. We hypothesize that the V883M mutant locks the enzyme into a conformation that

mimics an ATP bound state. This hypothesis is supported by low or no cooperativity under basal conditions and the inability of ATP to change the activity of the mutant enzyme. In addition, CNP alone markedly decreased the Km of GC-B-V883M, a phenomenon that requires ATP with the WT enzyme. Since cooperativity is maintained, this suggests that the mutation does not destroy the ability of GTP to bind to the allosteric site but rather modifies how GTP binding to the allosteric site affects the catalytic site. However, an alternative explanation is that the reduction in the Hill coefficient results from increased inhibition resulting from GTP binding to a site independent of the allosteric site. This third GTP binding site would explain the appearance of negative cooperativity while also allowing for the Km reduction resulting from the previously identified allosteric site. It is also possible that the V883M mutation could increase the affinity of GC-B for the products of the reaction (pyrophosphate and cGMP), which would result in reduced GC activity and apparent negative cooperativity.

Near linear cGMP production with time by HA-V883M-GC-B assayed both in the presence and absence of CNP indicated that HA-V883M-GC-B is resistant to desensitization. Experiments with alanine and glutamate substituted receptors indicated that unlike CNP activation of WT-GC-B, the increased activity observed with the V883M mutation does not require receptor phosphorylation, although activation was greater with the phosphorylated and phosphomimetic enzymes. The lack of dependence on phosphorylation for activity of the mutant enzyme likely contributes to its resistance to desensitization.

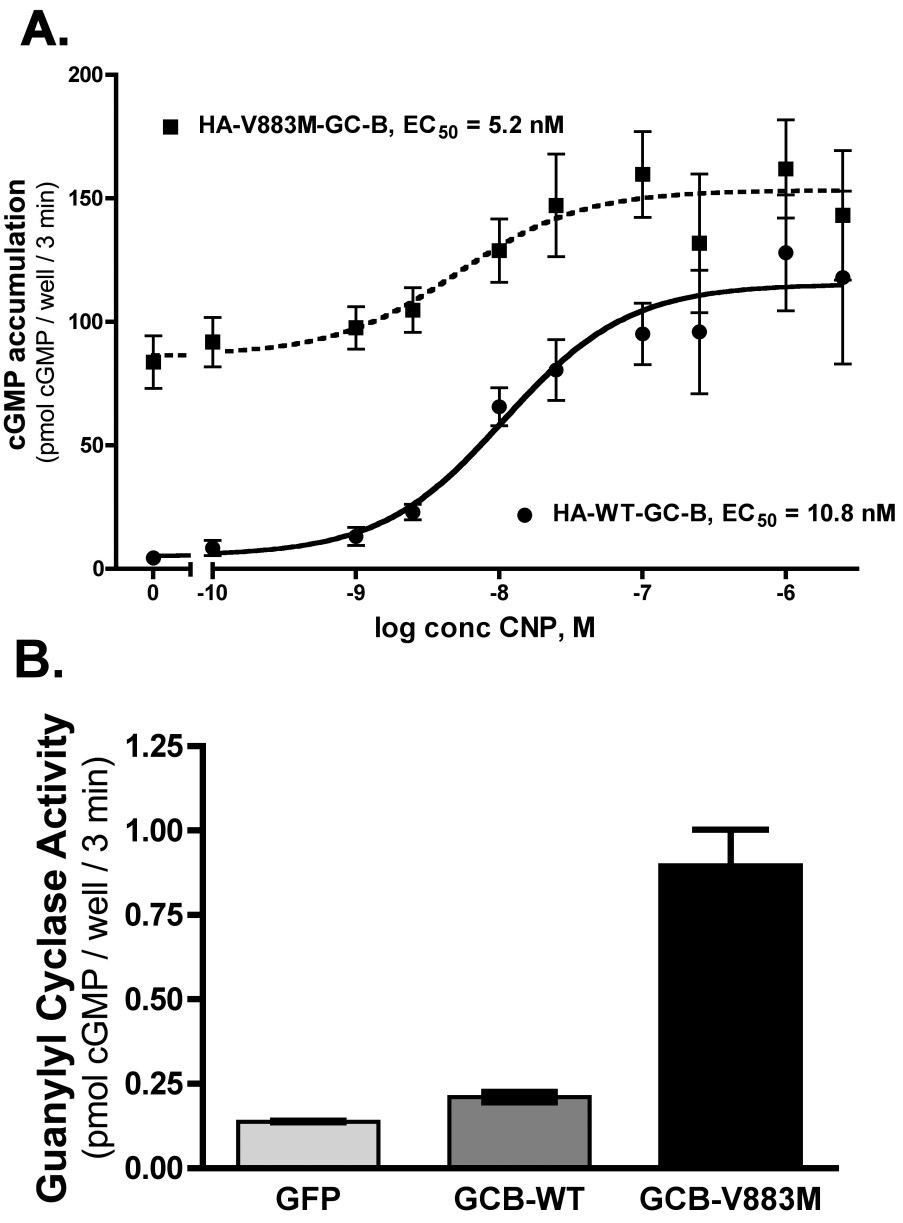
It is surprising how much the single amino acid substitution changes the regulation of GC-B (Fig. 6). In the absence of CNP and ATP, maximal velocity of WT-GC-B was low, affinity for substrate was low (high Km), and cooperative was significant and positive. In contrast, under the same conditions, maximal velocity of V883M-GC-B was high, affinity for substrate was low, and cooperativity was low and negative. Addition of ATP in the absence of CNP abolished positive cooperativity of the WT enzyme due to ATP replacing GTP at the allosteric site (Robinson and Potter 2012), but had no effect on the mutant enzyme under identical conditions. CNP alone increased maximal velocity of the WT enzyme, but it did not decrease the Km in the absence of ATP. In contrast, CNP alone failed to increase maximal velocity of the mutant enzyme but decreased the Km ten-fold in the absence of ATP. Finally, CNP reduced the cooperativity of both enzymes, but the WT enzyme went from positive to no cooperativity, whereas the mutant went from slightly negative cooperative to very negative cooperative.

In conclusion, we established a molecular mechanism for how a single amino acid substitution in GC-B activates the enzyme, which leads to abnormally long and fragile human bones. It will be interesting to determine the prevalence of this mutation in humans and other species.

FIGURES

FIGURE 1. Basal cGMP concentrations are markedly elevated in cells expressing V883M-GC-B.

A, 293 cells transiently expressing HA-WT-GC-B or HA-V883M-GC-B were incubated with the indicated concentrations of CNP for 3 min and then intracellular cGMP concentrations were determined. The EC_{50}s for the two enzymes were not significantly different, $p = 0.42$. B, ATDC5 cells were transiently transfected with plasmids expressing WT-GC-B or GC-B-V883M and cGMP concentrations were measured in basal (no CNP) serum-starved cells 2 days later. Cyclic GMP concentrations in cells expressing WT-GC-B were slightly higher than those observed in un-transfected cells ($p < 0.01$), but levels in cells expressing GC-B-V883M were 4.3-fold higher than those in cells expressing WT-GC-B ($p < 0.03$).


A.
200
150
100
50
0
cGMP accumulation
(pmol cGMP / well / 3 min)
HA-V883M-GC-B, EC50 = 5.2 nM
HA-WT-GC-B, EC50 = 10.8 nM
0
-10
-9
-8
-7
-6
log conc CNP, M
B.
Guanylyl Cyclase Activity
(pmol cGMP / well / 3 min)
1.25
1.00
0.75
0.50
0.25
0.00
GFP
GCB-WT
GCB-V883M

FIGURES 2. GC activity but not the protein level of V883M-GC-B was elevated in the absence of CNP.

Crude membranes from 293 cells transfected with plasmids expressing the indicated constructs were assayed for GC activity under the conditions indicated in the figure legend and text. Bars within the symbols indicate the range of duplicate determinations. This figure is representative of two independent assays. The inset shows a western blot of the indicated forms of GC-B purified and from transiently transfected 293 cells. The numbers on the left indicate the molecular weight of standards.

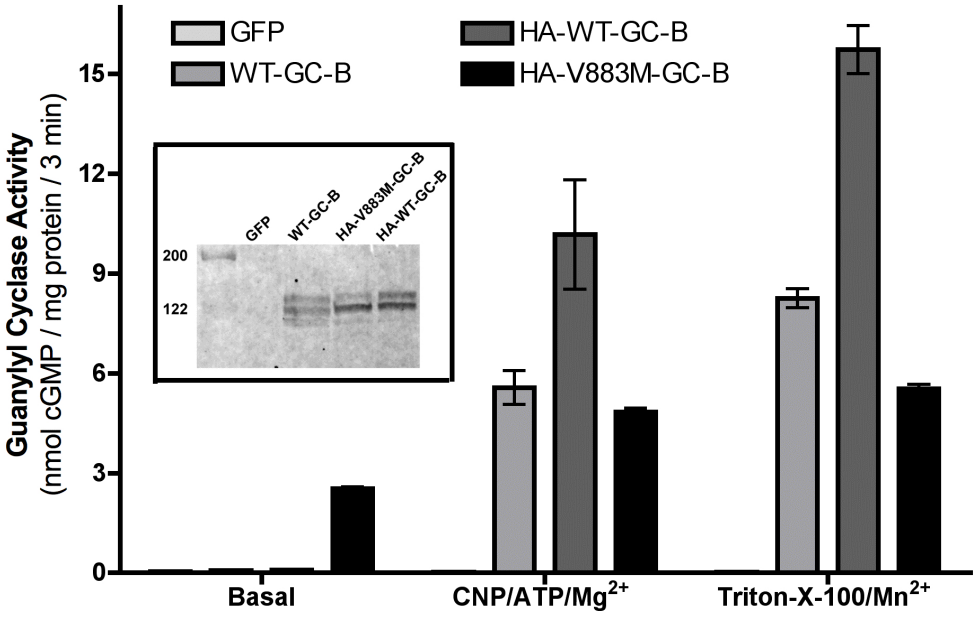
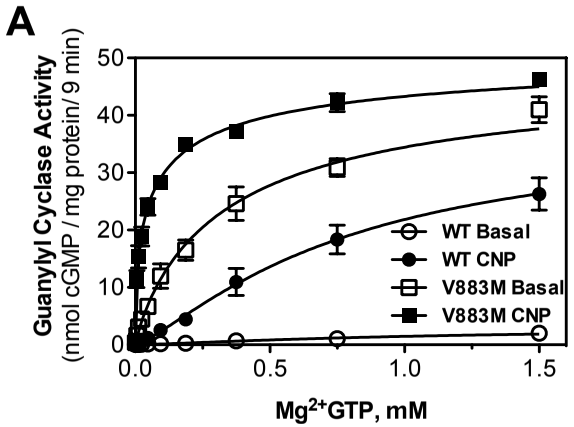
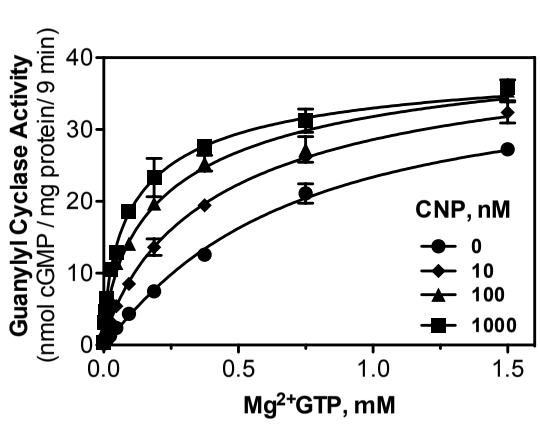

GFP
WT-GC-B
HA-WT-GC-B
HA-V883M-GC-B
Guanylyl Cyclase Activity
(nmol cGMP / mg protein / 3 min)
15
12
9
6
3
0
200
122
GFP
WT-GC-B
HA-V883M-GC-B
HA-WT-GC-B
Basal
CNP/ATP/Mg2+
Triton-X-100/Mn2+

FIGURE 3. Kinetic Characterization of V883M-GC-B.

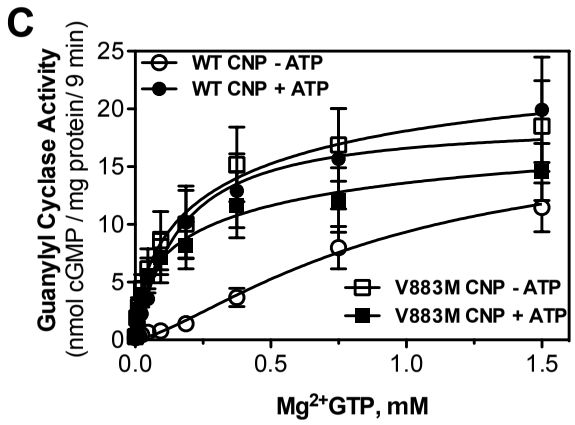
GC activity shown in panels A-D was measured for 9 min in crude membranes from 293 cells transiently expressing either HA-GC-B-WT or HA-GC-B-V883M. Bars within symbols indicate the SEM. Tables below each figure show Vmax, Km and Hill coefficient (n_H). A. Maximal velocity of HA-V883M-GC-B is elevated in the absence of CNP. GC activity was measured in the presence or absence of 1 μM CNP and the indicated concentrations of Mg^{2+}GTP where n = 4. The # indicates significantly different from HA-WT-GC-B - CNP at $p< 0.05$. The ## indicates significantly different from HA-V883M-GC-B - CNP at $p< 0.03$. B. CNP decreases the Hill coefficient and Km for HA-V883M-GC-B in a concentration-dependent manner in the absence of ATP**.** GC activity was measured in the presence or absence of increasing concentrations of CNP and the indicated concentrations of Mg^{2+}GTP where n = 4. * and ** indicate significantly different from no CNP values where $p < 0.05$ and 0.01, respectively. C. ATP does not affect the Hill coefficient or Km of HA-V883M-GC-B. GC activity was measured in the presence or absence of 0.1 mM ATP, 1 μM CNP and the indicated concentrations of Mg^{2+}GTP where n = 4. The * indicates significantly different from HA-WT-GC-B (-) ATP at $p< 0.05$. D. HA-V883M-GC-B is negative cooperative. GC activity was measured with the indicated concentrations of Mn^{2+}GTP and 1% Triton X-100 where n = 6. The * indicates significantly different from the corresponding value obtained for the WT enzyme at $p< 0.05$. *Inset.* Double reciprocal plots were generated from the raw data to demonstrate concave upward curves indicative of positive cooperativity or slightly downward curves indicative of negative cooperativity.



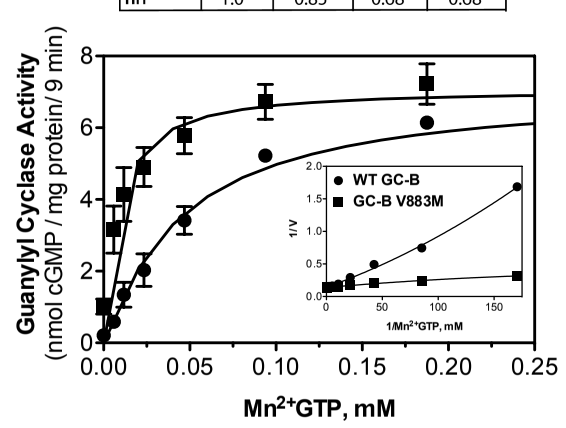
	WT-GC-B - CNP	WT-GC-B + CNP	GC-B-V883M - CNP	GC-B-V883M + CNP
Vmax	3.12	37.66	48.03	53.55
$K_m/S_{0.5}$	1.11	0.78	0.98	0.09 #
nH	1.3	1.3	0.9	0.5 ##



CNP, nM	0	10	100	1000
Vmax	38.02	43.81	45.63	41.35
$K_m/S_{0.5}$	0.65	0.48*	0.29**	0.13**
nH	1.0	0.85**	0.68**	0.68**



	WT-GC-B - ATP	WT-GC-B + ATP	GC-B-V883M - ATP	GC-B-V883M + ATP
Vmax	17.75	18.91	27.72	21.77
$K_m/S_{0.5}$	0.93	0.16 *	0.36	0.39
nH	1.40	1.07 *	0.62	0.54



	WT-GC-B	GC-B-V883M
Vmax	6.91	7.24
$K_m/S_{0.5}$	0.04	0.01 *
nH	1.3	0.9 *

FIGURE 4. V883M-GC-B is resistant to desensitization.

A. GC assays were conducted on crude membranes from 293 cells transfected with HA-WT-GC-B or HA-V883M-GC-B for the period of time indicated in the presence of 1 mM GTP, 1 mM ATP and 5 mM $Mg^{2+}Cl_2$ with or without 1 μM CNP. Each value represents 4 determinations. The asterisks indicate significantly different from corresponding values obtained in membranes expressing WT-GC-B at $p < 0.02$. B. Whole 293 cells transfected with HA-WT-GC-B or HA-V883M-GC-B were incubated with 1 μM CNP for the indicated times. Membranes were then prepared and assayed for GC activity in the presence of 1 mM GTP, 1 mM ATP and 5 mM $Mg^{2+}Cl_2$. N = 4. The asterisks indicate significance from the 0 time point value where $p < 0.05$. The bars within the symbols indicate SEM in all panels.

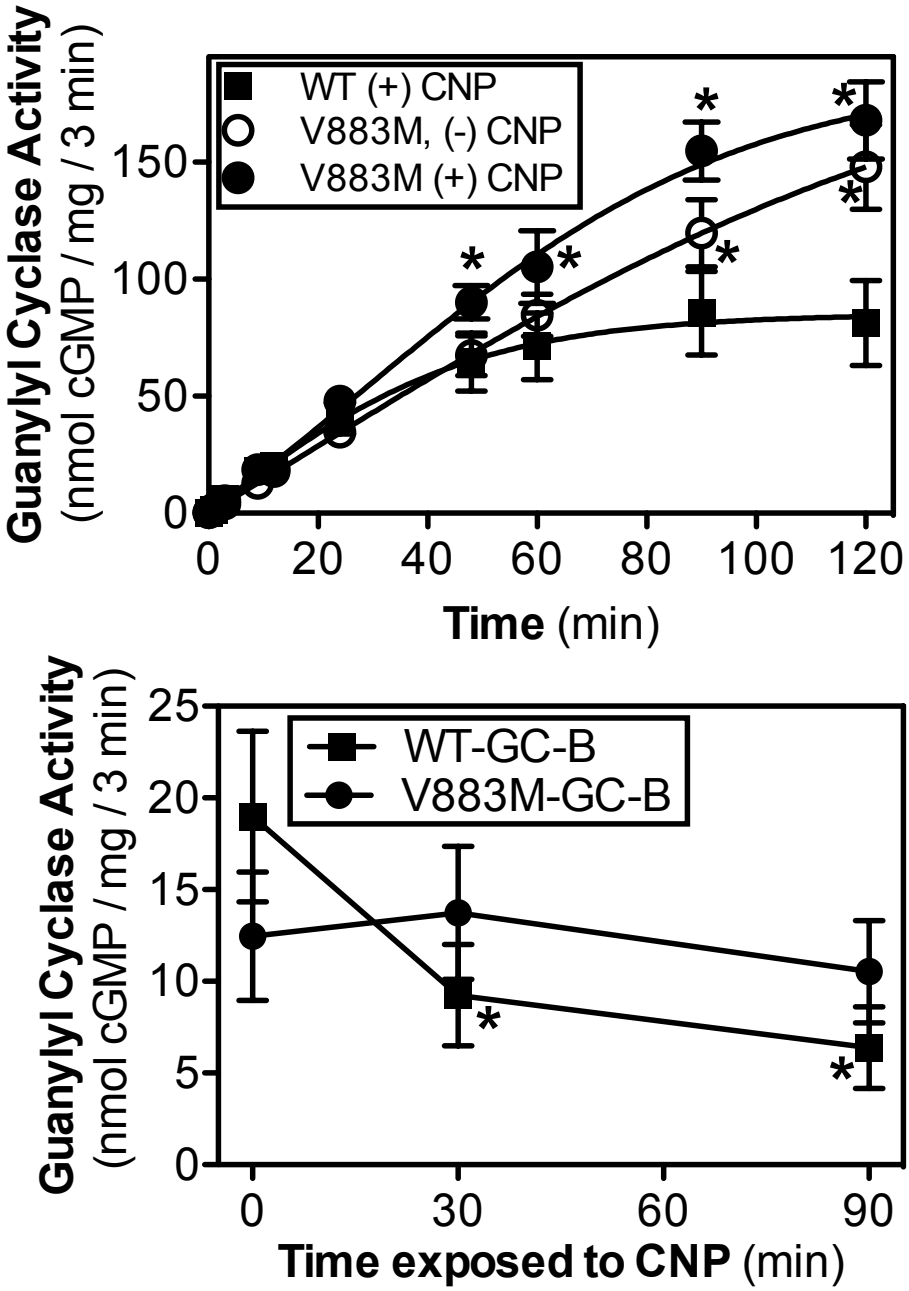

Guanylyl Cyclase Activity
(nmol cGMP / mg / 3 min)
WT (+) CNP
V883M, (-) CNP
V883M (+) CNP
150
100
50
0
0
20
40
60
80
100
120
Time (min)
Guanylyl Cyclase Activity
(nmol cGMP / mg / 3 min)
WT-GC-B
V883M-GC-B
25
20
15
10
5
0
0
30
60
90
Time exposed to CNP (min)

FIGURE 5. The GC-B-V883M mutation activates a dephosphorylated version of GC-B.

The V883M mutation was introduced into WT-GC-B, constitutively phosphorylated (7E) or constitutively desphosphorylated (7A) forms of GC-B. 293 cells were transiently transfected with plasmids expressing the indicated GC-B constructs. GC assays were performed for 3 min in the presence of 0.1 mM GTP, 1 mM ATP and 5 mM $Mg^{2+}Cl_2$ with or without 1 μM CNP. Each value represents 6 determinations. The bars within the symbols indicate SEM. The values above the brackets indicate the fold-difference above basal values.

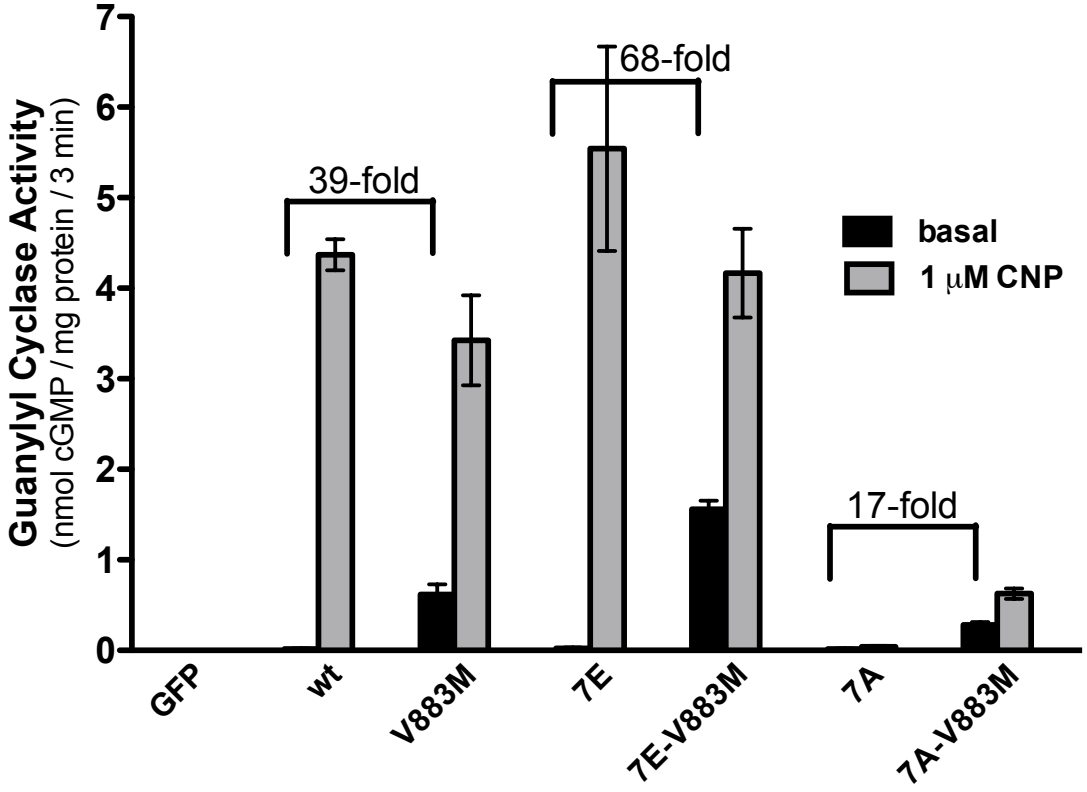
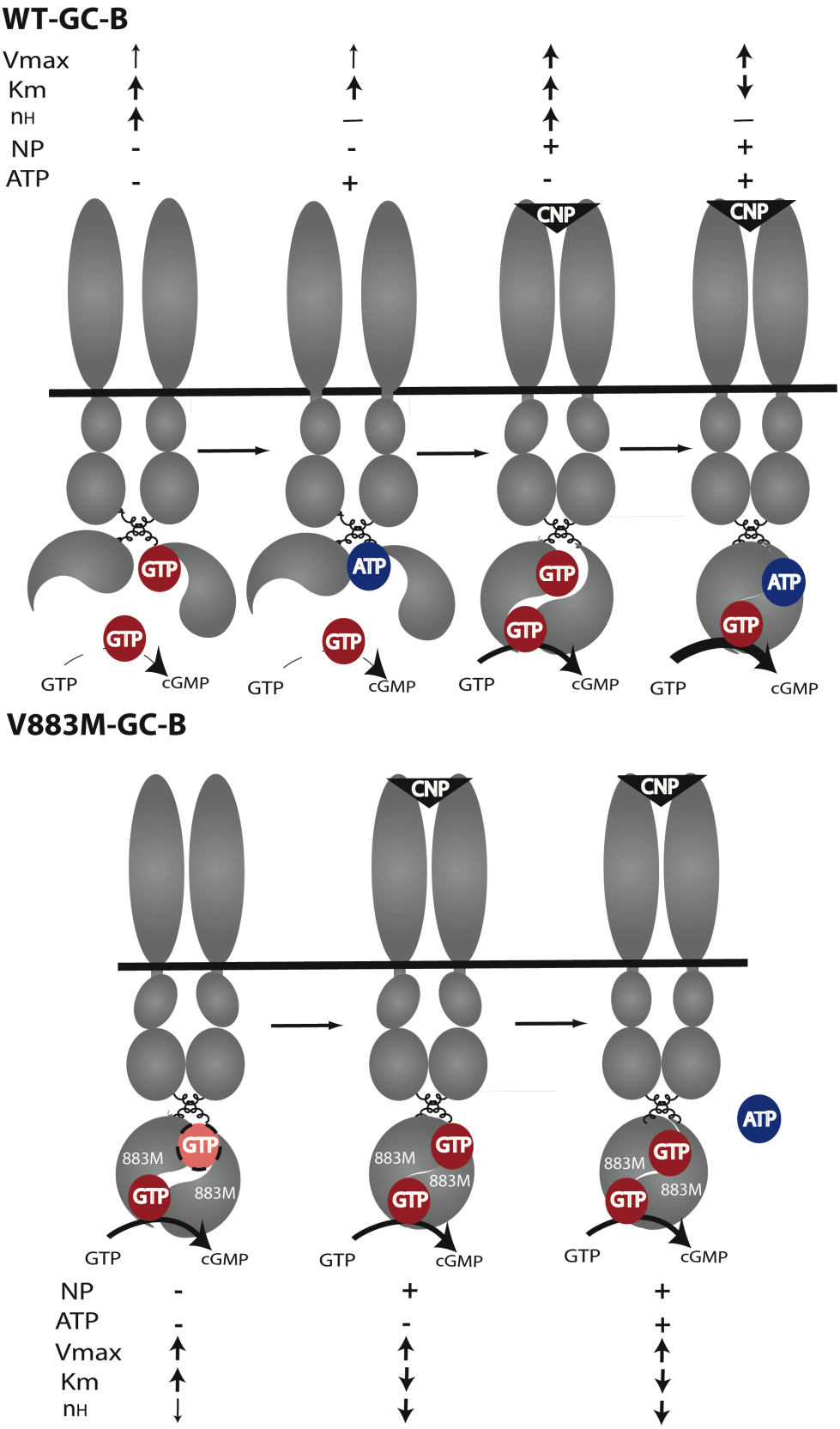

Guanylyl Cyclase Activity
(nmol cGMP / mg protein / 3 min)
0
1
2
3
4
5
6
7
39-fold
68-fold
17-fold
basal
1 μM CNP
GFP
wt
V883M
7E
7E-V883M
7A
7A-V883M

FIGURE 6. Activation models for wild type and mutant GC-B.

The models are described in detail under the discussion section. The abbreviations are: CNP, C-type natriuretic peptide; Km, Michaelis constant; nH, Hill coefficient; Vmax, maximal velocity. The orange circle in the first cartoon describing the 883M mutant indicates weak or no binding of GTP to the allosteric site since the n_H is 0.9 to 1.0.


WT-GC-B
Vmax
Km
nH
NP
ATP
CNP
GTP
ATP
cGMP
V883M-GC-B
883M

CHAPTER 7: ATP BINDING TO THE PYROPHOSPHATE SITE LINKS GUANYLYL CYCLASE ACTIVITY TO METABOLISM

This chapter is a reprint of a submitted publication with minor alterations:

Robinson, Jerid W. and Potter, Lincoln R. (2013) ATP binding to the pyrophosphate site links guanylyl cyclase activity to metabolism.

Jerid Robinson collected and interpreted all the data and also contributed heavily to the writing of the manuscript and interpretation of the data.

Guanylyl cyclases (GCs) regulate critical biologic functions by catalyzing the synthesis of cGMP and pyrophosphate from GTP. Reports suggest that ATP inhibits GCs by binding the pseudosymmetric site, but we found that low concentrations of ATP activate GC-A and GC-B by binding the pseudosymmetric site. Here, we show that higher, physiologic concentrations of ATP inhibit GC-A and GC-B by binding a lower affinity site in the catalytic domain. In contrast to activation, inhibition neither required a 2' ribosyl OH on ATP nor natriuretic peptide. ATP and ADP but not AMP were inhibitory, which indicated that the activation and inhibition sites are unique and that pyrophosphate was necessary for inhibition. Substrate-velocity studies demonstrated that inhibition was linear, mixed type. Increasing concentrations of both pyrophosphate and ATP proportionally inhibited GC activity without changing the IC_{50} of either inhibitor and parallel Dixon plots indicated that the two inhibitors were mutually exclusive. We conclude that low concentrations of ATP activate GC-A and GC-B by binding the pseudosymmetric site and that higher, cellular ATP concentrations inhibit these enzymes by binding the pyrophosphate product site.

INTRODUCTION

Guanylyl cyclases (GC) catalyze the conversion of GTP into the second messenger, cGMP and pyrophosphate in organisms ranging from bacteria to humans (Lucas et al. 2000, Potter 2011). Two major classes of GCs are known. Soluble heterodimeric, heme-containing enzymes are activated by nitric oxide (Friebe and Koesling 2009, Derbyshire and Marletta 2012), and particulate, single membrane-spanning homodimeric enzymes are activated by peptides (Friebe et al. 1998, Basu et al. 2010). GCs regulate a myriad of functions including blood pressure, bone growth, oocyte meiotic resumption, intestinal hydration, CO_2 detection, sexual arousal, platelet aggregation, phototransduction and metabolism (Salomon et al. 1974, Lucas et al. 2000, Kuhn 2003, Valentino et al. 2011).

Seven particulate GCs were identified in rats by molecular cloning and named alphabetically according to the order of discovery (Garbers 1999). Atrial natriuretic peptide (ANP) and B-type natriuretic peptide activate guanylyl cyclase-A (GC-A), which is also known as NPR1 or NPR-A. C-type natriuretic peptide (CNP) activates guanylyl cyclase-B (GC-B), which also known as NPR-B or NPR2 and is homologous to GC-A. GC-A and GC-B contain a large, peptide-binding extracellular domain, a single membrane-spanning region, and intracellular kinase homology regulatory domain, coiled-coil dimerization domain and GC catalytic domain (Potter and Hunter 2001).

We reported that the catalytic domains of GC-A and GC-B are asymmetric dimers that contain distinct catalytic and allosteric sites, similar to soluble GCs and

adenylyl cyclase (Robinson and Potter 2012). ATP increases the enzymatic activity of all transmembrane GCs (Friebe et al. 1998). ATP activates GC-A and GC-B with an EC_{50} of approximately 0.1 mM by binding an allosteric, pseudosymmetric site in the catalytic domain that reduces the Hill slope and the Michaelis constant (Km) (Antos and Potter 2007, Robinson and Potter 2012). Natriuretic peptide binding was not required to convert product formation from positive cooperative to linear but was required to reduce the Km. Structurally, the ribosyl 2' OH group of ATP but not the gamma phosphate was required for allosteric activation. In contrast, removing the comparable ribosyl 2' OH on GTP had no affect on cGMP production, demonstrating unique purine binding requirements for the allosteric and catalytic sites.

Since cellular ATP concentrations of 1-5 mM (Traut 1994) are high above the EC_{50} for ATP activation of GC-A and GC-B, changes in ATP concentrations are unlikely to affect the Km of these enzymes. However, cellular ATP concentrations in the millimolar range have been shown to inhibit GCs. Initial reports indicated that ATP inhibited soluble and particulate GCs with IC_{50}s of 0.4 and 1 mM, respectively, by a competitive mechanism (Kimura and Murad 1974). Later, separate and distinct ATP-dependent activation and inhibitory sites were identified in GC-C (Parkinson et al. 1994). For soluble GC, metabolic inhibitors were shown to reduce cellular ATP concentrations and increased GC activity as expected if ATP is a biologically relevant GC inhibitor (Ruiz-Stewart et al. 2004).

Although ATP was reported to inhibit GC by a mixed mechanism (Limbird and Lefkowitz 1975), exactly where ATP binds these enzymes has not been unequivocally

determined. Evidence for binding to the pseudosymmetric allosteric site or a site independent of the pseudosymmetric site has been reported for soluble GC (Hunt et al. 1994, Derbyshire et al. 2009). Here, we examined the question of whether ATP inhibits GC-A and GC-B, and if so, where it binds. We find that ATP inhibits both enzymes at concentrations found in cells by binding the pyrophosphate product site.

EXPERIMENTAL PROCEDURES

Reagents. The ^{125}I-cGMP radioimmunoassay kits were from Perkin Elmer (Waltham, MA). The pSVL-Kin^{-} plasmid expressing rat GC-A lacking the kinase homology domain was a kind gift from Dr. Michael Chinkers (Chinkers and Garbers 1989).

Cells. HEK 293T cells stably expressing either rat GC-A or rat GC-B and HeLa cells endogenously expressing human GC-A were maintained as previously described (Flora and Potter 2010).

Transient transfection. For Figure 5, 293 cells were transiently transfected by the HEPES-calcium-phosphate precipitation method as previously described (Yoder, Robinson et al. 2012). Data for all other figures were obtained using membranes from HEK 293 cells stably expressing the enzyme of interest or HeLa cells endogenously expressing human GC-A.

Membrane Preparation. Cells cultured on 10 cm plates were placed in serum-free media for at least 4 hours. Membranes were harvested at 4ºC by washing the plates twice with PBS. Cells were scraped off the plates and resuspended in phosphatase inhibitor buffer (PIB) containing 25 mM HEPES pH 7.4, 20% glycerol, 50 mM NaCl, 50 mM NaF, 2 mM EDTA, 0.5 μM microcystin-LR, and 1X Roche Complete protease inhibitor cocktail. The cell suspension was then sonicated for three 1 second bursts to lyse the cells. The lysates were then centrifuged at 20,000 x g for 15 min at 4^{o}C. The supernatant was aspirated and the pellet was resuspended with 0.5 mL PIB and centrifuged again at 20,000 x g for 15 min at 4^{o}C. The supernatant was aspirated, and the pellet was resuspended in PIB to yield a protein concentration between 5-8 mg/mL.

These crude membranes were assayed for GC activity without freezing.

Guanylyl cyclase assays. Crude membranes were assayed for GC activity for the time indicated in the figure legends. GC assays were performed at 37ºC in a buffer containing 25 mM HEPES pH 7.4, 50 mM NaCl, 0.1% BSA, 0.5 mM IBMX, 1 mM EDTA, 0.5 μM microcystin, 5 mM $MgCl_2$. Figures 1, 2A, and 4 were performed with 0.001 mM of the appropriate NP in the presence of 0.1 mM or 1 mM GTP with increasing ATP concentrations. Figure 2B was performed identically but without NP. Figure 6 was performed similarly to Figure 2A, but with only 0.1 mM GTP. Figures 3 and 5 were substrate-velocity assays performed with 1 μM NP and the absence or presence of fixed concentrations of ATP. Figure 7 was performed with 1 μM CNP and increasing concentrations of pyrophosphate at fixed ATP concentrations. All reactions were initiated by the addition of reaction cocktail to 20 μl of crude membranes for a total volume of 0.1 mL and stopped by the addition of ice-cold 0.4 mL ice-cold 50 mM sodium acetate buffer containing 5 mM EDTA. Cyclic GMP concentrations were determined by radioimmunoassay.

Statistical analysis. *S*tatistical analyses were performed with GraphPad Prism 5 software. All p-values were obtained using a paired t-test. Substrate-velocity curves were fitted using the allosteric sigmoidal model. Inhibition curves were fitted using the log (inhibitor) verses response model. Because enzymatic activity was not completely linear with time, the kinetic parameters are considered "apparent."

RESULTS

Low concentrations of ATP activate but higher physiologic concentrations inhibit GC-B. GC assays were conducted on 293T-GC-B membranes containing saturating concentrations of CNP and physiologic (0.1 mM) or supra-physiologic (1 mM) concentrations of GTP in the presence of increasing concentrations of ATP (Fig. 1). Membranes from these cells are ideal for studying the kinetic properties of GC-B because the enzyme is properly processed and highly stimulated by CNP in this model system (Yoder et al. 2010). Furthermore, the only detectable GC in these cells is the transfected enzyme, which allows unequivocal data interpretation (Robinson and Potter 2012).

Progressively increasing ATP concentrations to 0.75 mM increased GC-B activity in the presence of low (0.1 mM) or high (1 mM) GTP concentrations. Activation was greater at lower, physiologic GTP concentrations because ATP decreases the Km of the enzyme (Antos and Potter 2007) by binding an allosteric site in the catalytic domain (Robinson and Potter 2012). Hence, ATP is less effective at higher GTP concentrations. The EC_{50} for ATP activation of GC-A and GC-B was previously shown to be ~ 0.1 mM when assayed with endogenous enzymes at physiologic (0.1 mM) GTP concentrations (Antos and Potter 2007).

By extending the range of ATP, we observed that ATP above 1 mM inhibited GC-B in a concentration-dependent manner. When assayed with the lower, physiologic, GTP concentrations, inhibition was complete. Thus, the effect of ATP on GC-B was biphasic. At low concentrations, ATP activated the enzyme, but at higher physiologic

concentrations, ATP was inhibitory. The rate of decline in GC activity was similar between assays conducted with 0.1 or 1 mM GTP, which indicates that the ATP does not compete with GTP and that GTP does not increase the affinity of the inhibitory site for ATP as was shown for the allosteric and catalytic sites (Antos and Potter 2007, Robinson and Potter 2012).

Concentration response experiments starting at 1 mM ATP determined that IC_{50}s for CNP-activated GC-B were 2 to 3 mM when assayed in the presence 0.1 or 1 mM GTP (Fig. 2, upper panel). Basal (no CNP) GC-B activity was inhibited as well (Fig. 2, lower panel). IC_{50}s obtained under basal conditions were similar to those obtained in the presence of NP and were not affected by GTP concentrations. None of the IC_{50} values were significantly different from each other. These data indicate that ATP-dependent inhibition of GC-B does not require NP binding and is not sensitive to substrate concentration, which was not the case for ATP-dependent allosteric activation of GC-A and GC-B (Robinson and Potter 2012).

ATP inhibits GC-B and GC-A by a mixed mechanism. Substrate-velocity assays were conducted on membranes containing GC-B to determine the mechanism of inhibition. Experiments were repeated on membranes from 293 cells transfected with rat GC-A to determine if ATP also inhibits this highly related enzyme. The control concentration of ATP was 1 mM. As shown in Fig. 3, ATP concentrations above 1 mM increased the Km and decreased the maximal velocity of each GC in a concentration dependent manner, which is consistent with a mixed-type inhibitory mechanism that postulates that the inhibitor binds in the presence and absence of GTP to a non-substrate

binding site.

The data were regraphed as a double reciprocal plots (Fig. 3C and D). Of the three mixed-type inhibition systems described by Segel (Segel 1975), linear mixed-type inhibition, best described the ATP inhibition pattern. In this system, the enzyme (E) binds substrate (ES), inhibitor (EI) or both (ESI). With this model, whenever the inhibitor is bound, the enzyme is nonproductive, which explains the decreased maximal velocity. This model also postulates that the EI complex has a lower affinity for substrate than the free enzyme, which explains the increased K_m. As predicted for intersecting, linear noncompetitive inhibition, the control and inhibitor curves intersected above the 1/[MgGTP] axis. Furthermore, increasing the concentration of ATP caused a counter-clockwise rotation in the reciprocal plots as expected for this type of inhibition (Fig. 3C and D)

ATP inhibits endogenous human GC-A. The ability of ATP to inhibit endogenously expressed human GC-A was examined by performing ATP concentration-response GC assays on membranes from HeLa cells (Flora and Potter 2010). ATP inhibited human GC-A in the presence of 0.1 mM or 1 mM GTP with IC_{50} values of 2.0 mM and 2.9 mM, respectively, that were not significantly different (Fig. 4). These data indicate that physiologic ATP concentrations inhibit GC-A when endogenously expressed in a non-transformed cell.

The inhibitor site is in the catalytic domain. ATP was suggested to bind the GXGXXXG site in the KHD of GC-A (Goraczniak et al. 1992), although the mutation of this site had no effect on the ability of NP or ATP to activate GC-A or GC-B (Koller

et al. 1993, Antos and Potter 2007). To rule out the possibility that the inhibitory site is in the KHD of GC-A, substrate-velocity curves were conducted in the presence or absence of 1 mM ATP on membranes from 293 cells transiently expressing the KHD-deleted GC-A construct (Fig. 5). The resulting curves were sigmoidal with Hill slopes above 1.5, which indicates that the catalytic domain contains both the allosteric and catalytic sites as previously reported by the van den Akker and De Lean groups (Joubert et al. 2007, Pattanaik et al. 2009). However, in contrast to the full-length receptor, low ATP concentrations did not increase activity or change cooperativity either in the absence or presence of NP (data not shown). The reason for this is unclear, but it may indicate that the KHD is required to position the allosteric site in a conformation required to bind ATP or to properly transmit the ATP binding signal to the catalytic site. This is similar to the inability of YC-1 to activate constructs of sGC that only contain the catalytic domain (Derbyshire et al. 2009). In contrast, reduced activity of the KHD-deleted GC-A was observed in the presence of ATP, which is consistent with the inhibitory site residing in the catalytic domain, not the KHD.

Neither the ribosyl 2' OH, 3' OH or gamma phosphate are required for inhibition of GC-B by ATP. Previous studies determined that the 2' ribosyl OH group was required for allosteric activation of GC-A and GC-B (Robinson and Potter 2012). Therefore, the role of this functional group in the inhibition of GC-B by ATP was examined. GC assays were conducted in the presence of increasing concentrations of ATP, 2-deoxyATP or 3-deoxy-ATP starting at 0.4 mM using membranes from stably expressing 293T-GC-B cells (Fig. 6A). Neither the 2' OH nor the 3' OH was required

for inhibition of GC-B. The participation of phosphates in the inhibition process was also examined. We found that two phosphates were sufficient for inhibition, since ATP and ADP but not AMP inhibited GC-B (Fig. 6B). The reduced initial activity for AMP and ADP results from the decreased ability of these nucleotides to bind the allosteric activation site (Robinson and Potter 2012). These data indicate that the structural requirements for binding the inhibitory site are different than those required for binding the activation site.

ATP Inhibition is competitive with pyrophosphate. Since ATP and ADP but not AMP inhibited GC-B, we hypothesized that ATP inhibited the enzymes by binding the pyrophosphate product site. To investigate this possibility, we measured CNP-stimulated GC-B activity in the presence of increasing concentrations of ATP and pyrophosphate. We observed that inhibition increased proportionally with increasing concentrations of both compounds. Importantly neither the IC_{50} for pyrophosphate nor ATP was affected by the presence of the other inhibitor (Fig. 7A). We also graphed the data as a Dixon plot (reciprocal velocity verses pyrophosphate concentration) and found that the responses were straight lines indicating that the ESI complex is not catalytically active. Furthermore, the lines representing activities measured with increasing ATP concentrations were parallel, indicating mixed-type inhibition by two mutually exclusive molecules that compete for the same site (Fig. 7B). These data are consistent with ATP inhibiting GC-B by binding the pyrophosphate product site.

DISCUSSION

We investigated if and how physiologic concentrations of ATP inhibit GC-A and GC-B. Our studies demonstrate for the first time that both enzymes are inhibited by concentrations of ATP normally found in cells. Also for the first time, we show that the inhibition occurred by binding to the post-catalytic pyrophosphate site that prevents GC-A and GC-B from resuming a conformation capable of binding and catalyzing GTP. Importantly, these data suggest that changes in cellular ATP levels can fine-tune cGMP production. For example, reduced ATP concentrations resulting from ischemia could relieve ATP-dependent inhibition of GC-A and increase vascular smooth muscle cell relaxation, which would increase blood flow to the oxygen-depleted tissue.

Previous reports indicated that ATP inhibits soluble and particulate GCs, but different groups suggested different binding sites. ATP inhibited a sGC catalytic construct (Derbyshire et al. 2009), which indicated that the inhibitory site is in the catalytic domain. Our data showing that ATP inhibited a KHD deleted construct, is also consistent with the inhibitory site for ATP residing in the catalytic domain of GC-A.

An older report suggested that ATP competes with GTP for binding at the catalytic site (Limbird and Lefkowitz 1975), but this is inconsistent with mixed inhibition data observed by our group and others (Parkinson et al. 1994, Derbyshire et al. 2009, Robinson and Potter 2012). Another group suggested that ATP inhibits sGC by binding the pseudosymmetric site (Hunt et al. 1994). They found that nucleosides with at least two phosphates inhibited sGC, which is consistent with our data. Evidence for binding to the pseudosymmetric site was based a point mutation postulated to reside in

the pseudosymmetric domain that eliminated ATP-dependent inhibition. However, an analogous mutation in GC-A abolished catalytic activity, so this mutation is not restricted to the allosteric site in GC-A (Robinson and Potter 2012). Furthermore, inhibition by binding the pseudosymmetric site is not consistent with our previous data showing that ATP binding to the pseudosymmetric site activates GC-A and GC-B by reducing the Michaelis constants (Robinson and Potter 2012).

YC-1 is an allosteric enhancer that increases the potency and efficacy of NO by binding the pseudosymmetric site of sGC (Derbyshire and Marletta 2012). Because YC-1 reduced the affinity of 2'-dADP for a site in the catalytic domain, researchers concluded that YC-1 was competitive with 2'-dADP binding to the pseudosymmetric site (Makino et al. 2012). However, direct binding studies were not conducted, so it is possible that YC-1 binding to the pseudosymmetric site decreased affinity of a separate site for 2'-dADP. In fact, YC-1 binding reduced the Km for GTP, which would be expected to increase the Ki for 2'-dADP binding to the pyrophosphate site, since binding to the substrate and products sites are mutually exclusive. Consistent with this idea, we reported that ATP binding to the pseudosymmetric site increased affinity of the catalytic sites of GC-A and GC-B for GTP as demonstrated by reduced Michaelis constants (Antos and Potter 2007, Robinson and Potter 2012).

Pyrophosphate release from the product site of adenylyl cyclase was shown to be slow and partially rate limiting (Chang et al. 1990). Furthermore, pyrophosphate was suggested to enhance inhibition by P-site inhibitors by stabilizing an enzyme product complex. Pyrophosphate alone also inhibited a truncated catalytic construct of GC-A with an IC_{50} of 1.4 mM and enhanced inhibition by 2'd3-GMP, a P-site inhibitor of GC (Joubert et al. 2007). Similar results were reported for inhibition of sGC by 2'd3-GMP and the pyrophosphate analog foscarnet (Makino et al. 2012). Inhibition by pyrophosphate binding to its product site was deemed physiologically unlikely since cellular concentrations of pyrophosphate are in the 0.3 mM range. Since intracellular ATP concentrations are in the millimolar range, we suggest that ATP is ideally suited to bind this site.

Data supporting two independent ATP regulatory sites in GCs is accumulating. The biphasic effect of ATP at different concentrations supports this notion as does results using ATP analogs like 2'd-ATP containing potent inhibitory but weak allosteric effects. Furthermore, the ability of ATP and ADP but not AMP to inhibit GC activity is consistent with the need for two phosphates. Most importantly, GC assays conducted with increasing concentrations of ATP and pyrophosphate are consistent with both compounds binding the same site.

We propose a model where the GTP binding site is composed of two separate parts - one that binds the guanine and alpha phosphate of GTP and another that binds the beta and gamma phosphates. Upon catalysis, cGMP and pyrophosphate are formed and bind the two distinct product sites. However, due to high cellular ATP concentrations,

when pyrophosphate dissociates, ATP occupies the pyrophosphate site, which prevents reformation of the substrate-binding site until ATP dissociates and allows the enzyme to assume the conformation capable of binding GTP (Fig. 8). This effect would decrease maximal velocity. Furthermore, since the amount of effective enzyme would be decreased, higher GTP concentrations would be required for the remaining uninhibited enzyme to produce the same amount of product, which would increase the apparent Michaelis constant.

In conclusion, we show that physiologic concentrations of ATP inhibit GC-A and GC-B by a mixed-type mechanism that is competitive with pyrophosphate. We propose that ATP binds to the pyrophosphate site, which forms a non-productive enzyme and prevents the transition back to the conformational state that binds and catalyzes GTP. Finally, we note that these conclusions are not only consistent with our data but are also consistent with the majority of data published by other investigators. Thus, ATP binding to the pyrophosphate site reconciles findings from the independent laboratories studying multiple members of the GC family.

FIGURES

FIGURE 1. ATP activates GC-B at low concentrations but inhibits GC-B at higher, cellular concentrations.

GC activity was measured in membranes from 293T cells stably expressing GC-B in the presence of 0.1 or 1.0 mM GTP, 1 mM CNP and the indicated ATP concentrations. Vertical bars within symbols represent SEM where n = 5.

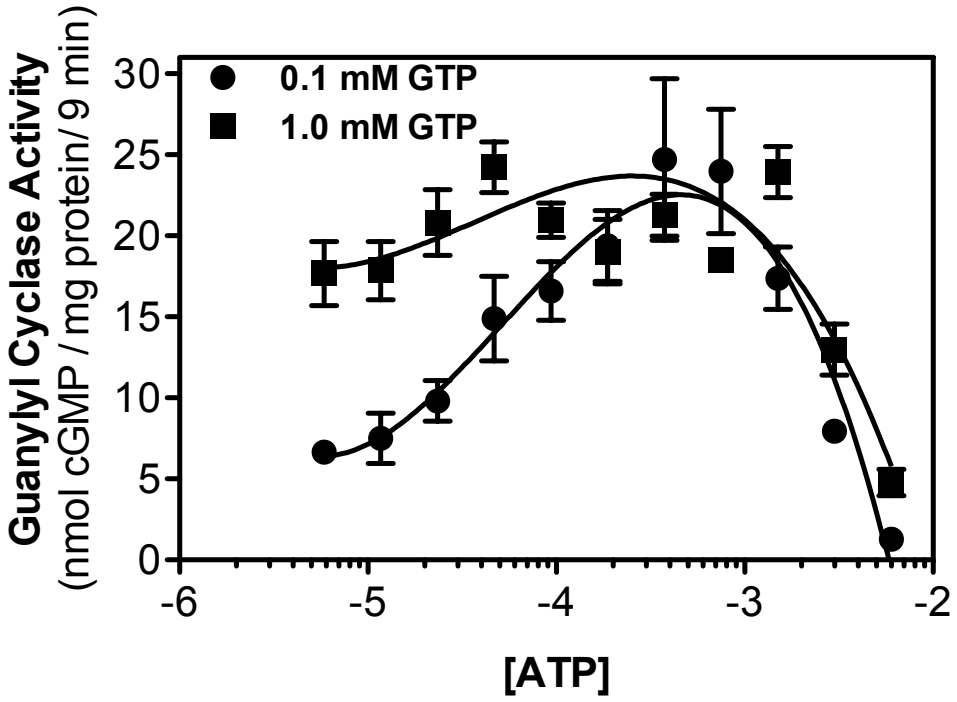
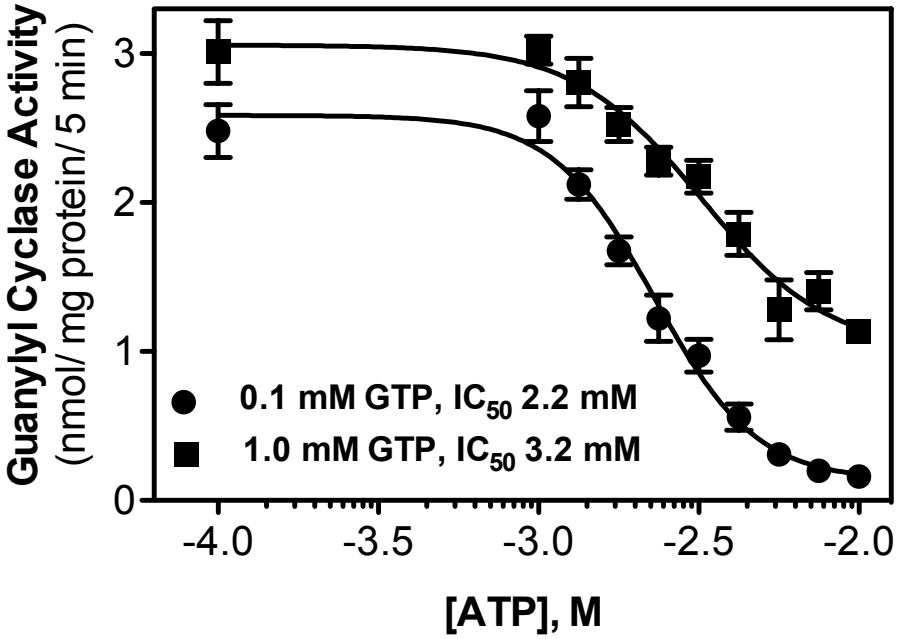

Guanylyl Cyclase Activity
(nmol cGMP / mg protein/ 9 min)
0.1 mM GTP
1.0 mM GTP
30
25
20
15
10
5
0
-6
-5
-4
-3
-2
[ATP]

FIGURE 2. ATP inhibits GC-B at physiologic ATP concentrations.

GC activity was measured in crude membranes prepared from 293T cells stably expressing GC-B with physiologic (0.1 GTP) or supra-physiologic (1.0 mM GTP) substrate concentrations in the presence of the indicated concentrations of ATP. A. ATP inhibits CNP-stimulated GC-B. B. ATP inhibits GC-B under basal (no CNP) conditions. The vertical bars in the symbols represent SEM where n = 4. IC_{50} values were not significantly different from one another.


Guanylyl Cyclase Activity
(nmol/ mg protein/ 5 min)
0.1 mM GTP, IC50 2.2 mM
1.0 mM GTP, IC50 3.2 mM
[ATP], M

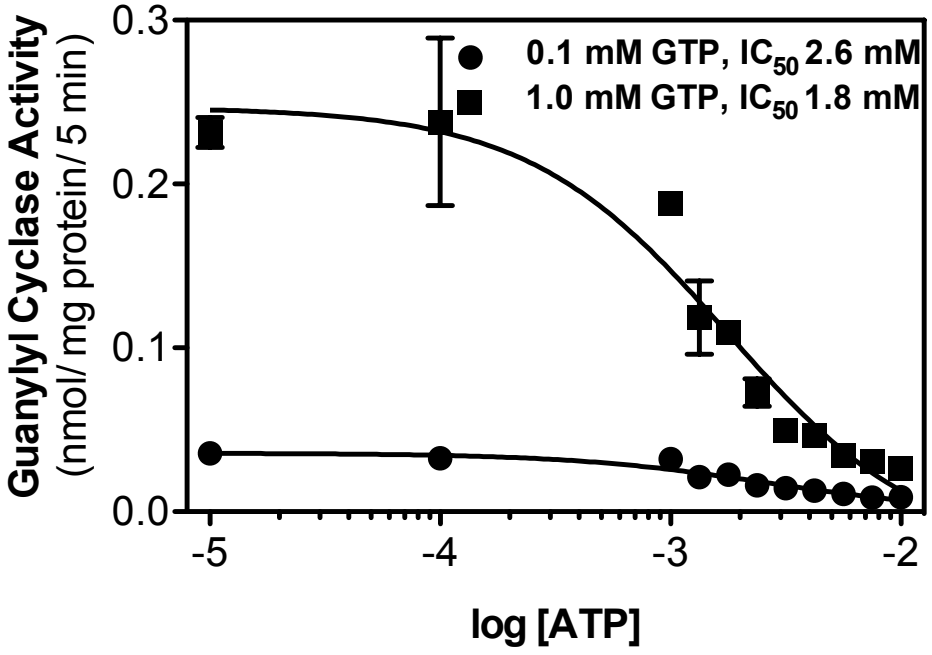
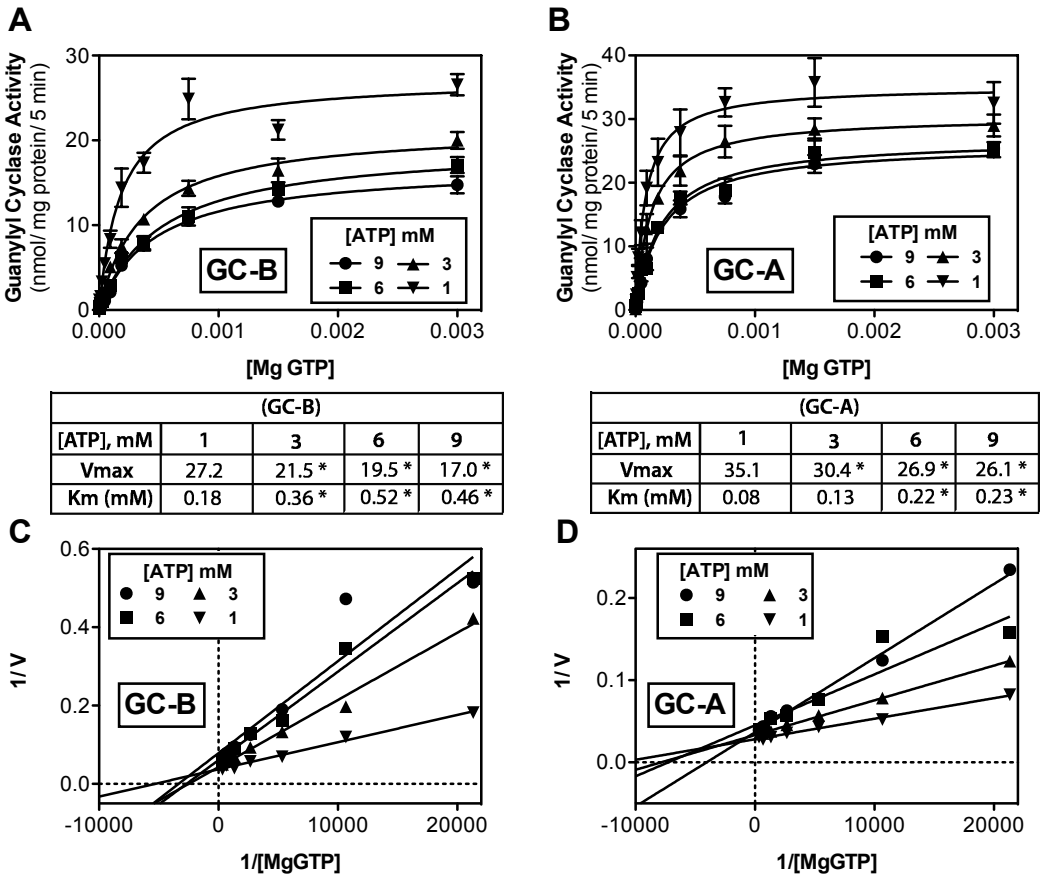

Guanylyl Cyclase Activity
(nmol/ mg protein/ 5 min)
0.1 mM GTP, IC50 2.6 mM
1.0 mM GTP, IC50 1.8 mM
log [ATP]

FIGURE 3. ATP is a linear, mixed-type inhibitor of GC-B and GC-A.

GC activity was measured in membranes from 293T cells stably expressing either rat GC-B (A and C) or rat GC-A (B and D) with increasing concentrations of GTP and the indicated concentrations of ATP. A and B. Substrate-velocity data were plotted and tables of resulting data were presented below. C and D. Data from A and B were graphed as double reciprocal plots. Vertical bars within the symbols represent SEM where n = 3. * in the tables indicates significantly different from 1 mM ATP treatment where $p \leq 0.05$.



(GC-B)				
[ATP], mM	1	3	6	9
Vmax	27.2	21.5 *	19.5 *	17.0 *
Km (mM)	0.18	0.36 *	0.52 *	0.46 *

(GC-A)				
[ATP], mM	1	3	6	9
Vmax	35.1	30.4 *	26.9 *	26.1 *
Km (mM)	0.08	0.13	0.22 *	0.23 *

FIGURE 4. ATP inhibits endogenous human GC-A.

ANP-stimulated GC activity was measured in crude membranes prepared from HeLa cells endogenously expressing human GC-A with increasing concentrations of ATP at the indicated concentrations of GTP. IC_{50} values were 2.0 and 2.9 mM for 0.1 and 1.0 mM GTP, respectively *Inset*. Data represented as percent of maximum activity to emphasize similar IC_{50} values. Vertical bars within the symbols represent range of duplicate determinations where n = 2.

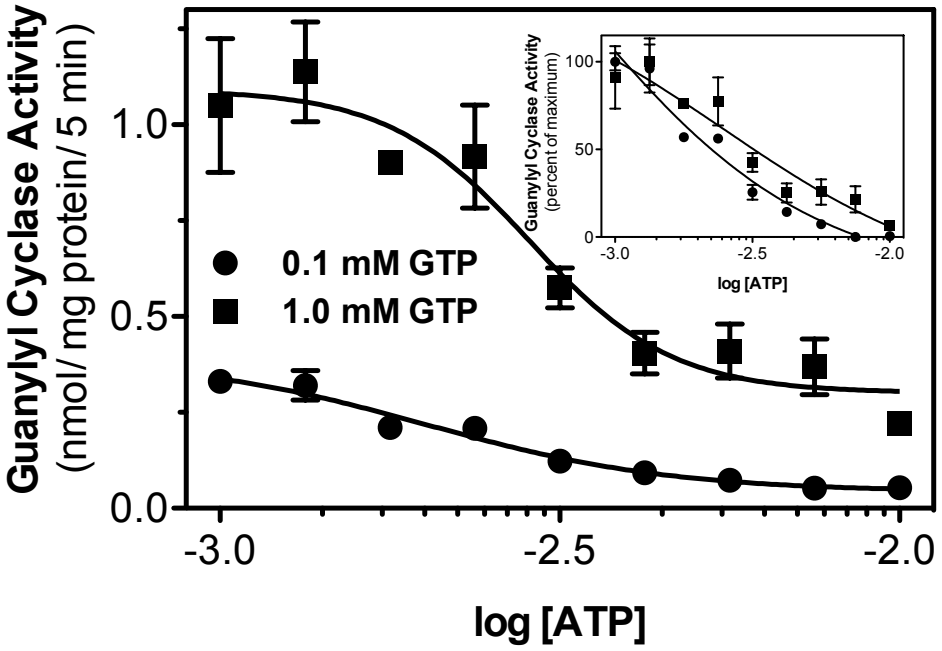

Guanylyl Cyclase Activity
(nmol/ mg protein/ 5 min)
1.0
0.5
0.0
-3.0
-2.5
-2.0
log [ATP]
0.1 mM GTP
1.0 mM GTP
Guanylyl Cyclase Activity
(percent of maximum)
100
50
0
-3.0
-2.5
-2.0
log [ATP]

FIGURE 5. The ATP inhibitory site is in the catalytic domain.

GC activity was measured in membranes from 293 cells transiently expressing a construct of GC-A lacking the kinase homology domain (ΔKHD). The assay was conducted in the presence or absence of 1 mM ATP and the indicated GTP concentrations. The vertical bars within the symbols represent SEM where n = 3.

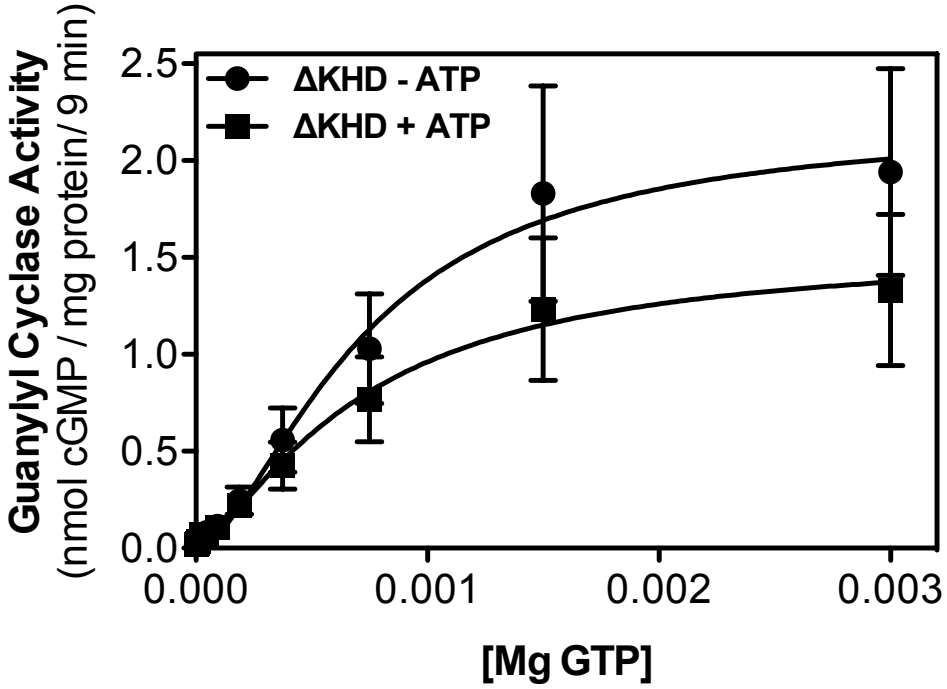

Guanylyl Cyclase Activity
(nmol cGMP / mg protein/ 9 min)
2.5
2.0
1.5
1.0
0.5
0.0
ΔKHD - ATP
ΔKHD + ATP
0.000
0.001
0.002
0.003
[Mg GTP]

FIGURE 6. Nucleotide inhibition does not require a 2' or 3' ribosyl OH group or a gamma phosphate.

GC activity was measured in membranes from 293T cells stably expressing GC-B for 9 min. A. Inhibition does not require ribosyl OH groups. GC activity was measured in the presence of 0.1 mM Mg^{2+}GTP and increasing concentrations of the indicated adenine nucleotides. Activity was normalized to percent of maximum to emphasize the similar inhibitory concentrations of the nucleotides. B. ATP and ADP, but not AMP, inhibited GC-B. Vertical bars within the symbols represent SEM where n = 3.

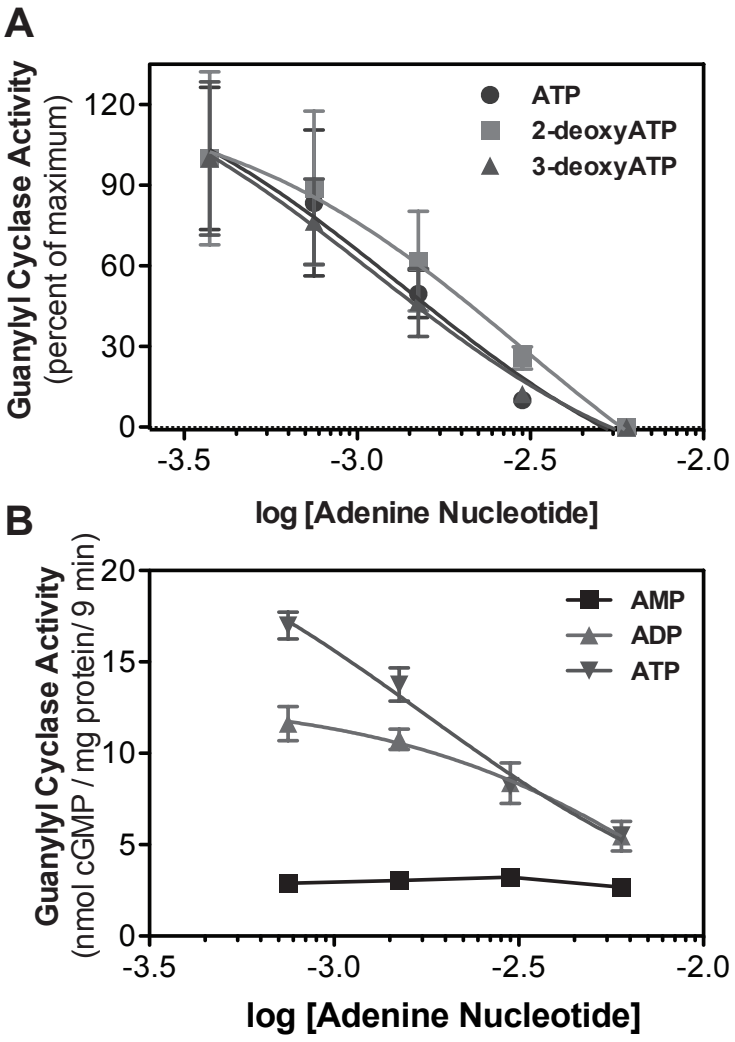

A
Guanylyl Cyclase Activity
(percent of maximum)
120
90
60
30
0
-3.5
-3.0
-2.5
-2.0
log [Adenine Nucleotide]
ATP
2-deoxyATP
3-deoxyATP
B
Guanylyl Cyclase Activity
(nmol cGMP / mg protein/ 9 min)
20
15
10
5
0
-3.5
-3.0
-2.5
-2.0
log [Adenine Nucleotide]
AMP
ADP
ATP

FIGURE 7. Pyrophosphate and ATP inhibit GC-B in a competitive, mutually exclusive manner.

GC activity was measured in membranes from 293T cells stably expressing GC-B in the presence of the indicated concentrations of ATP, pyrophosphate (PPi), and 1 mM GTP and 1 mM CNP. A. Raw data were plotted. IC_{50}s for 1.5, 3, 6, and 9 mM ATP were 1.8, 1.6, 1.9 and 1.8 mM, respectively, which were not significantly different from each other. B. The data were graphed as a Dixon plot, which indicated parallel lines, indicating mixed-type inhibition by two mutually exclusive inhibitors that compete for the same site. Vertical bars within the symbols represent SEM where n = 3.

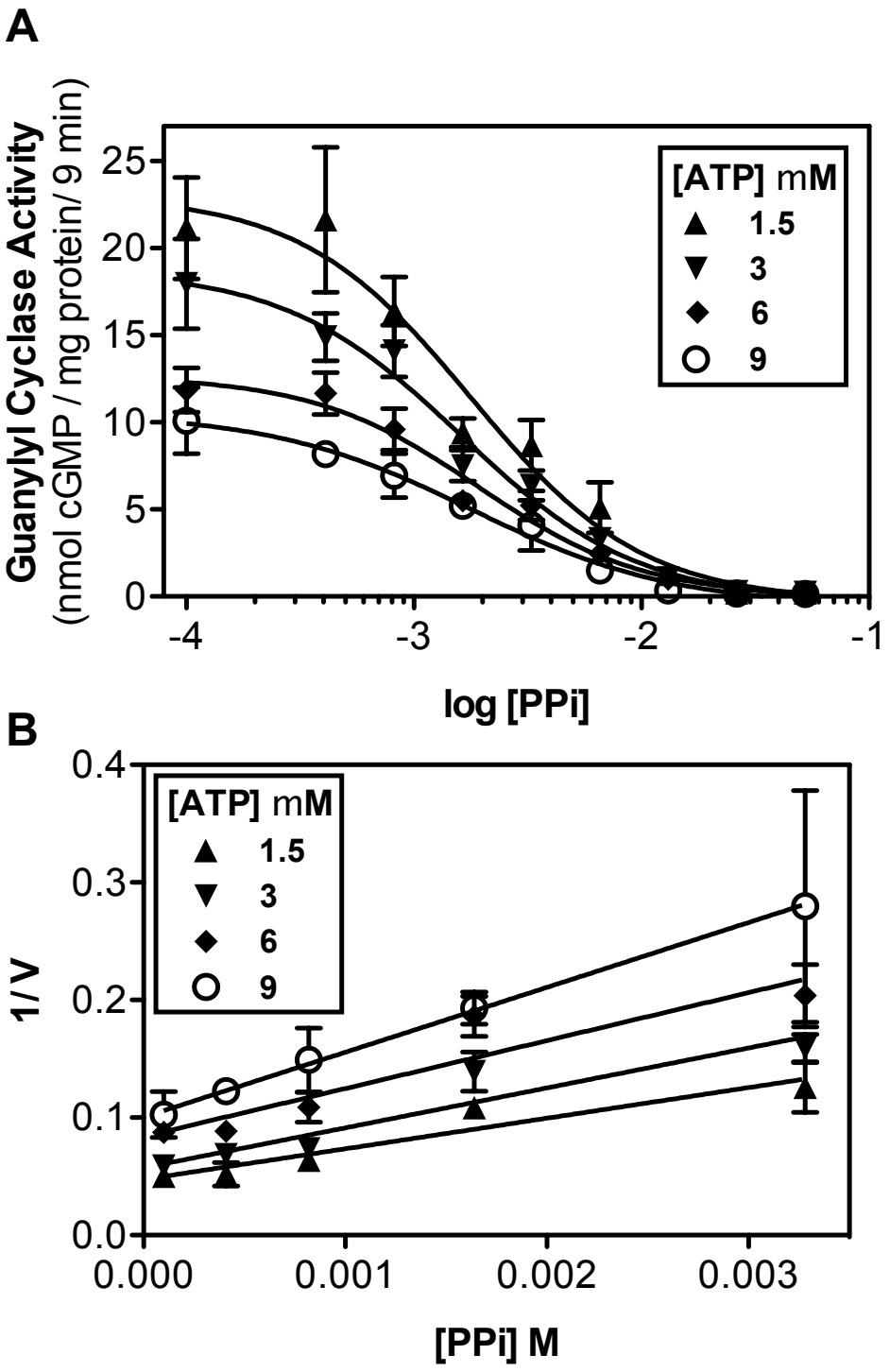
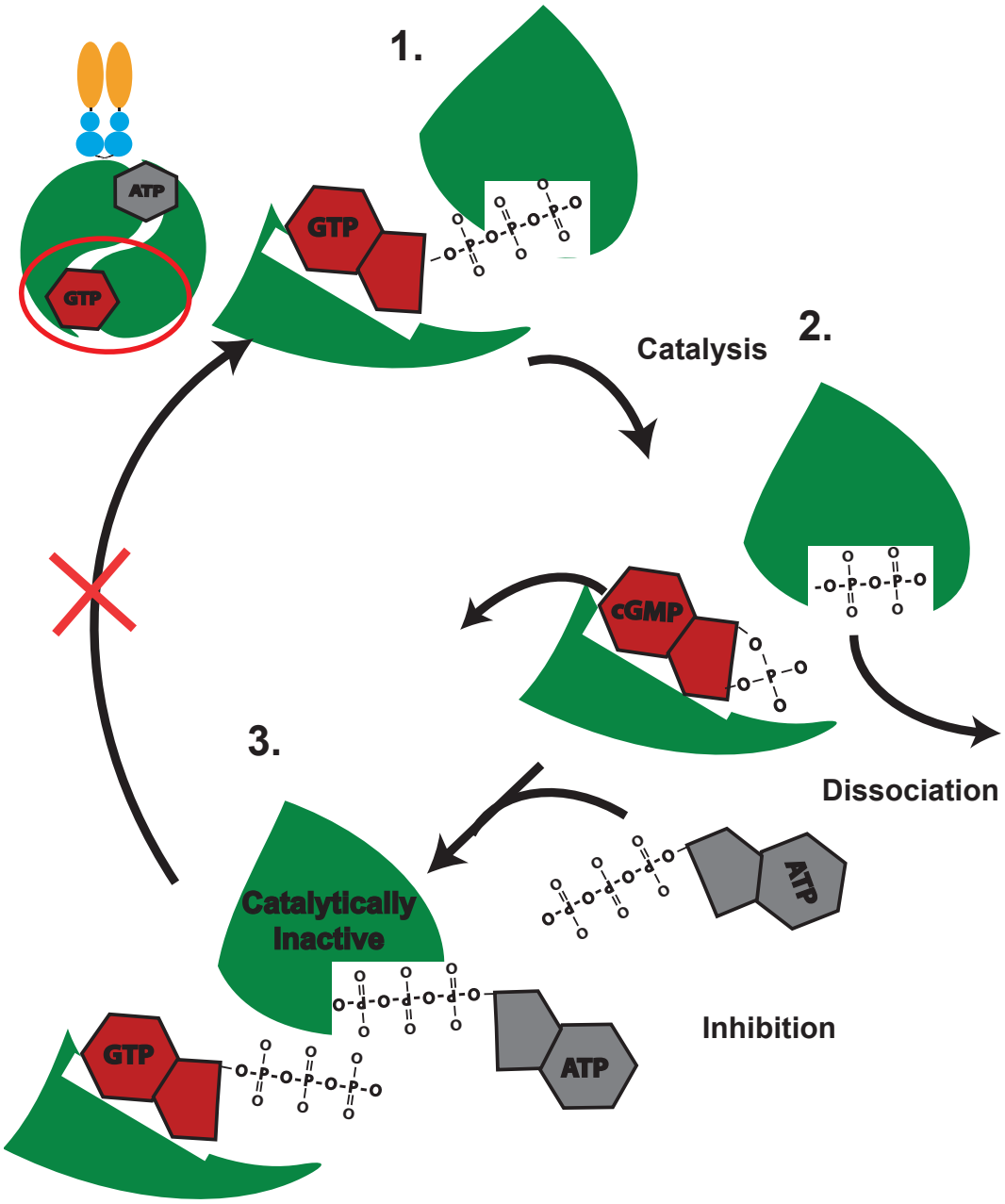

A
Guanylyl Cyclase Activity
(nmol cGMP / mg protein/ 9 min)
25
20
15
10
5
0
-4
-3
-2
-1
log [PPi]
[ATP] mM
1.5
3
6
9
B
1/ V
0.4
0.3
0.2
0.1
0.0
0.000
0.001
0.002
0.003
[PPi] M
[ATP] mM
1.5
3
6
9

FIGURE 8. Working Model Depicting ATP Inhibition of GC-A and GC-B.

The red circle indicates the catalytic site that is exploded to the right. (1.). Residues from each monomer bind GTP in the catalytic site. (2.) During catalysis, GTP is cleaved into cGMP and pyrophosphate, which dissociate randomly and independently from the catalytic site. (3.) ATP binds the pyrophosphate product site, forming a non-catalytic, dead end enzyme, which decreases maximal velocity. ATP binding to the pyrophosphate site also increases the Michaelis constant because more GTP is needed to generate higher specific activity from the remaining functional enzyme. Both effects result in mixed-type inhibition.


1.
ATP
GTP
GTP
Catalysis
2.
cGMP
Dissociation
3.
Catalytically
Inactive
ATP
Inhibition
GTP
ATP

CHAPTER 8: CONCLUSIONS AND FUTURE DIRECTIONS

DISCUSSION

My first project in the laboratory was to identify which isoform of PKC mediated the phosphorylation-dependent dephosphorylation of GC-B in response to phorbol ester (PMA) treatment. Our group showed that GF109203X, a broad range PKC inhibitor, prevents PKC-dependent inhibition of GC-B (Abbey-Hosch et al. 2005). In order to determine the subset of potential PKC isoforms responsible for the inhibition, I used Gö6976, a selective inhibitor of the conventional group of PKC isoforms. The conventional isoforms are activated by both calcium and phorbol esters. As GC-A and GC-B are inhibited by calcium and phorbol esters, the conventional group was hypothesized to mediate the PKC-dependent inhibition. We found that Gö6976 not only did not block PMA-dependent inhibition of GC-B, it inhibited activity on its own. This was the first report of an intracellular inhibitor of GC-A and GC-B. We found that Gö6976 inhibited GC-B activity in a rapid, potent, reversible manner that was not dependent on ligand stimulation. Gö6976 reduced the potency of CNP-stimulated activity and also increased the EC_{50} for CNP activation. Inhibition was also phosphorylation independent, as a construct of GC-B in which all known phosphorylation sites are mutated to the phosphomimetic glutamic acid was still inhibited by Gö6976. Finally, we found that Gö6976 inhibited GC-B activity in both whole cells and broken cell membrane preparations, which is consistent with a direct inhibitory effect. This study was published in the *British Journal of Pharmacology* in 2011 (Robinson et al. 2011).

Next, we published a follow-up paper in the *Journal of Biological Chemistry* in which we determined the mechanism of Gö6976 inhibition. We found that Gö6976 was a competitive inhibitor of GC-A and GC-B, as it increased the Michaelis constant without affecting maximal velocity. Additionally, we discovered that increasing ATP concentrations caused a dose-dependent increase in the magnitude of Gö6976 inhibition. Conversely, Gö6976 decreased the EC_{50} for ATP activation nearly five-fold. The reciprocal effect of Gö6976 and ATP suggested for the first time that binding to the catalytic site increased affinity to the allosteric site and vice versa. This conclusion led to my most significant contribution to the field of membrane guanylyl cyclases: determining that ATP activates GC-A and GC-B by binding to an allosteric purine binding site in the catalytic domain that increases the affinity of GC-A and GC-B for GTP in the presence of natriuretic peptide (NP).

At the beginning of my graduate career, the prevailing theory of ATP activation of GC-A, GC-B and GC-C was that ATP bound to a site in the kinase homology domain that increased the maximal velocity of these enzymes (Goraczniak et al. 1992, Duda et al. 1993, Duda et al. 1996, Joubert et al. 2005, Burczynska et al. 2007). Laura Antos, a previous student in the laboratory, found that ATP reduced the Michaelis constant (Km) of GC-A and GC-B but did not increase the maximal velocity of the enzymes (Antos et al. 2005, Antos and Potter 2007, Robinson and Potter 2012). My work showed that ATP binds a site in the catalytic domain of mGCs that converts them from positive cooperative substrate-velocity profiles to linear profiles. This concentration-dependent loss of cooperativity suggested that ATP was competing for binding to the second

purine-binding site. I also demonstrated that ATP binds in the absence of NP, but that NP is required for ATP to transmit this activation signal to the catalytic site to reduce the Km.

Additionally, I characterized the two purine-binding sites in these enzymes and found that the 2'-ribosyl OH group was required for maximal allosteric activation but was not required for binding to the catalytic site and that only two phosphates were required for activation of the allosteric site. By introducing mutations into the catalytic domain that were predicted to inactivate the catalytic site if it were symmetrical, I demonstrated that the binding requirements for the allosteric and catalytic sites were different, which provided the first evidence of asymmetry in the purine-binding sites for mGCs. Interestingly, the crystal structure of an asymmetric, homodimeric AC from *M. tuberculosis* is consistent with our data (Sinha et al. 2005). The AC has two purine binding sites: one that is catalytic and another that is allosteric since it can bind ATP as demonstrated by positive cooperativity. Taken together, these data indicate that GC-A and GC-B are asymmetric homodimers with distinct catalytic and allosteric sites that bind GTP and ATP, respectively. Although GTP can bind the allosteric site in enzyme assays lacking ATP, in cells ATP occupies the allosteric site because ATP concentrations are an order of magnitude higher than GTP concentrations (Traut 1994). Furthermore, since cellular ATP concentrations are much higher than the EC_{50} for ATP activation of these enzymes, it is unlikely that changes in cellular ATP concentrations affect the Km of these enzymes. Nonetheless, ATP binding to the allosteric site is necessary for normal enzyme activation because without the reductions in the Km

observed with ATP, the enzymes would be so inefficient that the cGMP formed in response to NP binding would be inconsequential. We published these findings in *Science Signaling* in 2012.

Another aspect of my research focused on the ability of physiologic concentrations of ATP to inhibit GC-A and GC-B. Early studies on detergent activated mGCs concluded that ATP inhibited activity by directly competing with GTP at the substrate-binding site (Limbird and Lefkowitz 1975). Subsequent studies on sGCs reported that ATP inhibited the enzymes by binding the pseudosymmetric site that is analogous to the allosteric site of GC-A and GC-B (Chang et al. 2005, Roy et al. 2008, Derbyshire et al. 2009). Surprisingly, although there was extensive data regarding the inhibition of both ACs and GCs by the products of the reactions, cGMP and pyrophosphate, no previous studies examined whether ATP bound the pyrophosphate product site in GCs. Since, we had shown that ATP binding to the pseudosymmetric allosteric site activated GC-A and GC-B, we investigated the possibility that ATP inhibited these enzymes by binding the pyrophosphate product site. We found that ATP competed for binding to the pyrophosphate site, which reduced the affinity of the enzymes for GTP as well as reduced the maximal velocity. This inhibition pattern is consistent with the mixed inhibition observed by others for ATP-dependent inhibition of GCs (Chang et al. 2005, Roy et al. 2008, Derbyshire et al. 2009). However, since we determined that pyrophosphate and ATP compete for the same site, we concluded for the first time that ATP inhibits GCs by binding the pyrophosphate product site. We have written a manuscript that will be submitted to the *Journal of Biological Chemistry*

describing this data, which is included as chapter 7 in my thesis.

As I became more able to interpret kinetic experiments, I initiated several side projects. GC-B is phosphorylated on multiple serine and threonine residues, and phosphorylation is necessary for activity. When I joined the laboratory, six phosphorylation sites for GC-B were known, four serines and two threonines. Based on mutational data, we found a putative seventh site at Ser-489. Work by Dr. Andrea Yoder in our lab verified the six known phosphorylation sites by mass spectrometry (Yoder et al. 2010). However, she was unable to provide mass-spectrometric conformation of the seventh site. By performing substrate-velocity experiments, I verified that phosphorylation of the new site was required for normal activity of the enzyme and that mutation of this site to alanine markedly increased the Km of the enzyme. These results were recently published in *PLoS One* (Yoder, Robinson et al. 2012) in 2012. I was a co-first author on this paper.

Recently, a single amino acid mutation in GC-B was identified in three generations of individuals with abnormally long and possibly fragile bones (Miura et al. 2012). We found that this Val to Met mutation at position 883 constitutively activated GC-B in the absence of CNP by increasing the maximal velocity of the enzyme. Surprisingly, CNP decreased the Michaelis constant of the enzyme ten-fold in the absence of ATP without further increasing maximal velocity. This was novel because WT-GC-B requires ATP for CNP-mediated reductions in the Km. We concluded that the V883M mutation locked GC-B in a conformation that mimicked the ATP-bound state. Furthermore, Dr. Deborah Dickey found that the mutant did not desensitize like

the WT enzyme, and the mutant enzyme activity was not phosphorylation-dependent. This data has been submitted to *Bone*.

Finally, in collaboration with Dr. Laurinda Jaffe at the University of Connecticut I investigated how luteinizing hormone inhibits GC-B activity. In the mammalian ovary, GC-B is active most of the time. GC-B activation maintains oocytes in meiotic prophase by reducing cAMP degradation by phosphodiesterase 3B (Holt et al. 2013). Luteinizing hormone (LH) initiates resumption of meiosis by inhibiting GC-B activity. We found that LH inhibits GC-B activity in cultured mouse follicles within 20 minutes, and that this inhibition did not affect protein concentrations (Robinson et al. 2012). These findings were published in *Developmental Biology*. We are currently investigating whether the LH-dependent inhibition requires the dephosphorylation of GC-B by a specific phosphatase.

While I have made significant contributions to the field of membrane guanylyl cyclases, there are still many unanswered questions regarding the activation and inhibition mechanisms of these enzymes. First, my initial project never answered which PKC isoform is responsible for phorbol ester dependent inhibition because the PKC inhibitor inhibited GC-B. In the future, different inhibitors that do not inhibit GC-B should be tested. Alternatively, siRNA approaches can be used to determine the specific PKC isozyme(s) required to inhibit GC-B.

Although we have shown that ATP binds into the allosteric site, we have not shown the amino acids involved in ATP binding, nor do we know the conformational changes induced by ATP in the presence of natriuretic peptide that increases affinity of

the enzymes for GTP. Mutational and modeling approaches can be used to determine the conformational changes involved upon ATP binding, and crosslinking studies employing azido-ATP and azido-GTP analogs would identify the amino acids that physically interact with these nucleotides. We have initiated crosslinking studies with ATP, using the photoaffinity labeled 8-azido ATP to crosslink to full-length GC-B. In contrast to previous attempts to crosslink ATP, we have used a photoaffinity analog that activates, not inhibits GC-B (Joubert et al. 2005). Once crosslinked, we can use mass spectrometry to determine which amino acids bind ATP. By using a KHD-deleted construct, we can investigate the amino acids responsible for ATP binding to the pyrophosphate product site.

Although we know that ATP inhibits GC-A and GC-B by binding into the post-catalytic pyrophosphate site, we have not shown that this inhibition mechanism takes place under physiologically relevant scenarios. It will be interesting to see whether GC-A or GC-B are inhibited in metabolic diseases where cellular ATP concentrations are high - such as diabetes and obesity. Conversely, in situations of low ATP, such as ischemia, it will be important to determine if the activity of these enzymes is increased.

Finally, we are just beginning to study the effect of end product inhibition of GC-A and GC-B. We have found that GC-A and GC-B inactivate as a function of time, and this inactivation is independent of phosphorylation and does not involve enzyme degradation. Within 10 minutes of NP stimulation, enzyme velocity falls. This effect has been shown in crude membrane preparations, and has been simulated using pyrophosphate and the GMP analog, 2'd3-GMP. With respect to physiologic regulation,

this feedback inhibition would be an effective method for shutting down enzyme in a manner that is dependent on the amount of product synthesized. Manipulation of this feedback inhibition could potentially be a point of pharmacological intervention. Future studies will examine the mechanism and physiological significance of the inhibition.

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