An Intracellular Adenine Nucleotide Binding Site Inhibits Guanylyl Cyclase C by a Guanine Nucleotide-Dependent Mechanism†

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Received October 11, 1995; Revised Manuscript Received December 11, 1995⊗

ABSTRACT: Guanylyl cyclase C (GCC), the receptor for the *Escherichia coli* heat-stable enterotoxin (ST), is inhibited by 2-substituted adenine nucleotides in an allosteric fashion. In confluent cultures of Caco 2 intestinal epithelial cells, extracellular 2-methylthioadenosine triphosphate (2MeSATP) had no effect on basal or ST-stimulated cyclic GMP (cGMP) accumulation. However, this nucleotide inhibited cGMP accumulation in digitonin-permeabilized Caco-2 human colon carcinoma cells, demonstrating that allosteric inhibition of GCC by adenine nucleotides is mediated by an intracellular adenine nucleotide binding site rather than purinergic receptors. The role of guanine nucleotides in the regulation of GCC by adenine nucleotides was examined. Increasing GTP concentrations from 5 to 100 μM increased the potency of 2MeSATP inhibition of GCC 20-fold, with a shift in the K_i from 447 to 22 μM, respectively. Also, the hydrolysis-resistant analogue, guanosine 5′-*O*-(3-thiotriphosphate) (GTPγS), supported 2MeSATP inhibition of GCC with a potency which was 10-fold greater than GTP. In addition, GTP alone, in the absence of adenine nucleotides and at concentrations greater than 1 mM, inhibited GCC through a mechanism convergent with 2MeSATP. Guanine nucleotides supported adenine nucleotide inhibition of GCC at low concentrations and directly inhibited this enzyme at high concentrations when these studies were conducted with receptors expressed in Caco-2 cells, native rat intestine, or cloned rat GCC heterologously expressed in 293 monkey kidney cells. These observations demonstrate that adenine nucleotide inhibition of GCC is mediated through an intracellular mechanism which is guanine nucleotide-dependent.

Guanylyl cyclase C (GCC)[1] is a member of the particulate guanylyl cyclase family of receptors which is localized in the brush border of intestinal mucosa cells (Guerrant et al., 1980; Rao et al., 1980; Schulz et al., 1990; deSauvage et al., 1991; Fulle & Garbers, 1994). GCC is a receptor for ST and an endogenous ligand, guanylin, which is secreted as a pro-peptide from enterochromaffin cells in the intestine (Schulz et al., 1990; deSauvage et al., 1991; Fulle & Garbers, 1994; Cetin et al., 1994). Occupancy of the extracellular ligand binding domain activates the cytoplasmic cyclase catalytic domain, increasing intracellular cGMP, and altering intestinal fluid and electrolyte secretion (Field et al., 1978; Guerrant et al., 1980; Rao et al., 1980; Giannella, 1981; Dreyfus & Robertson, 1984; Huott et al., 1988; Schulz et al., 1990; deSauvage et al., 1991; Fulle & Garbers, 1994).

Adenine nucleotides play a prominent role in regulating basal and ligand-stimulated GCC-associated guanylyl cyclase activity. Thus, ATP potentiates the activation of guanylyl cyclase by ST (Gazzano et al., 1991a; Vaandrager et al., 1993a). Previous studies suggest that occupancy of the intracellular kinase-homology domain of GCC with ATP stabilizes the active conformation of the catalytic domain, inhibiting desensitization of ligand-activated guanylyl cyclase (Vaandrager et al., 1993a,b). However, the role of ATP in coupling receptor occupancy and catalytic activation has been confounded by the observation that adenine nucleotides inhibit GCC when manganese, rather than magnesium, serves as the substrate cofactor (Gazzano et al., 1991a,b; Marala et al., 1991). Recently, adenine nucleotide-dependent inhibition of basal and ligand-stimulated GCC was demonstrated to be, in part, allosteric, independent of the substrate cation cofactor, and pharmacologically distinct from the activation of GCC by ATP (Parkinson et al., 1994).

The precise mechanisms mediating allosteric inhibition of GCC by adenine nucleotides remain unclear. Earlier studies demonstrated that this inhibition was associated with a shift in the kinetics of GCC from positive to negative cooperativity (Parkinson et al., 1994). These observations are consistent with the hypothesis that adenine nucleotide inhibition of GCC is mediated by a guanine nucleotide-dependent mechanism. Similarly, previous studies suggested that this inhibition might be mediated by purinergic receptors, since 2-substituted adenine nucleotides, selective agonists of P_{2Y} receptors,

† These studies were supported by grants from the NIH (DK43805) and NSF (IBN9205717) and by W. W. Smith Charitable Trust and Targeted Diagnostics and Therapeutics Inc.

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⊗ Abstract published in *Advance ACS Abstracts,* February 15, 1996.

[1] Abbreviations: ATCC, American Type culture collection; AMPS, adenosine 5′-*O*-(thiomonophosphate); 2ClATP, 2-chloroadenosine triphosphate; cGMP, cyclic GMP; ST, *E. coli* heat-stable enterotoxin; EDTA, ethylenediaminetetraacetic acid; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; G proteins, guanine nucleotide binding proteins; GDPβS, guanosine 5′-*O*-(3-thiodiphosphate); GTPγS, guanosine 5′-*O*-(3-thiotriphosphate); GCA, GCB, and GCC, guanylyl cyclase A, B, and C; IBMX, isobutylmethylxanthine; ICB, 10 mM HEPES containing 0.14 M KCl, 0.01 M NaCl, and 2.4 mM $MgCl_2$; K_i, concentration of adenine nucleotide yielding half-maximal inhibition of GCC; 2MeSADP, 2-methylthioadenosine diphosphate; 2MeSATP, 2-methylthioadenosine triphosphate; NaOAc, sodium acetate; NaOH, sodium hydroxide; TEED, 50 mM Tris-HCl (pH 7.6) containing 1 mM dithiothreitol, 1 mM EDTA, 1 mM EGTA, and 1 mM phenylmethanesulfonyl fluoride.

0006-2960/96/0435-3213$12.00/0

exhibited the greatest specificity and potency for inhibition of GCC (Boyer et al., 1989). These receptors bind extracellular adenine nucleotides and initiate a cascade involving activation of a G protein and, subsequently, phospholipase C (Boyer et al., 1989). Thus, one working hypothesis suggests that extracellular adenine nucleotides bind to a purinergic receptor which may mediate allosteric inhibition of GCC by activating a GTP binding protein. It is notable that a role for guanine nucleotides and proteins binding these nucleotides in regulating mammalian receptor guanylyl cyclases has not been demonstrated previously.

The current studies examine the role of extracellular receptors in mediating adenine nucleotide inhibition of GCC. They demonstrate that adenine nucleotides require access to the intracellular compartment to inhibit GCC signaling, ruling out a role for purinergic receptors in this process. Also, these studies examine the role of guanine nucleotides in regulating the allosteric inhibition of GCC by adenine nucleotides. They demonstrate that the potency of adenine nucleotides to inhibit GCC is dependent on the concentration of GTP and is enhanced by hydrolysis-resistant GTP analogues. Furthermore, GTP, in the absence of adenine nucleotides, also inhibits GCC. Guanine nucleotides regulate adenine nucleotide inhibition of GCC in human intestinal cells and in monkey kidney epithelial cells heterologously expressing rat GCC. Thus, the shift in GCC from positive to negative cooperativity observed previously in the presence of adenine nucleotides is consistent with a sequential model in which activation of an intracellular ATP binding site initiates inhibition of GCC by activating a GTP-dependent allosteric mechanism.

MATERIALS AND METHODS

Cell Culture and Membrane Preparation. Caco-2 cells (ATCC) were grown in an atmosphere of 5% CO_2 at 37 °C in DMEM/F12 (GIBCO, Grand Island, NY) supplemented with 10% fetal bovine serum and subcultured upon confluence (Cohen et al., 1993; Vaandrager et al., 1992). 293 cells stably transfected with rat GCC were grown under identical conditions with 200 μg/mL G418 (Schulz et al., 1990; Vaandrager et al., 1993a,b). Confluent cultures were washed 3 times with TEED buffer to remove residual culture media. Cells were removed from 150 cm^2 flasks by scraping into 5−10 mL of TEED buffer. The suspended cells were homogenized by 10 strokes with a Dounce homogenizer at 10 000 rpm. Homogenates were centrifuged at 100000*g* × 60 min, and the resulting pellet was resuspended in TEED at a protein concentration of 1−2 mg/mL. Membranes were stored in aliquots at −70 °C. These membranes contained guanylyl cyclase activity stimulated by ST, characteristic of GCC. In contrast, this guanylyl cyclase activity was not stimulated by atrial natriuretic peptide nor C-type natriuretic peptide (10^{-5} M) in the presence or absence of 1 mM ATP, confirming the absence of GCA and GCB in these preparations (Vaandrager et al., 1992; Fulle & Garbers, 1994).

Guanylyl Cyclase Assay. Guanylyl cyclase activity was assayed as described previously (Parkinson et al., 1994). Membranes were incubated at 37 °C in a final volume of 100−400 μL in the presence of 50 mM Tris-HCl (pH 7.6) containing 500 μM IBMX, 7.5 mM creatine phosphate/20 μM creatine phosphokinase, GTP, manganese or magnesium chloride (3 mM in excess of nucleotide), and adenine nucleotide, where indicated. Reactions were initiated by addition of membrane, incubated 5 min, and terminated by adding sodium acetate to a final concentration of 50 mM (pH 4.0) and boiling for 5 min. Following acetylation, samples were diluted with 50 mM sodium acetate (pH 4.0) containing 20 mM $CaCl_2$ (Patrinellis et al., 1994). Cyclic GMP was quantified by radioimmunoassay, as described previously (Parkinson et al., 1994). Radioimmunoassays were performed in triplicate and enzyme reactions in duplicate, and results are representative of at least 3 experiments. Enzyme activity was linear with respect to protein concentration and time for all experiments.

Assay of cGMP Accumulation in Intact and Permeabilized Caco-2 Cells. Caco-2 cells were seeded on 24-well plates (Corning Life Sciences, Corning, NY) and grown to confluence. Prior to use, cells were washed 3 times with ICB, followed by preincubation for 30 min in ICB containing a nucleotide regeneration system (7.5 mM creatine phosphate/20 μM creatine phosphokinase), 2 mM 2MeSATP where indicated, and 20 μM digitonin where indicated. Reactions were initiated by the addition of 0.5 mM Mg^{2+}-GTP, and 1 μM ST where indicated, incubated for 15 min, and terminated by collecting the extracellular contents into a final volume of 500 μL of 50 mM NaOAc (pH 4.0; extracellular cGMP) and adding 200 μL of 1 M NaOH to the remaining contents of each well (intracellular cGMP). Intracellular fractions were collected, acidified with 25 μL of 10 M HCl, and centrifuged for 15 min at 4 °C, and recovered supernates were neutralized in a final volume of 500 μL with 50 mM NaOAc (pH 4.0). Extracellular and intracellular fractions were acetylated and cGMP quantified by radioimmunoassay, as described above (Parkinson et al., 1994).

Miscellaneous. Protein concentration was quantified by the method of Bradford (Bio-Rad, Melville, NY) using bovine serum albumin as standard. All nucleotides were obtained from Sigma, except 2MeSATP and 2MeADP, which were from Research Biochemicals International (Natick, MA). Data are representative of at least 3 experiments, each performed in duplicate or triplicate, unless otherwise indicated.

RESULTS

Adenine Nucleotides Inhibit GCC in Caco-2 Human Intestinal Epithelial Cells in an Allosteric Fashion. Previously, adenine nucleotides were demonstrated to inhibit GCC in rat intestinal mucosa membranes in a concentration-dependent and saturable fashion when either magnesium or manganese served as the cation cofactor (Parkinson et al., 1994). Inhibition of GCC-associated guanylyl cyclase activity was mediated by both a competitive mechanism, with an increase in the $S_{0.5}$, and a noncompetitive allosteric mechanism, with a decrease in the V_{max}. It was suggested that the 2-substituted adenine nucleotides, 2MeSATP and 2ClATP, may allosterically inhibit GCC by interacting with extracellular purinergic P_2 receptors, since those nucleotides are potent agonists for these receptors (Parkinson et al., 1994).

In order to further investigate this hypothesis, the human intestinal epithelial cell line Caco-2 was employed as a model. These cells express GCC only, compared to other receptor guanylyl cyclases, and, therefore, represent a system in which the regulation of GCC alone may be examined in intestinal cells *in vitro* (Vaandrager et al., 1993b). Guanylyl

Table 1: Effects of Adenine Nucleotides on the Kinetic Parameters of Guanylyl Cyclase C in Caco-2 Membranes Employing Mn^{2+}-GTP as the Substrate

[2MeSATP] (mM)	V_{max}[a]	$S_{0.5}$[b]
none	136 ± 18[c]	0.2 ± 0.02
1	24 ± 2	1.4 ± 0.5

[a] V_{max} = picomoles of cGMP produced per minute per milligram of protein. [b] $S_{0.5}$ = mM Mn^{2+}-GTP. [c] Mean ± SD (n = 3).

cyclase activity in Caco-2 membranes increased in a concentration-dependent and saturable fashion when Mn^{2+}-GTP was employed as the substrate and 2MeSATP (1 mM) reduced the V_{max} of this enzyme >80% (Table 1). Similarly, this 2-substituted adenine nucleotide decreased the V_{max} of basal and ST-stimulated guanylyl cyclase activity about 50% in Caco-2 membranes when magnesium was employed as the cation cofactor (data not shown). These data demonstrate that 2MeSATP inhibits human guanylyl cyclase C, in part, through a noncompetitive allosteric mechanism, in close agreement with earlier observations of the effects of adenine nucleotides on rat GCC (Parkinson et al., 1994). As demonstrated previously, inhibition of GCC by adenine nucleotides is optimal employing manganese as the cation cofactor, presumably because this cation constitutively activates that enzyme in a ligand-independent fashion (Waldman & Murad, 1987; Marala et al., 1991; Parkinson et al., 1994). Therefore, Mn^{2+}-GTP was employed as the substrate in the studies described herein.

Regulation of GCC by Adenine Nucleotides Is Mediated by an Intracellular Mechanism. (A) Adenine Nucleotides Do Not Affect ST-Stimulated cGMP Accumulation in Intact Cells. The role of extracellular receptors in mediating adenine nucleotide inhibition of GCC was defined by examining the effect of 2MeSATP on ST-induced cGMP accumulation in intact Caco-2 cells. Thus, if adenine nucleotides inhibit GCC by binding to purinergic receptors, then they should decrease cGMP accumulation when applied extracellularly to intact Caco-2 cells. ST increased cGMP concentrations in Caco-2 cells >100-fold, in close agreement with previous reports (Cohen et al., 1993; Vaandrager et al., 1992). 2MeSATP (2 mM) did not alter the accumulation of cGMP induced by ST in intact Caco-2 cells when this nucleotide was included in incubations up to 15 min (Figure 1A, left panel). Caco-2 cells are well-differentiated and polarized, demonstrating distinct apical and basolateral surfaces (Pinto et al., 1983). In confluent cultures, experimental agents might only have access to the apical surface of cells. If purinergic receptors were localized in basolateral membranes, their interaction with adenine nucleotides might be limited *in vitro*, resulting in no apparent effect of these nucleotides on cGMP accumulation in intact cells. In contrast, receptor-mediated effects could be observed in membranes prepared from these cells since such preparations contain apical and basolateral components. Therefore, the effects of 2MeSATP on ST-induced cGMP accumulation was examined employing Caco-2 cells grown to confluence on Millicell-PCF inserts (Millipore, Bedford, MA), which permits access of experimental agents to the basolateral and/or apical surfaces of attachment-dependent cells (Figure 1B). ST-induced cGMP accumulation was maximal when this ligand was applied to the apical, compared to the basolateral, surface of Caco-2 cells, consistent with the previous observations that GCC is expressed almost exclusively in apical membranes of intestinal mucosal cells (Guarino et al., 1987; Almenoff et al., 1993). 2MeSATP had no effect on basal or ST-induced accumulation of cGMP when applied apically or basolaterally to confluent cultures of Caco-2 cells (Figure 1B).

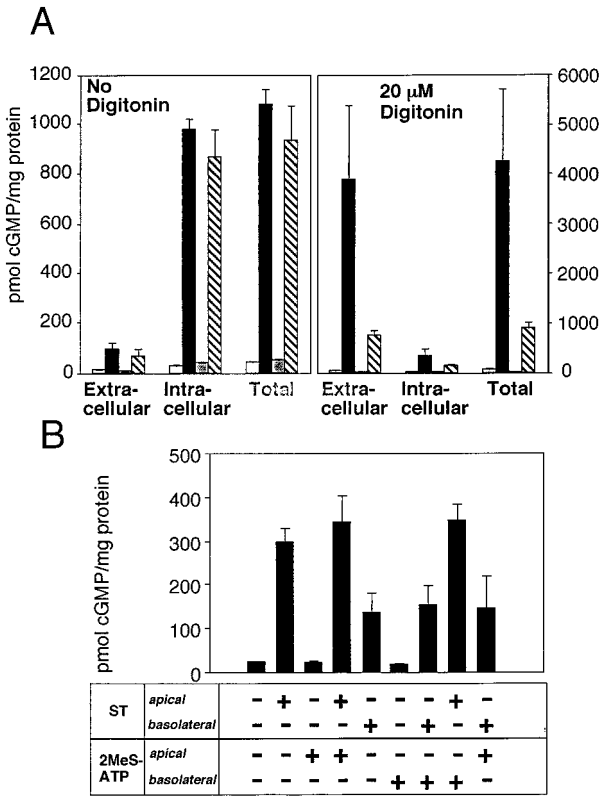

FIGURE 1: Effect of 2MeSATP on ST-induced accumulation of cGMP in intact and digitonin-permeabilized Caco-2 cells. Panel A: Effect of 2MeSATP (2 mM) on basal and ST (1 μM)-induced cGMP accumulation in intact (no digitonin) or permeabilized (20 μM digitonin) confluent Caco-2 cultures. Cultures were preincubated for 15 min with ICB containing IBMX (120 μM) and a nucleotide triphosphate regeneration system, in the absence or presence of 20 μM digitonin. Assays were initiated by the addition of 500 μM Mg^{2+}-GTP and either buffer alone (open bars) or buffer containing 1 μM ST (closed bars). Some cultures received 2 mM 2MeSATP during preincubation, prior to initiation in the absence (shaded bars) or presence (hatched bars) of ST. Panel B: Effect of 2MeSATP (2 mM) on basal and ST-induced accumulation of cGMP in intact Caco-2 cells. Cells were grown to confluence on filters and preincubated as above, and the assay was initiated by the addition of ST (1 μM) to either the apical or the basolateral surface of the cells. Results are expressed as the mean ± SEM of 3 separate experiments performed in duplicate.

(B) Adenine Nucleotides Inhibit ST-Stimulated cGMP Accumulation in Digitonin-Permeabilized Caco-2 Cells. The above data suggest that 2MeSATP inhibition of GCC is not mediated by apical or basolateral extracellular purinergic receptors but, rather, by an intracellular adenine nucleotide-dependent mechanism. Thus, the ability of 2MeSATP to inhibit ST-induced accumulation of cGMP was compared in intact and digitonin-permeabilized Caco-2 cells (Figure 1A). Accumulation of cGMP was detected primarily in the intracellular compartment of intact Caco-2 cells, demonstrating that the integrity of the plasma membrane was preserved during these studies. In the presence of 20 μM digitonin, Caco-2 cells remained attached to the plate, and cGMP accumulation was detected primarily in the extracellular media, demonstrating that the cells were efficiently permeabilized. As demonstrated earlier, 2MeSATP had no effect

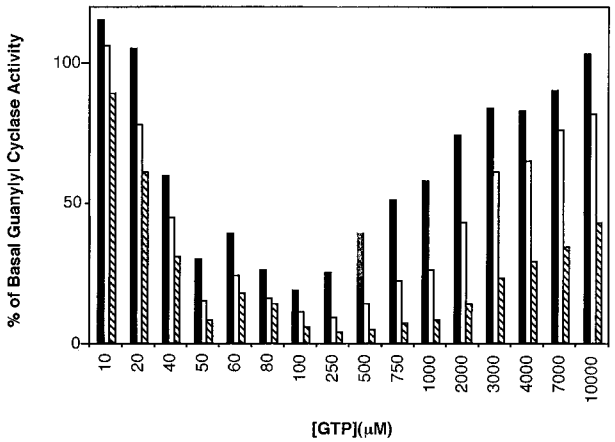

FIGURE 2: Effect of increasing substrate (Mn^{2+}-GTP) concentration on the inhibition of GCC in Caco-2 membranes induced by 2MeSATP. Guanylyl cyclase activity in Caco-2 membranes was measured using a range of Mn^{2+}-GTP concentrations in the absence or presence of 100 μM (filled bars), 300 μM (open bars), or 1 mM (striped bars) 2MeSATP. Resultant activities were plotted as the percent of basal (in the absence of 2MeSATP) guanylyl cyclase activity, to demonstrate the biphasic effect of GTP on the potency of 2MeSATP to inhibit GCC. Results are representative of 4 experiments using 2 separate membrane preparations.

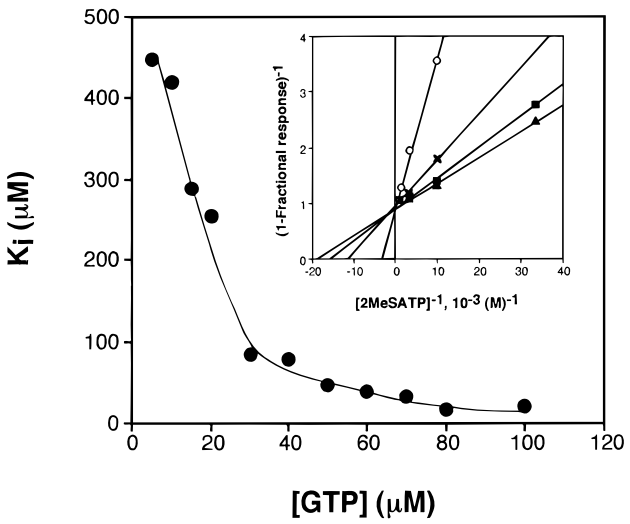

FIGURE 3: Effect of GTP on the K_i of 2MeSATP inhibition of GCC. Guanylyl cyclase activity was measured at selected GTP concentrations in the presence of varying concentrations of 2MeSATP. K_i values were obtained from these data by determining the *x*-axis intercept of the double-reciprocal plots of guanylyl cyclase activity versus 2MeSATP concentration at each GTP concentration [isotherms obtained with 20 μM (open circles), 50 μM (crosses), 70 μM (closed squares), and 100 μM (closed triangles) GTP demonstrated in inset]. Values shown are the mean of 2 to 6 experiments at each GTP concentration using 3 different membrane preparations.

on ST-induced cGMP accumulation in intact Caco-2 cells (Figure 1A, left panel). In contrast, this adenine nucleotide inhibited ligand-stimulated cyclic nucleotide accumulation approximately 80% in digitonin-permeabilized Caco-2 cells (Figure 1A, right panel). Permeabilization of Caco-2 cells did not result in loss of cytosolic factors which block 2MeSATP inhibition of GCC, since the effects of this adenine nucleotide on concentrated cell homogenates were identical to those on membrane preparations (data not shown). These data demonstrate that 2MeSATP inhibits GCC when this nucleotide has access to the cytoplasmic, but not the extracellular, compartment. They strongly support the suggestion that adenine nucleotide inhibition of GCC is mediated by an intracellular adenine nucleotide binding site rather than extracellular purinergic receptors.

Adenine Nucleotide Inhibition of GCC Is Guanine Nucleotide-Dependent. (A) Guanine Nucleotides Potentiate Inhibition of GCC by Adenine Nucleotides. Allosteric inhibition of rat GCC by adenine nucleotides is associated with a shift in substrate kinetics from positive to negative cooperativity (Parkinson et al., 1994). One possible explanation for this observation is that adenine nucleotide inhibition of GCC is a guanine nucleotide-dependent process. Therefore, the effect of GTP upon inhibition of human GCC by 2MeSATP was examined in Caco-2 membranes. Inhibition of GCC by 2MeSATP was dependent on the concentration of Mn^{2+}-GTP in the incubation (Figure 2). Indeed, the ability of 2MeSATP to inhibit guanylyl cyclase increased in a concentration-dependent fashion as the Mn^{2+}-GTP concentration increased from 10 to 100 μM. These data strongly support the suggestion that adenine nucleotide inhibition is, in large part, allosteric, since increasing substrate concentrations potentiate greater inhibition (Parkinson et al., 1994). In contrast, if the effects of 2MeSATP were competitive, increasing concentrations of GTP should reduce, not potentiate, inhibition of GCC over this concentration range. In addition, these studies support the suggestion that inhibition of GCC by adenine nucleotides requires guanine nucleotides since significant inhibition could not be detected at concentrations of Mn^{2+}-GTP <20 μM, although cGMP production was linear with respect to time at those substrate concentrations. At concentrations of substrate >250 μM, the ability of 2MeSATP to inhibit GCC apparently decreased in a concentration-dependent fashion. This latter observation will be examined in further detail below.

Table 2: K_i for 2MeSATP Inhibition of GCC Endogenously Expressed in Caco-2 Membranes in the Presence of Increasing Concentrations of Mn^{2+}-GTP or Mn^{2+}-GTPγS

guanine nucleotide concn (μM)	K_i for 2MeSATP (μM)	
	GTP	GTPγS
5	ND[a]	76 ± 20
10	420 ± 174[b]	55 ± 19
20	255 ± 153	26 ± 8
50	53 ± 23	27 ± 5
100	22 ± 6	ND

[a] Not determined. [b] Mean ± SD (n = 3).

Regulation of adenine nucleotide inhibition of GCC by guanine nucleotides was further examined by quantifying the potency of 2MeSATP to inhibit this enzyme at increasing concentrations of Mn^{2+}-GTP (Figure 3). The potency of 2MeSATP to inhibit GCC was quantified by determining its K_i (Figure 3, inset). Mn^{2+}-GTP increased the potency of 2MeSATP in a concentration-dependent fashion, decreasing the K_i of this adenine nucleotide 20-fold (Figure 3, Table 2). The isotherm of the alterations of the K_i of 2MeSATP versus substrate concentration was curvilinear, with the greatest change in K_i occurring at Mn^{2+}-GTP concentrations ≤30 μM (Figure 3). These data further demonstrate that adenine nucleotide inhibition of GCC is mediated by an allosteric mechanism which is regulated by guanine nucleotides. Indeed, at low GTP concentrations (<20 μM), the isotherm appears asymptotic to the *y*-axis ($K_i = \infty$ when GTP concentrations are infinitely low), suggesting that GTP may be absolutely required for 2MeSATP inhibition of GCC. Enzyme activity was linear with respect to time at all

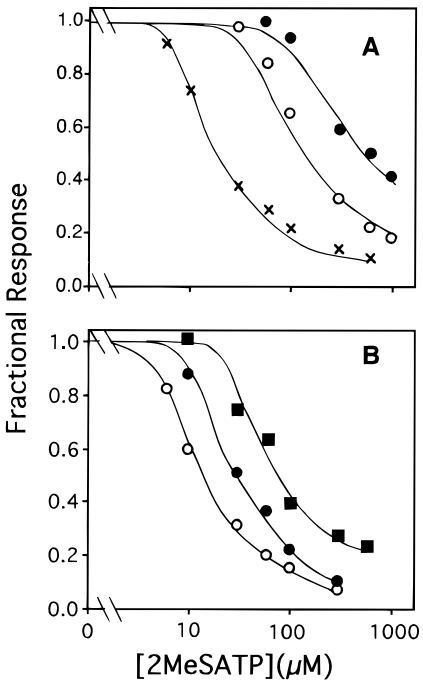

FIGURE 4: Effect of 2MeSATP on GCC employing either Mn^{2+}-GTP or Mn^{2+}-GTPγS as substrate. Guanylyl cyclase activity was measured in the presence of varying concentrations of 2MeSATP with Mn^{2+}-GTP (upper panel) or Mn^{2+}-GTPγS (lower panel) supplied as substrate. Values from the experiment shown are expressed as a fraction of the guanylyl cyclase activity in the absence of 2MeSATP. Concentrations of substrate used were 5 μM (closed squares), 10 μM (closed circles), 20 μM (open circles), and 50 μM (crosses). Basal activities for 10, 20, and 50 μM GTP were 3.46, 10.98, and 56.55 pmol of cGMP min^{-1} (mg of protein)$^{-1}$, respectively. Basal activities for 5, 10, and 20 μM GTPγS were 8.05, 29.77, and 88.00 pmol of cGMP min^{-1} (mg of protein)$^{-1}$, respectively. Results are representative of 3 experiments employing 3 separate membrane preparations.

substrate concentrations examined, demonstrating that these observations reflect guanine nucleotide regulation of GCC rather than catalytic activity limited by substrate availability.

(B) Nonhydrolyzable Analogues of GTP Are Most Potent in Supporting 2MeSATP Inhibition of GCC. At concentrations of substrate <100 μM, the ability of 2MeSATP to inhibit GCC is potentiated by increasing GTP concentrations. One mechanism by which guanine nucleotides regulate a variety of signaling processes involves their interaction with G proteins which couple ligand−receptor interaction to effector activation (Gilman et al., 1987; Birnbaumer et al., 1990; Neer, 1994). A central feature of processes mediated by such proteins is their potentiation by nonhydrolyzable GTP analogues, such as GTPγS (Gilman et al., 1987; Birnbaumer et al., 1990; Neer, 1994). Interestingly, Mn^{2+}-GTPγS was a more potent substrate, compared to Mn^{2+}-GTP, for GCC in Caco-2 membranes [$S_{0.5}$: 24 ± 7 μM (GTPγS), 108 ± 5 μM (GTP)]. However, GCC exhibited a lower V_{max} employing Mn^{2+}-GTPγS, compared to Mn^{2+}-GTP, as the substrate [77 ± 12 versus 153 ± 22 pmol of cGMP min^{-1} (mg of protein)$^{-1}$, respectively]. Similar results were obtained when magnesium was employed as the substrate cofactor (data not shown). Catalytic activity was linear with respect to time at all concentrations of Mn^{2+}-GTPγS examined in these studies.

Since Mn^{2+}-GTPγS supported catalytic activity, the effect of this guanine nucleotide on 2MeSATP inhibition of GCC was compared to Mn^{2+}-GTP (Figure 4A,B, Table 2).

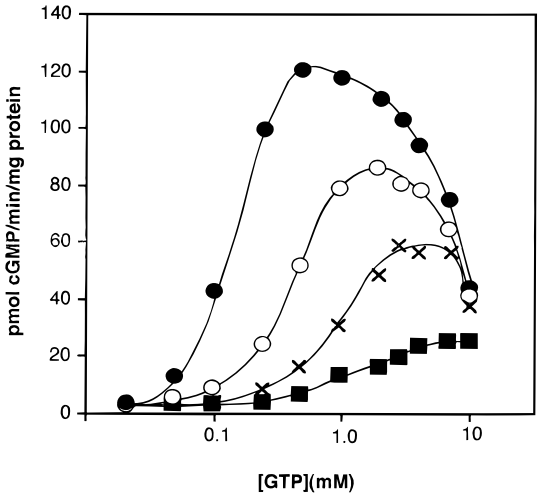

FIGURE 5: Effect of 2MeSATP on GCC catalytic activity in the presence of high concentrations of Mn^{2+}-GTP. Guanylyl cyclase activity in Caco-2 membranes was measured using a range of substrate concentrations in the absence (closed circles) or presence of 100 μM (open circles), 300 μM (crosses), or 1 mM (closed squares) 2MeSATP. Results are representative of 4 experiments using 2 separate membrane preparations.

2MeSATP inhibited GCC in a concentration-dependent fashion when either GTP or GTPγS was employed as the substrate. The potency of 2MeSATP to inhibit GCC was enhanced with increasing concentrations of either guanine nucleotide, in close agreement with results presented above (Figures 2 and 3, Table 2). The K_i of 2MeSATP decreased an order of magnitude when the GTP concentration was increased from 10 to 50 μM (Figure 4A, Table 2). However, GTPγS exhibited a greater potency in supporting adenine nucleotide inhibition of GCC compared to GTP. Indeed, the K_i for 2MeSATP was shifted an order of magnitude lower in the presence of 10 μM GTPγS compared with 10 μM GTP (Figure 4A,B, Table 2). The enhanced potency of 2MeSATP to inhibit GCC in the presence of GTPγS does not reflect increased catalytic activity at lower substrate concentrations since concentrations of guanine nucleotide yielding comparable basal catalytic activities (20 μM GTP, 5 μM GTPγS) resulted in significantly different K_i's for 2MeSATP (Table 2). These data demonstrate that 2MeSATP inhibition of GCC is supported by the hydrolysis-resistant analogue GTPγS, which is 5−10-fold more potent compared to GTP. That the nonhydrolyzable guanine nucleotide analogue GTPγS preferentially supports this inhibition suggests a role for a G protein possessing nucleotide phosphohydrolase activity in the regulation of GCC (Gilman et al., 1987; Birnbaumer et al., 1990; Neer, 1994).

(C) Guanine Nucleotides Inhibit GCC in the Absence of Adenine Nucleotides. At concentrations of GTP >250 μM, 2MeSATP exhibited a reduced ability to inhibit GCC in Caco-2 membranes (Figure 2). The decreased ability of 2MeSATP to inhibit GCC at higher substrate concentrations could reflect the ability of guanine nucleotides alone to inhibit that enzyme by a mechanism which is convergent with that of adenine nucleotides. Thus, the effect of higher concentrations of Mn^{2+}-GTP on guanylyl cyclase activity in Caco-2 membranes was examined. As demonstrated previously, catalytic activity increased in a concentration-dependent fashion, achieving an apparent V_{max} at 500 μM GTP (Figure 5). However, at concentrations >1 mM, GTP decreased the V_{max} of GCC in a concentration-dependent fashion (Figure

5). In some experiments, the maximum activity of GCC was reduced 85% by GTP alone. This inhibition produced by increasing substrate concentrations is quantitatively similar to that achieved in the presence of 1 mM 2MeSATP. These observations demonstrate that at higher concentrations, GTP inhibits GCC in the absence of adenine nucleotides. Similar results with concentrations of GTP >1 mM were obtained with rat intestinal membranes (data not shown).

(D) Inhibition of GCC by Guanine Nucleotides Is Mediated by a Mechanism Which Is Convergent with That by Adenine Nucleotides. In the presence of increasing concentrations of Mn^{2+}-GTP which directly inhibit GCC, a concentration-dependent reduction in the inhibition produced by 2MeSATP was observed (Figure 2). Thus, maximum concentration-dependent inhibition of GCC by 2MeSATP was observed at concentrations of substrate which yielded apparent maximum catalytic activity (500 μM). However, at concentrations of Mn^{2+}-GTP >1 mM, which inhibited GCC in the absence of adenine nucleotides, there was a substantial reduction in the ability of 2MeSATP to inhibit this enzyme. Indeed, at 10 mM GTP, which yielded a 70% decrease in the V_{max} of GCC in the absence of 2MeSATP, little inhibition of this enzyme by 2MeSATP was observed (Figure 5). These data suggest that inhibition of GCC by GTP alone and that obtained with 2MeSATP in the presence of GTP occur through a common mechanism, since these effects were not additive. Furthermore, the superphysiological concentration of GTP (10 mM) required to observe significant inhibition of GCC is 100-fold in excess of the optimal GTP concentration (0.1 mM) required to support 2MeSATP inhibition of GCC (Figure 3). Therefore, these observations suggest that GTP acts downstream of 2MeSATP to inhibit GCC, since 2MeSATP is dependent on the presence of GTP while GTP can independently inhibit GCC.

Adenine Nucleotides Inhibit Recombinant Rat GCC Heterologously Expressed in 293 Cells in a Guanine Nucleotide-Dependent Fashion. The above and previous studies demonstrated that rat and human GCC are allosterically inhibited by adenine nucleotides in a guanine nucleotide-dependent fashion in native intestinal cells (Parkinson et al., 1994). Guanine nucleotide-dependent adenine nucleotide inhibition also was examined employing recombinant rat GCC heterologously expressed in 293 monkey kidney cells (Schulz et al., 1990; Vaandrager et al., 1993a,b). Indeed, 2MeSATP inhibited recombinant rat GCC in a concentration-dependent fashion (Figure 6A). Inhibition of recombinant GCC by adenine nucleotide was mediated by both competitive and allosteric mechanisms since the $S_{0.5}$ increased but the V_{max} decreased in the presence of 2MeSATP, in close agreement with results obtained with native intestinal rat GCC and human GCC expressed in Caco-2 cells (Table 3; Figure 5; Parkinson et al., 1994). The potency of inhibition of GCC by adenine nucleotide was guanine nucleotide-dependent, and the K_i of 2MeSATP decreased from 223 ± 25 to 107 ± 21 μM (n = 5) as the GTP concentration increased from 10 to 100 μM, supporting the suggestion that inhibition by adenine nucleotides was mediated, in part, by an allosteric mechanism (Figure 6A). In addition, concentrations of GTP >1 mM decreased the V_{max} of GCC exogenously expressed in 293 cells in the absence of adenine nucleotides (Figure 6B). Furthermore, the effects of adenine and guanine nucleotides on rat GCC expressed in 293 cells were not additive, suggesting that inhibition by these nucleotides was mediated by a convergent mechanism (Figure 6B). These data support the observations obtained with native intestinal membranes and Caco-2 cells that adenine nucleotides inhibit GCC, in part, through an allosteric guanine nucleotide-dependent mechanism. They also support the suggestion that guanine nucleotides influence this inhibitory pathway downstream from the action of adenine nucleotides. Interestingly, although the effects of adenine and guanine nucleotides on heterologously expressed recombinant rat GCC were qualitatively similar to those on native human and rat GCC endogenously expressed, they were quantitatively attenuated in 293 cells compared to results obtained with both rat intestinal membranes and Caco-2 cells. These quantitative differences may reflect the overexpression of GCC in 293

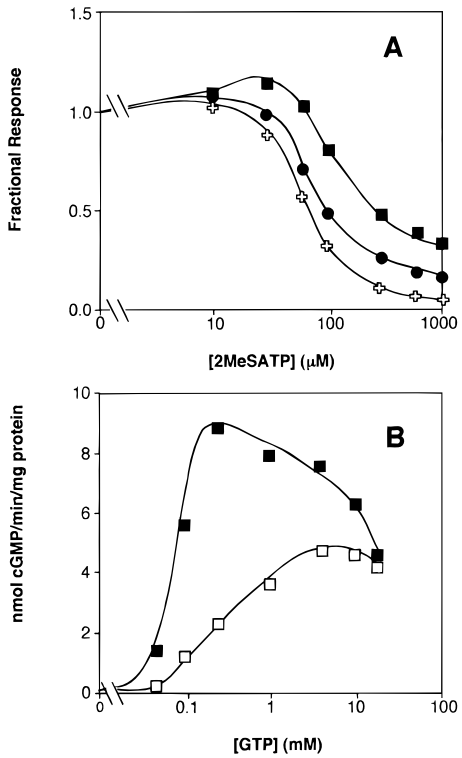

FIGURE 6: Effect of 2MeSATP on rat GCC heterologously expressed in 293 monkey kidney cells. Panel A: Concentration response of recombinant GCC to 2MeSATP employing 3 different concentrations of GTP. Guanylyl cyclase activity was measured in the presence of varying concentrations of 2MeSATP and either 10 μM (closed squares), 20 μM (closed circles), or 100 μM (crosses) Mn^{2+}-GTP. Values are expressed as a fraction of the guanylyl cyclase activity in the absence of 2MeSATP. Basal activities for 10, 50, and 100 μM Mn^{2+}-GTP were 114, 633, and 3476 pmol of cGMP min^{-1} (mg of protein)$^{-1}$, respectively. Results are representative of 3 experiments employing 3 separate membrane preparations. Panel B: Effect of 2MeSATP on recombinant GCC catalytic activity in the presence of high concentrations of Mn^{2+}-GTP. Guanylyl cyclase activity was measured using a range of substrate concentrations in the absence (open squares) or presence (closed squares) of 2 mM 2MeSATP. Results are representative of 4 experiments using 2 separate membrane preparations.

Table 3: Effects of Adenine Nucleotides on the Kinetic Parameters of Rat Guanylyl Cyclase C Heterologously Expressed in Monkey Kidney 293 Cells Employing Mn^{2+}-GTP as the Substrate

[2MeSATP] (mM)	V_{max}[a]	$S_{0.5}$[b]
none	7.81 ± 0.71[c]	0.16 ± 0.05
1	3.60 ± 0.72	1.24 ± 0.77

[a] V_{max} = nanomoles of cGMP produced per minute per milligram of protein. [b] $S_{0.5}$ = mM Mn^{2+}-GTP. [c] Mean ± SD (n = 3).

cells, in which the V_{max} of the enzyme is 50−100 times higher than in membranes from Caco-2 cells. Alternatively, those differences may reflect species-specific regulation of human and rat GCC by nucleotides. The V_{max} of GCC in rat intestinal membranes is lowered >70% by 10 mM GTP (data not shown), suggesting that quantitative differences are not due to species variation in the responsiveness of the enzyme. Finally, quantitative differences may reflect limited expression of factors required for nucleotide inhibition in 293 kidney compared to intestinal cells.

DISCUSSION

Previous studies demonstrated that GCC exhibited dual regulation by adenine nucleotides. ATP and its analogues are required for maximal activation of guanylyl cyclase activity by ligands of this protein including ST (Chinkers et al., 1991; Fulle & Garbers, 1994). This effect is presumably mediated by adenine nucleotide interaction with the intracellular kinase homology domain, a cognate domain found in all receptor guanylyl cyclases identified to date (Chinkers & Garbers, 1989; Goraczniak et al., 1992; Fulle & Garbers, 1994). Although the precise molecular mechanism is unclear, ATP appears to potentiate ligand activation of GCC by delaying ST-induced desensitization of this protein (Vaandrager et al., 1993a). Adenine nucleotides also inhibit basal and ligand-activated GCC (Gazzano et al., 1991b; Marala et al., 1991; Parkinson et al., 1994). Most adenine nucleotides inhibit Mn^{2+}-stimulated enzyme activity, whereas only the 2-substituted analogues, 2MeSATP and 2ClATP, inhibit GCC when either divalent cation is employed as the substrate cofactor (Parkinson et al., 1994). Inhibition of GCC by these nucleotides is mediated, in part, by an allosteric mechanism, since they markedly decrease the V_{max} of this enzyme (Parkinson et al., 1994). The inhibition of GCC by adenine nucleotides appears to be mediated by a site on the enzyme which is pharmacologically distinct from that mediating nucleotide activation (Parkinson et al., 1994). Indeed, nucleotides which are specific activators of GCC, such as AMPS, did not alter the potency of 2MeSATP to inhibit GCC. Similarly, 2MeSATP did not alter the potency of AMPS to potentiate the activation of this enzyme by ST.

These studies demonstrated that GCC is allosterically regulated by adenine nucleotides, regardless of the divalent cation employed, by a mechanism which is pharmacologically distinct from activation by those nucleotides. It was suggested that adenine nucleotide inhibition might be mediated by purinergic receptors, since the 2-substituted nucleotides, 2MeSATP and 2ClATP, are specific inhibitors of GCC and are highly specific ligands for the P_{2y} purinergic receptors (Boyer et al., 1989). The present studies examined the role of cell surface receptors in mediating inhibition of GCC by adenine nucleotides. These studies demonstrated that 2-substituted adenine nucleotides did not alter GCC activity in intact intestinal cells. However, once these nucleotides accessed the cytoplasmic compartment in digitonin-permeabilized intestinal cells, they were potent inhibitors of basal and ST-stimulated GCC activity. These studies excluded a role for cell surface purinergic receptors in mediating inhibition of GCC by adenine nucleotides. Rather, they suggest that an intracellular adenine nucleotide binding site mediates this effect. The precise nature of this adenine nucleotide binding site is currently being examined. Potential candidates for these sites include intracellular protein kinases. However, 2MeSADP and AMPPNP, which are not kinase substrates, inhibit GCC in a comparable fashion compared with 2MeSATP (data not shown). Alternatively, 2MeSATP and other inhibitory adenine nucleotides could inhibit a kinase required for enzyme activity or activate a phosphatase to inhibit GCC catalytic activity. Other posttranslational modifications of GCC must also be considered.

Previous studies also demonstrated that adenine nucleotide inhibition of GCC was associated with a characteristic shift in the kinetics of the enzyme from positive to negative cooperativity (Parkinson et al., 1994). This shift in the kinetics of GCC induced by adenine nucleotides suggested that inhibition of guanylyl cyclase by 2MeSATP was GTP-dependent. Thus, if 2MeSATP inhibition of GCC is guanine nucleotide-dependent, then an increase in substrate concentration would increase both the velocity of catalysis and the amount of inhibition induced by adenine nucleotides. An increase in both catalysis and inhibition could result in an apparent rate of increase of enzyme velocity that diminishes as a function of increasing substrate concentration. A diminishing rate of increase in the apparent velocity of catalysis as a function of substrate concentration would produce the curvilinear isotherms obtained by double-reciprocal analysis characteristic of negative cooperativity (Parkinson et al., 1994).

The present studies examined the role of guanine nucleotides in adenine nucleotide inhibition of GCC. At very low GTP concentrations, adenine nucleotides did not significantly inhibit GCC, although enzyme activity which was linear with respect to time could be measured at these substrate concentrations. As the GTP concentration increased up to 100 μM, there was a concomitant increase in the inhibition of GCC induced by 2MeSATP. Indeed, increasing GTP concentrations increased the potency of 2MeSATP to inhibit GCC about 20-fold. These studies demonstrate that inhibition of GCC by adenine nucleotides is allosteric, since inhibition is potentiated, rather than inhibited, by substrate. In addition, they demonstrate that adenine nucleotide inhibition of GCC is mediated by a guanine nucleotide-dependent mechanism, since inhibition is insignificant in the absence of, but potentiated by, increasing concentrations of GTP.

The observation that the potency of 2MeSATP to inhibit GCC is increased by GTP is consistent with the shift in substrate kinetics by adenine nucleotides from positive to negative cooperativity. In the absence of 2MeSATP, the catalytic rate of GCC increases correspondingly with the substrate concentration. When 2MeSATP is present, increasing GTP concentrations increase the catalytic rate simultaneously with the potency of 2MeSATP to inhibit catalysis. The resulting double-reciprocal plot of this relationship where GTP acts as both substrate and a required mediator of inhibition would be concave down, representing negative cooperativity. Indeed, this is the pattern of GCC kinetics observed in the presence of 2MeSATP (Parkinson et al., 1994).

The above studies demonstrate that adenine nucleotide inhibition of GCC is guanine nucleotide-dependent. Many intracellular guanine nucleotide-dependent functions are mediated by G proteins. Thus, G proteins are ubiquitous regulators of signal transducing mechanisms, including those mediated by receptor tyrosine kinases and adenylyl cyclases, which are structurally homologous with receptor guanylyl cyclases, including GCC (Fulle & Garbers, 1994). One

characteristic feature of G protein-regulated phenomena is their preferential utilization of nonhydrolyzable analogues of GTP, such as GTPγS (Gilman, 1987; Birnbaumer et al., 1990; Neer, 1995). G protein activity is maximal when GTP occupies the nucleotide binding site and minimal when GDP occupies that site. The activity of these proteins is regulated by their nucleotide phosphohydrolase activity, which converts GTP to GDP, terminating the G protein-mediated event. Nonhydrolyzable GTP analogues are preferred because they occupy the guanine nucleotide binding site, activating the G protein, but cannot be hydrolyzed to GDP. Thus, in the presence of GTPγS, G proteins remain in a constitutively activated conformation.

A role for G proteins in mediating adenine nucleotide inhibition of GCC was examined by employing GTPγS. In these studies, GTPγS fulfills the role of substrate and guanine nucleotide required for adenine nucleotide inhibition of GCC. The hydrolysis-resistant GTP analogue more efficiently supported 2MeSATP inhibition of GCC. The enhanced potency of 2MeSATP in the presence of GTPγS, compared with GTP, suggests a role for G proteins possessing nucleotide phosphohydrolase activity in this regulatory pathway (Gilman, 1987; Birnbaumer et al., 1990; Neer, 1995). Furthermore, these data suggest that the conformation of this regulatory protein which is active in supporting adenine nucleotide inhibition is bound to GTP rather than a metabolite (Gilman, 1987; Birnbaumer et al., 1990; Neer, 1995). The precise nature of the G protein which mediates adenine nucleotide inhibition of GCC remains unclear and is currently being examined. Preliminary studies indicate that GCC expressed in membranes preincubated with pertussis or cholera toxin retain the ability to be inhibited by 2MeSATP (data not shown). These data suggest that toxin-sensitive heterotrimeric G proteins, such as G_s and G_i, may not mediate adenine nucleotide inhibition of GCC (Neer, 1995). However, they do not rule out the possibility that this inhibition is mediated by toxin-insensitive heterotrimeric or other G proteins (Neer, 1995). Alternatively, 2MeSATP could initiate a change in affinity for a guanine nucleotide-dependent allosteric regulatory site directly on GCC. This is consistent with the cooperative nature of that enzyme.

The present studies demonstrate that higher concentrations of GTP diminish the ability of adenine nucleotides to inhibit GCC. This does not likely reflect competitive inhibition since (1) GTP, the catalytic substrate, appears to be required for this inhibition, (2) increasing concentrations of GTP up to 100 μM promote increasing inhibition by 2MeSATP, (3) GTPγS, also a catalytic substrate, preferentially potentiates inhibition of GCC by 2MeSATP, and (4) 2MeSATP dramatically reduces the V_{max} of GCC. Rather, these observations likely reflect the ability of higher concentrations of GTP to inhibit GCC. The present studies are the first to demonstrate substrate inhibition of a member of the family of receptor guanylyl cyclases. The precise molecular mechanism mediating this inhibition remains unclear. The current working hypothesis is that GTP directly interacts with and activates a G protein which mediates adenine nucleotide inhibition of GCC. That adenine nucleotides require GTP for inhibition of GCC but guanine nucleotides inhibit GCC in the absence of adenine nucleotides supports this hypothesis of sequential nucleotide inhibition. Alternatively, GTP might inhibit GCC by directly interacting with this protein. Presumably, this interaction would occur at a site on the protein other than the active site, since occupancy of that site should promote catalysis. The primary structure of GCC has been deduced, and potential nucleotide binding sites have been localized in the catalytic domain (active site) and the kinase homology domain (Schulz et al., 1990; deSauvage et al., 1991; Fulle & Garbers, 1994). The kinase homology domain has been suggested to mediate adenine nucleotide potentiation of ligand activation of receptor guanylyl cyclases (Chinkers & Garbers, 1989; Goraczniak et al., 1992; Fulle & Garbers, 1994). Previous studies demonstrated that adenine nucleotide activation and inhibition of GCC occur at pharmacologically distinct sites (Parkinson et al., 1994). Taken together, these data suggest that the kinase homology domain is not the site at which GTP inhibits GCC. Thus, a site on GCC which directly interacts with GTP to inhibit this enzyme is not readily obvious from structural studies of this protein, supporting the suggestion that the effects of guanine nucleotide on GCC may be mediated by a separate GTP binding protein.

The above discussion suggests a model for the mechanisms mediating guanine nucleotide-dependent adenine nucleotide inhibition of GCC. In this model, adenine nucleotide inhibition is initiated by associating with an intracellular adenine nucleotide binding factor. It is unlikely that adenine nucleotides inhibit GCC by binding directly to the enzyme for the reasons discussed above concerning lack of an identifiable nucleotide binding site on this protein to mediate that inhibition. The activity of the adenine nucleotide binding factor is mediated by a guanine nucleotide binding factor which directly inhibits GCC. The suggestion that the guanine nucleotide binding factor is acting downstream from the adenine nucleotide binding factor is supported by the observation that (1) GTP inhibits GCC in the absence of adenine nucleotides but 2MeSATP requires GTP to inhibit this enzyme and (2) adenine and guanine nucleotides inhibit GCC in a convergent fashion. That GTPγS, compared to GTP, is preferred to support adenine nucleotide inhibition suggests that the guanine nucleotide binding factor is bound to GTP in the active conformation. The nature of these nucleotide binding factors remains undefined and is currently being investigated. Indeed, it remains unclear if separate factors are required for adenine and guanine nucleotide regulation, or whether these nucleotides interact with a single component regulating GCC. If adenine and guanine nucleotide-dependent inhibition of GCC is mediated by distinct regulatory proteins, these proteins may be ubiquitously expressed since rat GCC exogenously expressed in 293 monkey kidney cells exhibited guanine nucleotide-dependent adenine nucleotide inhibition which was qualitatively similar to that observed in native intestinal cells from rat and human.

Previous studies suggested that guanylyl cyclases may be regulated by guanine nucleotides and G proteins. Thus, one defining characteristic of receptor guanylyl cyclases is positively cooperative substrate kinetics (Waldman & Murad, 1987). Indeed, as the concentration of GTP is increased, receptor guanylyl cyclases catalyze the conversion of this nucleotide to cGMP more efficiently. The precise mechanisms underlying positive cooperativity of receptor guanylyl cyclases has not been precisely elucidated, and whether G proteins play a role remains unclear. Also, studies suggest that membrane-bound guanylyl cyclase in *Dictyostelium* may be regulated by pertussis toxin-sensitive heterotrimeric G proteins (Hadwiger et al., 1994). Furthermore, previous

studies suggested that pertussis toxin treatment inhibited the ability of ST to activate GCC in rat intestinal membranes, implying a role for a heterotrimeric G protein in receptor−effector coupling in this system (Epstein et al., 1986). However, these studies have been difficult to reproduce in other laboratories (Crane et al., 1989). The studies presented herein demonstrate that in mammalian systems GCC is allosterically inhibited in a guanine nucleotide-dependent fashion by adenine nucleotides and suggest a role for nucleotide binding proteins in mediating that inhibition. Whether allosteric inhibition of GCC by nucleotides is a general mechanism regulating other members of the family of receptor guanylyl cyclases is currently being examined.

ACKNOWLEDGMENT

We thank Drs. Stephanie Schulz and David Garbers for their generous gift of 293 cells stably expressing rat GCC and Sajay Churi for technical assistance.

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BI9524326