Nucleotide Inhibitors and Activators of Retinal Guanylyl Cyclase†

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Received July 8, 1993; Revised Manuscript Received January 12, 1994®

ABSTRACT: The restoration of the dark state in retinal rod cells following illumination is due in part to resynthesis of cGMP. Retinal guanylyl cyclase specifically catalyzes the cyclization of GTP into cGMP *in vivo*. The reaction has been shown to involve the inversion of the configuration on the phosphate atom as demonstrated by conversion of the (S_P) isomer of GTPαS to (R_P)-cGMPS by guanylyl cyclase [Senter, P. D., Eckstein, F., Mülsch, A., & Böhme, E. (1983) *J. Biol. Chem. 258*, 6741–6745]. Since (R_P-cGMPS is not a substrate for retinal phosphodiesterase, we were able to measure cyclase activity with greater reliability using this novel assay as opposed to other published procedures. This assay has allowed us to reinvestigate the effects of adenylyl nucleotides on cyclase activity and to search for selective inhibitors of the rod-specific enzyme. We have measured the cyclase activity using homogenates of rod outer segments and a reconstituted system composed of guanylyl cyclase in washed rod outer segment membranes and the purified guanylyl cyclase activating protein. Our results indicate that 100–200 μM ATP (and other adenylyl nucleotides) stimulates guanylyl cyclase activity approximately 2-fold and that the observed stimulation of enzyme activity is independent of the free calcium concentration. In contrast to other particulate guanylyl cyclases, which are synergistically stimulated by a peptide ligand and ATP, guanylyl cyclase activating protein does not potentiate the effect of ATP, suggesting that retinal guanylyl cyclase may be regulated differently. ATP changes the V_{max} of retinal guanylyl cyclase without changing the K_m for (S_P)-GTPαS. We also found that guanosine tetrakisphosphate is a competitive inhibitor of retinal guanylyl cyclase with a K_i = 31 μM. Furthermore, organic cations competitively inhibit guanylyl cyclase activity in the following order: phosphodiesterase inhibitor dipyridamole (K_i = 65 μM), benzamidine (K_i = 229 μM), octylamine (K_i = 362 μM), and polylysine (K_i = 685 μM).

Transduction of a light impulse into a visual sensory signal, quenching of the signal, and restoration of the dark condition is accomplished through a cascade of enzymatically controlled reactions. The signal-amplifying cascade of phototransduction begins when light is absorbed by rhodopsin and the photoexcited rhodopsin molecule binds and activates a G protein, transducin [reviewed by Chabre and Deterre (1989), Hargrave and McDowell (1992), and Lagnado and Baylor (1992)]. A single absorbed photon generates several thousand GTP-bearing α-subunits of transducin (Gray-Keller et al., 1990), resulting in amplification of the light signal. The α-subunit of transducin stimulates phosphodiesterase (PDE) activity, which results in the hydrolysis of cGMP molecules and closure of cGMP-gated cation channels located in the plasma membrane. Quenching of photolyzed rhodopsin, transducin, and PDE is accomplished through a series of reactions that terminate the activation pathway of phototransduction [reviewed by Palczewski and Benovic (1991) and Hofmann et al. (1992)]. In order for the cation channels to reopen, cGMP must be resynthesized. It is generally accepted that calcium plays an important role in this process through its regulation of guanylyl cyclase (Lolley & Racz, 1982; Sather et al., 1988; Koch & Stryer, 1988). In the dark state, the free calcium concentration is high (approximately 500 nM) and guanylyl cyclase activity is low (Hodgkin & Nunn, 1988). Upon photoexcitation, the combined effects of the cGMP-gated cation channels (closure of the channels impedes calcium flow into the cytosol) and the Na^+-K^+/Ca^{2+} exchanger (continues to extrude calcium) [reviewed by Lagnado and Baylor (1992)] lead to a decrease in the intracellular Ca^{2+} concentration and, subsequently, activation of guanylyl cyclase. The molecular mechanism of this activation is unknown; however, the process involves a 23-kDa retinal guanylyl cyclase activating protein (GCAP) (Gorczyca et al., 1994).

The cytoplasmic domains of particulate guanylyl cyclases were described to contain a kinase homology motif that is separate from the catalytic domain [reviewed by Wong and Garbers (1992)]. One of the photoreceptor-specific guanylyl cyclases has been cloned and shown to share topological features with other members of the particulate guanylyl cyclase family (Shyjan et al., 1992). Although ATP and nonhydrolyzable analogs of ATP stimulate other membrane-bound guanylyl cyclases (Kurose et al., 1987; Chinkers & Garbers, 1989; Schulz et al., 1990; Koller et al., 1991), earlier reports suggest that retinal guanylyl cyclase is inhibited by ATP (Sitaramayya et al., 1991). It is difficult to reconcile these results describing the structural and functional properties of the rod outer segment (ROS) enzyme, and thus, several interpretations of the available data are possible: (a) the rod guanylyl cyclase clone that has been sequenced does not encode the enzyme involved in restoration of the dark level of cGMP during phototransduction, (b) the regulation of retinal guanylyl cyclase may be different, or (c) the results indicating that the cyclase was not affected by ATP may be incorrect, particularly since the cyclase activity was measured by an assay prone to

† This research was supported by NIH Grants EY08061, EY02048, and EY01730, by the Graduate School Fund Project from the University of Washington, by the Human Frontiers in Science Program, and, in part, by an award from Research to Prevent Blindness, Inc. K.P. is the recipient of a Jules and Doris Stein Research to Prevent Blindness Professorship.

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® Abstract published in *Advance ACS Abstracts,* February 15, 1994.

0006-2960/94/0433-3217$04.50/0

considerable error due to rapid metabolism of the product cGMP by ROS PDE.

In this paper, we address whether additional factors, such as nucleotides, polyanions, and polycations, influence guanylyl cyclase activity in ROS homogenates and guanylyl cyclase in washed membranes in the presence of a purified 23-kDa retinal guanylyl cyclase activating protein. The measurements were done using a reliable assay for guanylyl cyclase that is based on the stereospecific metabolism of thionucleotides. We also screened for nucleotide inhibitors of guanylyl cyclase.

MATERIALS AND METHODS

Isolation of Bovine Rod Outer Segments. Unless otherwise stated, all procedures were performed using ice-cold solutions. Fresh bovine eyes were obtained from a local slaughterhouse and retinas were dissected under dim red light to avoid rhodopsin bleaching. ROS were isolated using the discontinuous sucrose gradient method (Papermaster, 1982). ROS were homogenized in 50 mM Hepes, pH 7.8, 60 mM KCl, and 20 mM NaCl at a final concentration of 8 mg of rhodopsin/mL; this is referred to as *native ROS membrane homogenate. Washed ROS* were prepared by homogenization of native ROS prepared from 100 retinas in 35 mL of water containing 20 μg/mL leupeptin at 0–5 °C and centrifuged at 47000*g* for 30 min. The resulting pellet was collected and resuspended in 35 mL of water and centrifuged a second time (at 47000*g* for 30 min). The pellet was collected and resuspended in the Hepes buffer (as described above) at a final concentration of 8 mg of rhodopsin/mL. Rhodopsin concentration was determined by the method of Wald and Brown (1953).

Purification of Guanylyl Cyclase Activating Protein. GCAP was extracted from ROS with water and loaded on a DEAE-Sepharose column (5 × 50 mm; Pharmacia, Piscataway, NJ) in the presence of 5 mM BTP (1,3-bis[[tris(hydroxymethyl)methyl]amino]propane), pH 7.5, and 50 mM NaCl. GCAP was eluted with a linear NaCl gradient (100–350 mM) in 5 mM BTP, pH 7.5. Elution of protein was monitored by absorption at 280 nm and aliquots from selected fractions were tested for the presence of GCAP by their ability to stimulate guanylyl cyclase activity in a low Ca^{2+} solution (45 nM) using washed ROS membrane homogenate. Fractions that contained GCAP, which had been eluted by ~220 mM NaCl, were combined and loaded on a hydroxylapatite column (7.5 × 100 mm, Pentax Column SH-0710M, Asahi Optical Co., Ltd., Japan) which had been equilibrated with 100 mM NaCl in 10 mM BTP, pH 7.5. The GCAP fractions were eluted with a linear gradient of KH_2PO_4 (0–60 mM) and linearly decreasing concentration of NaCl (from 100 to 0 mM) in 10 mM BTP, pH 7.5, using a quaternary HPLC pump system (Hewlett-Packard, Model 1050). GCAP was eluted at approximately 30 mM KH_2PO_4 and 50 mM NaCl. The fractions containing GCAP eluted from the hydroxylapatite column were combined and concentrated to approximately 0.5 mL. Acetonitrile was then added to yield a final concentration of 15%, and the sample was loaded on a C-4 column (4.6 × 150 mm; W-Porex 5 C4, Phenomenex, Torrence, CA) equilibrated with 30% acetonitrile in 5 mM BTP, pH 7.5. GCAP was eluted with a linear gradient of acetonitrile (30–60%) in 5 mM BTP, pH 7.5. The results of GCAP purification were described by Gorczyca et al. (1994).

Guanylyl Cyclase Assay. The cyclase reaction was initiated by mixing ROS homogenates with the (S_P) isomer of GTPα^{35}S (19 000–25 000 dpm/nmol; New England Nuclear, Boston, MA) in a final volume of 64 μL and final concentrations of 50 mM Hepes buffer, pH 7.8, containing 30 μM rhodopsin, 0.65–1.3 mM (S_P)-GTPα^{35}S, 60 mM KCl, 20 mM NaCl, 10 mM $MgCl_2$, 0.4 mM EGTA, and 0.16 mM $CaCl_2$ (calculated [free calcium] = 45 nM) under room light or dim red light at 30 °C. Different GTPαS concentrations did not significantly change the calculated free calcium concentration. The reaction was terminated after 8–15 min by adding 15 μL of 0.4 N HCl. The sample was vortexed and then centrifuged in an Eppendorf centrifuge for 4 min at 16000*g*. Immediately, 50 μL of the supernatant was neutralized and residual guanylyl cyclase activity was quenched with 0.5 mL of 200 mM Tris/HCl, pH 7.4, containing 50 mM EDTA. The sample was vortexed and 0.5 mL was mixed with 150 mg of neutral alumina oxide (ICN alumina TSC04512; ICN Biomedicals Inc., Costa Mesa, CA). The sample was vortexed for 8 min and centrifuged for 5 min as above. The amount of (R_P)-cGMP^{35}S formed in the reaction was determined by measuring the radioactivity in 0.35 mL of the supernatant.

Nucleotides. 8-bromo-guanosine tetrakisphosphate was synthesized from guanosine tetrakisphosphate by direct bromination (Haley, 1977). Other nucleotides and chemicals were purchased from Sigma Chemical Co. (St. Louis, MO). (S_P)-GDPα^{35}S was made by hydrolysis of (S_P)-GTPα^{35}S with bovine myosin (Sigma Chemical Co., St. Louis, MO) in the presence of 2 mM $CaCl_2$ and 4 mM $MgCl_2$. GMPS was made by hydrolysis of (R_P)- or (S_P)-cGMPS with trypsin-activated retinal PDE (Zimmerman et al., 1985).

Separation of Nucleotides Using Nucleosil and Mono Q Columns. Nucleotides were separated on a Nucleosil 10 SB column (10 μm, 250 × 4 mm, Macherey-Nagel, Germany) using a quarternary HPLC pump system (Hewlett-Packard, Model 1050). The column was equilibrated with 100 mM potassium phosphate buffer, pH 5.4. Nucleotides were eluted with a linear gradient of 100 mM potassium phosphate buffer, pH 2.6, containing 800 mM KCl at a flow rate of 1 mL/min for 30 min. Alternatively, nucleotides were separated by HPLC on a Mono Q column (5 × 50 mm; Pharmacia, Piscataway, NJ). The column was equilibrated with 5 mM BTP, pH 7.5. Nucleotides were eluted with a linear gradient of 300 mM sodium chloride in the BTP buffer at a flow rate of 1.9 mL/min for 20 min. Nucleotides separated by each method were detected by monitoring absorption at 260 nm.

Calculation of Free Calcium Concentration. The predicted concentration of free calcium ions [Ca^{2+}] in the reaction mixture was estimated using the computer program Chelator 1.00 (Schoenmakers et al., 1992) that takes into account the EGTA content, pH, [$CaCl_2$], [$MgCl_2$], nucleotide concentrations, ionic strength, and temperature of the system. Low calcium buffer ([Ca^{2+}] = 45 nM) was composed of 50 mM Hepes buffer, pH 7.8, containing 60 mM KCl, 20 mM NaCl, 10 mM $MgCl_2$, 0.65–1.3 mM (S_P)-GTPαS, 0.4 mM EGTA, and 0.16 mM $CaCl_2$ at 30 °C. High calcium buffer ([Ca^{2+}] = 1 μM) was composed of 50 mM Hepes buffer, pH 7.8, containing 60 mM KCl, 20 mM NaCl, 10 mM $MgCl_2$, 0.65–1.3 mM (S_P)-GTPαS, 0.4 mM EGTA, and 0.375 mM $CaCl_2$.

RESULTS

Assay for Retinal Guanylyl Cyclase. Retinal guanylyl cyclase specifically catalyzes the conversion of GTP into cGMP *in vivo*. The activity of guanylyl cyclase in homogenized ROS is relatively low even when stimulated by calcium levels that mimic those induced by light *in vivo*. By comparison, basal PDE activity (500 nmol min^{-1} mg^{-1}) is almost 100 times higher than that of guanylyl cyclase, and inadvertent exposure of ROS preparations to low bleaches can stimulate PDE from severalfold to full activity (100 000 nmol min^{-1} mg^{-1}) (Baehr

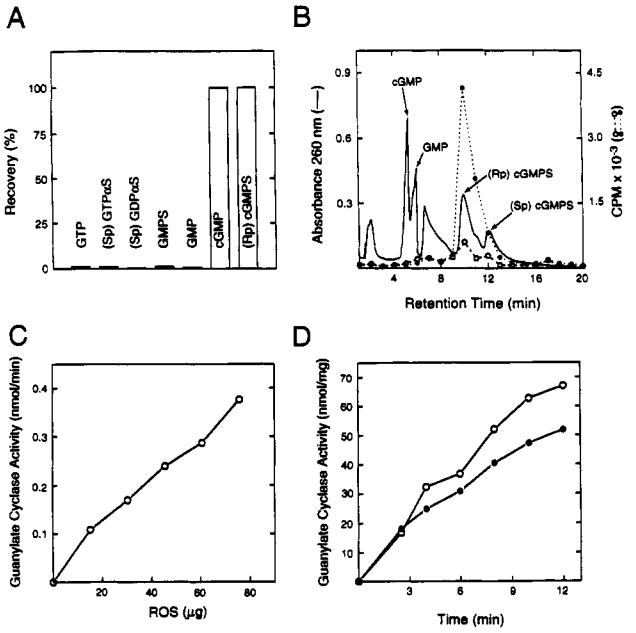

FIGURE 1: Characterization of guanylyl cyclase assay. (A) Recovery of nucleotides from neutral alumina gel. Each nucleotide (0.6–2 μmol) in 0.55 mL of 0.2 M Tris/HCl buffer, pH 7.4, containing 50 mM EDTA was mixed with 150 mg of neutral alumina, vortexed for 8 min, and centrifuged at 16000*g* for 5 min, and the resulting supernatant was tested for the presence of nucleotides by spectrophotometric or radioactive measurements. (B) Coelution profile of (R_P)-cGMP^{35}S with nucleotide standards on Mono Q column. Homogenized ROS (10 μL, 8 mg/mL) in the guanylyl cyclase assay buffer (see Materials and Methods) were mixed with (S_P)-GTPα^{35}S (final concentration 0.65 mM) and incubated at 30 °C for 8 min. The reaction was (1) quenched with 15 μL of 0.4 N HCl; (2) neutralized with 0.5 mL of 0.2 M Tris/HCl buffer, pH 7.4, containing 50 mM EDTA, (3) mixed with 150 mg of neutral alumina, (4) vortexed for 8 min, and (5) centrifuged at 16000*g* for 5 min. The supernatant was then diluted 3-fold with 5 mM BTP buffer, pH 7.5, containing 13.3 μM GMP, 13.2 μM cGMP, 12.5 μM (S_P)-cGMPS, and 13.2 μM (R_P)-cGMPS and analyzed using a quaternary pump system HPLC on a Mono Q column (5 × 50 mm, Pharmacia) equilibrated with 5 mM BTP buffer, pH 7.5. Nucleotides were eluted with a linear gradient of 300 mM sodium chloride in 10 mM BTP buffer, pH 7.5, at a flow rate of 1.9 mL/min for 20 min and were detected by monitoring absorption at 260 nm (—) or by radioactivity measurements (•••). Closed circles and open circles represent measurements of cGMP^{35}S made at 45 nM and 1 μM free calcium, respectively. (C) Guanylyl cyclase activity as a function of ROS concentration. Guanylyl cyclase activity was measured (see Materials and Methods) in samples containing increasing amounts of homogenized ROS. (D) Time course of cGMP^{35}S formation under dark conditions and ambient room light. Guanylyl cyclase activity was measured in ROS homogenates under dim red light (open circles) or under room illumination (closed circles).

et al., 1979). Retinal guanylyl cyclase can use the (S_P) isomer of GTPαS to generate (R_P)-cGMPS with inversion of the configuration on the phosphate atom (Senter et al., 1983; Koch et al., 1990). Since (R_P)-cGMPS is a poor substrate for PDE (Zimmerman et al., 1985), one can employ (S_P)-GTPαS to assay guanylyl cyclase activity *in vitro* without concern for hydrolysis of the reaction product by endogenous PDE. Unreacted substrate (S_P)-GTPαS and other nucleotide byproducts bind to the alumina gel (Figure 1A), whereas cGMPS, like cGMP, remains unbound and is identified by coelution with an authentic chemically synthesized standard from Mono Q (Figure 1B) or Nucleosil 10 SB (data not shown) columns. Predictably, cGMPS production by ROS homogenates was dose- and calcium-dependent (Figure 1B). Thus, increased concentration of the enzyme produces proportionally more (R_P)-cGMPS in the studied range (Figure 1C). In our assay, up to 0.2 mM (R_P)-cGMPS does not inhibit cyclase activity. The assay is linear up to 15 min and is only slightly sensitive to bleaching of rhodopsin (Figure 1D). The activity difference between ROS homogenates incubated in light and dark conditions may result from the light-dependent GTPase activity of transducin (Yamanaka et al., 1985) or from the phosphorylation activity of rhodopsin kinase, as each enzyme can utilize (S_P)-GTPαS as a substrate. Using this assay, we determined that the effect of several reagents commonly used in other guanylyl cyclase assays may influence enzymatic activity: (a) EGTA higher than 1.5 mM inhibits guanylyl cyclase with an IC_{50} of approximately 3.5 mM, (b) enzyme activity is optimal at $MgCl_2$ concentrations ranging from 5 to 15 mM, (c) $MnCl_2$ can substitute for $MgCl_2$, but even at its optimal concentration (3 mM $MnCl_2$), specific enzyme activity is approximately 10-fold lower than it is in the presence of magnesium ions, (d) NaCl inhibits the cyclase activity (IC_{50} = 120 mM) such that at 500 mM approximately 5% guanylyl cyclase activity remains, (e) IBMX (3-isobutyl-1-methylxanthine) and dipyridamole (known inhibitors of retinal PDE; Gillespie & Beavo, 1989) inhibit retinal guanylyl cyclase with a K_i of approximately 1.5 mM and 65 μM, respectively, and (f) sodium nitroprusside (from 1 μM to 10 mM) has no effect on retinal guanylyl cyclase activity, suggesting that the contribution of a soluble guanylyl cyclase to overall cGMP production is negligible (data not shown). To eliminate possibilities that other enzymes involved in the nucleotide metabolism may influence the cyclase activity (for example by generating diphosphate nucleotides and monophosphate nucleotides), we reconstituted the guanylyl cyclase system by using extensively washed disc membranes and purified guanylyl cyclase activating protein (GCAP). The most significant advantage of this reconstituted system over detergent-purified guanylyl cyclase is its high enzymatic activity. Furthermore, trace amounts of detergent interfere with the function of this hydrophobic activator and make detergent-purified retinal guanylyl cyclase reconstitution system unstable. To determine the possible metabolism of GTPαS, washed ROS or purified guanylyl cyclase activating protein was incubated under the condition of the assay, and the GTPαS and other nucleotides were analyzed on a Mono Q column as described in Materials and Methods. Using washed ROS, less than 4% of the nucleotide was converted to cGMPS or GDPαS. Guanylyl cyclase activating protein alone does not catalyze decomposition of GTPαS, but when mixed with guanylyl cyclase in native ROS membranes, a severalfold Ca-dependent increase in cGMPS production was observed. These results verify the measurements performed in homogenates of both native and washed ROS.

Effect of Adenylyl Nucleotides on Guanylyl Cyclase Activity. Particulate guanylyl cyclases contain a protein kinase-like domain, and they are activated by ATP (Wong & Garbers, 1992). We reinvestigated the effect of ATP on retinal guanylyl cyclase under a variety of experimental conditions. In the presence of ATP, the production of (R_P)-cGMPS is almost double that produced under control conditions (Figure 2, Tables 1 and 2). The stimulatory effect of ATP does not appear to be due to phosphorylation of the cyclase or components of its regulation (Table 2), because ADP and AMP-PNP also stimulate retinal guanylyl cyclase with optimal concentrations in the 100–500 μM range. Nucleotide concentrations greater than 500 μM have an inhibitory effect, probably due to competition with GTP for the catalytic site. Addition of 0.2 mM ATP stimulates guanylyl cyclase activity in homogenates of ROS at least 60% over a range of 10^{-6}–10^{-8}

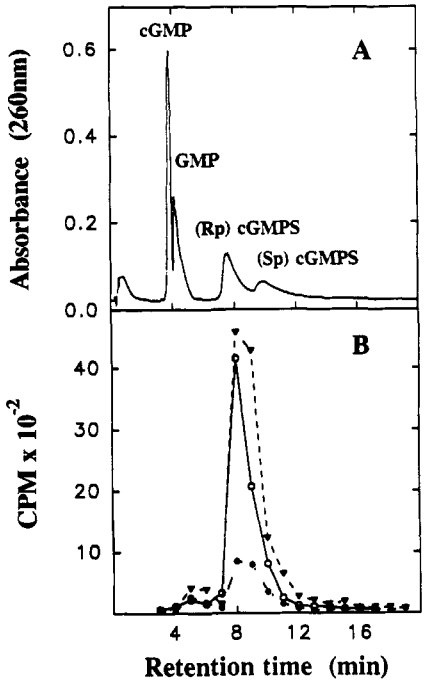

FIGURE 2: Elution profile of (R_P)-cGMP^{35}S produced by retinal guanylyl cyclase in the presence and absence of ATP on a standardized Mono Q column. (A) (R_P)-cGMP^{35}S produced in standard conditions (45 nM free calcium concentration) was separated from the substrate and byproducts on an alumina oxide gel as described in Materials and Methods. The sample was diluted 3-fold with 5 mM BTP buffer, pH 7.5, containing 13.3 μM GMP, 13.2 μM cGMP, 12.5 μM (S_P)-cGMPS, and 13.3 μM (R_P)-cGMPS and analyzed using a quaternary pump system HPLC on a Mono Q column (5 × 50 mm, Pharmacia) equilibrated with 5 mM BTP buffer, pH 7.5. Nucleotides were eluted with a linear gradient of 300 mM sodium chloride in 10 mM BTP buffer, pH 7.5, at a flow rate of 1.9 mL/min for 20 min and detected at 260 nm. (B) Radioactive profile obtained on the sample assayed for guanylyl cyclase activity in control conditions (open circles) and in the presence of 0.2 mM ATP (open triangles) or 0.2 mM guanosine tetrakisphosphate (GTetP) (closed circles). The radioactive product (R_P)-cGMP^{35}S comigrated with authentic (R_P)-cGMPS.

Table 1: Kinetic Parameters for GTPαS and Its Components or Analogues[a]

nucleotide	K_m (μM)	V_{max} (nmol min^{-1} mg^{-1})	K_i[b] (μM)
(Sp)-GTPαS in ROS	381 ± 116	7.8 ± 0.6	
(Sp)-GTPαS in ROS + ATP[c]	450 ± 81	11.1 ± 1	
(Sp)-GTPαS in reconst[d]	107 ± 15	2.2 ± 0.1	
(Sp)-GTPαS in reconst[d] + ATP[c]	106 ± 16	2.6 ± 0.1	
GTetP[e]			31 ± 5
GTetP[e] in reconst[d]			38 ± 5

[a] The K_m and V_{max} for (Sp)-GTPαS was determined from a Lineweaver–Burk plot for the formation of (Rp)-cGMPS as a function of (Sp)-GTPαS (Segel, 1975) in the presence or absence of 200 μM ATP. [b] The activity of guanylyl cyclase was plotted as a function of the concentration of the inhibitors at 45 nM free calcium concentration (see Materials and Methods). IC_{50} was determined from the plot and K_i was derived by using the approach described by Cheng and Prusoff (1973). [c] ATP concentration was 200 μM. [d] Reconstituted system was composed of 30 μM rhodopsin in washed disc membranes and 100 nM GCAP. [e] GTetP = guanosine tetrakisphosphate.

M Ca^{2+} indicating that the effect of ATP is independent of [Ca^{2+}] (Figure 3).

Similar results were obtained using washed ROS in the presence of guanylyl cyclase activating protein (Figure 3, inset). Additionally, this stimulatory effect is seen for guanylyl cyclase solubilized in 2% Triton X-100 or 5% Lubrol (data not shown). ATP increases the V_{max} of the enzyme with a slight increase in K_m for (S_P)-GTPαS (Table 1). The effect of ATP on K_m is very likely a result of competition between product(s) of transphosphorylation by ATP (*e.g.*, GTP or other polyphosphate nucleotides) and (S_P)-GTPαS for the catalytic site. In the reconstituted system, the K_m for (S_P)-GTPαS is lower (Table 1) than in ROS and may be due to the binding of this thionucleotide to the enriched nucleotide binding milieu in the homogenates. The ATP effect in the reconstituted system was also reflected in the changes in V_{max}, similar to the measurements of ROS homogenates (Table 1). The stimulatory effect of ATP on guanylyl cyclase observed here are consistent with earlier reports that ATP stimulates particulate guanylyl cyclases such as atrial natriuretic factor-stimulated cyclase (Kurose et al., 1987) or *Escherichia coli* heat-stable enterotoxin (ST)-stimulated intestinal guanylyl

Table 2: Activation and Inhibition of Guanylyl Cyclase[a]

activators and inhibitors	EC_{max} (μM)	IC_{50} (mM)
Guanylyl Nucleotides		
GMP-PNP		0.5
GTP		1
8-Br-GTetP		1.3
8-Br-GMP		>5
3′-GMP		>5
8-Br-cGMP		no effect
Adenylyl Nucleotides		
AMP		no effect
ADP	0.2	>5
ATP	0.15	>5
AMPNP	0.5	>5
AMP-PNP	0.1	1.8
ATetP[b]	0.1	1.2
Polyphosphates and Other Compounds		
PP_i		0.7
PNP_i		0.7
PPP_i		0.8
$PPPP_i$		2.8
TTP		no effect
UTP		no effect
ribose 5-P		no effect

[a] The activity of guanylyl cyclase was plotted as a function of the concentration of the inhibitors, at 45 nM free calcium concentration (see Materials and Methods). EC_{max} (the concentration at which maximal stimulatory effect was observed) and IC_{50} (the concentration at which 50% of inhibition was observed) were determined from the plot. [b] ATetP = adenosine tetrakisphosphate.

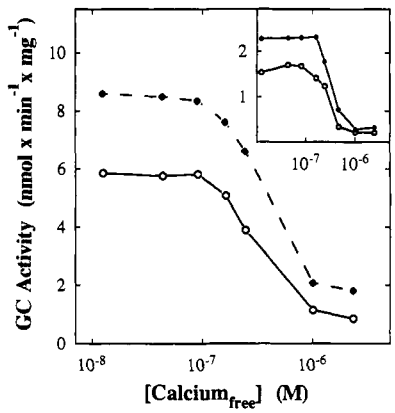

FIGURE 3: Calcium titration of guanylyl cyclase activity in ROS homogenate in the presence or absence of ATP. The Ca^{2+}-sensitivity of guanylyl cyclase was assayed in ROS homogenates in the absence (open circles) or presence (closed circles) of 0.2 mM ATP. Inset: Calcium titration of guanylyl cyclase activity in washed ROS homogenate in the presence of guanylyl cyclase activating protein (rhodopsin and GCAP concentrations were 30 μM and 100 μM, respectively) without (open circles) or with 0.2 mM ATP (closed circles). The free calcium concentrations were adjusted with an EGTA/Ca^{2+} buffer as described in Materials and Methods.

Table 3: Cations and Anions as Inhibitors of Guanylyl Cyclase[a]

cations and anions	K_i (μM)	IC_{50} (μM)
dipyridamole	65	
benzamidine	229	
octylamine	362	
polylysine	685	
heparin[b]		1400

[a] The K_i was determined from a Dixon plot (Segel, 1975) at 45 nM free calcium concentration (see Materials and Methods). [b] The activity of guanylyl cyclase was plotted as a function of the concentration of the inhibitors, at 45 nM free calcium concentration (see Materials and Methods). IC_{50} was determined from the plot.

cyclase (Gazzano et al., 1991).

Inhibitors of Retinal Guanylyl Cyclase. To further characterize inhibitors of guanylyl cyclase, we compared the structures of various nucleotides (or their fragments) and tested their potency on inhibition of guanylyl cyclase. Monophosphorylated nucleotides cGMP, 8-Br-cGMP, and GMP have little or no effect on guanylyl cyclase activity (Table 2). The effective inhibitors are highly phosphorylated derivatives of guanosine, with guanosine tetrakisphosphate being the most potent. Guanosine tetrakisphosphate is a competitive inhbitor of retinal guanylyl cyclase with K_i = 31 μM, when K_m for (S_P)-GTPαS is 381 ± 116 μM and V_{max} is 7.8 ± 0.6 nmol min^{-1} (mg of rhodopsin)$^{-1}$ in ROS homogenates and in the reconstituted system (Table 1). Guanosine tetrakisphosphate would be a useful inhibitor to study the role of guanylyl cyclase in ROS since it is slowly metabolized to GDP in ROS homogenates (data not shown). Nucleotides such as UTP, TTP, or phosphorylated ribose did not affect guanylyl cyclase activity. Pyrophosphate (IC_{50} = 0.7 mM) and tripolyphosphate (IC_{50} = 0.8 mM) were the most potent polyphosphate inhibitors (Table 2). Similar results were obtained by Yang and Wensel (1992) for pyrophosphate. Inhibition by polyphosphate and polyanions may be a general property of guanylyl cyclases, since heparin also partially inhibits guanylyl cyclase activity at IC_{50} = 1.4 mM (Table 3). A possible mechanism would involve competition between negatively charged molecules and three phosphate ions of GTP. Inhibition of guanylyl cyclase by cations is also of interest. We found that a cationic inhibitor of proteolysis, benzamidine, competitively inhibits guanylyl cyclase with K_i = 229 μM. We extended this observation to other cations such as octylamine (K_i = 362 μM) and polylysine (K_i = 685 μM) and an inhibitor of PDE, dipyridamole (K_i = 65 μM) (Table 3).

DISCUSSION

Phototransduction processes are driven by the consumption of energy, where ATP, GTP, and NADPH are rapidly metabolized and resynthesized. For example, GTP is converted to cGMP by retinal guanylyl cyclase, further cGMP is hydrolyzed by light-stimulated cGMP PDE, and subsequently, GMP is phosphorylated to GTP in a two-step reaction by guanylyl kinase (Hall & Kühn, 1986) and nucleoside diphosphate kinase [reviewed by Agarwal et al. (1978)]. Our modification of the guanylyl cyclase assay is based on the observation of Senter et al. (1983) and Zimmerman et al. (1985) that guanylyl cyclase does utilize the (S_P) α-thio analog of GTP and forms a product which is poorly degradable by PDE. Therefore, it was possible to assay the cyclization step specifically.

Guanylyl cyclases from many systems are modulated by ATP. We found that retinal guanylyl cyclase is also activated by ATP. The prime candidate of retinal-specific guanylyl cyclase was recently identified by partial protein sequencing (Margulis et al., 1993). By comparison with other particulate guanylyl cyclases, this ROS-specific guanylyl cyclase would be predicted to have four distinct segments: (1) a large extracellular domain, which is typically involved in binding a peptide ligand; (2) a single membrane-spanning domain, which may be involved in the interaction with cytoskeleton elements in ROS; (3) a kinase homology domain; and (4) the catalytic domain. This complex structure provides opportunity for rigid regulation of its activity. The kinase-like domain is involved in potentiating the effect of a peptide ligand on particulate guanylyl cyclases. The peptide or ATP alone has only a modest (approximately 2-fold stimulation) effect on the enzymatic activity, whereas both synergistically stimulate cyclase activity more than 10-fold (Chinkers et al., 1991; Gazzano et al., 1991). The activation of retinal-specific guanylyl cyclase by ATP is modest and GCAP does not potentiate this effect. This suggests that GCAP may interact and modulate cyclase activity via the catalytic sites rather than through the "extracellular domain". This would be a novel mechanism for the regulation of the cyclase activities, possibly extending beyond phototransduction.

An important observation is that guanosine tetrakisphosphate competitively inhibits guanylyl cyclase with K_i = 31 μM; this is the most potent inhibitor known of guanylyl cyclase. This observation opens possibilities for further refinement and modification of this structure in a search for more potent guanylyl cyclase inhibitors.

ACKNOWLEDGMENTS

We wish to thank Dr. Peter B. Detwiler for helpful discussions during the course of this work, Drs. John C. Saari and Mary Ann Asson-Batres for critical review of the manuscript, Dr. Maria Rudnicka-Nawrot for help at the initial phase of this work, and Dr. Theo J. M. Schoenmakers from the University of Nijmegen (Netherlands) for the computer program entitled "Chelator". (S_P)- and (R_P)-cGMPS was a generous gift from Drs. W. Stec and J. Baraniak (Polish Academy of Science, Lodz, Poland).

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