Interactions of Nucleotide Analogues with Rod Outer Segment Guanylate Cyclase†

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ABSTRACT: Light activation of cyclic GMP hydrolysis in rod outer segments is mediated by a G-protein which is active in the GTP-bound form. Substitution of GTP with a nonhydrolyzable GTP analogue is thought to leave the G-protein in a persistently activated state, thereby prolonging the hydrolysis of cyclic GMP. Restoration of cyclic GMP concentration in the cell also depends upon GTP since it is the substrate for guanylate cyclase, but little is known about the effects of GTP analogues on this enzyme. We report here the effects of the analogues of GTP and ATP as inhibitors and substrates of rod disk membrane guanylate cyclase. The rate of cyclic GMP synthesis from GTP in rod disk membranes was about 50 pmol min^{-1} (nmol of rhodopsin)$^{-1}$. Analogues of GTP and adenine nucleotides competitively inhibited the cyclase activity. The order of inhibition, with magnesium as metal cofactor, was ATP > GMP-PNP > AMP-PNP ≈ GTP-γ-S; with manganese, AMP-PNP was more inhibitory than GTP-γ-S. The inhibition constants, with magnesium as cofactor, were 0.65–2.0 mM for GTP-γ-S, 0.4–0.8 mM for GMP-PNP, 1.5–2.3 mM for AMP–PNP, and 0.07–0.2 mM for ATP. The fraction of cyclase activity inhibited by analogues was similar at 1 and 0.03 μM calcium. Besides inhibition of cyclase, the analogues also served as its substrates. GTP-γ-S substituted GTP with about 85% efficiency while GMP-PNP and ATP were about 5 and 7% as efficient, respectively. As substrates, GTP and GMP-PNP were about twice as effective at 0.03 μM calcium than at 1 μM, while GTP-γ-S was not affected significantly by calcium. For all three substrates, cyclase activity was higher with manganese than with magnesium, but with either cofactor, the order of effectiveness remained the same, GTP > GTP-γ-S ≫ GMP-PNP. These results show that in addition to prolonging the activation of G-protein and thus the hydrolysis of cyclic GMP, GTP analogues also adversely influence the synthesis of cyclic GMP. This effect, whose magnitude varies depending upon the analogue, has to be taken into account in evaluatig the effects of the analogues on the light response in rod photoreceptors.

Dark-adapted vertebrate rod outer segments (ROS) have a steady inward current carried mostly by sodium (Korenbrot & Cone, 1972; Yoshikami & Hagins, 1972) and about 15% by calcium (Yau & Nakatani, 1985). This current is regulated by cyclic GMP (Nicol & Miller, 1978; Cobbs et al., 1985), and there is general agreement now that cyclic GMP binding regulates a channel protein conducting this current (Fesenko et al., 1985; Zimmerman et al., 1985; Tanaka et al., 1987; Hanke et al., 1988). A light flash on the outer segment activates an enzyme cascade that results in hydrolysis and depletion of cyclic GMP and consequent decrease in the dark current [for a recent review, see Pugh and Lamb (1990)]. This decrease is transient, recovering, after a near-saturating flash, with a time constant of about 4 s in toad rods (Pugh & Cobbs, 1986) and somewhat faster in rat rods (Penn & Hagins, 1972). The various steps in the activation of the enzyme cascade leading to cyclic GMP hydrolysis are the photolysis of rhodopsin to metarhodopsin II (R*), R*-catalyzed exchange of GDP for GTP on G-protein (GTP binding protein, transducin, G_t, G_v) (Godchaux & Zimmerman, 1979; Fung & Stryer, 1980; Liebman & Pugh, 1982; Bennett, 1982; Pugh & Cobbs, 1986), and the binding of G-protein·GTP (G*) to cyclic GMP phosphodiesterase (PDE), converting the PDE to an active enzyme (Fung et al., 1981). This enzyme cascade causing the suppression of dark current is therefore GTP-dependent. GTP is also essential for the recovery of the dark current in that it is the substrate for the formation of cyclic GMP by guanylate cyclase.

The dark current is restored when free cyclic GMP in the rod outer segment returns to preflash concentration. It is logical to assume that the light-activated cyclic GMP hydrolysis is terminated before the concentration of the nucleotide is restored. Since rhodopsin kinase (Sitaramayya & Liebman, 1983; Wilden et al., 1986; Bennett & Sitaramayya, 1988) and GTPase (Dratz et al., 1987; Sitaramayya et al., 1988; Vuong & Chabre, 1990) are thought to be involved in terminating light-activated cyclic GMP hydrolysis, inhibitors of these enzymes should be useful in testing this assumption. Analogues of GTP such as GTP-γ-S and GMP-PNP are not hydrolyzed by G-proteins and serve as effective inhibitors of GTPase activity, leaving the G-proteins in a persistently activated state (Pfeuffer & Helmreich, 1975; Cassel & Selinger, 1977; Yamanaka et al., 1985). In electrophysiological studies by Kondo and Miller (1988), and Lamb and Matthews (1988) in which GTP analogues were injected into rods, light-suppressed dark current recovered, though much slower than in control cells. Kondo and Miller (1988) felt that in analogue-injected cells activated by a dim light flash, guanylate cyclase may be activated to a higher level and that the rate of the cyclic GMP synthesis may surpass the rate of its hydrolysis [for a recent review of events leading to the activation of cyclase, see Pugh and Lamb, (1990)]. While G-protein was shown not to hydrolyze GTP-γ-S (Yamanaka et al., 1985), there were no biochemical reports on the effects of these analogues on RDM

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guanylate cyclase. In this study, we have investigated the effects of analogues of GTP and ATP on cyclase activity. The results show that there is a wide variation among the analogues in inhibiting guanylate cyclase as well as in serving as its substrates. The implication of these observations for the kinetics of light response in photoreceptor cells is discussed.

Experimental Procedures

Cyclase Preparations. All the guanylate cyclase activity measurements were done either on washed rod disk membranes (RDM) or on preparations purified 100–300-fold from RDM by a procedure improved from an earlier reported protocol (Hakki & Sitaramayya, 1990).

(A) Washed Rod Disk Membranes. These were prepared as described earlier by Hakki and Sitaramayya (1990). Briefly, RDM isolated from fresh bovine retinas were washed thoroughly to remove soluble and peripheral proteins. The washing sequence was as follows: 3 times in 20 mM MOPS, 2 mM $MgCl_2$, 1 mM DTT, and 0.5 mM PMSF, pH 8.0; twice in Tris buffer (10 mM Tris, 5 mM DTT, 0.5 mM PMSF, and 12.5 μg/mL each of aprotinin, benzamidine, and leupeptin, pH 7.5) containing 50 μM GTP; once in Tris buffer without additions; once in Tris buffer with 200 mM KCl; once in Tris buffer with 200 mM KCl and 2 mM EDTA; and finally in the same buffer with 2 mM EDTA.

(B) Partially Purified Guanylate Cyclase. The purification protocol will be described in detail elsewhere. Briefly, washed RDM prepared as described above were regenerated with 11-*cis*-retinal, solubilized as described earlier (Hakki & Sitaramayya, 1990) but under infrared instead of room light, and filtered on a Biogel A 0.5m column in the dark. The cyclase activity eluted in the exclusion volume as noted earlier, but with much less contaminating rhodopsin. The specific activity of cyclase was about 17-fold enhanced in this step. The pooled cyclase fractions were incubated with hydroxyapatite for 3 h which removed some contaminating proteins, and this step resulted in a further purification of 4–5-fold. The preparation was then chromatographed on DEAE-cellulose as described earlier. The cyclase activity eluted essentially in one peak at 250–260 mM KCl. The specific activity of the enzyme eluting from DEAE-cellulose was 110–320-fold higher than that in the solubilized RDM, and this preparation was free of GTPase, phosphodiesterase, and nucleotidase activities. The purified enzyme was supplemented with protease inhibitors and stored at 4 or −50 °C. At −50 °C, the activity remained unchanged in 2 weeks and lost 48% of activity after 20 weeks. At 4 °C, the activity decreased about 10% in 2 weeks.

Guanylate Cyclase Assay. Two procedures were used to measure cyclase activity. Both methods gave comparable results. When [α-^{32}P]GTP was used as substrate, the [^{32}P]-cGMP formed in the assay was separated from other nucleotides by thin-layer chromatography and counted as described earlier (Hakki & Sitaramayya, 1990). This assay was used in measuring the kinetics of cyclase in the presence or absence of nucleotide analogues. Formation of cyclic AMP from ATP was measured in a similar assay using [α-^{32}P]ATP as substrate. The concentrations of the nucleotides and metal ions in the assays are indicated at appropriate places in the text and tables. The amounts of Mg, Mn, and Ca added to the assays to achieve the desired free metal concentrations were calculated by using the software "Max Chelator", version 4.12, provided by Chris Patton, Hopkins Marine Station, Pacific Grove, CA. The other components in the assay are 25 mM Tris, 1 mM DTT, 1 mM IBMX, and 1 mM cyclic GMP, pH 7.0.

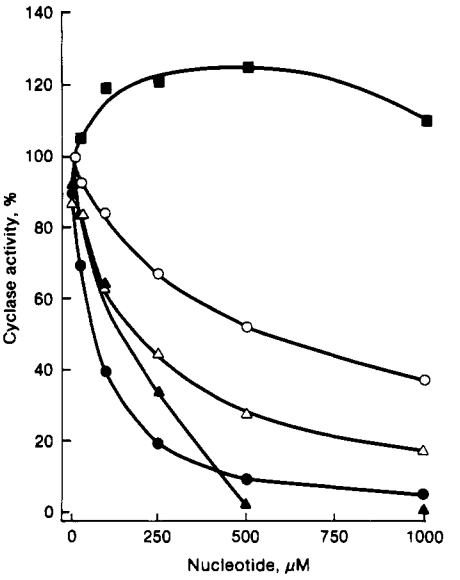

FIGURE 1: Influence of ATP and nucleotide analogues on purified guanylate cyclase. Cyclase activity was measured with 2 mM manganese and 250 μM GTP in the assays. The nucleotide analogues or ATP was included at the concentrations indicated. GTP-γ-S (○); AMP-PNP (△); GMP-PNP (●); ATP (▲); GDP-β-S (■).

In assays designed to test if GTP analogues themselves served as substrates for cyclase, the amount of cyclic GMP formed from nonradioactive analogues was measured by radioimmunoassay as described in detail by Nambi et al. (1982). As a reference, identical assays were also run using cold GTP as substrate.

Materials. GTP-γ-S (catalog no. 220647 and 1110349) and GDP-β-S (catalog no. 528536) were from Boehringer Mannheim, and according to the manufacturer, they were 86, 92, and 94% pure, respectively. AMP-PNP (catalog no. A2647), ATP (catalog no. A-5394), and GMP-PNP (catalog no. G-8639) were from Sigma Chemical Co. and were 95, 99–100, and 87% pure, respectively, according to Sigma. The concentrations of these nucleotides referred to in the text are nominal and not corrected for the purity. Inorganic pyrophosphate is a competitive inhibitor of cyclase activity (Hakki & Sitaramayya, 1990). Inhibition of cyclase by ATP and analogues of ATP and GTP as shown in Figure 1 could have been in part due to possible contamination of these nucleotides with pyrophosphate. While an estimate of the pyrophosphate content of the nucleotides used in our experiments is not available, product analyses provided by the manufacturers indicated that the impurities other than metals and nucleotides including inorganic phosphate, pyrophosphate, etc. accounted for less than 1% in ATP and AMP-PNP, less than 2.7% in GMP-PNP, and less than 3% in GTP-γ-S. [α-^{32}P]ATP and [α-^{32}P]GTP were obtained from ICN. Other chemicals were all from Sigma Chemical Co. Fresh bovine eyes were purchased from MOPAC, Souderton, PA.

Results

Influence of Various Nucleotides on Guanylate Cyclase Activity. The nucleotides chosen are those that were most commonly injected into rods in the electrophysiological studies (Sather & Detwiler, 1987; Kondo & Miller, 1988; Lamb & Matthews, 1988; Erickson et al., 1990). The effects of various concentrations of these nucleotides on guanylate cyclase activity at 250 μM GTP and 2 mM Mn^{2+} are shown in Figure 1. GDP-β-S, a strong inhibitor of G-protein activation (Eckstein et al., 1979), had a mild stimulatory effect on cyclase

Table I: Inhibition of Purified Cyclase Activity by ATP and Nucleotide Analogues with Mg^{2+} or Mn^{2+} as Metal Cofactors[a]

additions	pmol of cyclic GMP formed in assay	
	Mg^{2+}	Mn^{2+}
control	6.78 ± 0.60 (100)	349 ± 3.8 (100)
GTP-γ-S		
100 μM	6.52 ± 0.02 (96)	311 ± 3.2 (89)
250 μM	6.12 ± 0.70 (90)	245 ± 3.2 (70)
500 μM	5.20 ± 0.02 (77)	178 ± 6.2 (51)
1000 μM	3.00 ± 0.40 (44)	92 ± 1.6 (26)
ATP		
100 μM	3.90 ± 0.20 (58)	242 ± 4.4 (69)
250 μM	1.80 ± 0.60 (27)	146 ± 3.8 (42)
500 μM	ND	32 ± 3.2 (9)
1000 μM	ND	ND
control	35.9 ± 4.7 (100)	965 ± 31 (100)
GTP-γ-S		
250 μM	31.3 ± 1.8 (87)	696 ± 26 (72)
1000 μM	21.8 ± 1.3 (61)	332 ± 7 (34)
ATP		
250 μM	13.5 ± 0.6 (38)	444 ± 21 (46)
1000 μM	0.6 ± 0.3 (2)	1 (<1)
GMP-PNP		
250 μM	28.0 ± 0.4 (78)	169 ± 2 (17)
1000 μM	17.3 ± 1.7 (48)	32 ± 5 (3)
AMP-PNP		
250 μM	32.5 ± 1.8 (91)	370 ± 5 (38)
1000 μM	22.7 ± 0.8 (63)	141 ± 3 (15)

[a] The data shown in the two sections of this table are obtained with two different purified preparations of cyclase. Assays contained 250 μM GTP, 2 mM Mg^{2+} or Mn^{2+}, and the indicated concentration of ATP or the nucleotide analogue. the numbers in parentheses show activity as percent of control. ND refers to assays in which less than 1 pmol of cyclic GMP was formed.

Table II: Inhibition Constants for Analogues and ATP[a]

nucleotide tested	K_i (mM)
GTP-γ-S	0.65–2.0, 1.34–1.6, 2.0
GMP-PNP	0.8, 0.36
ATP	0.074–0.11, 0.128, 0.2
AMP-PNP	1.5–2.3

[a] Partially purified cyclase preparations were assayed with magnesium as cofactor. For each nucleotide tested, inhibition constants obtained with different batches of purified guanylate cyclase are shown.

activity. The other nucleotides tested were all inhibitory in the order ATP > GMP-PNP > AMP-PNP > GTP-γ-S. While GMP-PNP appears to be more inhibitory than ATP in the experiment shown in Figure 1, the results from several experiments presented in Tables I and II show that ATP inhibits cyclase more than GMP-PNP. The strong inhibition of the guanylate cyclase activity by ATP could have been due to phosphorylation by a membranous kinase that was retained in the purified cyclase preparation. To test this possibility, the cyclase preparation was preincubated for 15 min at 37 °C with 100 μM ATP followed by assay for the cyclase activity. The control preparation was not preincubated but contained 100 μM ATP in the assay. There was no significant difference between the activities of these two preparations, suggesting that ATP was competing with GTP and not acting as a substrate for a kinase under the conditions used here. Experiments on inhibition kinetics, discussed later, also showed that ATP was a competitive inhibitor of guanylate cyclase.

Inhibition with Mn^{2+} and Mg^{2+} as Cofactors. Guanylate cyclase from RDM as well as other sources has been shown to have higher activity with Mn·GTP as substrate than with Mg·GTP (Krishnan et al., 1978; Nambi & Sharma, 1981; Brandwein et al., 1982). However, the concentration of manganese in the nervous tissue and body fluids is 2–3 orders of magnitude lower than that of magnesium (Biology Data Book, 1974), and therefore nucleotides are probably present in the cells as magnesium complexes. Analysis of the energy-dispersive X-ray spectra of freeze-dried bullfrog rods showed no measurable manganese (M. Foster and W. A. Hagins, personal communication). In one preparation analyzed by Foster and Hagins, the concentration of Mg was 14.7 ± 0.6 mM, and that of Mn was 0.8 ± 1.5 mM. In another preparation where the retina was treated with 50 μM ouabain and 50 μM IBMX before freeze-drying, the Mg concentration was 8.2 ± 0.9 mM and that of Mn −0.6 ± 1.9 mM. In both cases, the Mn concentration was below the detection limit of the method used. We compared the effects of the various nucleotides on cyclase activity using 2 mM Mg^{2+} or Mn^{2+} as cofactor at 250 μM GTP. As shown in Table I, the cyclase activity was severalfold higher with Mn^{2+} than with Mg^{2+}, but with either metal as cofactor, all the tested nucleotides inhibited cyclase activity. For all nucleotides except for ATP, the inhibition was greater with Mn^{2+} than with Mg^{2+}. With Mg^{2+} as metal cofactor, the kinetics of inhibition were investigated for each analogue. The inhibition was competitive in all cases (data not shown). The inhibition constants are listed in Table II.

Table III: Inhibition of Rod Disk Membrane Guanylate Cyclase by Analogues[a]

GTP analogue	pmol of cyclic GMP min^{-1} (nmol of rhodopsin)$^{-1}$
control	43.43 ± 2.14
GTP-γ-S	
2 mM	23.46 ± 2.18
5 mM	20.37 ± 1.55
10 mM	17.19 ± 1.02
GMP-PNP	
2 mM	22.64 ± 1.95
5 mM	15.44 ± 1.89
10 mM	11.71 ± 0.82

[a] Assays were done with washed rod disk membranes as the source of guanylate cyclase. The GTP and Mg^{2+} concentrations were 1 and 5 mM, respectively. When assays were done at 2 mM GTP, the activity in the control increased from 43.43 ± 2.14 to 49.32 ± 4.43 pmol of cyclic GMP produced min^{-1} (nmol of rhodopsin)$^{-1}$.

Effects of Higher Concentrations of Analogues at 1 mM GTP. The concentration of GTP in rod outer segments is about 1–2 mM (Robinson & Hagins, 1979; Biernbaum & Bownds, 1985). In some studies where the analogues were injected into the rod outer segments, the concentration of analogue in the pipet was 2–10 mM (Kondo & Miller, 1988; Lamb & Matthews, 1988), though it is not clear if the concentration of analogue in the cell reached that in the pipet. We measured the effects of high concentrations of analogues on the cyclase activity at 1 mM GTP and 5 mM Mg^{2+}. As shown in Table III, 2, 5, and 10 mM GTP-γ-S inhibited 46, 53, and 61% of cyclase activity, respectively. For the same concentrations of GMP-PNP, the inhibition was 48, 65, and 73%.

Effect of Calcium on Inhibition of Cyclase Activity by GTP Analogues and ATP. Changes in the free calcium concentration were reported to influence guanylate cyclase activity (Lolley & Racz, 1982; Pepe et al., 1986) through mediation by a soluble protein (Koch & Stryer, 1988). Though the RDM used in the present experiments were washed to remove soluble proteins, the inhibition of cyclase activity by the nucleotides as shown in Figure 1 and Tables I–III could have been due to changes in free calcium concentration caused by the addition of nucleotides. To test this possibility, cyclase activity was measured at 1 and 0.03 μM Ca^{2+}, concentrations at which cyclase was shown to exhibit lower and 15-fold higher activity, respectively (Koch & Stryer, 1988). As shown in

Table IV: Effect of Calcium on Inhibition of Guanylate Cyclase Activity by GTP Analogues and ATP[a]

additions	pmol of cyclic GMP formed in assay	
	1 μM Ca^{2+}	0.03 μM Ca^{2+}
	Experiment 1	
none (control)	121	226
GTP-γ-S	92	175
GMP-PNP	60	113
ATP	7	5
	Experiment 2	
none (control)	207	419
GTP-γ-S	141	266
GMP-PNP	125	258
ATP	19	35

[a] Assays were done on washed RDM. The controls contained 1 mM GTP. Competing nucleotides were added at a final concentration of 2 mM. To obtain the desired free calcium and magnesium concentrations, assays with 1 mM nucleotide contained 1 mM EGTA, 5.06 mM $MgCl_2$, and 0.748 mM $CaCl_2$ (free [Ca], 1 μM; free [Mg], 4 mM) or 1 mM EGTA, 5.22 mM $MgCl_2$, and 0.0814 mM $CaCl_2$ (free [Ca], 0.03 μM; free [Mg], 4 mM). Assays with a total of 3 mM nucleotide contained 1 mM EGTA, 7.04 mM $MgCl_2$, and 0.749 mM $CaCl_2$ (free [Ca], 1 μM; free [Mg], 4 mM) or 1 mM EGTA, 7.21 mM $MgCl_2$, and 0.0814 mM $CaCl_2$ (free [Ca], 0.03 μM; free [Mg], 4 mM). The data shown are mean values of triplicate determinations.

Table V: Use of Nucleotides and Analogues as Substrates by Purified Guanylate Cyclase[a]

substrate	nmol of cyclic GMP or cyclic AMP min^{-1} (mg of protein)$^{-1}$	
	experiment 1	experiment 2
GTP	237 ± 82	265 ± 41
GTP-γ-S	206 ± 0	219 ± 54
GMP-PNP	10 ± 2	18 ± 3
ATP	16 ± 7	

[a] All assays contained 1 mM nucleotide or analogue and 5 mM magnesium.

Table IV, at 0.03 μM Ca^{2+}, the cyclase activity was about twice that at 1 μM. ATP and the analogues of GTP inhibited the cyclase activity at both concentrations of calcium. The fraction of activity inhibited by a given nucleotide was not significantly different at the two calcium concentrations.

Analogues as Substrates of Guanylate Cyclase. Though GTP-γ-S and GMP-PNP reduced the formation of cyclic GMP from GTP, they themselves could have served as cyclase substrates, with the result that the formation of cyclic GMP is unaffected. Table V shows the relative efficiency of these substrates at 1 mM with 5 mM Mg^{2+} as cofactor. GTP-γ-S is about 85% as good a substrate as GTP, but GMP-PNP substitutes for GTP with only about 5% efficiency. The cyclase also used ATP as substrate, producing cyclic AMP but only at about 7% efficiency compared to GTP. The cyclase preparation is clearly highly specific for GTP, though we cannot rule out the possibility that the partially purified guanylate cyclase preparation has a 7% contamination of adenylate cyclase.

Effects of Calcium and Manganese on the Effectiveness of GTP and GTP Analogues as Substrates of Guanylate Cyclase. Experiments shown in Table V were repeated under conditions where free calcium concentration was set at 1 or 0.03 μM. At 1 μM calcium, cyclase activity with GTP-γ-S was about 50% of that with GTP. With GMP-PNP, it was about 3%. At 0.03 μM calcium, their respective activities as substrates, compared with GTP, were about 23 and 2%. With Mn^{2+} as metal cofactor, all the nucleotides showed higher activity than with Mg^{2+}, but again GTP-γ-S was a better substrate than GMP-PNP (66% of GTP activity vs 15%).

Discussion

Guanylate cyclase activity was inhibited by ATP and the analogues of GTP and ATP. Was the inhibition due to changes in the free calcium brought about by added nucleotides? In experiments shown in Figure 1 and Tables I–III and V, free calcium concentration was not controlled. The solutions used in our experiments probably contained a finite amount of calcium contamination. If we assume that to be about 10 μM (Barkdoll et al., 1989), increasing the nucleotide concentration in the assay from 0.25 to 1.25 mM at fixed 2 mM free Mg^{2+} (as in Table I) should reduce the free calcium concentration from about 9 to about 6 μM. Changes in the calcium concentration in that range do not significantly alter the cyclase activity (Koch & Stryer, 1988). If calcium contamination was much lower and the nucleotides reduced it to a submicromolar level, cyclase activity should increase rather than decrease (Table IV) and result in an apparent analogue-dependent activation rather than inhibition. Therefore, the inhibition of cyclase activity by analogues is not attributable to changes in the free calcium concentration.

The guanylate cyclase activity of RDM assayed with 5 mM Mg and 2 mM GTP is about 0.05 nmol min^{-1} (nmol of rhodopsin)$^{-1}$ (Table III). Assuming that the rhodopsin concentration in the rod outer segment is 6 mM (Liebman, 1972) and that the cyclase activity is 15-fold-enhanced by the decrease in the internal free calcium concentration following a light flash (Lolley & Racz, 1982; Pepe et al., 1986; Ames et al., 1986; Hodgkin & Nunn, 1988; Koch & Stryer, 1988), this cyclase activity can produce about 70 μM cyclic GMP/s. Koch and Stryer (1988) reported a similar rate of 60 μM/s. From the data reported in the literature, we calculated that the maximal rate of cyclic GMP hydrolysis in light-activated ROS is between 0.5 and 14 mM/s[1] and therefore 7–200 times greater than the potential cyclase activity. Given this disparity in their activities, light-suppressed dark current can be restored by cyclase activity only after dim flashes that activate a small fraction of PDE activity. When a bright flash stimulates PDE activity in excess of ROS cyclase activity, cyclase activity alone cannot restore the ROS dark current. In the normal ROS, light-activated R*s and G*s are inactivated by rhodopsin kinase and GTPase activities, respectively (Liebman & Pugh, 1980; Dratz et al., 1987; Barkdoll et al., 1989), rapidly reducing the PDE activity and permitting cyclase activity to eventually outpace the PDE activity and restore the dark current. However, when rods injected with GTP analogues are activated by a bright light flash, the dark current should remain closed for hours because the G-proteins bind the analogues and cannot hydrolyze them over a period of several hours (Pfeuffer & Helmreich, 1975; Cassel & Selinger, 1977; Yamanaka et al., 1985). However, Kondo and Miller (1988) and Lamb and Matthews (1988) observed that dark current

[1] The turnover number for PDE is reported to be 2000–4000/s (Sitaramayya et al., 1986; Hurley & Stryer, 1982; Bennett & Clerc, 1989). The ratio of PDE to rhodopsin in rod outer segment is between 0.0155 and 0.01 (Baehr et al., 1979; Sitaramayya et al., 1986; Hamm & Bownds, 1986). The K_m for cyclic GMP is reported to be as low as 80 μM or as high as 1400 μM (Sitaramayya et al., 1986). The concentration of free cyclic GMP in the outer segment is probably about 4 μM [see Pugh and Lamb (1990)]. If we take the conditions that provide the lower limit of PDE activity (ratio to rhodopsin at 0.01, K_m at 1 mM, and turnover number of 2000/s), the hydrolytic rate of fully activated PDE in RDM will be 2000/s × 6 mM × 0.01 = 120 mM/s at saturating substrate concentration, which corresponds to 0.478 mM/s at 4 μM free cyclic GMP according to the Michaelis equation of $v = V_{max}[S]/K_m + [S]$. At the other extreme, assuming a K_m of 100 μM, the rate is 14.3 mM/s.

suppressed in response to "bright" and "intense" light flashes in analogue-injected cells recovered in 10–100 min. The eventual slow recovery of dark current in such cases could not have been due to cyclase alone since cyclase activation due to lowered calcium concentration occurs in less than 2 s following a light flash (Hodgkin et al., 1987; Nakatani & Yau, 1988). The observations of Kondo and Miller (1988) and Lamb and Matthews (1988) therefore suggest that the analogue-bound G-proteins do get inactivated in the cell either by simple dissociation of the analogue or by mechanisms yet unknown.

Both GTP-γ-S and GMP-PNP inhibit the cyclase activity, and under all the conditions that we tested, GMP-PNP was more inhibitory than GTP-γ-S. In addition, GTP-γ-S is about 85% as efficient a substrate as GTP while GMP-PNP is only about 5% as good (Table V). For cyclases from other tissues also, GTP-γ-S is a better substrate than GMP-PNP (Brandwein et al., 1982; Waldman et al., 1983). Given these observations, one would predict that when activated by light flashes of the same intensity, ROS injected with GTP-γ-S would recover the dark current faster than those treated with GMP-PNP. However, Lamb and Matthews (1988) found that dark current returns faster in GMP-PNP-injected ROS. Given this latter result, it appears that G*s with bound GTP-γ-S or GMP-PNP do get inactivated, with G*(GTP-γ-S) inactivated slower than G*(GMP-PNP).

ATP strongly inhibits the cyclase activity. Inhibition of rat lung guanylate cyclase by ATP was reported earlier (Brandwein et al., 1982). At the ROS concentrations of GTP and ATP (about 2 and 4 mM, respectively; Robinson & Hagins, 1979), and a K_i of 0.1 mM for ATP (Table II), cyclase activity will only be about 15% of its potential maximum activity. However, in the linear range of light activation, ATP-dependent mechanisms involving rhodopsin kinase, and possibly arrestin, strongly suppress PDE activity also (Liebman & Pugh, 1980; Zuckerman & Cheasty, 1986; Bennett & Sitaramayya, 1988). In the presence of ATP, the number of PDEs activated per R* is reduced by about 8-fold, and the duration of PDE activity is reduced by 4-fold (Barkdoll et al., 1989) or more (Sitaramayya et al., 1988). Thus, in normal ROS, ATP probably reduces cyclic GMP hydrolysis more than its synthesis. The extent to which analogues of GTP and ATP interfere with the effects of ATP on PDE activation, thereby influencing the recovery of dark current, is unknown. Metabolic transformation of nucleotide analogues by cyclase as shown here and possibly by nucleoside-diphosphate kinase as discussed by Lamb and Matthews (1988) should eventually remove the injected analogues. The effects of the products of such transformation, especially by nucleoside-diphosphate kinase, on G-protein, cyclase, and rhodopsin kinase, may again influence the kinetics of light response.

From the above observations, it appears that nucleotide analogues injected into rod outer segments have influences other than those on G-proteins. Their effects as inhibitors and substrates of cyclase and their possible influence on rhodopsin kinase need to be carefully considered in interpreting the effects of these analogues on photoreceptor light response.

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Registry No. GTP-γ-S, 37589-80-3; ATP, 56-65-5; GMP-PNP, 34273-04-6; AMP-PNP, 25612-73-1; GTP, 86-01-1; Mg, 7439-95-4; Mn, 7439-96-5; Ca, 7440-70-2; guanylate cyclase, 9054-75-5.

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Assignment of the Histidine Axial Ligands to the Cytochrome b_H and Cytochrome b_L Components of the bc_1 Complex from *Rhodobacter sphaeroides* by Site-Directed Mutagenesis†

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ABSTRACT: The cytochrome *b* subunit of the bc_1 complex contains two cytochrome components, cytochrome b_H and cytochrome b_L. Sequence comparisons of this polypeptide from a number of organisms have revealed four invariant histidines which have been postulated to be the heme ligands for the two protoheme IX prosthetic groups. In *Rhodobacter sphaeroides*, these correspond to His97, His111, His198, and His212. In this paper, the results of amino acid substitutions at each of these positions are reported. Replacement of His97 by either Asp or Asn and of His198 by Asn or Tyr resulted in loss of both cytochrome components. However, His111Asn, His111Asp, and His212Asp all resulted in the selective loss of cytochrome b_H and the retention of cytochrome b_L. Furthermore, flash kinetics studies show that the myxothiazol-sensitive quinol oxidase (Q_z) site associated with cytochrome b_L is still functional. These data support the assignment of the axial ligands to cytochrome b_H (His111 and His212) and cytochrome b_L (His97 and His198). This pairing is consistent with current models of the cytochrome *b* subunit with eight transmembrane α-helices.

The ubiquinol:cytochrome *c* oxidoreductases, also known as the bc_1 complexes, are central components in the energy-conserving electron-transfer chains of mitochondria, chloroplasts (where it is known as the b_6/f complex), and bacteria (Gabellini, 1988; Hauska et al., 1988; Crofts, 1985). These complexes manifest remarkable structural and functional similarities. All the bc_1 (and b_6f) complexes contain a cytochrome *b* subunit which appears to contribute major structural elements of both a quinol oxidase site (Q_z or Q_o) and a quinone reductase site (Q_c or Q_i). Two spectroscopically distinct cytochrome *b* components with different midpoint potentials (b_L and b_H) are found in all the bc_1 and b_6f complexes.

In a proposed Q-cycle mechanism (Mitchell, 1976; Crofts, 1985; Crofts et al., 1983), the low-potential cytochrome b_L component is located close to the quinol oxidase site, and the high-potential cytochrome b_H is close to the quinone reductase

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