Effects of Guanyl Nucleotides and Rhodopsin on ADP-ribosylation of the Inhibitory GTP-binding Component of Adenylate Cyclase by Pertussis Toxin*  

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Hormonal inhibition of adenylate cyclase is mediated by a guanyl nucleotide binding protein, Gi, which is composed of α, β, and γ subunits (Giα, Giβ, Giγ). Pertussis toxin blocks hormonal inhibition by catalyzing the ADP-ribosylation of Giα. With purified Gi subunits, but without nucleotides, it was observed that toxin-catalyzed ADP-ribosylation of Giγ was negligible in the absence of Giα. ATP, previously shown to increase ADP-ribosylation in membranes, enhanced the ADP-ribosylation of Giα in the absence, more than in the presence, of Giγ. Prior studies (Kanaho, Y., Tsai, S.-C., Adamik, R., Hewlett, E. L., Moss, J., and Vaughan, M. (1984) J. Biol. Chem. 259, 7976–7981) had demonstrated that rhodopsin, the retinal photon receptor protein, can replace inhibitory hormone receptors, and stimulate the hydrolysis of GTP by Giα in the presence of Giγ. Photolyzed rhodopsin, but not the inactive, dark protein, inhibited ADP-ribosylation of Giα in the presence of Giγ. ADP-ribosylation of Giγ, in the presence of Giα, and photolyzed (but not dark) rhodopsin was increased by guanosine 5'-O-(2-thiodiphosphate) or GDP, but not by (β,γ-methylene)guanosine triphosphate or guanosine 5'-O-(3-thiotriphosphate). Presumably, photolyzed rhodopsin and nucleoside triphosphate analogues activate Giα, whereas with dark rhodopsin and nucleoside diphosphates Giα is in the inactive state. The latter appears to be the preferred substrate for pertussis toxin. These observations are consistent with other evidence that rhodopsin and inhibitory hormone receptors are functionally similar.

The hormone-sensitive adenylate cyclase system is a multicOmponent, membrane-associated complex responsible for the synthesis of cAMP (1–3). Upon interaction with their specific agonists, inhibitory and stimulatory receptors, acting through different coupling proteins, decrease or increase, respectively, the activity of the catalytic unit (3). These coupling proteins, Gi1 and Gi2, involved in inhibition and stimulation, respectively, are heterotrimeric consisting of different α subunits (Giα, Giβ, Giγ) linked noncovalently to identical βγ subunits, Giβ, (3, 4); Giα (41 kDa) and Giγ (45 kDa) are GTP-binding proteins (3). Activation of Giα by the agonist-receptor complex appears to result from dissociation of the inactive, Giα, Giγ, to yield Giα, which, when GTP is bound directly stimulates the catalytic unit (3). Activation is terminated with hydrolysis of bound GTP and reassociation of Giα, GDP with Giγ (3, 5). The mechanism of activation by Giα is less clear, although, like Giγ, Giα is activated by dissociation of the α and βγ subunits (3). Inhibition is believed to occur either via a direct effect of Giα, GTP on the catalytic unit, with GTP hydrolysis terminating the suppression of activity, or as a result of the increase in free Giγ (3, 6). The latter, through mass action, would lead to increased formation of Giα,Giγ, and decreased stimulation (3). Conceivably, regulation may occur by both mechanisms. Conversion of the active form of Giα as well as of Giγ to an inactive species results from hydrolysis of bound GTP to GDP (7–9).

The retinal rod outer segment system consisting of the photon receptor rhodopsin, the GTP-binding protein transducin, and the cGMP phosphodiesterase is similar to the hormone-sensitive adenylate cyclase (10). Transducin also shares structural analogies with the GTP-binding proteins of cyclase (12, 15, 16). Transducin is a heterotrimer with α, β, and γ subunits of 39, 35, and ~10 kDa, respectively (12). The amino acid composition and protease digestion patterns of transducin Tα, and transducin Tβ are similar to those of Giα and Giγ, respectively (15). In addition, rhodopsin appears to be similar in some functional properties to the inhibitory hormone receptor (16). Photolyzed rhodopsin stimulates the GTPase activity of both Tα and Giα, the presence of Tβ, or Giγ (16). Thus, rhodopsin is capable of recognizing and activating the inhibitory components of cyclase (16).

Pertussis toxin (islet-activating protein) is believed to exert its effects on cells by catalyzing the ADP-ribosylation of Giα, (17). This modification of Giα inactivates it and abolishes hormonal inhibition of cyclase (17, 18, 21–23). Transducin also serves as a pertussis toxin substrate (24–26); ADP-ribosylated Tα no longer stimulates the phosphodiesterase;

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†The abbreviations used are: Gi and Giα, inhibitory and stimulatory guanyl nucleotide-binding regulatory components of adenylate cyclase; Giα, Giβ, Giγ, α subunits of Giα, Giβ, Giγ; Gi2, β and γ subunits of Giα, Giβ, and Giγ; Tα, and Tβ, subunits of transducin; Gpp(NH)p, guanyl-5'-yl imidodiphosphate; Gpp(CH2)p, (β,γ-methylene)guanosine triphosphate; GTPαS, guanosine 5'-O-(3-thiotriphosphate); GDPβS, guanosine 5'-O-(2-thiotriphosphate); CHAPS, 3-(3-cholamidopropyl)-dimethylammonio)-1-propane sulfonate.

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GTPase activity is decreased as is binding to rhodopsin (25, 26). Pertussis toxin has thus been useful in understanding the molecular basis for the rhodopsin-transducin interaction. Since rhodopsin can functionally replace the inhibitory hormone receptor in the interaction with Gi (16), we examined the effect of rhodopsin in its inactive (dark-adapted) and active (photolyzed) states, on the ADP-ribosylation by pertussis toxin of Gi, in the presence of GTP or other guanyl nucleotides. These studies demonstrate that the preferred toxin substrate is Gi,. ADP-ribosylation is decreased by agents that favor activation of G, such as Gpp(NH)p and photolyzed rhodopsin.

EXPERIMENTAL PROCEDURES

**Materials**—Sodium cholate (Sigma) was purified by the procedure of Ross and Schatz (27). NAD, thymidine, GTP, GMP, ATP, and phosphatidylcholine were purchased from Sigma; [γ-32P]GTP and [α-32P]NAD from New England Nuclear; Gpp(NH)p, GTPyS, and GDPβS from Boehringer Mannheim; NaF, AlCl₃, and MgCl₂ from Fisher; CHAPS from Calbiochem-Behring; Gpp(Ch)p and GDP from P-L Biochemicals.

**Purification of Proteins**—Rhodopsin, purified under dim red light as described by Hong and Hubbell (28), except that rod outer segment membranes were not lyophilized, was incorporated in phosphatidylcholine vesicles. Transducin was purified by the procedure of Kuhn (29).

G₉ and G₈ were prepared from rabbit liver membranes by the method of Sternweis et al. (30) with minor modifications; 10 μM AlCl₃ was used instead of ATP in TED/AMF (20 mM Tris, pH 8.0, containing 2 mM EDTA, 1 mM dithiothreitol, 10 μM AlCl₃, 6 mM MgCl₂, 10 mM fluoride) buffer (31). On electrophoresis of the G₉ preparations, a 41-kDa band accounted for ~25% of the stained protein (16). From comparison of its density with bovine serum albumin standards, the concentration of G₉ in the preparation used for experiments reported here was 7.2 μg/ml. In the experiments described in the legends to Figs. 1 and 4, a fraction of highly purified G₉ was obtained during gradient elution of the heptylamine column. The protein exhibited a predominant band at 41 kDa (Fig. 1); by densitometry, purity was estimated at 85%. On electrophoresis of G₈, a major band of 35 kDa was seen with a lighter band just behind the dye front. Purity was estimated as ~90%. By densitometry, the concentration of the 35-kDa proteins in the G₈ preparations was 18.5 μg/ml. Concentrations of other proteins were determined by the method of Lowry et al. (32).

**GTpase Assay**—Transducin (T₉, 2.5 μg and Tᵦ, 3.0 μg) was incubated at 4°C for 30 min in a total volume of 75 μl containing 0.1% sodium cholate, 15 μg of phosphatidylcholine, and 1.5 μg of rhodopsin (dark or light). The assay was initiated by adding 25 μl of reaction mixture to give final concentrations of 20 mM Tris-HCl (pH 7.5), 5 mM MgCl₂, 1 mM dithiothreitol, 0.1 mM EDTA, 2 μM [γ-32P]GTP (5-8 x 10⁶ cpm/assay), and 1 mM ATP. After incubation at 30°C for 10 min, 0.5 ml of 12% Norit-A in 20 mM phosphate buffer (pH 7.5) was added followed by centrifugation, and determination of radioactivity in the supernatant (16).

**[32P]ADP-ribosylation of Proteins and Electrophoresis**—Toxin-catalyzed [32P]ADP-ribosylation of proteins was carried out as described (34). For labeling with pertussis toxin, a sample of G₉ (36 ng in 5 μl) and G₈ (93 ng in 5 μl) in TED/AMF buffer (pH 8.0) containing 0.05% cholate was incubated at 30°C for 30 min in a total volume of 100 μl containing phosphatidylcholine vesicles without or with rhodopsin (1.5 μg of protein), 20 μg of ovalbumin, 20 μM thymidine, 20 μM [32P]NAD (2 μCi), 2 mM MgCl₂, 1.4 μg of activated pertussis toxin in 16 mM glycine and 0.5 mM dithiothreitol, 50 mM sodium phosphate buffer (pH 7.5), and nucleotides (10 μM) as indicated. Reaction was terminated by addition of trichloroacetic acid and proteins were prepared for electrophoresis in 12% gels (33). Autoradiograms of the gels are given in Figs. 2–5.

**RESULTS**

Without added nucleotide, in the absence of G₉π, there was very little ADP-ribosylation of G₉ by pertussis toxin whether or not photolyzed rhodopsin was present; labeling was increased by G₉π and was greater without than with rhodopsin (Fig. 2). As reported with membrane preparations (17), ATP increased ADP-ribosylation of G₉π; the effect of ATP was greater in the absence of G₉π. In the presence of 0.5 mM ATP, labeling was still increased by G₉π and little affected by rhodopsin (Fig. 2). Whereas photolyzed rhodopsin (in the absence of added nucleotide) decreased ADP-ribosylation of G₉π in the presence of G₉π (Figs. 2 and 3), dark rhodopsin had little effect (Fig. 3). With G₉π and G₈ in the absence of rhodopsin, labeling was increased somewhat by all the guanyl nucleotides tested and to a lesser extent by ATP, GTP, GDP, and GDPβS were more effective than Gpp(NH)p, Gpp(Ch)p, and GTPyS. ATP was effective at much higher concentrations (Fig. 4, upper). With rhodopsin, [32P]ADP-ribosylation in the absence of nucleotide was very low and was enhanced by GDP = GDPβS = GTP > Gpp(NH)p = Gpp(Ch)p = GTPyS (Fig. 4, lower). The effect of GDPβS was similar in photolyzed or dark rhodopsin (Fig. 5). Similarly, the differences between GDPβS and Gpp(NH)p were more apparent with photolyzed rhodopsin (Fig. 5).

**DISCUSSION**

Exposure of cells to pertussis toxin results in the apparent inactivation of G, following intoxication, there is 1) a de-
ADP-ribosylation of Adenylate Cyclase \( G_i \) by Pertussis Toxin

**Fig. 2.** Effect of \( G_m \) and rhodopsin on \([^{32}P]ADP-ribosylation of G_i. G_m was incubated with pertussis toxin with or without \( G_m \), photolyzed rhodopsin and 0.5 mM ATP as indicated. The peak areas determined by densitometry are, from left to right: 0, 2.7, 0, 0.4, 1.0, 3.1, 0.5, and 2.3. D.F., dye front.

**Fig. 3.** Effect of photolyzed and dark rhodopsin with and without \( G_m \) on \([^{32}P]ADP-ribosylation of G_i. G_m was incubated with pertussis toxin with or without \( G_m \), photolyzed rhodopsin and 0.5 mM ATP as indicated. The peak areas determined by densitometry are, from left to right: 0, 2.9, 0, 3.4, 0.0, 2.4, 0.1, and 0.6. D.F., dye front.

**Fig. 4.** Effect of guanyl nucleotides with and without photolyzed rhodopsin on \([^{32}P]ADP-ribosylation of a highly purified \( G_i \). G_m was incubated with pertussis toxin and \( G_m \), without (upper) or with (lower) photolyzed rhodopsin and nucleotide as indicated. Nucleotide concentrations were 10 \( \mu \)M except where ATP (a) 10 \( \mu \)M and (b) 0.5 mM were used. The peak areas are, upper panel from left to right: 1.6, 2.0, 2.5, 2.6, 5.2, 5.3, 5.1, 3.2, 6.0, and lower panel from left to right, 0, 0.1, 0.2, 0.3, 0.4, 1.7, 2.4, 0, 1.0. D.F., dye front.

**Fig. 5.** Effects of Gpp(NH)p and GDP\(\beta\)S with and without rhodopsin. G_m was incubated with pertussis toxin and \( G_m \), with or without 10 \( \mu \)M nucleotide and rhodopsin in the light or dark as indicated. The peak areas are: 1.5, 4.3, 4.5, 4.7, 4.5, 0.7, 2.7, 2.9, and 2.5. D.F., dye front.

Increased effect of inhibitory agonist on cAMP content of cells or on adenylate cyclase activity in membrane preparations (21–23), 2) a loss of inhibitory agonist-stimulated GTPase activity (34), and 3) a decrease in affinity of receptors for agonist but not antagonist (35, 36). All of these effects result from the ADP-ribosylation of G_m (17, 18). Based on studies in a number of laboratories, it would appear that toxin-catalyzed ADP-ribosylation promotes the stabilization of the inactive species of G_m containing G_m·G_d. As shown here, the preferred substrate for pertussis toxin is G_m·G_d. ADP-ribosylation of G_m was less than one-tenth that of G_m. G_m, whether or not the inhibitory hormone receptor analogue, rhodopsin, was present.

ADP-ribosylation in the reconstituted G_m, G_d system with and without rhodopsin was influenced by ATP and guanyl nucleotides. Without rhodopsin, GDP\(\beta\)S increased slightly the \([^{32}P]ADP-ribosylation of G_m and was slightly more effective than Gpp(NH)p. In the presence of photolyzed rhodopsin, which decreased ADP-ribosylation, effects of guanyl nucleotides were more clearly differentiated. Gpp(NH)p, Gpp(CH_3)p, and GTP\(\gamma\)S slightly increased the ADP-ribosylation but were less effective than GDP, GTP, and GDP\(\beta\)S; a more than 4-fold increase in ADP-ribosylation was observed over that obtained in the absence of nucleotide. The similarity in effects of GTP and GDP reflects the hydrolysis of GTP to...
GDP; as demonstrated previously, in the presence of photolyzed rhodopsin, transducin catalyzed the hydrolysis of GTP to GDP (16). Since GDP/PS is believed to interact with the inactive G\textsubscript{a}.G\textsubscript{o} complex, while binding of Gpp(NH)p or GTP\textsubscript{y}S promotes the formation of the active G\textsubscript{a} species with bound nucleotide (3, 37), these data further support the hypothesis that pertussis toxin catalyzes the ADP-ribosylation of the inactive form of G\textsubscript{a}.

It has been reported that ATP enhanced the pertussis toxin-catalyzed reaction in membranes (17), although other investigators have not observed an ATP dependence for ADP-ribosylation of purified G\textsubscript{a} (38). In the present studies, with purified G\textsubscript{a} plus G\textsubscript{b}, ATP increased only slightly the extent of ADP-ribosylation. In the absence of G\textsubscript{b}, however, ADP-ribosylation of G\textsubscript{a} was enhanced to a significant extent by 0.5 mM ATP. Thus, the presence of an ATP effect on ADP-ribosylation may depend on whether or not G\textsubscript{b} is associated with G\textsubscript{a}.

Rhodopsin can substitute for an inhibitory hormone receptor in the GTPase reaction catalyzed by G\textsubscript{a} (16). Photolyzed rhodopsin is similar in function to the agonist-occupied receptor, whereas the dark-adapted protein appears to be analogous to the inactive receptor. Studies by Van Dop et al. (25) were consistent with the hypothesis that pertussis toxin-catalyzed ADP-ribosylation of transducin T\textsubscript{a}.T\textsubscript{b} was favored in dark-adapted rod outer segment membranes. Similarly with G\textsubscript{a}, G\textsubscript{b}, as substrate, ADP-ribosylation by toxin was suppressed by photolyzed rhodopsin to a much greater extent than by dark rhodopsin. Thus, the effects of light on the ADP-ribosylation of transducin in the presence of rod outer segments was similar to that observed in the present system when G\textsubscript{a} was used as the toxin substrate and purified rhodopsin served as the photon acceptor. Although previously an effect of light on the toxin-catalyzed ADP-ribosylation of transducin was not observed with rod outer segments, using the purified rhodopsin-transducin system, photolysis proved to be inhibitory.\textsuperscript{2} In some cases, an effect was obtained with dark rhodopsin; the small effect of dark rhodopsin is in agreement with the observation that its effect on transducin GTPase activity was only one-tenth that of photolyzed rhodopsin (data not shown). With photolyzed or dark rhodopsin, GDP/PS promoted the toxin-catalyzed reaction to a greater extent than did Gpp(NH)p; GDP/PS was also more effective in the rhodopsin-transducin system (25). Thus, these studies further document the structural and functional similarities of rhodopsin and the inhibitory hormone receptor.

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\textsuperscript{2} P. A. Watkins, Y. Kanaho, J. Moss, unpublished observations.

REFERENCES