Inhibition of the GTPase activity of transducin by an NAD⁺:arginine ADP-ribosyltransferase from turkey erythrocytes

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The bacterial toxins, cholera toxin and pertussis toxin, inhibit the light-stimulated GTPase activity of bovine retinal rod outer segments by catalysing the ADP-ribosylation of the α -subunit (T_α) of transducin [Abood, Hurley, Pappone, Bourne & Stryer (1982) *J. Biol. Chem.* **257**, 10540–10543; Van Dop, Yamanaka, Steinberg, Sekura, Manclark, Stryer & Bourne (1984) *J. Biol. Chem.* **259**, 23–26]. Incubation of retinal rod outer segments with NAD⁺ and a purified NAD⁺:arginine ADP-ribosyltransferase from turkey erythrocytes resulted in approx. 60% inhibition of GTPase activity. Inhibition was dependent on both enzyme and NAD⁺, and was potentiated by the non-hydrolysable GTP analogues guanosine 5'-[β -imido]triphosphate (p[NH]ppG) and guanosine 5'-[β -methylene]triphosphate (p[CH₂]ppG). The transferase ADP-ribosylated both the T_α and T_β subunits of purified transducin. T_α (39 kDa), after ADP-ribosylation, migrated as two distinct peptides with molecular masses of 42 kDa and 46 kDa on SDS/polyacrylamide-gel electrophoresis. T_β (36 kDa), after ADP-ribosylation, migrated as a 38 kDa peptide. With purified transducin subunits, it was observed that the GTPase activity of ADP-ribosylated T_α , reconstituted with unmodified T_β and photolysed rhodopsin, was decreased by 80%; conversely, reconstitution of T_α with ADP-ribosyl- T_β resulted in only a 19% inhibition of GTPase. Thus ADP-ribosylation of T_α , the transducin subunit that contains the guanine nucleotide-binding site, has more dramatic effects on GTPase activity than does modification of the critical 'helper subunits' T_β . To elucidate the mechanism of GTPase inhibition by transferase, we studied the effect of ADP-ribosylation on p[NH]pp[³H]G binding to transducin. It was shown previously that modification of transducin by cholera toxin, which like transferase ADP-ribosylates arginine residues, did not affect guanine nucleotide binding. ADP-ribosylation by the transferase, however, decreased p[NH]pp[³H]G binding, consistent with the hypothesis that cholera toxin and transferase inhibit GTPase by different mechanisms.

INTRODUCTION

Transducin (T), a guanine nucleotide-binding protein of retinal rod cells, regulates signal coupling during the early stages of visual excitation by linking the photon receptor, rhodopsin, with a cyclic GMP phosphodiesterase (Pober & Bitensky, 1979; Stryer *et al.*, 1981). Transducin is a heterotrimer with subunits of 39 kDa (T_α), 36 kDa (T_β) and approx. 8 kDa (T_γ) (Kühn, 1980; Fung *et al.*, 1981). T_α contains the guanine nucleotide-binding site and is a GTPase, although manifestation of this activity requires the presence of T_β and photolysed rhodopsin (Fung *et al.*, 1981; Fung, 1983). Transducin is homologous to the stimulatory (G_s) and inhibitory (G_i) guanine nucleotide-binding regulatory proteins of adenylate cyclase (Manning & Gilman, 1983); these proteins also consist of α (45 and 41 kDa respectively), β (35 kDa) and γ (5 kDa) subunits (Northup *et al.*, 1980; Hildebrandt *et al.*, 1984; Bokoch *et al.*, 1983; Gilman, 1984). G_s and G_i , like transducin, are GTPases (Cassel & Selinger, 1976; Koski & Klee, 1981; Gilman, 1984).

Cholera toxin activates adenylate cyclase by ADP-

ribosylating the α -subunit of G_s (Cassel & Pfeuffer, 1978; Gill & Meren, 1978; Johnson *et al.*, 1978; Gilman, 1984); this covalent modification inhibits the GTPase activity of G_s and thus preserves the activated G_s -GTP complex (Cassel & Selinger, 1977). Similarly, cholera toxin ADP-ribosylates T_α in ROS, resulting in the inhibition of light-stimulated GTPase activity (Abood *et al.*, 1982). Pertussis toxin (also known as islet-activating protein) affects adenylate cyclase by ADP-ribosylating G_i (Katada & Ui, 1982a,b; Bokoch *et al.*, 1983; Gilman, 1984); the rise in GTPase activity normally caused by agonists acting through inhibitory receptors is thereby prevented (Burns *et al.*, 1983). T_α is also ADP-ribosylated by pertussis toxin, leading to a decrease in light-stimulated GTPase activity (Van Dop *et al.*, 1984a; Watkins *et al.*, 1984). Thus, although the amino acid ADP-ribosylated in T_α by the two toxins differs (Van Dop *et al.*, 1984b; West *et al.*, 1985), modification by either toxin decreases the light-stimulated ROS GTPase activity (Abood *et al.*, 1982; Van Dop *et al.*, 1984a; Watkins *et al.*, 1984).

An NAD⁺:arginine ADP-ribosyltransferase that catalyses a reaction similar to that of cholera toxin has been

Abbreviations used: GTP[γ S], guanosine 5'-[γ -thio]triphosphate; GDP[β S], guanosine 5'-[β -thio]diphosphate; p[NH]ppA, adenosine 5'-[β -imido]triphosphate; p[NH]ppG, guanosine 5'-[β -imido]triphosphate; p[CH₂]ppG, guanosine 5'-[β -methylene]triphosphate; ROS, retinal rod outer segments; T, transducin; G_i , inhibitory guanine nucleotide-binding subunit of adenylate cyclase; G_s , stimulatory guanine nucleotide-binding subunit of adenylate cyclase.

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purified from turkey erythrocytes (Moss & Vaughan, 1978; Moss *et al.*, 1979, 1980). This enzyme ADP-ribosylates numerous proteins in both crude mixtures and purified protein preparations (Moss & Vaughan, 1978; Watkins & Moss, 1982; Moss *et al.*, 1984); however, the role of this transferase in the regulation of cellular metabolism remains unclear. As reported in the present paper, the erythrocyte transferase ADP-ribosylates transducin and this covalent modification results in decreased GTPase activity.

A preliminary report of some of the present work has been published (Watkins & Moss, 1984).

EXPERIMENTAL

Materials

NAD⁺:arginine ADP-ribosyltransferase was purified to homogeneity from turkey erythrocyte cytosol as previously described (Moss *et al.*, 1980); 1 unit of enzyme catalyses the transfer of 1 μ mol of ADP-ribose to agmatine/min. p[NH]ppG, p[CH₂]ppG, GTP[γ S] and GDP[β S] were purchased from Boehringer-Mannheim (Indianapolis, IN, U.S.A.); GTP, ATP (prepared by phosphorylation of adenosine), agmatine and thymidine were from Sigma Chemical Co. (St. Louis, MO, U.S.A.); p[NH]ppA was from ICN (Cleveland, OH, U.S.A.); NAD⁺ was from P-L Biochemicals (Piscataway, NJ, U.S.A.); [α -³²P]NAD⁺ (10–50 Ci/mmol) and [γ -³²P]GTP (10–50 Ci/mmol) were from New England Nuclear (Boston, MA, U.S.A.); p[NH]pp[³H]G (5–15 Ci/mmol) was from Amersham (Arlington Heights, IL, U.S.A.); reagents for gel electrophoresis were from Bio-Rad Laboratories (Rockville Centre, NY, U.S.A.). [α -³²P]ADP-ribose was prepared by incubation of [α -³²P]NAD⁺ with erythrocyte transferase in 50 mM-potassium phosphate buffer, pH 7.0, containing 0.2 M-NaCl; the product was purified by h.p.l.c. on a Dupont SAX column (4.6 mm $\times$ 250 mm) by isocratic elution with 150 mM-KH₂PO₄.

Determination of protein

Protein was determined either by the method of Lowry *et al.* (1951), with bovine plasma albumin as standard, or by the method of Bradford (1976), with bovine plasma γ -globulin as standard.

Preparation of ROS, transducin, transducin subunits and rhodopsin

ROS membranes were prepared from dark-adapted bovine retinas (Watkins *et al.*, 1984) under dim red light by the method of Fung & Stryer (1980). Dark-adapted-retinal ROS, which were stored at -20°C , were photolysed by exposure to room light for at least 10 min immediately preceding incubation; all subsequent procedures were performed under normal room light. Transducin was extracted from photolysed ROS with 100 μ M-GTP as described by Kühn (1980) and purified to homogeneity. T _{α} was separated from T _{$\beta\gamma$} on Blue Sepharose CL-6B (Kanaho *et al.*, 1984). Rhodopsin, purified to homogeneity from dark-adapted retinas by solubilization in 100 mM-dodecyltrimethylammonium bromide and chromatography on hydroxyapatite, was reconstituted in phosphatidylcholine vesicles (Hong & Hubbell, 1973).

ADP-ribosylation of transducin and GTPase assay

Photolysed ROS (85–100 μ g/assay) were incubated at 30 $^{\circ}\text{C}$ with the NAD⁺:arginine ADP-ribosyltransferase (0.4–44 munits/assay) in a reaction mixture containing 50 mM-potassium phosphate buffer, pH 7.5, 20 mM-thymidine, 0.2 M-NaCl, 1 mM-NAD⁺ and other additions as indicated. After incubation, samples were removed for assay of GTPase activity (Watkins *et al.*, 1984). In [³²P]ADP-ribosylation experiments, purified transducin subunits were used instead of ROS, and 1 mM-[α -³²P]NAD⁺, 2–3 μ Ci/assay, replaced unlabelled NAD⁺. Reactions were terminated with ice-cold 10% (w/v) trichloroacetic acid; precipitated proteins were sedimented at 2200 g for 15 min, solubilized in 1% (w/v) SDS in the presence of 50 mM-dithiothreitol, and subjected to electrophoresis on 15% polyacrylamide gels containing 0.1% SDS (Laemmli, 1970). Kodak XAR film was exposed to dried gels for 4–24 h. For quantitative determinations of [³²P]ADP-ribose incorporation, Coomassie Blue-stained gels were sliced and radioactivity of individual bands was measured by liquid-scintillation spectrometry. Densitometry of stained gels with an LKB model 2202 densitometer was used to estimate the relative proportions of 46 kDa and 42 kDa T _{α} . The results of five or six determinations are expressed as mol of ADP-ribose incorporated/mol of protein (means $\pm$ S.D.).

p[NH]pp[³H]G binding

Transducin subunits (2.5 μ g of T _{α} , 2.5 μ g of T _{$\beta\gamma$}) were incubated in a total volume of 60 μ l for 1 h at 30 $^{\circ}\text{C}$ with or without transferase (8.2 munits) in medium containing 50 mM-potassium phosphate buffer, pH 7.5, 20 mM-thymidine, 1.2 M-NaCl and 1 mM-NAD⁺. Samples were cooled on ice for 5 min, and photolysed rhodopsin (1.5 μ g) was added. Binding of p[NH]pp[³H]G at 4 $^{\circ}\text{C}$ was initiated with 25 μ l of buffer containing (final concentrations) 20 mM-Tris/HCl buffer, pH 7.5, 5 mM-MgCl₂, 1 mM-dithiothreitol, 0.1 mM-EDTA and 1 μ M-p[NH]pp[³H]G (1.7×10^5 c.p.m.). After the indicated time at 4 $^{\circ}\text{C}$, radioactivity bound to transducin was trapped on 0.65 μ m-pore-size type DA Millipore filters.

RESULTS

Incubation at 30 $^{\circ}\text{C}$ of photolysed ROS membranes with erythrocyte NAD⁺:arginine ADP-ribosyltransferase resulted in inhibition of GTPase activity (Figs. 1 and 2). Inhibition was time-, transferase- and NAD⁺-dependent (Figs. 1 and 2). At suboptimal transferase concentrations, several guanine nucleotides promoted inhibition of GTPase activity by the enzyme, whereas adenine nucleotides (ATP, p[NH]ppA) had little effect (Table 1). The non-hydrolysable GTP analogue p[NH]ppG at a concentration of 1 μ M enhanced inhibition by transferase (Table 1); p[CH₂]ppG had a similar effect but at higher concentrations (Table 1). GDP was inactive (Table 1). GTP[γ S] was itself inhibitory; no additional effect of transferase was seen (Table 1). In these experiments, incubations with nucleotides and transferase were done at high concentrations of ROS and subsequently diluted into GTPase assays to minimize nucleotide effects on the assay itself.

When ROS were incubated with [³²P]NAD⁺ and transferase, numerous proteins were [³²P]ADP-ribo-

Fig. 1. Time- and ADP-ribosyltransferase-dependence of the inhibition of ROS GTPase activity by turkey erythrocyte NAD⁺:arginine ADP-ribosyltransferase

(a) Time course. Photolysed ROS (approx. 85 μ g of protein) were incubated at 30 °C with 1 mM-NAD⁺, transferase (23 munits) and 1 μ M-p[NH]ppG in a total volume of 60 μ l. At the indicated times, GTPase activity of triplicate samples was determined. Results are expressed as percentage of activity of control samples incubated for the same time but without transferase. Control activities were 690 and 560 pmol/min per mg of ROS protein at 0 and 180 min respectively. (b) Transferase-dependence. Photolysed ROS were incubated as in (a) for 60 min with the indicated concentrations of transferase. GTPase activity of triplicate samples was determined and is expressed as percentage of that of control incubations without transferase.

sylated (results not shown); thus correlation of decreased GTPase activity with covalent modification of a specific ROS protein was not possible. Since transducin was the most likely light-activated GTPase to be affected by the

Fig. 2. Effects of NAD⁺ on inhibition by erythrocyte transferase of ROS GTPase activity

Photolysed ROS (approx. 85 μ g of protein) were incubated for 60 min at 30 °C with transferase (41 munits), 1 μ M-p[NH]ppG and the indicated NAD⁺ concentration in 60 μ l. GTPase activity of triplicate samples was determined; results are expressed as percentage of activity of control incubations without transferase but with the appropriate NAD⁺ concentration. NAD⁺ alone had no significant effect on GTPase activity.

transferase, effects of covalent modification on the activity of this protein were examined. Both the T _{α} and T _{β} subunits of purified transducin were [³²P]ADP-ribosylated in the presence of transferase and [³²P]-NAD⁺ (Fig. 3). ADP-ribosylation of T _{β} increased its apparent size on SDS/polyacrylamide-gel electrophoresis from 36 kDa to 38 kDa; all radioactivity was associated with the 38 kDa band (Fig. 3). Approx. 0.30 $\pm$ 0.08 mol of ADP-ribose was incorporated/mol of T _{β} under these conditions. Unmodified T _{α} migrated as a single band of approx. 39 kDa; after incubation with transferase and [³²P]NAD⁺, however, two bands of approx. 42 kDa and 46 kDa were observed by autoradiography and protein staining (Fig. 3). On the basis of densitometry of the Coomassie Blue-stained protein bands, incorporation of ADP-ribose into these two peptides was 1.37 $\pm$ 0.07 mol/mol of 46 kDa T _{α} and 0.73 $\pm$ 0.23 mol/mol of 42 kDa T _{α} . Incorporation of radioactivity into the 42 kDa and 46 kDa proteins proceeded rapidly; after about 30 min of incubation, no further change in the labelling of either protein was observed for up to 4.5 h at 30 °C (results not shown). Further addition of transferase, [³²P]NAD⁺ or both during incubation failed to alter either the Coomassie Blue staining pattern or the relative radioactivity in the two bands, suggesting that the 42 kDa protein was not simply an intermediate in the formation of the 46 kDa protein. When T _{α} was incubated with [³²P]ADP-ribose (rather than [³²P]NAD⁺), with or without transferase, there was no incorporation of radioactivity and the protein remained as a 39 kDa species; thus non-enzymic ADP-ribosylation of T _{α} did not occur. To determine the mechanism of inhibition of GTPase activity, we examined with purified T _{α} and T _{β} subunits

Table 1. Effect of nucleotides on inhibition of ROS GTPase activity by the NAD⁺:arginine ADP-ribosyltransferase

Photolysed ROS (approx. 100 μ g of protein) were incubated for 60 min with 1 mM-NAD⁺, without or with transferase (1.5 munits) and the indicated nucleotide in a final volume of 60 μ l. GTPase activity of triplicate samples was determined.

Addition	Concn. (μ M)	GTPase activity (pmol/min per mg of protein)	
		Without transferase	With transferase
None		627	488 (78 %)
p[NH]ppG	1	423	239 (56 %)
p[CH ₂]ppG	1	628	475 (76 %)
	100	513	278 (54 %)
GDP	1	600	476 (79 %)
	10	542	397 (73 %)
GTP[γ S]	1	166	146 (88 %)
	10	139	133 (96 %)
ATP	5	602	466 (78 %)
	0.5	612	490 (80 %)
p[NH]ppA	1	633	469 (74 %)
	10	646	467 (72 %)

whether ADP-ribosylation of transducin had an effect on binding of p[NH]pp[³H]G. T _{α} and T _{$\beta\gamma$} were incubated with transferase in the absence of p[NH]ppG. As shown in Fig. 4, p[NH]pp[³H]G binding after reconstitution with photolysed rhodopsin was decreased by approx. 60%. To determine whether inhibition of GTPase by transferase was more dependent on ADP-ribosylation of

Fig. 4. Effect of transferase on p[NH]ppG binding to transducin

Transducin subunits (2.5 μ g of T _{α} , 2.5 μ g of T _{$\beta\gamma$}) were incubated for 1 h at 30 °C without (●) or with (▲) 8.2 munits of transferase; photolysed rhodopsin (1.5 μ g) was added before assay of p[NH]pp[³H]G binding as described in the Experimental section.

T _{α} or T _{$\beta\gamma$} , the individual subunit preparations were first incubated with or without transferase before assay of GTPase in a reconstituted system with liposomes containing photolysed rhodopsin (Table 2). Reconstitution of ADP-ribosyl-T _{α} with unmodified T _{$\beta\gamma$} yielded only 20% of the GTPase activity measured with unmodified T _{α} -T _{$\beta\gamma$} (Table 2). Conversely, 81% of control GTPase activity was measured in a reconstituted system with unmodified T _{α} and ADP-ribosyl-T _{$\beta\gamma$} (Table 2). When both T _{α} and T _{$\beta\gamma$} were ADP-ribosylated, GTPase activity was only 11% of control activity (Table 2).

Fig. 3. [³²P]ADP-ribosylation of transducin and its subunits by NAD⁺:arginine ADP-ribosyltransferase

Purified transducin (T, 16 μ g), T _{α} (10 μ g) or T _{$\beta\gamma$} (12 μ g) was incubated for 2.5 h with 1 mM-[α -³²P]NAD⁺ and 1 μ M-p[NH]ppG, with or without transferase (44 munits), in a final volume of 60 μ l. After termination of the reaction with trichloroacetic acid, precipitated proteins were electrophoresed on 15% polyacrylamide gels containing SDS. Lanes marked '-' or '+' transferase show the Coomassie Blue staining pattern; lanes marked 'A' are autoradiograms of the preceding ('+') lane. Markers at the left indicate the mobilities of molecular-mass standards.

Table 2. Inhibition of reconstituted GTPase activity following ADP-ribosylation of purified transducin subunits by NAD⁺:arginine ADP-ribosyltransferase

Purified T_α (90 μg) or T_{βγ} (30 μg) was incubated in a total volume of 300 μl for 60 min at 30 °C with 1 mM-NAD⁺ and 1 μM-p[NH]ppG, without or with transferase (123 units). After addition of agmatine (to inhibit further ADP-ribosylation of transducin subunits, final concn. 5 mM), 30 μl of T_α plus 40 μl of T_{βγ} were reconstituted with photolysed rhodopsin (1.5 μg) and assayed in duplicate for GTPase activity.

Subunits	GTPase activity	
	(pmol/ 20 min per μg of T _α)	(%)
T _α + T _{βγ}	7.0	100
ADP-ribosyl-T _α + T _{βγ}	1.4	20
T _α + ADP-ribosyl-T _{βγ}	5.7	81
ADP-ribosyl-T _α + ADP-ribosyl-T _{βγ}	0.8	11

DISCUSSION

The present report demonstrates that ADP-ribosylation of transducin by an NAD⁺:arginine ADP-ribosyltransferase purified from turkey erythrocytes results in a decrease in GTPase activity. It is well documented that arginine residues in proteins can be critical for enzymic activity (Lange *et al.*, 1974; Forster & Harrison, 1974; Borders & Riordan, 1975; Powers & Riordan, 1975); in most instances, the importance of the arginine residue was established by the use of chemical reagents such as phenylglyoxal, which display a selective reactivity for the guanidino group (Takahashi, 1968; Arnone *et al.*, 1971; Daemen & Riordan, 1974; Lange *et al.*, 1974; Borders & Riordan, 1975; Powers & Riordan, 1975). With glutamine synthetases from sheep brain and chicken heart, it was shown that modification catalysed by the erythrocyte transferase resulted in the loss of the Mg²⁺-dependent activity (Moss *et al.*, 1984). Thus the enzymic modification may be useful for identifying critical arginine residues. In the case of transducin, modification of T_α was critical to inhibition of GTPase. Although T_{βγ} and photolysed rhodopsin have been shown to be necessary for expression of T_α GTPase activity (Fung *et al.*, 1981; Fung, 1983), ADP-ribosylation of T_{βγ} had only a small effect on GTPase activity.

Van Dop *et al.* (1984a) reported a slight decrease in the mobility on SDS/polyacrylamide-gel electrophoresis of T_α that had been ADP-ribosylated by pertussis toxin. After modification by the erythrocyte transferase, both T_α and T_β exhibited decreased mobility. Since prolonged incubation of T_α with the erythrocyte transferase failed to alter the extent of incorporation of ADP-ribose into the 42 kDa or 46 kDa peptides, it is unlikely that the 42 kDa species (containing approx. 1 mol of ADP-ribose/mol of protein) is an 'intermediate' in the formation of the 46 kDa species (containing approx. 2 mol of ADP-ribose/mol of protein). The separation of two distinct ADP-ribosylated species of transferase-treated T_α is consistent with the hypothesis that T_α may consist of two non-identical peptides of very similar

molecular mass that co-purified from ROS. More likely, however, minor changes in primary, secondary or tertiary structure of T_α during purification or storage could have altered the availability of sites for ADP-ribosylation by the transferase.

The bacterial toxins cholera toxin and pertussis toxin inhibit the GTPase activity of transducin by ADP-ribosylating T_α, but neither toxin modifies T_{βγ} in ROS. Whereas cholera toxin ADP-ribosylated an arginine residue in T_α (Van Dop *et al.*, 1984b), the acceptor site for pertussis toxin-catalysed ADP-ribosylation was a cysteine residue (West *et al.*, 1985).

Inhibition of ROS GTPase activity by cholera toxin but not pertussis toxin required the presence of p[NH]ppG (Abood *et al.*, 1982; Van Dop *et al.*, 1984a; Watkins *et al.*, 1984). In the present study, p[NH]ppG enhanced the inhibitory effect of transferase on GTPase activity, but only at suboptimal amounts of the transferase. Inhibition of GTPase by cholera toxin results from a decrease in the single turnover rate of GTP hydrolysis without an effect on p[NH]ppG binding (Navon & Fung, 1984). In contrast, pertussis toxin is believed to inhibit GTPase by blocking the agonist-dependent binding to guanine nucleotides (Asano *et al.*, 1984; Bruni *et al.*, 1985). In this respect, it appears that the mechanism of transferase-catalysed GTPase inhibition is more like that of pertussis toxin than that of cholera toxin.

The difference in mechanism of action of cholera toxin and the erythrocyte transferase is interesting since both are NAD⁺:arginine ADP-ribosyltransferases. Both cholera toxin and the transferase ADP-ribosylate transducin; however, purified transducin is a poor substitute for cholera toxin (Abood *et al.*, 1982), whereas it is readily ADP-ribosylated by the transferase. Thus, with purified transducin, arginine residues available to the erythrocyte transferase are not readily accessible to the toxin. Under these conditions, the toxin is active, since the free amino acid as well as T_α in ROS is readily ADP-ribosylated. These studies are consistent with the hypothesis that different arginine residues are modified by cholera toxin and the erythrocyte transferase with different effects on the action of transducin.

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