Immunological and Biochemical Differentiation of Guanyl Nucleotide Binding Proteins: Interaction of $G_{o\alpha}$ with Rhodopsin, Anti-$G_{o\alpha}$ Polyclonal Antibodies, and a Monoclonal Antibody against Transducin α Subunit and $G_{i\alpha}$

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ABSTRACT: Guanyl nucleotide binding proteins couple agonist interaction with cell-surface receptors to an intracellular enzymatic response. In the adenylate cyclase system, inhibitory and stimulatory effects are mediated through guanyl nucleotide binding proteins, G_i and G_s, respectively. In the visual excitation complex, the photon receptor rhodopsin is linked to its target, cGMP phosphodiesterase, through transducin (G_t). Bovine brain contains another guanyl nucleotide binding protein, G_o. The proteins are heterotrimers of α, β, and γ subunits; the α subunits catalyze receptor-stimulated GTP hydrolysis. To examine the interaction of $G_{o\alpha}$ with $\beta\gamma$ subunits and rhodopsin, the proteins were reconstituted in phosphatidylcholine vesicles. The GTPase activity of $G_{o\alpha}$ purified from bovine brain was stimulated by photolyzed, but not dark, rhodopsin and was enhanced by bovine retinal $G_{t\beta\gamma}$ or by rabbit liver $G_{\beta\gamma}$. $G_{o\alpha}$ in the presence of $G_{\beta\gamma}$ is a substrate for pertussis toxin catalyzed ADP-ribosylation; the modification was inhibited by photolyzed rhodopsin and enhanced by guanosine 5′-*O*-(2-thiodiphosphate). ADP-Ribosylation of $G_{o\alpha}$ by pertussis toxin inhibited photolyzed rhodopsin-stimulated, but not basal, GTPase activity. It would appear from this and prior studies that $G_{o\alpha}$ is similar to $G_{t\alpha}$ and $G_{i\alpha}$; all three proteins exhibit photolyzed rhodopsin-stimulated GTPase activity, are pertussis toxin substrates, and functionally couple to $G_{t\beta\gamma}$. $G_{o\alpha}$ (39K) can be distinguished from $G_{i\alpha}$ (41K) but not from $G_{t\alpha}$ (39K) by molecular weight. A monoclonal antibody against $G_{t\alpha}$ that cross-reacts well with $G_{i\alpha}$ but relatively poorly with $G_{o\alpha}$ and polyclonal antibodies directed to $G_{o\alpha}$ but not $G_{i\alpha}$ and $G_{t\alpha}$ can be used to distinguish the two 39-kilodalton proteins.

Guanyl nucleotide binding proteins are involved in the regulation of cell growth and responses to external stimuli (Gilman, 1984; Lefkowitz et al., 1984; Kataoka et al., 1985). In the hormone-sensitive adenylate cyclase system, agonists either stimulate or inhibit catalytic activity by binding to receptors coupled to stimulatory or inhibitory guanyl nucleotide binding proteins, termed G_s[1] or G_i, respectively (Gilman, 1984; Lefkowitz et al., 1984). G_i and G_s are responsible for modulation of catalytic unit activity and thus formation of cAMP from ATP (Gilman, 1984; Lefkowitz et al., 1984). In the visual excitation system, the light receptor rhodopsin is coupled to its target enzyme, cGMP phosphodiesterase, through a guanyl nucleotide binding protein, transducin (Stryer et al., 1981; Fung & Stryer, 1980, Fung et al., 1981; Fung, 1983). G_i, G_s, and transducin are similar in structure and enzymatic properties. All three proteins are heterotrimers, composed of α, β, and γ subunits (Gilman, 1984; Fung et al., 1981; Hildebrandt et al., 1984); the α subunits bind GTP and related guanyl nucleotides. The molecular weights of the α subunits, $G_{i\alpha}$ (41K), $G_{s\alpha}$ (42K–47K), and $G_{t\alpha}$ (39K), are similar (Gilman, 1984; Fung et al., 1981; Northup et al., 1980, Sternweis et al., 1981; Bokoch et al., 1983, 1984). G_i and G_s share common $\beta\gamma$ subunits of molecular weights 36K and ~10K, respectively (Gilman, 1984; Hildebrandt et al., 1984; Manning & Gilman, 1983). $G_{t\beta\gamma}$ is similar in molecular weight to the $G_{\beta\gamma}$ components (Fung et al., 1981), and $G_{t\beta}$ is similar in amino acid composition, protease digestion pattern, and sequence to G_β (Manning & Gilman, 1983; Sugimoto et al., 1985; Fong et al., 1986; Codina et al., 1986).

The α subunits of G proteins bind guanyl nucleotides and catalyze the hydrolysis of GTP to GDP + P_i (Northup et al., 1982, 1983; Katada et al., 1984; Brandt et al., 1983; Kanaho et al., 1984; Sunyer et al., 1984; Kahn & Gilman, 1984; Milligan & Klee, 1985). Guanyl nucleotide binding and GTPase activity are enhanced by receptors in the presence of agonist (Brandt et al., 1983; Kanaho et al., 1984; Asano et al., 1984a,b; Cassel & Selinger, 1976; Cerione et al., 1985).

Several types of experimental evidence indicate that T_α and $G_{i\alpha}$ are very similar to each other, while $G_{s\alpha}$ is a more distinct protein. Isoproterenol acting through the β-adrenergic receptor increases GTP hydrolysis by G_s and, to some extent, by $G_{i\alpha}$ (Brandt et al., 1983; Cerione et al., 1985; Asano et al., 1984a). Rhodopsin enhances the GTPase activity of $G_{t\alpha}$ and $G_{i\alpha}$, and, to a significantly lesser degree, that of $G_{s\alpha}$ (Kanaho et al., 1984; Cerione et al., 1985). Thus, $G_{t\alpha}$ is more similar in function as well as in structure to $G_{i\alpha}$ than $G_{s\alpha}$ (Manning & Gilman, 1983; Kanaho et al., 1984; Cerione et al., 1985).

$G_{t\alpha}$, $G_{i\alpha}$, and $G_{s\alpha}$ differ in their ability to serve as substrates for bacterial toxin ADP-ribosyltransferases. Cholera toxin and *Escherichia coli* heat-labile enterotoxin catalyze the ADP-ribosylation of $G_{s\alpha}$, leading to activation of adenylate cyclase (Gilman, 1984; Moss et al., 1984). Pertussis toxin ADP-ribosylates $G_{i\alpha}$, resulting in its inactivation and, hence, may in fact increase cAMP formation (Moss et al., 1984; Katada & Ui, 1982). Both types of toxins modify $G_{t\alpha}$ (Abood et al.,

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[1] Abbreviations: G protein, guanyl nucleotide binding protein; G_s and G_i, stimulatory and inhibitory guanyl nucleotide binding proteins of adenylate cyclase, respectively; $G_{o\alpha}$ and $G_{i\alpha}$, α subunit of G_o and G_i, respectively; $G_{\beta\gamma}$, β and γ subunits of G_o and G_i; $G_{t\alpha}$, α subunit of transducin; $G_{t\beta\gamma}$, β and γ subunits of transducin; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; GTPγS, guanosine 5′-*O*-(3-thiotriphosphate); GDPβS, guanosine 5′-*O*-(2-thiodiphosphate); kDa, kilodalton(s); Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride; EDTA, ethylenediaminetetraacetic acid.

1982; Van Dop et al., 1984; Watkins et al., 1984).

Recently, Sternweis and Robishaw (1984) and Neer et al. (1984) described in bovine brain a guanyl nucleotide binding protein of 39 kDa, termed $G_{o\alpha}$, which is ADP-ribosylated by pertussis toxin. On the basis of deduced amino acid sequences of $G_{i\alpha}$, $G_{t\alpha}$, and $G_{o\alpha}$ determined from their respective cDNA clones (Lochrie et al., 1985; Medynski et al., 1985; Tanabe et al., 1985; Yatsunami & Khorana, 1985; Angus et al., 1986; Nukada et al., 1986a,b; Robishaw et al., 1986; Van Meurs et al., 1987), $G_{o\alpha}$ is most similar to $G_{i\alpha}$ with 82% homology considering conservative substitutions, while homology to two transducin clones is 76% and 78%, respectively (Van Meurs et al., 1987). $G_{s\alpha}$ is considerably more different from $G_{o\alpha}$ with only 50% homology (Van Meurs et al., 1987). Thus, the family of pertussis toxin substrates appears to be more homologous to each other than to the cholera toxin substrate $G_{s\alpha}$. We questioned whether specific receptor recognition domains might be conserved among the pertussis toxin substrates. Pertussis toxin substrates $G_{i\alpha}$ and $G_{t\alpha}$, but to a lesser extent the choleragen substrate $G_{s\alpha}$, interact with rhodopsin. The current study was designed to test whether the pertussis toxin substrate $G_{o\alpha}$, similar to $G_{t\alpha}$ and $G_{i\alpha}$, is recognized by rhodopsin.

Materials and Methods

Materials. ATP, GTP, NAD, and soybean and lima bean trypsin inhibitors were purchased from Sigma Chemical Co. (St. Louis, MO); GTPγS and GDPβS were from Boehringer-Mannheim (Indianapolis, IN); [γ-^{32}P]GTP, [^{32}P]NAD, and [^{35}S]GTPγS were from New England Nuclear (Boston, MA). Sodium cholate from Sigma Chemical Co. was purified by the procedure of Ross and Schatz (1976). Heptylamine–Sepharose 4B was prepared as described (Shaltiel, 1974; Cuatrecasas, 1970). Pertussis toxin was purchased from List Biological Laboratories (Campbell, CA).

Purification of Proteins. Rhodopsin purified by a slight modification of the method of Hong and Hubbell (1973) was incorporated into phosphatidylcholine vesicles. The purified rhodopsin preparation exhibited one major band by SDS–PAGE. Contamination of rhodopsin with $G_{t\alpha}$ or $G_{t\beta\gamma}$ was estimated by the addition of either $G_{t\beta\gamma}$ or $G_{t\alpha}$, respectively, and assay of GTPase activity as well as by immunoblotting with anti-$G_{t\alpha}$ antibodies (Halpern et al., 1986). With rhodopsin free of $G_{t\alpha}$ or $G_{t\beta\gamma}$, both $G_{t\alpha}$ and $G_{t\beta\gamma}$ should be required for GTPase activity. The data for the rhodopsin preparations are included in the appropriate figure and table legends. Transducin, $G_{t\alpha}$, and $G_{t\beta\gamma}$ were purified by the procedures of Kühn (1980) and Shinozawa et al. (1980).

$G_{o\alpha}$ was purified from bovine brain by the method of Sternweis and Robishaw (1984) with some modifications. Briefly, membranes were extracted with 20 mM Tris-HCl (pH 8.0)/1 mM EDTA/1 mM dithiothreitol/1 mM NaN_3 and 0.25 M sucrose (buffer A) containing 1% sodium cholate and lima bean and soybean trypsin inhibitors (each 1 μg/mL). After centrifugation, the supernatant was applied to a DEAE-Sephacel column (5 × 60 cm) (Pharmacia Fine Chemicals, Piscataway, NJ) which was eluted with a gradient from 0 to 225 mM NaCl (1.8–1.8 L). Fractions were assayed for the adenylate cyclase stimulatory activity (Kanaho et al., 1984) and [^{35}S]GTPγS binding activity (Sternweis & Robishaw, 1984). Those fractions containing proteins with major [^{35}S]GTPγS binding activity that included <20% of total adenylate cyclase stimulatory activity were pooled, concentrated, and applied to a column (2 × 86 cm) of Ultrogel AcA 34 (LKB) equilibrated with buffer A containing 1% cholate, 100 mM NaCl, and both trypsin inhibitors. Fractions from this column that bound [^{35}S]GTPγS, which were well separated from fractions containing adenylate cyclase stimulatory activity, were pooled and applied to a heptylamine–Sepharose column (2 × 31 cm). The column was eluted with a gradient of buffer A containing 0.25% cholate and 200 mM NaCl to buffer A containing 1% cholate and 90 mM NaCl (400 mL of each). A first peak of protein with GTPγS binding activity was separated from a second large protein peak with more GTPγS binding activity. SDS–PAGE (12% polyacrylamide gels) revealed that the first peak contained $G_{o\alpha}$ of >70% purity. This preparation was used for the experiments in Figure 1 and Table I, and a portion was concentrated and applied to Ultrogel AcA 44 (1.2 × 62 cm). Apparently homogeneous $G_{o\alpha}$, further purified on hydroxylapatite, was used in Figures 2–4 and Tables II–IV for the assay of GTPase activity and ADP-ribosylation by pertussis toxin.

$G_{\beta\gamma}$ was purified from rabbit liver membrane extract by the procedures of Sternweis et al. (1981) and Kanaho et al. (1984). $G_{\beta\gamma}$ (~80% pure) was eluted from DEAE-Sephacel with buffer A containing 0.6% Lubrol and 25 mM NaCl.

GTPase Activity. $G_{o\alpha}$, $G_{t\alpha}$, $G_{\beta\gamma}$, or $G_{t\beta\gamma}$ as indicated was incubated at 4 °C for 5 min in a total volume of 75 μL containing 15 μg of phosphatidylcholine without or with 4.5 μg of rhodopsin. In each experiment, all assays contained the same concentrations of components added with the subunits. Assays were initiated by adding 25 μL of reaction mixture to give final concentrations of 20 mM Tris-HCl, pH 7.5, 5 mM $MgCl_2$, 1 mM dithiothreitol, 0.1 mM EDTA, and 5 μM [γ-^{32}P]GTP [(5–8) × 10^5 cpm]. 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 radioassay of ^{32}P in the supernatant (Kanaho et al., 1984). Means of values from duplicate assays are reported. Blanks from incubations in the absence of rhodopsin and G protein subunits have been subtracted in all cases. GTP hydrolysis was constant throughout the assay period.

[^{32}P]ADP-Ribosylation of Proteins and Electrophoresis. Toxin-catalyzed [^{32}P]ADP-ribosylation was carried out as described (Burns et al., 1983). $G_{o\alpha}$ with $G_{\beta\gamma}$ or $G_{t\beta\gamma}$ in buffer A containing 0.05% cholate was incubated for 60 min at 30 °C in a total volume of 100 μL (Burns et al., 1983). Proteins were precipitated with and washed once with cold trichloroacetic acid and then solubilized and separated by SDS–PAGE (12% polyacrylamide gel) as described (Laemmli, 1970). Proteins were stained with Coomassie blue and gels exposed to Kodak X-Omat film. For estimation of stoichiometric ADP-ribosylation of the $G_{o\alpha}$ subunit by pertussis toxin and [^{32}P]NAD, the concentration of stained $G_{o\alpha}$ band in gels was measured by densitometry with bovine serum albumin as the standard protein. The same gel was then sliced in 2 × 2 mm sections, and sections were counted for the ^{32}P-labeled $G_{o\alpha}$ band. The result was calculated as moles of ADP-ribose per mole of $G_{o\alpha}$ subunit.

Protein Determination. Protein was determined by the method of Lowry et al. (1951) using crystallized bovine serum albumin (Miles Laboratories) as a standard or by a dye binding assay (Bio-Rad, Rockville Center, NY). Protein determinations by the methods of Lowry et al. (1951) or by Coomassie blue gave similar values; the result from amino acid analysis was 50% that of both colorimetric determinations.

Preparation of Monoclonal Antibodies. Hybridoma cells were grown in serum-free medium (Nutridoma, Boehringer-Mannheim). Monoclonal antibodies were purified (Ey et al., 1978) from medium from spent cultures, concentrated (ca. 10-fold), and stored in 40% glycerol at −20 °C.

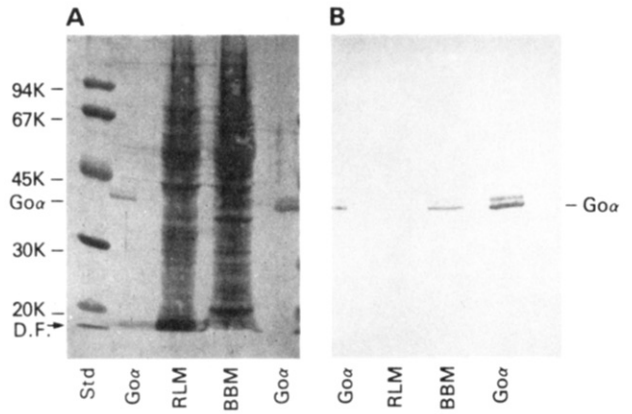

FIGURE 1: Reaction of polyclonal antibodies against $G_{o\alpha}$ on Western blot. Proteins were precipitated with trichloroacetic acid, dissolved in 1% SDS and 5% mercaptoethanol, and subjected to electrophoresis in a 12% polyacrylamide gel (Laemmli, 1970) at 200 V. (a) Western blot (Towbin et al., 1979) of the gel stained with amido black dye; (b) Western blot of the duplicate gel incubated with polyclonal antibodies against $G_{o\alpha}$. From left to right, (panel A) lane 1, standard proteins [phosphorylase *b*, 94 kDa; bovine serum albumin (BSA), 67 kDa; ovalbumin, 45 kDa; carbonic anhydrase, 30 kDa; soybean trypsin inhibitor, 20 kDa]; lane 2, 0.5 μg of $G_{o\alpha}$; lane 3, 100 μg of rabbit liver membrane; lane 4, 100 μg of bovine brain membrane; lane 5, 3.5 μg of $G_{o\alpha}$; (panel B) lane 1, 0.5 μg of $G_{o\alpha}$; lane 2, 100 μg of rabbit liver membrane; lane 3, 100 μg of bovine brain membrane; lane 4, 3.5 μg of $G_{o\alpha}$.

Preparation of Polyclonal Antibodies to $G_{o\alpha}$. Before immunization was initiated, rabbits were bled 3 times at weekly intervals to obtain preimmune serum. $G_{o\alpha}$ from AcA 44 (see above) in 0.25 M sucrose/100 mM NaCl/20 mM Tris-HCl (pH 7.6)/1 mM EDTA/1 mM dithiothreitol/0.2% cholate (300 μg/mL) was emulsified with an equal volume of Freund's complete adjuvant. Each of two rabbits received 1 mL of emulsion. After bleeding 3 weeks later, the rabbits received 75 μg of $G_{o\alpha}$ in Freund's incomplete adjuvant. They were bled 9 days later and 5 days after that injected again with 75 μg of $G_{o\alpha}$ in incomplete adjuvant. Blood was obtained 9 days later and then every 7–9 days for 1.5 months. The third and subsequent immune sera from both rabbits displayed similar reactivity with $G_{o\alpha}$. Cross-reactivity with $G_{t\alpha}$ was slightly higher in the later samples but was still very low. (Titers to $G_{o\alpha}$ were at least 10^3.)

The anti-$G_{o\alpha}$ polyclonal antibodies exhibited a high specificity for $G_{o\alpha}$; the antibody cross-reacted with a protein having a molecular weight similar to that of $G_{o\alpha}$ when analyzed by Western blotting against rabbit liver and bovine brain membranes (Figure 1A,B). Monoclonal antibodies to $G_{t\alpha}$ were prepared as described (Halpern et al., 1986).

For assessment of reactivity of immune and preimmune sera, both crude brain membranes and apparently homogeneous $G_{o\alpha}$ from chromatography on hydroxylapatite (see above) were used.

RESULTS

As noted on SDS–PAGE, $G_{o\alpha}$ migrated as a protein of 39 kDa clearly different from $G_{i\alpha}$ (41 kDa) but not distinguishable in size from $G_{t\alpha}$ (Figure 2). Immediately following purification, one band corresponding to $G_{o\alpha}$ was visualized (Figure 1). Storage (at −70 °C) and freeze–thawing of the sample resulted in the generation of $G_{o\alpha l}$ (Figure 2). A monoclonal antibody against $G_{t\alpha}$ reacted to almost the same extent with $G_{i\alpha}$ but much less with $G_{o\alpha}$ (Halpern et al., 1986). Antiserum against $G_{o\alpha}$ did not cross-react with $G_{i\alpha}$ and $G_{t\alpha}$ (Figure 2). Since anti-$G_{o\alpha}$ antibodies did not react with $G_{t\alpha}$ (Figure 2B) and anti-$G_{t\alpha}$ antibodies did not recognize a protein in $G_{o\alpha}$ (Figure 2C), it would appear that the preparations of $G_{t\alpha}$ and $G_{o\alpha}$ are not cross-contaminated.

Table I: Effect of Photolyzed Rhodopsin and $G_{t\beta\gamma}$ on GTP Hydrolysis Catalyzed by $G_{o\alpha}$[a]

photolyzed rhodopsin	GTPase activity (pmol/10 min)	
	no $G_{t\beta\gamma}$	plus $G_{t\beta\gamma}$
none	7.6	8.4
4.5 μg	10.2	34.7

[a] GTPase activity of $G_{o\alpha}$ (1.6 μg) was assayed with or without $G_{t\beta\gamma}$ (2.5 μg) and/or photolyzed rhodopsin (4.5 μg) with 12 μg of phosphatidylcholine. GTP hydrolysis by $G_{t\beta\gamma}$ without and with rhodopsin was 0.5 and 5.0 pmol/10 min, respectively. These values have been subtracted.

Table II: Effect of Dark and Photolyzed Rhodopsin on GTP Hydrolysis Catalyzed by $G_{o\alpha}$[a]

rhodopsin	GTPase activity (pmol/10 min)	
	no $G_{\beta\gamma}$	plus $G_{\beta\gamma}$
dark	7.4	9.9
photolyzed	7.4	18.2

[a] GTPase activity of $G_{o\alpha}$ (1.0 μg) was assayed with or without $G_{\beta\gamma}$ (1.3 μg) and dark or photolyzed rhodopsin (~4 μg) with 12 μg of phosphatidylcholine. GTPase activity of $G_{\beta\gamma}$ in the presence of dark and light rhodopsin was 3.5 and 2.8 pmol/10 min, respectively. The blanks have been subtracted. The effects of $G_{\beta\gamma}$ do not appear to result from stimulation of contaminating $G_{t\alpha}$ in rhodopsin. Rhodopsin (5 μg) analyzed for $G_{t\alpha}$ by immunoblotting did not exhibit any reactivity (<0.1 μg of $G_{t\alpha}$).

Table III: Effect of $G_{t\beta\gamma}$ or $G_{\beta\gamma}$ on ADP-Ribosylation of $G_{t\alpha}$ and $G_{o\alpha}$ by Pertussis Toxin[a]

expt	additions	[^{32}P]ADP-ribosylation (density units)	
		$G_{t\alpha}$	$G_{o\alpha}$
1	none	1.1	0.1
	$G_{t\beta\gamma}$, 2.5 μg	4.7	1.3
2	none	0.6	0.1
	$G_{\beta\gamma}$, 1.3 μg	7.6	1.4

[a] $G_{t\alpha}$ and $G_{o\alpha}$ with or without $G_{t\beta\gamma}$ or $G_{\beta\gamma}$ were [^{32}P]ADP-ribosylated as described in Figure 4. Densitometry of autoradiograms yielded the values recorded in arbitrary units. In experiment 1, amounts of $G_{t\alpha}$ and $G_{o\alpha}$ were 2.5 and 1.6 μg, respectively; in experiment 2, 1 μg of both α subunits was added. To determine the extent of ADP-ribosylation, the proteins were identified by SDS–PAGE and nitrocellulose transfer. Autoradiograms yielded bands that coincided with immunoreactive material.

The GTPase activity of $G_{o\alpha}$, like that of $G_{t\alpha}$ (Fung, 1983) and $G_{i\alpha}$ (Kanaho et al., 1984), was stimulated by photolyzed rhodopsin plus $G_{t\beta\gamma}$; rhodopsin alone increased activity somewhat, whereas $G_{t\beta\gamma}$ had very little effect (Table I). In the presence of photolyzed rhodopsin, the GTPase activity of $G_{o\alpha}$ was increased by $G_{\beta\gamma} > G_{t\beta\gamma}$ (Figure 3A). GTP hydrolysis by $G_{t\alpha}$ was also stimulated in the presence of photolyzed rhodopsin by $G_{\beta\gamma} > G_{t\beta\gamma}$ (Figure 3B). Basal GTPase activity of $G_{o\alpha}$ was greater than that of $G_{t\alpha}$ (Figure 3A,B); maximal activities in the presence of $G_{\beta\gamma}$ were similar (Figure 3A,B). In the presence of photolyzed rhodopsin, the GTPase activity of $G_{o\alpha}$ was linear with time in the presence or absence of $G_{\beta\gamma}$ (data not shown). $G_{\beta\gamma}$ had little effect in the presence of dark rhodopsin (Table II).

As has been shown for $G_{t\alpha}$ (Watkins et al., 1985) and $G_{i\alpha}$ (Tsai et al., 1984), ADP-ribosylation of $G_{o\alpha}$ was increased by $G_{t\beta\gamma}$ or $G_{\beta\gamma}$ (Neer et al., 1984) (Table III). GTP and GDPβS increased ADP-ribosylation of $G_{t\alpha}$ and $G_{o\alpha}$ both in the dark and in the light. ADP-Ribosylation of $G_{o\alpha}$, with dark or

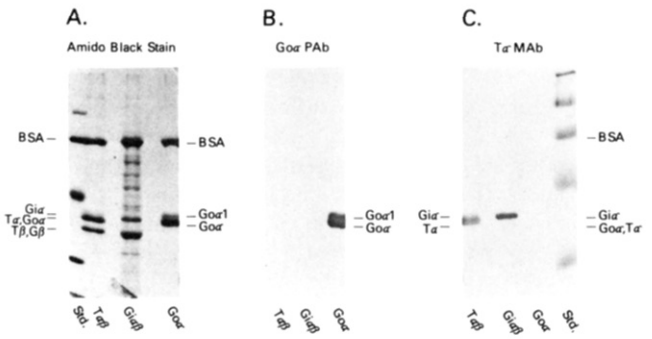

FIGURE 2: Reaction of $G_{o\alpha}$ antiserum and $G_{t\alpha}$ monoclonal antibody with $G_{t\alpha}$, $G_{o\alpha}$, and $G_{i\alpha}$ on Western blot. Protein samples plus 5 μg of bovine serum albumin as carrier were precipitated, solubilized, subjected to SDS–PAGE, and transferred to nitrocellulose paper (Towbin et al., 1979). (Panel A) Protein was stained with amido black; nitrocellulose blots as noted in panel A were treated with polyclonal antibodies against $G_{o\alpha}$ (panel B) or monoclonal antibody to $G_{t\alpha}$ (panel C). Left to right (panel A): lane 1, standard proteins as in Figure 1; lane 2, 4.0 μg of $G_{t\alpha}$ and 4.0 μg of $G_{t\beta\gamma}$; lane 3, 1.5 μg of $G_{i\alpha}$; lane 4, 5 μg of $G_{o\alpha}$ (lower major band); minor upper band ($G_{o\alpha 1}$) was observed when $G_{o\alpha}$ (no guanyl nucleotide present) was frozen and thawed during storage; $G_{o\alpha 1}$ and $G_{o\alpha}$ were ADP-ribosylated by pertussis toxin and [^{32}P]NAD as described under Materials and Methods. Left to right (panels B and C): lane 1, 4 μg of $G_{t\alpha}$; lane 2, 1.5 μg of $G_{i\alpha}$; lane 3, 5 μg of $G_{o\alpha}$. As noted by the amido black stain for protein in panel A, similar amounts of $G_{t\alpha}$, $G_{i\alpha}$, and $G_{o\alpha}$ were transferred to nitrocellulose. Thus, the differences in antibody reactivity with $G_{o\alpha}$, $G_{i\alpha}$, and $G_{t\alpha}$, noted in panels B and C, reflect antibody specificity.

Table IV: Effect of Pertussis Toxin Catalyzed ADP-Ribosylation on GTP Hydrolysis Catalyzed by $G_{o\alpha}$[a]

rhodopsin	GTPase activity (pmol/10 min) no PT	plus PT
dark	2.6	2.7
photolyzed	7.5	3.5

[a] $G_{o\alpha}$ (1 μg) with $G_{\beta\gamma}$ (1.3 μg) was incubated 1 h at 30 °C without or with activated pertussis toxin (PT) (total volume 50 μL). Dark or photolyzed rhodopsin (4.5 μg with 15 μg of phosphatidylcholine in 5 μL) and a reaction mixture (45 μL) to give final concentrations of 20 mM Tris-HCl (pH 7.4), 5 mM $MgCl_2$, 1 mM dithiothreitol, 0.1 mM EDTA, and 5 μM [γ-^{32}P]GTP were then added to initiate assay of GTPase activity at 30 °C for 10 min; 1.04 mol of ADP-ribose was incorporated per mole of $G_{o\alpha}$ based on quantification of $G_{o\alpha}$ by amino acid analysis. The stoichiometric ADP-ribosylation is in contrast to earlier reports showing modification of only a fraction of the available $G_{o\alpha}$ (Neer et al., 1984).

photolyzed rhodopsin, was decreased by GTPγS which had little effect on labeling of $G_{t\alpha}$ (Figure 4). The GTPase of $G_{o\alpha}$ observed in the presence of $G_{\beta\gamma}$ and dark rhodopsin was not altered by pertussis toxin catalyzed ADP-ribosylation, whereas stimulation by photolyzed rhodopsin was virtually abolished (Table IV).

Discussion

$G_{o\alpha}$, like $G_{i\alpha}$ (Kanaho et al., 1984; Tsai et al., 1984), can replace $G_{t\alpha}$ in reconstituting photolyzed rhodopsin-stimulated GTPase activity in the presence of $G_{t\beta\gamma}$ or $G_{\beta\gamma}$. The activity of $G_{o\alpha}$ in the reconstituted system was similar to that of $G_{t\alpha}$, consistent with the conclusion that the receptor site on rhodopsin can interact functionally with either protein. In both instances, the $\beta\gamma$ subunits were necessary for optimal expression of GTPase. Activity observed with $G_{\beta\gamma}$ was higher than that with $G_{t\beta\gamma}$ whether $G_{t\alpha}$ or $G_{o\alpha}$ represented the other component. As concluded earlier for $G_{t\alpha}$ and $G_{i\alpha}$ (Kanaho et al., 1984; Tsai et al., 1984), ADP-ribosylation of $G_{o\alpha}$ by pertussis toxin was increased by the addition of $G_{t\beta\gamma}$ or $G_{\beta\gamma}$. In the presence of $G_{t\beta\gamma}$, modification was greater with dark

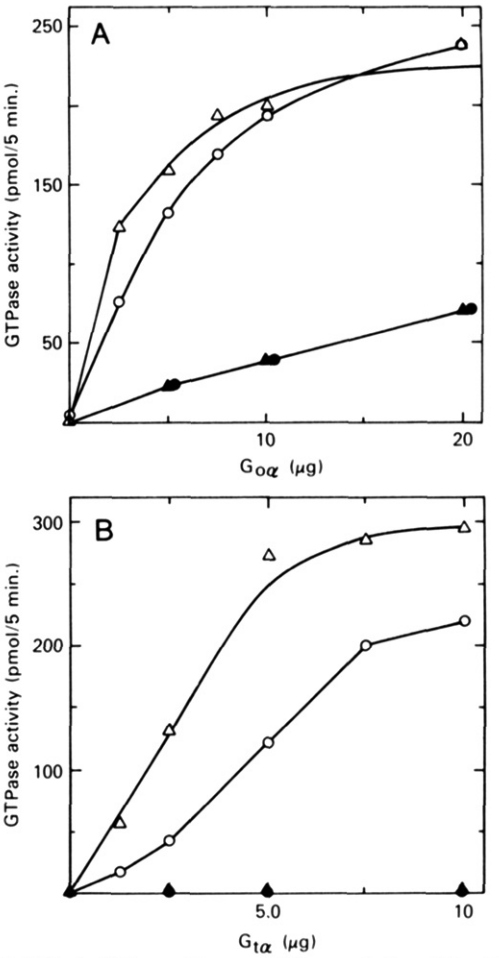

FIGURE 3: Effect of $G_{\beta\gamma}$ or $G_{t\beta\gamma}$ on GTPase activity of $G_{o\alpha}$ (A) and $G_{t\alpha}$ (B) with or without photolyzed rhodopsin. (A) The indicated amounts of $G_{o\alpha}$ were assayed in the presence (△, ○) of 1 μg of rhodopsin reconstituted in phospholipid vesicles or in its absence (▲, ●) and 0.9% sodium cholate with 1.5 μg of $G_{\beta\gamma}$ (△, ▲) or 1.5 μg of $G_{t\beta\gamma}$ (○, ●). (B) The indicated amounts of $G_{t\alpha}$ were assayed with 1 μg of rhodopsin reconstituted in phospholipid vesicles (△, ○) or in its absence (▲, ●) and with 0.13% sodium cholate with 1.5 μg of $G_{\beta\gamma}$ (△, ▲) or 1.5 μg of $G_{t\beta\gamma}$ (○, ●). The low concentrations of cholate did not inhibit GTPase activity of $G_{t\alpha}$, but 0.9% cholate abolished the activity completely.

rhodopsin than it was with light rhodopsin. The inhibitory effect of light-stimulated rhodopsin on ADP-ribosylation of $G_{o\alpha}G_{\beta\gamma}$ was less than noted with $G_{t\alpha}G_{\beta\gamma}$; these data are compatible with the finding that $G_{o\alpha}$ in the absence of $G_{\beta\gamma}$ is a better substrate for toxin-catalyzed ADP-ribosylation than is $G_{t\alpha}$ (Watkins et al., 1985); thus, following rhodopsin-stimulated dissociation of α from $\beta\gamma$, the $G_{o\alpha}$ subunit still serves as an ADP-ribose acceptor, whereas $G_{t\alpha}$ is less amenable to modification. With light or dark rhodopsin, GTP (which was presumably hydrolyzed to GDP) and GDPβS increased ADP-ribosylation of $G_{o\alpha}$. The inhibitory effect of GTPγS on pertussis toxin catalyzed ADP-ribosylation of $G_{o\alpha}$ was enhanced in the presence of photolyzed rhodopsin. Thus, with $G_{o\alpha}$ as well as with $G_{t\alpha}$ and $G_{i\alpha}$, photolyzed rhodopsin may promote the binding of a guanyl nucleotide which renders the protein less susceptible to ADP-ribosylation. Further studies are necessary to determine to what extent these effects result from dissociation of $\alpha\cdot\beta\gamma$ or occupation of the guanyl nucleotide binding site on α by a nonhydrolyzable GTP analogue.

Rhodopsin stimulation of GTP hydrolysis by $G_{t\alpha}G_{t\beta\gamma}$ is decreased following ADP-ribosylation by the toxin, and this is the case also for $G_{o\alpha}$ as shown here. Pertussis toxin catalyzed ADP-ribosylation, however, did not appear to alter basal

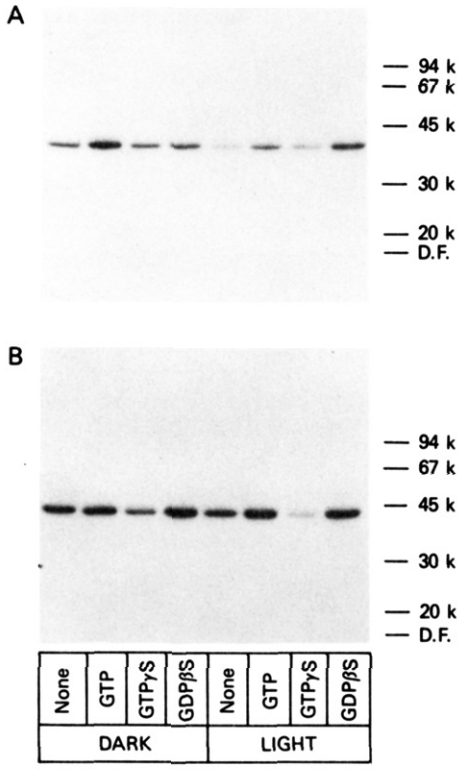

FIGURE 4: Effects of guanyl nucleotides on ADP-ribosylation of $G_{t\alpha}$ and $G_{o\alpha}$ with dark-adapted or light-activated rhodopsin. $G_{t\alpha}$ (2.5 μg) or $G_{o\alpha}$ (2.5 μg) with 2.5 μg of $G_{t\beta\gamma}$, 1.5 μg of rhodopsin in phosphatidylcholine vesicles (4.7 μg), and 10 μM nucleotide as indicated was ADP-ribosylated by pertussis toxin as described (Burns et al., 1983), except that ATP and GppNHp were omitted. Autoradiograms of dried gels are shown. (A) $G_{t\alpha}$ plus $G_{t\beta\gamma}$; peak areas are (from left to right) 4.6, 10.8, 4.6, 5.5, 1.9, 4.3, 2.8, and 6.3. (B) $G_{o\alpha}$ plus $G_{t\beta\gamma}$; peak areas are (from left to right) 4.7, 6.1, 3.1, 8.9, 3.8, 8.3, 1.0, and 7.8.

GTPase activity. Since the extent of ADP-ribosylation as determined by amino acid analysis would appear to be stoichiometric (1.04 mol of ADP-ribose incorporated per mole of $G_{o\alpha}$) and since prior studies by West et al. (1985) demonstrated that in $G_{t\alpha}$ only one cysteine at the carboxy terminus serves as an ADP-ribose acceptor, it would appear that basal GTPase of $G_{o\alpha}$ is unaffected by ADP-ribosylation.

The physiological role of G_o has not been determined. The present report is consistent with the hypothesis that it can couple to receptors involved in cyclase inhibition (Florio & Sternweis, 1985). Agonists, e.g., muscarinic, that inhibit adenylate cyclase can also have effects on phospholipid turnover, phosphodiesterase activity, cGMP accumulation, and Ca^{2+} flux (Michell, 1975; Berridge, 1981; Meeker & Harden, 1982; Sokolovsky et al., 1983). Since for some of these agents structurally only one type of receptor has been identified (Birdsall et al., 1978; Haga, 1980; Venter, 1983), it has been proposed that different effects of an agonist–receptor complex are dependent on the coupling protein (Birdsall, 1984; Brown & Brown, 1984). In this scheme, a number of different coupling proteins might be expected to interact with a single class of receptor. As shown here, G_o can interact functionally with the photon receptor rhodopsin, which mimics an inhibitory receptor in its ability to stimulate GTP hydrolysis by G_i (Kanaho et al., 1984). Thus, in the cell, G_o and G_i may couple to the same receptor, although they may mediate different effects.

It is clear on the basis of the deduced amino acid sequence analysis that the G proteins represent a family of highly homologous proteins. In view of the apparent similarities in structure and function of G_o, transducin, and G_i, it is important to be able to distinguish between three related proteins. Primary identification of the proteins can be made by pertussis toxin catalyzed ADP-ribosylation in the presence of $G_{\beta\gamma}$ or by their ability to express photolyzed rhodopsin- and $G_{t\beta\gamma}$-stimulated GTPase activity. Differentiation of the pertussis toxin substrates can then be made on the basis of immunological criteria. The polyclonal antibodies that react with $G_{o\alpha}$ but not $G_{i\alpha}$ and monoclonal antibodies that react with $G_{t\alpha}$ and $G_{i\alpha}$ but poorly with $G_{o\alpha}$ can be useful for distinguishing these proteins on Western blots after SDS-PAGE. The use of the toxins and receptor should facilitate further understanding of the action of these guanyl nucleotide binding regulatory proteins.

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