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Interaction of Retinal Transducin with Guanosine Triphosphate Analogues: Specificity of the γ-Phosphate Binding Region†

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ABSTRACT: The interaction of six hydrolysis-resistant analogues of GTP with transducin, the signal-coupling protein in vertebrate photoreceptors, was investigated. GppNHp and $GppCH_2p$ differ from GTP at the bridging position between the β- and γ-phosphate groups. The other analogues studied (GTPγF, GTPγOMe, GTPγOPh, and GTPγS) differ from GTP in containing a substituent on the γ-phosphorus atom or at a nonbridging γ-oxygen atom. Competition binding experiments were carried out by adding an analogue, [α-^{32}P]GTP, and a catalytic amount of photoexcited rhodopsin (R*) to transducin and measuring the amount of bound [γ-^{32}P]GTP. The order of effectiveness of these analogues in binding to transducin was

GTPγS > GTP ≫ GppNHp > GTPγOPh > GTPγOMe > $GppCH_2p$ > GTPγF

A second assay measured the effectiveness of GTPγS, GppNHp, and $GppCH_2p$ in eluting transducin from disc membranes containing R*. The basis of this assay is that transducin is released from disc membranes when it is activated to the GTP form. The relative potency of these three analogues in converting transducin from a membrane-bound to a soluble form was 1000, 75, and 1, respectively. Stimulation of cGMP phosphodiesterase activity served as a third criterion of the interaction of these analogues with transducin. The order of effectiveness of these analogues in promoting the transducin-mediated activation of the phosphodiesterase was

GTPγS > GTP ≫ GppNHp > GTPγOPh ≫ $GppCH_2p$ > GTPγOMe > GTPγF

GTPγS was more than a 1000 times as potent as GTPγF in activating the phosphodiesterase. For GTPγOPh, GTPγOMe, and GTPγF, the order of effectiveness in stimulating the phosphodiesterase was the same as previously reported for the activation of adenylate cyclase in pigeon erythrocyte membranes [Pfeuffer, T., & Eckstein, F. (1976) *FEBS Lett. 67*, 354–358] but the opposite of that found with bacterial elongation factor G [Eckstein, F., Bruns, W., & Parmeggiani, A. (1975) *Biochemistry 14*, 5225–5232]. Thus, transducin and the stimulatory G protein have similar binding sites for the γ-phosphoryl group of GTP, whereas that of elongation factor G is significantly different.

The photoexcitation of rhodopsin in vertebrate retinal rod outer segments (ROS)[1] triggers a cascade that results in the hydrolysis of many molecules of cGMP (Miller, 1981; Chabre, 1985; Stryer, 1986). Transducin, a multisubunit peripheral membrane protein, is the information carrier in the activation of the cyclic GMP phosphodiesterase (PDE) (Fung et al., 1981). Transducin consists of a 39-kDa α chain that binds guanyl nucleotides, a 36-kDa β chain, and an 8-kDa γ chain (Kühn, 1980). In the dark, transducin is bound to the disc membrane in the GDP form (T–GDP). Photoexcited rhodopsin (R*) catalyzes the exchange of GTP for GDP bound to the α subunit (Godschaux & Zimmerman, 1979). About 500 molecules of T_α–GTP are formed per R* at low light levels

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[1] Abbreviations: ROS, rod outer segment; T, transducin; PDE, cGMP phosphodiesterase; R*, urea-stripped disc membranes containing photolyzed rhodopsin; DTT, dithiothreitol; GppNHp, guanosine 5′-(β,γ-imidotriphosphate); $GppCH_2p$, guanosine 5′-(β,γ-methylenetriphosphate); GTPαS, guanosine 5′-*O*-(1-thiotriphosphate); GTPβS, guanosine 5′-*O*-(2-thiotriphosphate); GTPγS, guanosine 5′-*O*-(3-thiotriphosphate); GTPγF, guanosine 5′-*O*-(3-fluorotriphosphate); GTPγOMe, guanosine 5′-*O*-(3-methyl triphosphate); GTPγOPh, guanosine 5′-*O*-(3-phenyl triphosphate); EF-G, elongation factor G; EF-Tu, elongation factor Tu; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride; SDS, sodium dodecyl sulfate.

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(Fung & Stryer, 1980). T_α–GTP, released from the $\beta\gamma$ subunit of transducin, then activates the phosphodiesterase by relieving an inhibition imposed by its small subunit (Shinozawa et al., 1980; Hurley & Stryer, 1982). The rapid hydrolysis of cGMP by activated PDE (Yee & Liebman, 1978) markedly lowers the cytosolic level of free cGMP, which closes light-sensitive channels in the plasma membrane (Fesenko et al., 1985; Yau & Nakatani, 1986). On a slower time scale, T_α hydrolyzes bound GTP to form T_α–GDP, which reassociates with $T_{\beta\gamma}$ to return to the inactive dark state.

Several lines of evidence indicate that transducin belongs to a family of signal-coupling proteins that includes the G proteins of the hormonally regulated adenylate cyclase cascade. First, there are mechanistic similarities, such as the catalysis of GTP–GDP exchange by an activated receptor, the relief of an inhibitory constraint in the effector enzyme by the GTP form of the signal-coupling protein, and the return to the inactive state by GTP hydrolysis (Bitensky et al., 1981; Stryer et al., 1981; Schramm & Selinger, 1984). Second, cholera toxin ADP-ribosylates the stimulatory G protein (G_s) and renders it persistently activated (Cassell & Pfeuffer, 1978; Moss & Vaughan, 1979), whereas pertussis toxin ADP-ribosylates the inhibitory G protein (G_i) and prevents it from interacting with the activated receptor (Bokoch et al., 1983). Both toxins have very similar effects on transducin (Abood et al., 1982; Van Dop et al., 1984; Navon & Fung, 1984). Third, transducin and the G proteins have the same $\alpha\beta\gamma$ subunit structure. Peptide mapping and gene cloning studies have shown that their β chains are very similar and that their α chains display extensive regions of homology (Manning & Gilman, 1983; Gilman, 1984; Robishaw et al., 1986). Brain is rich in G_o, a related but distinct member of the G protein family (Sternweis & Robishaw, 1984; Neer et al., 1984), which may control phospholipase C in the phosphoinositide cascade. A more distant relative of this family is the *ras* protein, which also cycles between GDP and GTP states (Gibbs et al., 1985; Sweet et al., 1984). Oncogenic forms of the *ras* protein have diminished GTPase activity, suggesting that the *ras* protein participates in the control of cell proliferation. Amino acid sequence homologies between the *ras* protein and elongaton factor Tu from *Escherichia coli* have suggested that the GTP binding sites of these proteins are similar (Leberman & Egner, 1984; Halliday, 1984).

Hydrolysis-resistant analogues of GTP have been invaluable in elucidating the biological roles of guanyl nucleotide binding proteins and their mechanism of action. GppNHp and GTPγS have been used to form persistently activated states of both the G proteins (Pfeuffer & Helmreich, 1975) and transducin (Wheeler & Bitensky, 1977; Leibman & Pugh, 1982), thus demonstrating that the hydrolysis of GTP is a necessary step in deactivation. GTPγS has also been used to study the interaction between microtubule-associated proteins and tubulin in the guanyl nucleotide exchange reaction during microtubule polymerization (Hamel & Lin, 1984). Furthermore, thio analogues of GTP and GDP with chiral centers at the α- and β-phosphorus position have revealed facets of the guanyl nucleotide binding site of elongation factor Tu (Wittinghofer et al., 1982; Leupold et al., 1983) and transducin (Yamanaka et al., 1985) and the degree of evolutionary kinship between them. We report here studies of the interaction of transducin with a series of hydrolysis-resistant analogues of GTP that probe the γ-phosphate binding region.

Materials and Methods

GTPγS was from Boehringer Mannheim. GTP, cGMP, GppNHp, and $GppCH_2p$ were from Sigma. GTPγF, GTPγOMe, and GTPγOPh were synthesized as described (Eckstein et al., 1975). [α-^{32}P]GTP was from Amersham. The purity of nucleotides was checked by anion-exchange HPLC. When necessary, nucleotides were repurified on a Varian AX-10 anion-exchange column, with a gradient of 0–320 mM triethylammonium bicarbonate, pH 7.8. Dark-adapted bovine retinas were from J. A. Lawson, Lincoln, NE.

ROS were isolated from bovine retinas in the dark and stored at −70 °C (Fung & Stryer, 1980). Urea-stripped rhodopsin membranes were prepared from ROS and stored similarly (Yamanaka et al., 1985). Transducin was purified from ROS and stored at −20 °C in 50% glycerol (Fung et al., 1981; Fung, 1983).

The elution of transducin from R* membranes was measured with a centrifugation assay (Yamanaka et al., 1985). Solutions of transducin and carbonic anhydrase (an internal standard) were combined with R* and then diluted 2-fold with guanyl nucleotide to final concentrations of 0.5 and 10 μM, respectively, of transducin and R*. The buffer contained 5 mM Tris-HCl, pH 7.4, 50 mM NaCl, 2 mM $MgCl_2$, 1 mM DTT, and 10% (w/v) glycerol. After 5 min, reaction mixtures (100 μL) were centrifuged in a Beckman airfuge (2 min at 30 psi at room temperature), and 90 μL of the supernatants was removed and analyzed on 12% SDS–polyacrylamide gels (Laemmli, 1970). Gels were stained with Coomassie blue and quantitated with carbonic anhydrase as a normalization standard.

The binding of guanyl nucleotides to transducin was measured with a nitrocellulose filter assay (Yamanaka et al., 1985). [α-^{32}P]GTP was combined with varying concentrations of nonradioactive guanyl nucleotides in buffer A (20 mM Tris-HCl, pH 7.4, 120 mM NaCl, 30 mM KCl, 2 mM $MgCl_2$, 1 mM DTT). Reactions were initiated by the addition of a transducin and R* mixture, from a freshly prepared stock solution. Final concentrations of transducin, R*, and [α-^{32}P]GTP were 0.1, 2.0, and 0.5 μM, respectively. After 1 min at 23 °C, 80 μL was removed into 3 mL of ice-cold buffer A (minus DTT) and passed through a nitrocellulose filter (Gelman GN-6). Filters were washed twice with 3 mL of buffer A and counted in Aquasol scintillation cocktail.

Phosphodiesterase assays were performed with the pH meter method of (Liebman & Evanzcuk, 1982). Reactions (2-mL final volume) contained 20 mM 3-(*N*-morpholino)propanesulfonic acid (MOPS), pH 8.0, 150 mM KCl, 2 mM $MgCl_2$, 2 mM cGMP, and bleached ROS membranes containing 5 μM rhodopsin. Following equilibration of the reaction mixture in a stirred plastic cuvette into which a pH electrode was palced, cGMP hydrolysis was initiated by the addition of guanyl nucleotide. The initial rate of hydrolysis was measured by monitoring the production of protons. Following complete hydrolysis of 2 mM cGMP, the pH dropped from 8.00 to 7.73.

Protein concentrations were measured according to the method of Bradford (1976), with bovine serum albumin as standard. Guanyl nucleotide concentrations were measured by using $\epsilon_{253} = 1.37 \times 10^4\ M^{-1}\ cm^{-1}$ at neutral pH. Rhodopsin concentrations in membranes were determined in a solution of 1.5% lauryldimethylamine oxide by using $\epsilon_{500} = 4 \times 10^4\ M^{-1}\ cm^{-1}$.

Results

Relative Affinity of Transducin for GTP Analogues. Several hydrolysis-resistant analogues of GTP were employed in an effort to better understand the structural requirements for inducing the conformational change in transducin required for PDE activation. These analogues differ from GTP by a modification at either (1) the bridging position between the

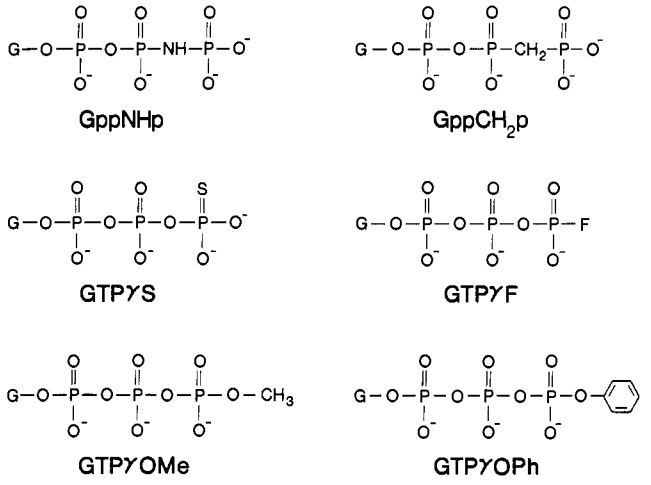


FIGURE 1: Structural formulas of guanyl nucleotide analogues.

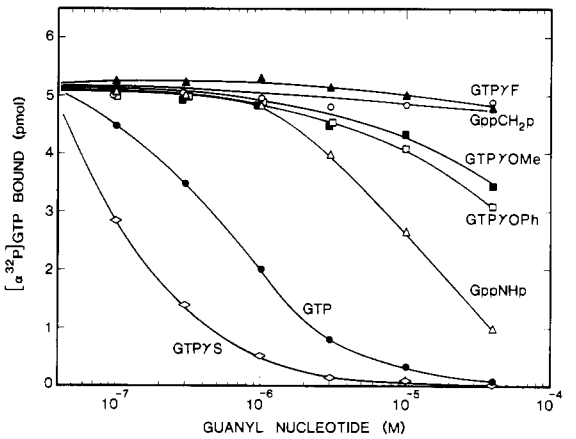
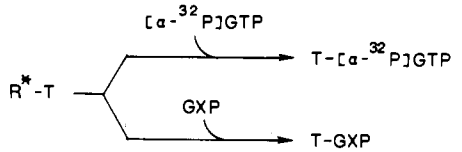


FIGURE 2: Competition between GTP analogues and [α-^{32}P]GTP in binding to transducin. A series of nucleotide solutions containing [α^{32}P]GTP (0.5 μM, 20 Ci/mmol, final reaction concentration) and varying amounts of nonradioactive GTP analogues were mixed with transducin and R* (0.1 and 2.0 μM, respectively). After 30 s, the amount of [α-^{32}P]GTP bound to transducin was measured in a filter binding assay. Nonradioactive nucleotides added were GTPγS (◇), GTP (●), GTPγF (○), GTPγOMe (■), GTPγOPh (□), GppCH$_2$p (▲), and GppNHp (△).

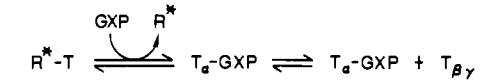
β- and γ-phosphorus atoms or (2) a nonbridging oxygen attached to the γ-phosphorus atom (Figure 1).

The effectiveness of these analogues in competing with GTP for binding to transducin was measured in the following way: R*–T was first formed and then added to a mixture of 0.5 μM [α-^{32}P]GTP and different concentrations of nonradioactive nucleotides. After 30 s, the amount of [α-^{32}P]GTP bound to transducin was measured:



As shown in Figure 2, GTPγS is extremely effective in competing with [α-^{32}P]GTP and reduces the binding of the radioactive nucleotide by 50% when present at 0.12 μM. About 8 μM GppNHp is required to reduce the binding of [α-^{32}P]-GTP by 50%. At 40 μM, GTPγOPh and GTPγOMe inhibit [α-^{32}P]GTP binding by 40% and 33%, respectively, which give extrapolated half-inhibition constants of 80 μM and 130 μM. GppCH$_2$p and GTPγF are almost without effect.

Release of Transducin from Disc Membranes by GTP Analogues. The effectiveness of GTPγS, GppNHp, and GppCH$_2$p in releasing transducin from the disc membrane as a consequence of its dissociation into T_α and $T_{\beta\gamma}$ subunits was investigated. In this experiment, transducin (0.5 μM) was mixed with stripped disc membranes containing 10 μM R* to ensure that more than 95% of the transducin was initially bound. This mixture was combined with increasing concentrations of one of these three analogues and centrifuged after incubation. The amount of transducin in the supernatant was then measured:



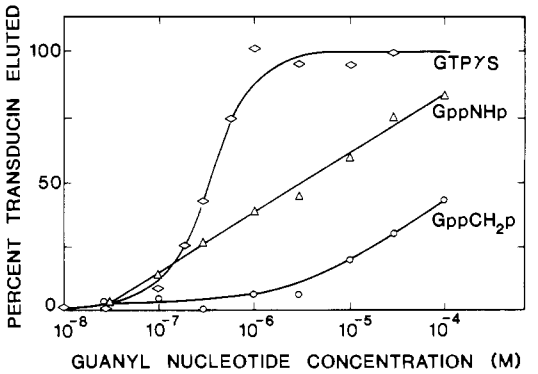
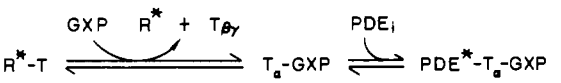


FIGURE 3: Differential solubilization of transducin by GTPγS, GppNHp, and GppCH$_2$p. Solutions containing transducin (0.5 μM), R* (10 μM), and the indicated concentrations of guanyl nucleotides were incubated for 5 min. The R* and R*–T complexes were pelleted by centrifugation in an airfuge. The supernatants were removed, and the transducin solubilized by GTPγS (◇), GppNHp (△), and GppCH$_2$p (○) was measured.

As shown in Figure 3, GTPγS elutes 50% of the transducin at a concentration of 0.3 μM, which is nearly equal to the concentration of transducin (0.5 μM). This stoichiometric release indicates that the dissociation constant of GTPγS is less than about 0.1 μM. Half-elution of transducin is obtained with 4 μM GppNHp, which means that GppNHp is at least 40-fold less effective than is GTPγS. GppCH$_2$p, which releases only 40% of the transducin at 100 μM, is about 1000-fold less effective than GTPγS. In the presence of 1 μM guanyl nucleotides, the proportion of transducin released by GTPγS, GppNHp, and GppCH$_2$p is 90%, 35%, and 5%, respectively. GTPγS is more effective than GTP in eliciting the release of transducin under these assay conditions, in part because of its resistance to hydrolysis.

Effectiveness of GTP Analogues in Promoting Transducin-Mediated Activation of cGMP Phosphodiesterase. The relative potencies of the six GTP analogues and GTP in activating the PDE are compared in Figure 4. These experiments measure the reaction



where PDE$_i$ and PDE* are the inhibited and activated forms of the enzyme. GTPγS is the most effective nucleotide, producing half-maximal initial PDE velocity when present at 0.1 μM. This compares with 0.2 μM for GTP (Yamanaka et al., 1985). GTPγOPh and GppNHp are at least 20-fold less effective than GTPγS. Both analogues produce half-maximal activities when present at 2–5 μM, in contrast to their 10-fold difference in binding affinities for transducin (Figure 2). This indicates that nucleotide binding does not necessarily lead to PDE activation. GppCH$_2$p and GTPγOMe are both very poor PDE activators and are required at concentrations

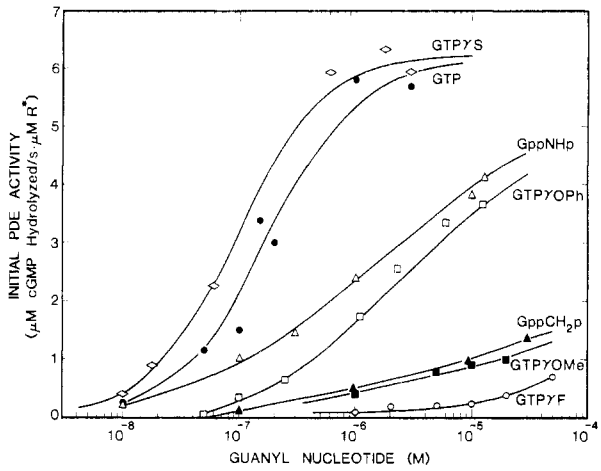


FIGURE 4: Relative effectiveness of GTP analogues in activating the cGMP phosphodiesterase. Bleached ROS (containing 5 μM R*) were equilibrated with 2 mM cGMP. GTP analogues were added to the final concentrations shown, and the initial rates of cGMP hydrolysis were measured as described under Materials and Methods. GTP (●), GTPγS (◇), GppNHp (△), GTPγOPh (□), $GppCH_2p$ (▲), GTPγOMe (■), and GTPγF (○).

greater than 300-fold that of GTPγS in producing a 10% activation. GTPγF is even less effective and is about 1000-fold less potent GTPγS.

Discussion

The present study provides an interesting demonstration of the differences between several GTP analogues in their interactions with transducin, as measured by nucleotide binding, subunit dissociation, and activation of the effector enzyme. If we first consider GppNHp and $GppCH_2p$, the degree to which these analogues mimic GTP in activating transducin can be understood in terms of their known electrostatic and structural characteristics. As previously noted (Yount et al., 1971), the bridging atom between the β- and γ-phosphorus atoms becomes more electronegative in going from $-CH_2-$ to $-NH-$ to $-O-$. The acidity of the terminal phosphate increases in the same order. The pK_a values have been recently determined by ^{31}P NMR to be 8.8 for the methylene analogues (Gerlt et al., 1983), by ^{17}O NMR to be 8.2 for the imido analogue (Reynolds et al., 1983), and by ^{31}P NMR to be 6.7 for ATP (Jaffe & Cohn, 1978). Furthermore, the P–C–P bond angle is considerably more acute (117°) than that of P–N–P (127°) and P–O–P (129°), and the distance between the phosphorus atoms decreases from 3.05 to 3.01 to 2.94 Å in this series. In each of these respects, $GppCH_2p$ differs the most from GTP. Similar characteristics are displayed by the cobalt(III) complexes of P–C–P and P–N–P model compounds, suggesting that the chelates of the corresponding nucleotides differ from that of GTP in much the same way as do their free anions (Harmony et al., 1984).

Of the GTPγ analogues tested, GTPγS is greatly preferred by transducin. This is consistent with the numerous reports in the literature describing the high degree of effectiveness of this analogues in mediating GTP-dependent functions (Pfeuffer & Helmreich, 1975; Liebman & Pugh, 1982; Leupold et al., 1983; Hamel & Lin, 1984; Yamanaka et al., 1985). The substitution of sulfur for oxygen at the γ-phosphorus atom produces less of a steric perturbation than the esterification by either a phenyl or a methyl group. By comparison to bond distances determined with crystals of model compounds, the P–S bond length in GTPγS is probably only 0.4–0.5 Å greater than the exo P–O bonds in GTP (Frey & Sammons, 1985). Furthermore, the pK_a for ATPγS has been determined by ^{31}P NMR to be about 5.3 (Jaffe & Cohn, 1978) and by ^{17}O NMR to be 5.8 (Gerlt et al., 1983). This enhanced acidity may account for the tight binding of ATPγS and GTPγS to nucleoside triphosphate binding proteins (Eckstein, 1985). Like GTP, GTPγS would have a net charge of 2– at the γ-phosphorus position at neutral pH, in contrast with a net charge of 1– for the other three GTPγ analogues (Figure 1).

Table I: Relative Potencies of Guanyl Nucleotides in Interacting with Transducin[a]

nucleotides	relative affinity (μM)	release from R* membranes (μM)	PDE activation (μM)
GTPγS	0.12	0.3	<0.1
GTP	0.5	nd[b]	0.2
GppNHp	8	4	2
GTPγOPh	~80	nd	5
GTPγOMe	~130	nd	>100
$GppCH_2p$	[c]	>100	>100
GTPγF	[c]	nd	>1000

[a] The data are from Figures 2–4 and correspond to concentrations required for 50% maximal effectiveness. [b] Not determined. [c] No detectable interaction was seen in this assay.

The almost total ineffectiveness of GTPγF in interacting with transducin is interesting in view of the structural similarities between this analogues and GTPγS. Because there is free rotation about the β,γ-pyrophosphate bond, neither F nor S would restrict the nucleotide from forming a β,γ-bidentate chelate with a metal ion. The major difference between these analogues is the loss of a negative charge in the case of GTPγF. Strong binding to transducin may require the presence of two negatively charged atoms on the γ-phosphate. One of these negative charges may be used to form a γ-monodentate chelate to a metal ion and the other to form a salt bridge with a group on the protein. GTPγOPh and, to a lesser extent, GTPγOMe may activate transducin weakly by providing an additional hydrophobic interaction to partially compensate for the loss of a negative charge.

The relative affinities of the six analogues for transducin generally parallel their effectiveness in releasing transducin from R* and in stimulating PDE (Table I and Figures 2–4). However, two exceptions should be noted. GTPγOPh is about 10-fold less effective than GppNHp in competing with GTP for binding to transducin, yet these analogues are quite similar in activating PDE. GTPγOPh and GTPγOMe have similar binding affinities, but GTPγOPh is at least 20-fold more potent in stimulating PDE. Thus, GTPγOPh binds weakly to transducin but once bound is highly effective in inducing activation of transducin, perhaps by triggering subunit dissociation.

Three of the GTPγ analogues studied here, GTPγOPh, GTPγOMe, and GTPγF, were first used to probe the guanyl nucleotide specificity of bacterial elongation factor G (EF-G) (Eckstein et al., 1975). Shortly afterward, these analogues were used to study the guanyl nucleotide dependent activation of adenylate cyclase in pigeon erythrocytes (Pfeuffer & Eckstein, 1976). A most interesting result is that the order of potency of the three analogues in the elongation factor system (GTPγF > GTPγOMe > GTPγOPh) is the reverse of that of the adenylate cyclase system (GTPγOPh > GTPγOMe > GTPγF). We find here that the order of effectiveness in the transducin system is the same as that in the adenylate cyclase system. This observation reinforces the notion that transducin, the stimulatory G protein (G_s), and the inhibitory G protein (G_i) are members of a homologous family of signal-coupling proteins (Stryer, 1986; Manning &

Gilman, 1983; Shinozawa et al., 1979; Hildebrandt et al., 1984).

Our results suggest that the γ-phosphate region of the guanyl nucleotide binding site of EF-G is significantly different from that of transducin, G_s, and G_i. It has recently been shown with diastereomers of GTPαS and GTPβS that transducin and EF-Tu have similar stereochemical preferences at the α-phosphorus but probably differ at the β-phosphorus (Leupold et al., 1983; Yamanaka et al., 1985). Our present results show that transducin and EF-G differ in how they bind at the γ-phosphorus group of GTP.

Finally, a third class of guanyl nucleotide binding proteins, the p21 proteins of the *ras* oncogene family (Gibbs et al., 1985), exhibits homologies with the vertebrate G proteins and EF-Tu (Halliday, 1983; Leberman & Egner, 1984; Lochrie et al., 1985). It will be interesting to determine whether the guanyl nucleotide binding specificity of the p21 *ras* proteins is similar to that of the vertebrate G proteins or to that of bacterial elongations factors.

Registry No. PDE, 9068-52-4; GTP, 86-01-1; GppNHp, 34273-04-6; $GppCH_2p$, 13912-93-1; GTPγS, 37589-80-3; GTPγF, 57817-57-9; GTPγOMe, 57817-58-0; GTPγOPh, 57817-64-8.

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