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A light-activated GTPase in vertebrate photoreceptors: Regulation of light-activated cyclic GMP phosphodiesterase

(retina/rhodopsin/cyclic nucleotides/adenylate cyclase/guanosine nucleotides)

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ABSTRACT We have been studying the mechanism by which light and nucleoside triphosphates activate the disc-membrane phosphodiesterase (oligonucleate 5'-nucleotidohydrolase; EC 3.1.4.1) in frog rod outer segments. GTP is orders of magnitude more effective than ATP as a cofactor in the light-dependent activation step. GTP and the analogue guanylyl-imidodiphosphate function equally as allosteric activators of photoreceptor phosphodiesterase rather than participating in the formation of a phosphorylated activator. Moreover, we have found a light-activated (5-fold) GTPase which participates in the modulation of photoreceptor phosphodiesterase. This GTPase activity appears necessary for the reversal of phosphodiesterase activation *in vitro* and may play a critical role in the *in vivo* regulation of light-sensitive phosphodiesterase. The K_m for GTP in the light-activated GTPase reaction is <1 μM. The light sensitivity of this GTPase (number of photons required for half-maximal activation) is identical to that of light-activated phosphodiesterase. The GTPase action spectrum corresponds to the absorption spectrum of rhodopsin. There is, in addition, a light-insensitive GTPase activity with a K_m for GTP of 90 μM. At GTP concentrations above 5 μM, there is no appreciable activation of GTPase activity by light. The substrate K_m values for guanylate cyclase, light-activated GTPase, and light-activated phosphodiesterase order an enzyme array that might permit light to simultaneously cause the hydrolysis of both the substrate and product of guanylate cyclase. These findings reveal yet another facet of light regulation of photoreceptor/cyclic GMP levels and also provide a striking analogy to the GTP regulation of nonphotoreceptor, hormone-sensitive adenylate cyclase.

In 1971 we found that light could regulate the levels of cyclic nucleotides in membrane suspensions prepared from vertebrate photoreceptors (1). Subsequent studies revealed that the same process was occurring in the intact retina and *in vivo* (2, 3). We later found that the level of cyclic nucleotides was regulated by a light-activated cyclic GMP (cGMP) phosphodiesterase (dGTP triphosphohydrolase; EC 3.1.5.1) and that activation of phosphodiesterase had an additional requirement for a nucleoside triphosphate (4). It was not known whether the nucleoside triphosphate could participate in the formation of a stable phosphorylated protein (which acts as a regulator) or whether the triphosphate serves as an allosteric modifier in the light-dependent activation of phosphodiesterase.

Here, we report that GTP is orders of magnitude more effective than ATP in the light-dependent activation of photoreceptor phosphodiesterase (oligonucleate 5'-nucleotidohydrolase; EC 3.1.4.1). Our data reveal that GTP functions as an allosteric modifier which gains access to its receptor as a consequence of illumination.

We also report a light-activated outer-segment GTPase which exhibits an identical light dependence and action spectrum as the phosphodiesterase (5). We find that this GTPase regulates the activity of phosphodiesterase *in vitro* and may play a critical role in its regulation *in vivo*.

METHODS AND MATERIALS

Preparation of Rod Outer-Segment Disc Membranes. *Rana catesbiana* (300–550 g) were dark-adapted (23°) overnight and decapitated. The dissected retinas were shaken and centrifuged in 46% (wt/wt) sucrose as described (5). The outer segments, which collect at the air/sucrose interface, were suspended in 20 mM Tris·HCl (pH 7.4) containing 1 mM dithiothreitol and 5 mM $MgSO_4$. All manipulations (except for intentional bleaching of disc membranes) were done using infrared sources and image converters (5). In those experiments where a wash step was included, membranes were diluted 1:100 in isotonic Tris·HCl (pH 7.4) containing 5 mM $MgSO_4$ and 1 mM dithiothreitol and sedimented at 20,000 × *g* for 10 min (4). This washing procedure was repeated twice.

Enzyme Assays. Except where indicated, assays were done in 150 mM Tris·HCl (pH 7.4) with 5 mM $MgSO_4$ and 1 mM dithiothreitol. Phosphodiesterase was measured as described (5). We selected cyclic AMP (cAMP) as substrate to achieve manageable rates. [The K_m for cGMP is 0.07 mM; the K_m for cAMP is 3 mM (6). We have established that the process of light activation is independent of which substrate is used to measure phosphodiesterase activity (6).] Assays were done at 30° for 3 min in a volume of 20 μl using 5 μg of disc-membrane protein, and an initial cAMP concentration of 2.5 mM containing 10 Ci of [^{3}H]cAMP/mol. Under these assay conditions we observe first-order kinetics. Comparisons of different activities (over the constant 3-min assay interval) were obtained by comparing the quantities ln ($cAMP_{initial}/cAMP_{final}$) for each activity. Determinations were done in duplicate and values accepted only when agreement was equal to or better than 5%.

For the GTPase assay, we measured production of $^{32}P_i$ by the method of Neufeld and Levy (7). Assays were done at 37° under linear conditions, as determined at four time intervals. No single determination in each group of four varied from linearity by more than 5%. In determining the K_m for the light-activated GTPase, we examined substrate concentrations over the range of 0.04–4 μM and analyzed our data by the method of Eadie (8). Substrate ([γ-^{32}P]GTP) specific activity was 220 Ci/mmol. In determining the K_m for the light-insensitive GTPase, we examined substrate concentrations over the range of 3–120 μM. Substrate specific activity was 340 Ci/mol. Assays were done in 100 μl that contained 10 μg of protein. Protein concentration was determined by the method of Lowry, with bovine albumin as standard (9).

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Abbreviations: cGMP, cyclic GMP; cAMP, cyclic AMP; AMPPCP, β,γ-methylene-ATP; AMPPNP, adenylyl-imidodiphosphate; GMPPNP, guanylyl-imidodiphosphate; $NaDodSO_4$, sodium dodecyl sulfate.

Light Quantitation. Varying degrees of rhodopsin photoisomerization were obtained by exposing stirred disc-membrane suspensions to attenuated (neutral density filtered) light for various time intervals. The filter array was calibrated to permit half-maximal activation with an exposure of 30 sec. The percent rhodopsin photoisomerized was directly measured (OD 500) in 1% hexadecyltrimethylammonium bromide for a bleach in excess of 5%, and linearly extrapolated for fractional percent bleaches.

The action spectrum was obtained on a Gilford 2000 Spectrophotometer masked against light leaks with black velvet. The sample holder and photomultiplier were removed and replaced by a camera shutter to provide measured durations of exposure. Frosted glass, placed over the shutter aperture, provided a uniformly diffuse light source. The intensity of the tungsten lamp at each wavelength was measured with a YSI radiometer. A 100-μl sample was contained in a self-masking cuvette with a 5-mm pathlength which was situated after the frosted glass. At each wavelength, a different duration of sample exposure was computed to normalize photon number.

Use of Sodium Dodecyl Sulfate ($NaDodSO_4$)/Polyacrylamide Gel Electrophoresis to Identify Phosphorylated Intermediates. Disc-membrane protein (200 μg) containing 0.1% photoisomerized rhodopsin was incubated in a 350-μl volume containing 0.5 μM [γ-^{32}P]GTP (specific activity, 220 Ci/mmol). The reaction was stopped with 2 ml of ice-cold 10% trichloroacetic acid. The spun pellet was twice dissolved with alkali and repelleted with fresh trichloroacetic acid. Finally, the pellet was washed in ethyl ether, dissolved in 100 μl of 1% $NaDodSO_4$ solution (with tracking dye), and applied to a 5.6% acrylamide gel (10). Gels were run, fixed, and sliced at 1-mm thickness. The radioactivity of each slice was measured (NEN-963 cocktail) in a Beckman L200 scintillation spectrometer. The phosphorylation reactions were stopped at intervals that corresponded with the various phases of phosphodiesterase activation and reversal of activation (Fig. 4A).

Materials. Radioisotopes [γ-^{32}P]ATP, [γ-^{32}P]GTP, and [^{3}H]cAMP were purchased from ICN. ATP, GTP, and cAMP were purchased from Sigma. β,γ-Methylene-ATP (AMPPCP), adenylyl-imidodiphosphate (AMPPNP), and guanylyl-imidodiphosphate (GMPPNP) were purchased from P & L Biochemicals.

RESULTS

Light-Activated Photoreceptor GTPase. In our efforts to identify a putative phosphorylated protein as an activator of phosphodiesterase we examined both [γ-^{32}P]ATP and [γ-^{32}P]GTP as possible phosphoryl donors. In the course of studying phosphorylation of outer-segment proteins by [γ-^{32}P]GTP we attempted to inventory all GTP-consuming reactions that take place in freshly prepared, unwashed suspensions of outer-segment disc membranes. When only 1 in 1000 rhodopsins are photoisomerized in the presence of 0.5 μM [γ-^{32}P]GTP, less than 3% of the radioactivity appears covalently linked to protein, as analyzed in $NaDodSO_4$/polyacrylamide gel electrophoresis. All of the remaining [γ-^{32}P]GTP radioactivity appears as P_i within 10 min of initiating the reaction. These studies revealed the presence of a light-activated GTPase in these preparations. The light sensitivity of this enzyme is strictly comparable to that shown by photoreceptor phosphodiesterase (Fig. 1). With both enzymes, half-maximal activation is observed when only 1 in 2000 rhodopsins is photoisomerized. The action spectrum for light-activated GTPase was identical with that of the absorption spectrum of rhodopsin, as is that of phosphodiesterase (5). The light-activated GTPase exhibits an explicit substrate preference for GTP. We do not find light-activated hydrolysis of ATP under the same conditions. For the light-sensitive reaction, the K_m for GTP was below 1 μM and the V_{max} in the suspension of unwashed disc membranes is 0.5 nmol of GTP hydrolyzed/min per mg of protein (Fig. 2A). At higher GTP concentrations, a light-insensitive GTPase is found, with a K_m for GTP of 90 μM and a V_{max} of 12 nmol of GTP hydrolyzed/min per mg of protein (Fig. 2B). Thus, above a GTP concentration of 5 μM, the GTPase activity is not enhanced by light. The pH optimum for these reactions is approximately 7.

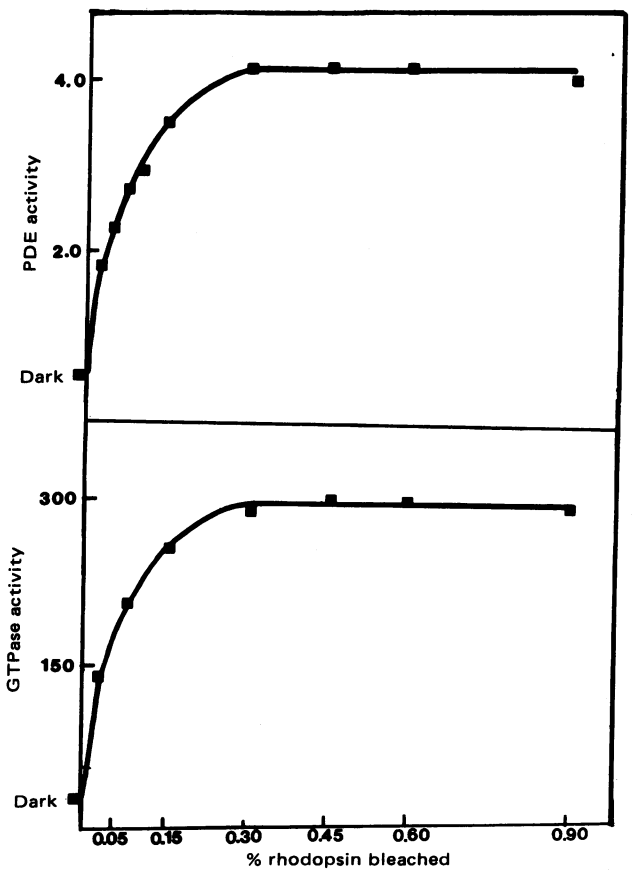

FIG. 1. A comparison of the light dependence of the phosphodiesterase (PDE) and GTPase reactions. Phosphodiesterase activity (determined in the presence of 0.5 μM unlabeled GTP) is expressed as μmol of cAMP hydrolyzed/min per mg of protein. GTPase activity is expressed as pmol of GTP hydrolyzed/min per mg of protein. Initial GTP concentration was 0.5 μM and the [γ-^{32}P]GTP specific activity was 220 Ci/mmol.

Activation of Photoreceptor Phosphodiesterase. Disc-membrane-bound phosphodiesterase requires both light and a nucleoside triphosphate for its activation (11). Half-maximal activation of phosphodiesterase (in suspensions of bleached disc membranes) is found at a GTP concentration of 0.07 μM, while with ATP, half-maximal activation requires a concentration of 20 μM (a ratio of 1:285) (Fig. 3). Indeed, phosphodiesterase activation by ATP might only reflect the recently reported contamination of commercial ATP with small quantities of GTP (12). Similarly, half-maximal activation of phosphodiesterase can also be achieved at 12 μM GDP (a ratio of 1:171). Again, these data suggest that the GDP effect reflects either contamination by, or enzymatic conversion to, GTP. Furthermore, nucleoside triphosphate analogues such as AMPPCP and AMPPNP, which are not enzymatically converted to (and are unlikely to be contaminated by) GTP, are completely inef-

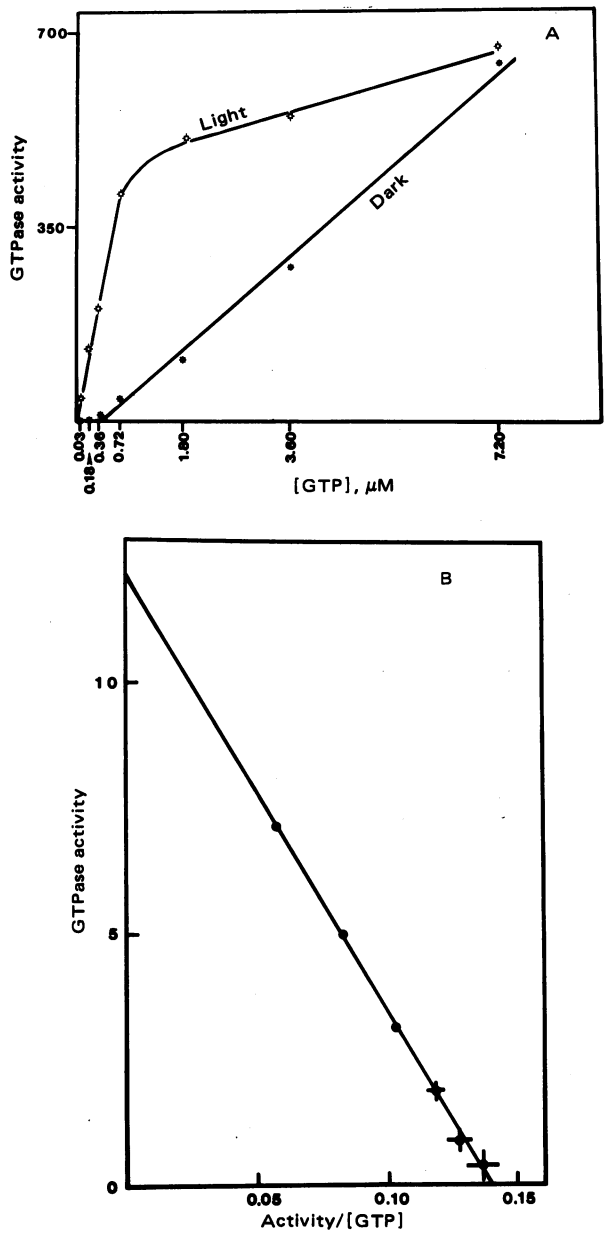

FIG. 2. Kinetic analyses of the light-activated and light-insensitive GTPase activities. (*A*) GTPase activity is shown as a function of GTP concentrations between 0.03 and 7.2 μM in the presence and absence of light. The light curve represents a membrane suspension in which only 0.05% of the rhodopsin is photoisomerized. GTPase activities are given as pmol of GTP hydrolyzed/min per mg of protein. (*B*) An Eadie plot is shown for the light-insensitive GTPase activity, which is observed at GTP concentrations between 3 and 120 μM. GTPase activities are given as nmol of GTP hydrolyzed/min per mg of protein.

fective. In contrast, the GTP analogue GMPPNP actually works as well as GTP in the light activation of phosphodiesterase (Table 1).

The sequence in which light and GTP participate in the activation of phosphodiesterase is critically ordered. If we add GTP to unilluminated suspensions of disc membranes and then wash the membranes (by sedimentation and resuspension twice) prior to illumination, no activation of phosphodiesterase is observed. If, however, we photoisomerize as few as 1 in 1000 of the rhodopsins in the presence of 1 μM GTP and then wash, the phosphodiesterase remains maximally activated. Thus, light must precede the GTP-dependent step in the activation sequence (Table 2).

Table 1. Efficacy of various nucleotides as cofactors for light activation of phosphodiesterase (PDE)

Addition	Concentration, μM	% PDE activation above basal*
GTP	1.0	800
	0.2	560
GMPPNP	1.0	800
	0.2	500
GDP	50	740
	25	340
ATP	100	600
	50	370
	10	190
AMPPCP	1000	16
AMPPNP	1000	32

Assays were done (3 min, 30°) with maximally bleached disc membranes.

* Basal activity was 0.55 μmol of cAMP hydrolyzed/min per mg of protein. Values are ±20% (SEM).

If one activates disc phosphodiesterase with a flash of light in the presence of 0.4 μM (or less) GTP, the activity declines to basal levels within 10 min at 23° and can be restored by the addition of a fresh increment of GTP. Repeated cycles of activation, decay, and reactivation can be observed (Fig. 4*A*). When activation or reactivation of phosphodiesterase is produced with larger increments of GTP (above 1 μM), the onset of decay is considerably delayed (Table 3). This "sawtooth" pattern of phosphodiesterase activation and decay provided a definitive criterion in the search for a putative phosphorylated intermediate in the GTP-dependent activation step. If GTP were to work by the formation of a (serine- or threonine-hydroxyl) phosphorylated protein, then one ought to detect, in $NaDodSO_4$ gel analyses, an isotopically labeled protein band that is phosphorylating and dephosphorylating with the same kinetics as the activation and deactivation of phosphodiesterase. Under conditions that could readily detect phosphorylation of proteins that were present at only 1% of the mole fraction of phosphodiesterase [based on the ratio, phosphodiesterase:rhodopsin = 1:900 (6)], no such protein is detected.

We have found that activation of phosphodiesterase by the higher concentrations (see above) of GDP shows the same decay phenomenon as that observed with the lower concentrations of GTP. In contrast, however, activation of phosphodiesterase

Table 2. Sequence of GTP and light requirements in the activation of photoreceptor phosphodiesterase

Treatment	Assay in dark*	Repeat assay†
Unilluminated membranes exposed to 1.0 μM GTP and then washed twice	70	70
Partially bleached‡ membranes exposed to 1.0 μM GTP and then washed twice	610	610

Membranes were washed by sedimentation and resuspension in isotonic buffers. Assays were for 3 min at 30°. Values are % phosphodiesterase activity above basal (±20% SEM). Basal activity was 0.55 μmol of cAMP hydrolyzed/min per mg of protein.

* The degree of photoisomerization produced by short exposures to light is maintained in the dark.

† After 15 min at room temperature, following maximal bleach.

‡ Partially bleached membranes are those in which 0.05% of the rhodopsin was photoisomerized.

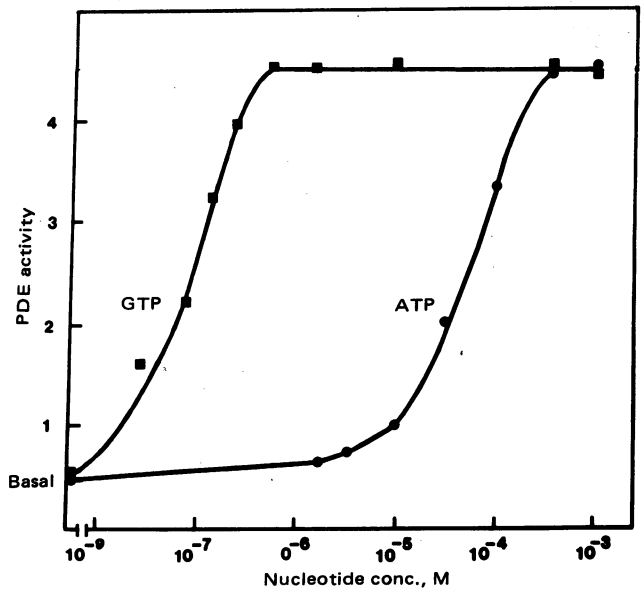

FIG. 3. A comparison of GTP and ATP as cofactors in the activation of phosphodiesterase (PDE). Activity is expressed as μmol of cAMP hydrolyzed/min per mg of protein.

by the analogue GMPPNP (using concentrations at which GTP-dependent activation is rapidly followed by decay) does not exhibit this decay (Table 3). If we illuminate suspensions of disc membranes in the presence of GTP and then wash the membranes (by sedimentation and resuspension in isotonic buffers), there results a full and persistent activation of phosphodiesterase which does not decay with time (Table 2).

Role of Light-Activated GTPase in Regulation of Phosphodiesterase Activity. When we prepare a persistently active photoreceptor phosphodiesterase by washing illuminated GTP-exposed membranes, we find that the washing step, which stabilizes phosphodiesterase activity, also markedly diminishes the light-activated GTPase activity (Table 4).

Furthermore, when we add the analogue GMPPNP (0.2 μM) (which is not a substrate for GTPase) to unwashed disc-membrane preparations, we observe maximal and persistent activation of phosphodiesterase that does not decay with time (Table 3). Under these conditions, independent measurements of light-activated GTPase show that it is completely inhibited by 0.2 μM GMPPNP, over a GTP concentration range from 0.04 to 0.7 μM.

Table 3. Influence of various nucleotide cofactors on the rate of reversal of phosphodiesterase (PDE) activation

Nucleotide cofactor	Concentration, μM	PDE activity* Early	Late
GTP	1.0	800	545
	0.6	660	250
	0.2	560	160
GDP	50	740	290
	25	340	170
GMPPNP	1.0	690	800
	0.2	330	500

Phosphodiesterase activity is expressed as the percent activity (±20% SEM) above basal. Basal activity was 0.55 μmol of cAMP hydrolyzed/min per mg of protein. Assays were for 3 min at 30°.

* Early activity was assayed during the time interval between 0 and 3 min after addition of the cofactor. Late samples were incubated for 10 min at 30°, and then activity was assayed during the time interval between 10 and 13 min after addition of the cofactor.

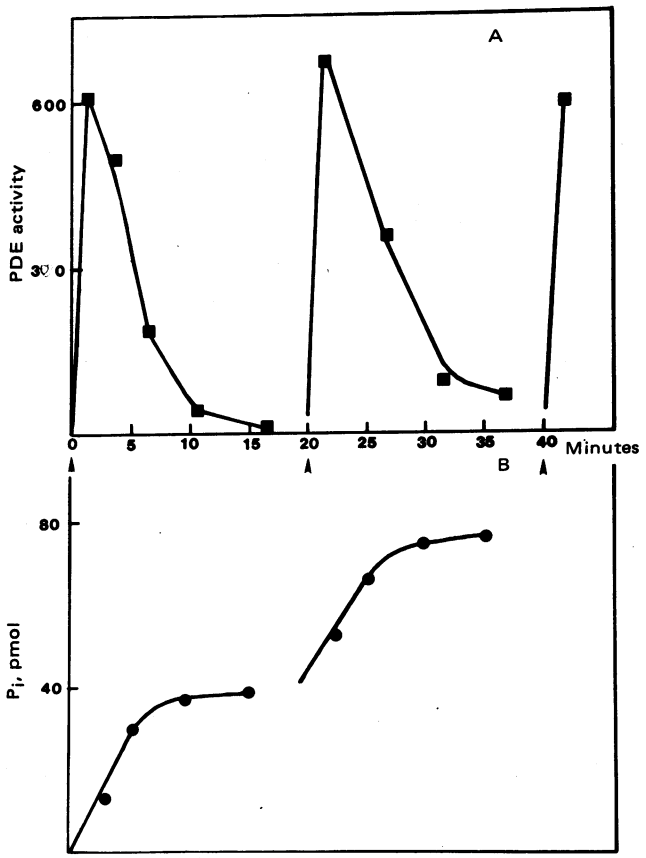

FIG. 4. A comparison of the rates of phosphodiesterase (PDE) activation/deactivation with the rates of P_i liberation by the light-activated GTPase. (*A*) Phosphodiesterase activity, shown as a function of time, is expressed as the percent activation above basal. (Basal activity was 0.55 μmol of cAMP hydrolyzed/min per mg of protein.) GTP is added at 0, 20, and 40 min to a concentration of 0.4 μM. Assays were done at 30°, and 0.05% bleached membranes were used for experiments described in both *A* and *B*. (*B*) Time course of the concomitant release of P_i during phosphodiesterase activation/deactivation. Reactions were done in a 100-μl volume and 40 pmol of [γ-^{32}P]GTP (specific activity 220 Ci/mmol) were added at 0, 20, and 40 min. The liberated P_i is expressed as pmol of P_i accumulated at each of the times shown.

In order to further evaluate the role of light-activated GTPase in the regulation of phosphodiesterase, we examined the time course of P_i production during repeated cycles of phosphodiesterase activation or decay. We found that the decay of phosphodiesterase activity correlates with the depletion of (submicromolar) GTP by light-activated GTPase (Fig. 4*B*).

Conclusions. We conclude that there are both a light-sensitive GTPase ($K_m < 1$ μM) and a light-insensitive GTPase ($K_m \cong 9$ μM) in vertebrate photoreceptor outer segments. (Neither enzyme hydrolyzes ATP.) Rhodopsin is the photopigment that permits light activation of the low K_m GTPase; this enzyme is half-maximally activated by the photoisomerization of 1 in 2000 rhodopsins.

The light activation of phosphodiesterase has a specific requirement for GTP: half-maximal activation is seen at 0.07 μM GTP or comparable amounts of GMPPNP. (Activation by GDP or ATP, which requires much higher concentrations, probably reflects contamination by GTP.) Photoisomerization of rhodopsin must precede the GTP-dependent step. Activation of phosphodiesterase by GTP appears to be an allosteric effect which does not depend on the formation of a phosphorylated intermediate. This activation shows a time-dependent decay

Table 4. Effects of washing on the light-activated GTPase activity

Treatment	Light-activated GTPase, specific activity*	% PDE activation above basal
Partially bleached† membranes exposed to 1 μM GTP and then washed twice	40 ± 20	610
Partially bleached† unwashed membranes	330 ± 20	—
Partially bleached† unwashed membranes to which 1 μM GTP was added	—	800

Phosphodiesterase (PDE) assays (3 min, 30°) were done with 5 mg of protein per ml. Basal PDE activity was 0.55 μmol of cAMP hydrolyzed/min per mg of protein. Values are ±20% SEM.

* GTPase assays (3 min, 37°) were done at an initial GTP concentration of 0.5 μM. GTPase activities are expressed as pmol of P_i liberated/min per mg of protein.

† Partially bleached are those in which 0.05% of the rhodopsin was photoisomerized.

which is linked to the light-activated GTPase activity. This decay is not observed when activation is produced by GMPPNP or when GTPase activity is impaired by washing the membrane suspensions. We emphasize that the decay of phosphodiesterase activity *in vitro* is seen only in the presence of, and correlates with, the activity of light-activated GTPase.

Furthermore, we conclude that the allosteric site at which GTP activates phosphodiesterase may correspond to the light-activated GTPase catalytic site for the following reasons: The concentrations of GTP required for half-maximal activation of phosphodiesterase (0.07 μM) and the K_m for light-activated GTPase (<1 μM) are of comparable magnitude. In addition, the analogue GMPPNP can maximally activate phosphodiesterase at concentrations (0.2 μM) that entirely inhibit light-activated GTPase activity.

DISCUSSION

The guanosine nucleotide-related enzymes of the photoreceptor are linked in a kinetic arrangement that may permit light to induce simultaneous hydrolysis of both the substrate and product of guanylate cyclase. Depending on the GTP concentrations that prevail *in situ*, the light-activated GTPase (K_m < 1 μM) may have a negative influence on the synthesis of cGMP (guanylate cyclase K_m is 0.3 mM) (13). Furthermore, the destruction of cGMP by GTP-activated phosphodiesterase can continue even after a cGMP synthesis has been impaired by falling GTP levels. (Half-maximal activation of phosphodiesterase requires only 0.07 μM GTP.) It is clear that if the K_m values were otherwise ordered, one could either lose phosphodiesterase activity before a significant decline in cGMP had been achieved, or continue inappropriately to produce cGMP in the face of its ongoing destruction.

There are at least two possible mechanisms that could account for the reversal of phosphodiesterase activation *in vivo*. The regeneration of rhodopsin could inactivate phosphodiesterase by removing the effects of illumination. Alternatively, as found in our *in vitro* studies, the hydrolysis of GTP by light-activated GTPase could remove that GTP which is required as a cofactor for the activation of phosphodiesterase. Whether the latter mechanism is the correct one depends upon whether light causes the *in vivo* GTP concentrations to fall below the critical cofactor requirement for phosphodiesterase activation.

The regulation of disc-membrane phosphodiesterase by light and GTP bears a striking resemblance to the regulation of nonphotoreceptor adenylate cyclase by hormones and GTP. The photoreceptor phosphodiesterase and a variety of adenylate cyclases are activated by a dual switch. For the photoreceptor phosphodiesterase system, this is light and GTP; for the adenylate cyclase system, a hormone and GTP (12, 14). It appears that the continual hydrolysis of GTP is not necessary for the activation of phosphodiesterase or adenylate cyclase (15) since in both cases GMPPNP serves equally well. There is also a similarity in the required order of the activation steps: the hormone (16) (or light) must precede GTP and therefore it allows GTP to bind to the activation site.

Finally, for both photoreceptor phosphodiesterase and hormonally responsive adenylate cyclase (15, 17) there are two ways to produce a persistently activated state. These are the use of the analogue GMPPNP, which is not hydrolyzed by GTPase, or the inhibition of GTPase activity (18). Further studies of these photosensitive and light-insensitive guanosine nucleotide-linked enzymes are needed in order to evaluate their role in photoreceptor physiology and to more fully characterize the biochemical significance and mechanistic features of enzyme regulation by GTP.

1. Bitensky, M. W., Gorman, R. E. & Miller, W. H. (1971) *Proc. Natl. Acad. Sci. USA* **68**, 561–562.
2. Goridis, C. & Virmaux, N. (1974) *Nature* **248**, 57–58.
3. Lolley, R. N. & Farber, D. B. (1975) *Exp. Eye Res.* **20**, 585–597.
4. Miki, N., Keirns, J. J., Marcus, F. R. & Bitensky, M. W. (1974) *Exp. Eye Res.* **18**, 281–297.
5. Keirns, J. J., Miki, N., Bitensky, M. W. & Keirns, M. (1975) *Biochemistry* **14**, 2760–2766.
6. Miki, N., Baraban, J. M., Keirns, J. J., Boyce, J. J. & Bitensky, M. W. (1975) *J. Biol. Chem.* **250**, 6320–6327.
7. Neufeld, A. & Levy, H. (1969) *J. Biol. Chem.* **244**, 6493–6497.
8. Eadie, G. S. (1942) *J. Biol. Chem.* **146**, 85–93.
9. Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951) *J. Biol. Chem.* **193**, 265–275.
10. Fairbanks, G., Steck, T. L. & Wallach, D. F. H. (1971) *Biochemistry* **10**, 2606–2617.
11. Bitensky, M. W., Miki, N., Keirns, J. J., Keirns, M., Baraban, J. M., Freeman, J., Wheeler, M. A., Lacy, J. & Marcus, F. R. (1975) *Adv. Cyclic Nucleotide Res.* **5**, 213–240.
12. Kimura, N. & Nagata, N. (1977) *J. Biol. Chem.* **252**, 3829–3835.
13. Goridis, C., Virmaux, N., Urban, P. F. & Mandel, P. (1973) *FEBS Lett.* **30**, 163–166.
14. Rodbell, M., Lin, M. C., Salomon, Y., Londos, C., Harwood, J. P., Martin, B. R., Rendell, M. & Berman, M. (1974) *Acta Endocrinol.* **77**, 11–37.
15. Schramm, M. & Rodbell, M. (1975) *J. Biol. Chem.* **250**, 2232–2237.
16. Schramm, M. (1975) *Adv. Cyclic Nucleotide Res.* **5**, 105–116.
17. Aurbach, G. D., Spiegel, A. M. & Gardner, J. D. (1975) *Adv. Cyclic Nucleotide Res.* **5**, 117–132.
18. Levinson, S. L. & Blume, A. J. (1977) *J. Biol. Chem.* **252**, 3766–3774.