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'-nucleotidohy- drolyase; 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 guanyl-

yl-imidodiphosphate function equally as allosteric activators of photoreceptor phosphodiesterase rather than participating in the formation of a phosphorylated enzyme. Moreover, we have found a light-activated (5-fold) GTPase activity in human outer segments GMP levels and also light-activated phosphodiesterase activation in vitro and may play a critical role in the in vivo regulation of light-sensitive phosphodiesterase. The Km 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 which is not a critical role in the in vivo regulation of light-sensitive phosphodiesterase.

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 vitro (2, 3). We later found that the level of cyclic nucleotides was regulated by a light-activated cyclic GMP (cGMP) phosphodiesterase (dGTP triphosphohydrolase; EC 3.1.4.1) 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'-nucleotidohydrolyase; EC 3.1.4.1) and 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 catesbeiana (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 MgSO4. 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 MgSO4 and 1 mM dithiothreitol and sedimented at 20,000 x 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 MgSO4 and 1 mM dithiothreitol. Phosphodiesterase was measured as described (5). We selected cyclic AMP (cAMP) as substrate to achieve manageable rates. The Km for cGMP is 0.07 mM; the Km 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 [3H]cAMP/mmol. 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 of cAMP (cAMPinitial/cAMPfinal) 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 32P3 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 Km 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 (Iy-32P3GTP) specific activity was 220 Ci/mmole. In determining the Km for the light-insensitive GTPase, we examined substrate concentrations over the range of 3-120 µM. Substrate specific activity was 340 Ci/mmol. 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).

Abbreviations: cGMP, cyclic GMP; cAMP, cyclic AMP; AMPPCP, β,γ-methylene-ATP; AMPFNP, adenylyl-imidodiphosphate; GMPFNP, guanylyl-imidodiphosphate; NaDodSO4, sodium dodecyl sulfate.

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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 path length 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 (NaDodSO4)/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-32P]GTP (specific activity, 220 Ci/mmole). The reaction was stopped with 2 ml of ice-cold 10% trichloroacetic acid. The spin 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% NaDodSO4 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-32P]ATP, [γ-32P]GTP, and [3H]cAMP were purchased from ICN. ATP, GTP, and cAMP were purchased from Sigma. β,γ-Methylene-ATP (AMPPCP), adenylyl-imidodiphosphate (AMPPNP), and guanylyl-imidodiphosphate (GMPNNP) 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-32P]ATP and [γ-32P]GTP as possible phosphoryl donors. In the course of studying phosphorylation of outer-segment proteins by [γ-32P]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-32P]GTP, less than 1% of the radioactivity appears covalently linked to protein, as analyzed in NaDodSO4/polyacrylamide gel electrophoresis. All of the remaining [γ-32P]GTP radioactivity appears as Pi 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.

Activation of Photoreceptor Phosphodiesterase. Disc-membrane-bound phosphodiesterase requires both light and a nucleotide 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 GDF (a ratio of 1:171). Again, these data suggest that the GDP effect reflects either contamination by, or enzymatic conversion to, GTP. Furthermore, nucleotide triphosphate analogues such as AMPPCP and AMPNNP, which are not enzymatically converted to (and are unlikely to be contaminated by) GTP, are completely inef-
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 rhodopins 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).

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. 4A). 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 NaDodSO4 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

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

<table>
<thead>
<tr>
<th>Addition</th>
<th>Concentration, μM</th>
<th>% PDE activation above basal*</th>
</tr>
</thead>
<tbody>
<tr>
<td>GTP</td>
<td>1.0</td>
<td>800</td>
</tr>
<tr>
<td>GMPPNP</td>
<td>0.2</td>
<td>560</td>
</tr>
<tr>
<td>GMPPNP</td>
<td>1.0</td>
<td>800</td>
</tr>
<tr>
<td>GDP</td>
<td>0.2</td>
<td>500</td>
</tr>
<tr>
<td>GDP</td>
<td>50</td>
<td>740</td>
</tr>
<tr>
<td>ATP</td>
<td>100</td>
<td>600</td>
</tr>
<tr>
<td>AMPPCP</td>
<td>1000</td>
<td>16</td>
</tr>
<tr>
<td>AMPPNP</td>
<td>1000</td>
<td>32</td>
</tr>
</tbody>
</table>

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).

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

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Assay in dark*</th>
<th>Repeat assay†</th>
</tr>
</thead>
<tbody>
<tr>
<td>Unilluminated membranes exposed to 1.0 μM GTP and then washed twice</td>
<td>70</td>
<td>70</td>
</tr>
<tr>
<td>Partially bleached membranes exposed to 1.0 μM GTP and then washed twice</td>
<td>610</td>
<td>610</td>
</tr>
</tbody>
</table>

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.
Early (±20% SEM) above 0.2 by light-activated 3. Under these membrane preparations, light-activated the stabilizes phosphodiesterase Activity. which does buffers), there results (by membranes of disc membranes, GTPase). 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

<table>
<thead>
<tr>
<th>Nucleotide cofactor</th>
<th>Concentration, μM</th>
<th>PDE activity*</th>
<th>Early</th>
<th>Late</th>
</tr>
</thead>
<tbody>
<tr>
<td>GTP</td>
<td>1.0</td>
<td>800</td>
<td>545</td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.5</td>
<td>660</td>
<td>250</td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.2</td>
<td>560</td>
<td>160</td>
<td></td>
</tr>
<tr>
<td>GDP</td>
<td>50</td>
<td>740</td>
<td>290</td>
<td></td>
</tr>
<tr>
<td></td>
<td>25</td>
<td>340</td>
<td>170</td>
<td></td>
</tr>
<tr>
<td>GMPPNP</td>
<td>1.0</td>
<td>690</td>
<td>800</td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.2</td>
<td>330</td>
<td>500</td>
<td></td>
</tr>
</tbody>
</table>

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.

In order to further evaluate the role of light-activated GTPase in the regulation of phosphodiesterase, we examined the time course of P1 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. 4B).

Conclusions. We conclude that there are both a light-sensitive GTPase (K_m < 1 μM) and a light-insensitive GTPase (K_m ≈ 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.
which is linked to the light-activated GTPase activity. This decay is not observed when activation is produced by GMPNNP 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 Kₘ for light-activated GTPase (<1 μM) are of comparable magnitude. In addition, the analogue GMPNNP 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 (Kₘ < 1 μM) may have a negative influence on the synthesis of cGMP (guanylate cyclase Kₘ = 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 Kₘ 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 vitro. 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 vitro 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 GMPNNP 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 GMPNNP, 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.