Light-induced Increases in cGMP Metabolic Flux Correlate with Electrical Responses of Photoreceptors*

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The metabolism of photoreceptor cGMP and the relationship of its light-sensitive regulation to rhodopsin photoisomerization and to the photoreceptor electrical response was examined in isolated, intact rabbit retinas. The dynamics of cGMP metabolism were assessed by measuring the rate of $^{18}$O incorporation from $^{18}$O-water into the α-phosphoryls of the guanine nucleotides. The photoreceptor electrical response was determined by measuring the aspartate-isolated mass receptor potential. Basal cGMP flux in dark-adapted retinas was 33 pmol cGMP·mg protein$^{-1}$·s$^{-1}$ which translates into a metabolic rate in the rod outer segment (ROS) of 1.7 mM/min in ATP equivalents. Photic stimulation increased this flux as much as 4.5-fold. With continuous illumination, increasing intensity caused increments in cGMP metabolic flux to a maximum of 4.5-fold, with corresponding increases in the electrical response over the same 3-log unit intensity range. Tight coupling between activation of guanylate cyclase and phosphodiesterase was indicated by either no changes in cGMP steady state concentrations or relatively small fluctuations represented by increases of 50% at lower light intensities and a 12% decrease at one of the highest intensities. A stoichiometry of about 10,000 molecules of cGMP generated and hydrolyzed per photon absorbed was calculated for the lowest light intensity when the increment in cGMP metabolic flux per photon was maximal.

Flashing light caused an increase in flux in proportion to frequency up to 1 Hz and a nearly proportional increase in the voltage time integral of the electrical response up to 0.5 Hz. This indicates that the temporal resolution, or “on”/“off” rate, of the cGMP metabolic response was as fast or faster than the temporal resolution of the electrical response. The concentration of cGMP remained relatively stable in spite of the marked acceleration of cGMP flux that occurred over the 32-fold range of frequencies tested.

Taken together these results show that the light-accelerated rate of cGMP synthesis tightly coupled to hydrolysis becomes a primary energy-utilizing system in the photoreceptor and represents a response that fulfills certain of the fundamental criteria required of a metabolic event playing an essential role in phototransduction.

A major feature of phototransduction that is still incompletely defined is the process by which the photosomeration of a molecule of rhodopsin leads to a change in the cation conductance of the outer segment of the photoreceptor cell. The metabolism of cGMP has been implicated in this signaling and amplification process because photosomeration has been shown to trigger a cascade of defined molecular events leading to the activation of a phosphodiesterase that hydrolyzes cGMP in the outer segment (1, 2). The functional significance of this phosphodiesterase activation has, however, not been elucidated. There are two different views of the importance that this increased rate of cGMP hydrolysis may have in phototransduction. The predominating concept is that light-promoted activation of this phosphodiesterase is of importance for rapidly lowering the concentration of photoreceptor cGMP (3, 4). This idea stems from the view that cyclic nucleotides impose regulatory influences through allosteric interactions that depend on critical changes in their cellular concentrations (5). The recent reports that cGMP can increase cation conductance determined by clamping of isolated patches of rod membranes (6–8) or by measurement with suction electrodes (8, 9) have reinforced interest in the primary role that cGMP steady state levels play in phototransduction.

The other quite different concept is that phosphodiesterase-promoted hydrolysis of cyclic nucleotides represents a biochemical event which in itself is of functional utility in promoting certain cellular processes (10, 11). This new view has been supported by observations that increasing intensities of photic stimulation produce incremental increases in photoreceptor cGMP metabolic flux which are independent of decreases in photoreceptor cGMP concentration (11). These results bear out the prediction that the rate of cGMP synthesis catalyzed by guanylate cyclase would be closely synchronized with the rate of phosphodiesterase-promoted hydrolysis to sustain the demands of the system for increased cGMP hydrolytic flux.

The studies to be described were conducted to obtain additional information about the metabolism of cGMP and its light-sensitive regulation in relation to photoreceptor function. The experiments were performed on an isolated rabbit retina preparation (12) in order to obtain this information under physiological conditions. The dynamics of photoreceptor cGMP metabolism were determined by measurement of the rate of phosphodiesterase-promoted incorporation of $^{18}$O from $^{18}$O-water into endogenous guanine nucleotide α-phos-
phoryls (11, 13). Electrical responses of the photoreceptors were determined by measurement of the PII1 of the mass electroretinogram of aspartate-blocked retinas (14-16). Rates of photoisomerization were estimated using three different approaches. By these analytical procedures it was possible to determine GMP hydrolytic rates in relation to the electrical output of the photoreceptors and to the rates of photoisomerization over a broad range of intensities and frequencies of photic stimulation.

The results of the investigations provide information on the following five points. First, in relatively dim light the increase in cGMP metabolic flux corresponds to about 10,000 molecules of GMP synthesized and hydrolyzed per photon absorbed. Second, the cGMP metabolic flux responses occur over at least as broad a range of light intensities as the electrical responses and the characteristics of both responses over this range are very similar. Third, the "on"/"off" rate of the cGMP metabolic flux response to a light stimulus is at least as fast as that of the electrical response. Fourth, under the conditions examined, the responses of the cyclase and phosphodiesterase to photic stimulation exhibit a degree of coupling that is adequate for a process governed by alterations in cGMP metabolic flux independent of decreases in cGMP steady state level. And fifth, the rapid rate of cGMP production and hydrolysis in response to photic stimulation represents a very active, high energy phosphate-requiring process utilizing millimolar equivalents of ATP per min. These results indicate that the accelerated rate of cGMP synthesis tightly coupled to hydrolysis represents a response to light stimulation that fulfills certain of the fundamental criteria required of a metabolic event playing an essential role in phototransduction.

EXPERIMENTAL PROCEDURES

Methods

The procedures used for isolating and incubating intact rabbit retinas have been described in detail by Ames and Nesbett (12). In brief, retinas were isolated from New Zealand White rabbits under darkroom conditions and incubated in darkness in medium that simulated cerebrospinal fluid. Temperature was maintained at 37 ± 0.1 °C. During the 20-s labeling period, the only modification of the medium composition was the substitution of approximately 40-45% of the O-water with O-water. During the labeling in O-water, the retina was held by forceps on the nerve stump and moved continuously through the medium. The incubation was terminated by immersion of the retina in 4 ml of ice-cold 0.5 N perchloric acid. The stumps were then immediately removed and discarded; the acidified retinal tissue was stored at -70 °C until the analyses described below were carried out.

The analytical procedure for extraction and determination of the tissue concentration of nucleotides and cyclic nucleotides and the methodology for measuring the O-content of the nucleotide α-, β-, and γ-phosphoryls has been previously described in detail (11, 13). The basic methodology involves a separation and isolation of the mono-, di-, and triphosphates of the adenine and guanine nucleotides by anion-exchange (Bio-Rad AG MP-1) chromatography employing trifluoroacetic acid as the eluant. The γ-phosphoryls of ATP and GTP and the β-phosphoryls of ADP and ATP were analyzed as glycerol-3-phosphate after they were enzymically transferred to glycerol. The β-phosphoryls of GDP and GTP were analyzed as P after they were enzymically treated with alkaline phosphatase and P1. The monophosphates representing the γ-phosphoryls of GTP, ATP, GDP, ADP, and the endogenous GMP and AMP were chemically degraded to glycerol-3-phosphate (17). Glycerol-3-phosphate or P1 which contained the phosphoryl moieties of interest were purified by chromatography on Bio-Rad AG 1-X4 and converted to their trimethylsilyl derivatives using bis(trimethylsilyl)trifluoroacetamide. These trimethylsilyl derivatives were analyzed for atom percent excess of O-water with respect to the percentage that contained one, two, or three atoms of O. The fraction, F, of the phosphoryl oxygens that had been replaced with O during the labeling period was calculated as:

\[ F = \frac{\%^{16}O + 2 \times \%^{17}O + 3 \times \%^{18}O}{3 \times \text{atom percent excess of } ^{18}O} \]

A correction for instances in which an atom of oxygen was replaced more than once with O (i.e. saturation effect) was made as follows:

\[ F' = 1 - \frac{t}{t} - F \]

in which t is the labeling time in seconds and F' is the fraction of oxygen being replaced per second. To determine the rate of O incorporation into nucleotide phosphoryls (mol O incorporated mg protein -1 s-1), the fraction of oxygens replaced, F' was multiplied by three times the concentration of nucleotide being analyzed for enrichment of O (to account for the three atoms of oxygen per phosphoryl). The values for GDP, GTP, and GMP were summed to yield the total rate of O incorporation into the guanine nucleotide α-phosphoryls; this value is a measurement of the rate of cGMP hydrolysis in the intact rabbit retina. The O-labeling rate of the phosphoryl of GDP was not always measured because the low concentration of this nucleotide, representing no more than 5% of the total guanine nucleotide pool, was sometimes below the limits of detection of the mass spectrometer. Previous studies (11) and those described here show that O labeling of the phosphoryl of this nucleoside monophosphate closely parallels that of the nucleoside di- and triphosphates, so omission of O in the phosphoryl of GDP results is no more than 5% underestimation of cGMP flux.

Photopic stimulation was provided by fluorescent lamps (Sylvania F4T5 CWX) with a phosphor exhibiting rapid (20 μs) rise and fall times. A pulse generator (Tektronix) controlled the tachistoscopic lamp driver (Gerbrands) to provide stimulus durations from 10 ms to 20 s. The stimulus intensity was varied by rheostat control of the current to the lamps and by neutral density filters which, in combination, provided an intensity range of 3.5 x 10^7 in 5% increments.

The procedure for the electrophysiological recordings involved measuring the mass ERG in a specially designed chamber that permitted the retina to be incubated under nearly the same conditions and to be exposed to the same photic stimulation as occurred during the O-labeling incubations. A central, hollow plastic mandrel was sealed to the floor of the chamber which contained 20 ml of gassed medium. The retina was placed, vitreous surface downward, over the mandrel and held gently in place by a silicone rubber O-ring so that it covered several perforations at the top of the mandrel and thus separated the fluid within, the mandrel from the fluid outside. One Ag-AgCl electrode with agar bridge was immersed in the fluid outside, and another was sealed within a short angulated silicon tube that penetrated the base of the mandrel so that it made contact only with the fluid within. The electrodes, with conventional DC amplification and recording (Gould), were very stable, usually showing only a few μV of drift over many min. The isolated preparation exhibited the responses expected from recordings in vivo. Aspartate (4 mM) was added to the medium to restrict the light-evoked response to the photoreceptor cells (18). The PII1 values thus obtained varied from about 25 μV in response to stimuli of 1 photon/2 μm2 to about 1100 μV in response to a maximum stimulus. Under control conditions and without mechanical disturbance, the amplitude of the PII1 remained quite constant for many hours.

Determination of Quantum Irradiance and Measurement of Rates of Photoisomerization

Quantum Irradiance—The amplitude of the PII1 of the mass electroretinogram was used to establish the relationship between the white light used in the labeling experiments and a monochromatic light with a wavelength near the peak of the rhodopsin absorption spectrum. The PII1 was measured over a range of intensities of the white light, and of the monochromatic light (obtained using a Baird Atomic interference filter; 10 nm half band width); and the irradiance (ps) of each stimulus was measured with a photometer (United Detector Technology). By comparing the response curves generated for both the white and the monochromatic light, it was determined that the white light was only 0.259 times as effective as monochromatic, 500-nm light in bleaching rhodopsin. Since 1 μW cm-2 of 500-nm light is equivalent to 5.61 x 10^6 photons μm^-2 s^-1, the product
0.259 ± 2.51 × 10^4, or 5.60 ± 10^4, was used to convert the white light, measured as μW·cm^-2, s^-1, to its equivalence in 500-nm photons·μm^-2·s^-1.

**Rate of Photoisomerization Determined from Collecting Area and Capture Efficiency**—The cross-sectional area of the ROS of the rabbit photoreceptor was determined in tangential photomicrographs of retinas fixed in 1% osmium tetroxide in isotonic medium to minimize volume changes (19) and was found to average 2.55 μm^2. Using this value, and an axial optical density of rhodopsin of 0.24 (0.016 O.D. units/μm at 500 nm, in an ROS that is 15 μm long), the effective collecting area was calculated to be 2.55 × (1.0×10^-3m) = 1.1 μm^2. A value of 0.67 was taken as the quantum efficiency of isomerization. In combination, these factors indicated that irradiance of 1 (500 nm) photon·μm^-2·s^-1 caused 1.1 × 0.67 = 0.74 photoisomerizations/ROS/s.

**Rate of Photoisomerization Determined by Spectrophotometric Analysis**—Direct spectrophotometric measurements of rhodopsin content were made as previously described (20) on retinas handled in precisely the same way as those used in the labeling experiments. One retina of each experimental pair served as a dark-adapted control and the other was exposed to the white light used in the labeling experiments, with intensity and duration increased by large, but well-defined, factors in order to bleach a measurable fraction (approximately 20%) of the rhodopsin. In some experiments, 0.3 m hydroxy-ylamine was added to the medium to prevent possible photoreisomerization of retinal, but it did not alter the results. The dark-and light-exposed retinas were labeled by 1.62 ± 0.09 (S.E.) nmol rhodopsin·mg protein^1. The bleaching coefficient, k, was calculated for each test-control pair from the fraction of rhodopsin remaining in the test retina (F) and the time of exposure to the light (t), using the relationship k = -1/t ln (1−F). The average value for k for the test stimulus of 16.4 μW·cm^-2 was 28.7 ± 4.5 × 10^-4 (S.E.)·s^-1. The corresponding expression for a stimulus of 1 (500 nm) photon·μm^-2·s^-1 is 2.6 × 10^-5·s^-1. The amount of rhodopsin that was bleached in an 1^9O labeling experiment was then calculated as the product of the rhodopsin content (1.6 nmol·mg protein^1), the bleaching coefficient (1.7 × 10^-5·s^-1·μW·cm^-2), and the amount of light (μW·cm^-2). Measurements expressed per milligram of protein to measurements expressed per ROS, the number of photoreceptors per milligram of protein was determined by dividing retinal area by protein content and by the area ascribable to a single photoreceptor. Retinal area averaged 3.5 cm^2; protein content averaged 4.5 mg, and the area ascribable to a photoreceptor as measured on tangential electron micrographs (19) was 4.4 μm^2, yielding a value of 17.7 × 10^6 photoreceptors/mg protein.

The results of the bleaching experiments were expressed in terms of molecules of rhodopsin bleached per ROS per photon·μm^-2·s^-1, by expressing rhodopsin content per ROS (1.6 nmol·mg protein^1) × 8 × 10^6 molecules·nmol^-1·17.7 × 10^6 photoreceptors·mg protein^-1 = 5.4 × 10^8) and multiplying this by the bleaching coefficient expressed per photon·μm^-2·s^-1 (2.6 × 10^-8·s^-1) to obtain the value of 1.4 molecules/Ros/photon·μm^-2.

**Rate of Photoisomerization from Relative Size of the III of the Mass Electroretinogram**—A consistent relationship has been observed between the number of photoisomerizations per ROS and the size (percent of maximum) of the electrophysiologic response, when the latter is measured at the lower, near linear end of the stimulus response curve. A single photoisomerization per ROS has been reported to cause about a 5% reduction in dark current (24-27). In experiments on retinas maintained under conditions similar to those used in the 1^9O labeling studies, we found that the stimulus required to elicit 5% of the maximum III averaged 1.08 photons·μm^-2·s^-1. Thus, on the basis of the published data, it can be estimated that 1 photon·μm^-2·s^-1 resulted in 0.9 photoisomerizations/ROS.

As a partial check on the accuracy of some of the measurements made, the concentration of rhodopsin in the ROS was calculated from the spectrophotometric measurement of rhodopsin content (1.62 × 10^8 pmol·mg protein^-1) and the volume of the ROS as determined from measurements of the retinal dimensions (2.55 μm^2 cross-sectional area and 15 μm length) and their number (17.7 × 10^6·mg protein^-1). The value for rhodopsin concentration thus obtained from our data was 2.4 μM which falls within the range of ROS rhodopsin concentrations (1.7-7.4 μM) obtained by others using quite different measurements and calculations (22).

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1 The abbreviations used are: ROS, rod out segment(s); PIII, component of the electroretinogram attributed to the photoreceptor light response.
Fig. 1. Relationship of cGMP hydrolysis to continuous photic stimulation of increasing intensity. Retinas were incubated in medium containing 40–45% $^{18}$O-water for 20 s in dark (A) or with varying intensities of continuous photic stimulation (C) and prepared for analysis of $^{18}$O content of nucleotide $\alpha$-phosphoryls as described under "Experimental Procedures." Panel A shows the rates of appearance of total $^{18}$O into the $\alpha$-phosphoryls of tissue GTP, including the molecular species containing 1, 2, and 3 atoms of $^{18}$O as a function of increasing intensity of photic illumination. Panels D–F show the rates of $^{18}$O labeling of the $\alpha$-phosphoryls of GTP with 1, 2, or 3 atoms of $^{18}$O, respectively, as a function of the intensity of the illuminating stimulus. Panel B shows the rates of incorporation of $^{18}$O into the $\alpha$-phosphoryls of GDP, including molecular species with one or two atoms of $^{18}$O as a function of illuminating intensity. Panel C shows the rates of appearance of $^{18}$O in the phosphoryl of GMP, represented by molecular species containing only one atom of $^{18}$O as a function of increasing intensity. Small amounts of ($\alpha$-$^{18}$O)GDP, ($^{18}$O$_2$)GMP and ($^{18}$O$_3$)GMP were detected, but the amounts were too small to provide reliable measurements and were, therefore, not included. Panel G shows the rates of cGMP hydrolysis represented by the total $^{18}$O incorporated into the $\alpha$-phosphoryls of GTP, GDP, and GMP (i.e., summation of Panels A–C) as a function of increasing intensity of continuous photic stimulation. The $^{18}$O labeling rates of the $\gamma$-guanine nucleotide phosphoryls (panels A–G) were corrected for the percentage of $^{18}$O-water determined to be present in the medium during incubation of the retina. They were also corrected for the loss of phosphoryl $^{18}$O predicted by the removal of the $\alpha\beta$ bridge during the process of conversion of ATP to GTP by guanylate cyclase as described under "Experimental Procedures.

The stoichiometry between the photoisomerization of rhodopsin and the hydrolytic flux of cGMP—The quantitative measurements of cGMP metabolic flux obtained by $^{18}$O labeling, when combined with measurements of the rhodopsin bleached in light-stimulated retinas, made it possible to determine the stoichiometry between the photoisomerization of rhodopsin and the metabolism of cGMP. Three methods were used to estimate the rate of photoisomerization (see "Experimental Procedures" for details). The first was based on a determination of the collecting area of the ROS of the rabbit photoreceptor and on published data for the quantum efficiency of photon capture; it gave a value of 0.74 photoisomerizations/ROS/photon $\mu$m$^{-2}$. The second method was based on spectrophotometric measurement of the amount of rhodopsin bleached by stimuli that were known multiples of those used in the labeling studies; it gave a value of 1.4 photoisomerizations/ROS/photon $\mu$m$^{-2}$. The third method was based on published data (24–27) relating the number of photoisomerizations per ROS to the electrical response expressed as a fraction of the maximal response; in conjunction with measurements in our preparation of the PIII response to known stimuli, it gave a value of 0.9 photoisomerizations/ROS/photon $\mu$m$^{-2}$. The lowest intensity of light used in the labeling experiments was 32 photons $\mu$m$^{-2}$ s$^{-1}$. Depending on which of the above methods was used to convert photons $\mu$m$^{-2}$ s$^{-1}$ to photoisomerizations per ROS, light of this intensity was estimated to produce 24, 45, or 29 photoisomerization/ROS/s.

Exposure to 32 photons $\mu$m$^{-2}$ s$^{-1}$ increased the rate of cGMP metabolized by 9.1 pmol mg protein$^{-1}$ s$^{-1}$ (Table I). Since there are 17.7 $\times$ 10$^6$ photoreceptors/mg of protein (see "Experimental Procedures"), this represents an increase of 308,000 molecules metabolized per ROS per s (9.1 $\times$ 10$^{-12}$ $\times$ 6 $\times$ 10$^6$/$17.7$ $\times$ 10$^6$ = 308,000). By dividing the increase in
reasonably reliable estimate of the stoichiometry when photo- 
the synthesis/hydrolysis of cGMP. It is possible there was 
between photoisomerization and hydrolysis, between photo-
stimuli, under nearly identical incubation conditions (but with 
same retinas; the electrical responses were recorded from a 
separate group of retinas that were exposed to the same photic 
data in Table I are equally valid for showing the stoichiometry 
some saturation even at the lowest intensity studied and that 
increased over the first three log units of light intensity and a 
plateau at approximately 500 photons. 

The metabolic rate by the rate of photoisomerization, the number of molecules of cGMP metabolized per quantum absorbed was found to be 12,800, 6,800, or 10,600, depending on the method used to determine the rate of photoisomerization. The average of these values, 10,100 molecules of cGMP metabolized per photoisomerization, appears to provide a reasonably reliable estimate of the stoichiometry when photoisomerization is in the range of 30 molecules/ROS/s, or 0.9 
fmol/mg of protein/s.

When the rate of photoisomerization was increased by increasing light intensity, there was a less-than-proportional increase in the metabolic rate (Table I), indicating saturation of one or more of the steps linking the bleaching of rhodopsin to the synthesis/hydrolysis of cGMP. It is possible there was some saturation even at the lowest intensity studied and that measurement at a still lower intensity would have demonstrated a stoichiometry of more than 10,000 molecules of cGMP synthesized/hydrolyzed per quantum.

Since the light-induced increases in metabolic flux were large compared to the changes in cGMP concentration, the data in Table I are equally valid for showing the stoichiometry between photoisomerization and hydrolysis, between photoisomerization and synthesis, or between photoisomerization and the flux, or metabolic rate, of cGMP.

Relationship of Light-induced Electrical and Biochemical Responses—The changes in the concentration and in the metabolic flux of cGMP in the retinas described in Fig. 1 that were exposed to continuous illumination of increasing intensities were compared with changes in the amplitude of the PIII of the aspartate-blocked mass electroretinogram. The measurements of concentration and flux were made on the same retinas; the electrical responses were recorded from a separate group of retinas that were exposed to the same photic stimuli, under nearly identical incubation conditions (but with 

In Fig. 2B, the light-evoked increase in cGMP flux and the amplitude of the PIII have both been expressed as percentages of the maximal responses observed and have been plotted against the stimulus intensity. The electrical response increased over the lower range of light intensities and began to plateau at approximately 500 photons \( \mu \text{m}^{-2} \cdot \text{s}^{-1} \) when 90% of the maximal response was achieved. Further increases in stimulus intensity of almost two log units produced only small additional increases in electrical output. The cGMP metabolic flux exhibited a similar but not identical response to increasing light intensities. Increases in cGMP metabolic rates occurred over the first three log units of light intensity and a maximum flux was achieved at 7540 photons \( \mu \text{m}^{-2} \cdot \text{s}^{-1} \). At greater intensities, covering an additional log unit, there was

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**Table I**

| Stimulus | Photoisomerization | Increase in cGMP metabolized | cGMP metabolized per photoisomerization |
|----------|--------------------|-----------------------------|----------------------------------------|
| photons | \( \mu \text{m}^{-2} \cdot \text{s}^{-1} \) | \( \mu \text{mol rhodopsin bleached} \) | mg protein \( \cdot \text{s}^{-1} \) | \( \frac{\mu \text{mol cGMP}}{\text{mg protein} \cdot \text{s}^{-1}} \) | molecules photon \(^{-1} \) |
| 32 | 0.90 | 9,100 | 10,100 |
| 164 | 4.6 | 25,200 | 5,478 |
| 433 | 12.2 | 58,600 | 4,803 |
| 2,270 | 63.8 | 91,600 | 1,436 |
| 7,540 | 212.1 | 121,600 | 573 |

* Retinas were exposed to 20 s of continuous photic stimulation over the range of intensities shown (Fig. 1).
* Difference between rate in darkness and rate during photic stimulation.
* Based on the average value of 10,100 molecules photon \(^{-1} \) for the 32 photon \( \mu \text{m}^{-2} \cdot \text{s}^{-1} \) stimulus (see text).

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**Fig. 2.** Responses of the concentration and the flux of cGMP to increasing light intensities compared to the response of PIII. Retinas were exposed to 20 s of continuous photic stimulation over the range of intensities shown (as in Fig. 1). Retinal cGMP concentration, \( ^{18} \text{O} \) content of guanine nucleotide \( \alpha \)-phosphoryl, and measurement of PIII were all determined as described under "Experimental Procedures." A, the concentration of cGMP expressed as percentage of the concentration in darkness (\( 77.7 \mu \text{mol mg} \cdot \text{protein}^{-1} \)), obtained by subtracting the rate in darkness (33.3 \( \mu \text{mol mg protein}^{-1} \cdot \text{s}^{-1} \)) from the rates during photic stimulation, expressed as percentage of the maximum increase (122 \( \mu \text{mol mg protein}^{-1} \cdot \text{s}^{-1} \)). PIII (●) is expressed as percentage of the maximum amplitude (1089 \( \mu \text{V} \)). C, the relationship between PIII and the cGMP flux from the experiment shown in B.
a relative decline of about 15% from the peak metabolic rate. This decline, as pointed out above, has been noted previously (11) with high intensity illumination.

In Fig. 2A, the concentration of cGMP in the photically stimulated retina has been expressed as a percentage of the concentration in darkness and plotted against stimulus intensity. In the dark-adapted state the concentration of cGMP was 77.7 pmol·mg protein⁻¹. At the two lowest intensities of illumination this level increased to 113 and 119 pmol·mg protein⁻¹. With greater intensities of photic stimulation, the cGMP concentration returned toward the level found in non-illuminated retinas, and then to slightly below it with a maximum reduction of 12% at 31,900 photons·μm⁻²·s⁻¹.

The data presented in Fig. 2B have been replotted in Fig. 2C to show more directly the relationship between the electrical response and cGMP flux. The curve generated shows that successive increases in cGMP flux (in response to increasing light intensities) were associated with progressively smaller increases in the amplitude of the PIII. This curve is equivalent to a Michaelis-Menten hyperbola that is representative of a saturable process. It suggests that cGMP flux is a determinant of the conductance change and that saturation occurs at some step(s) between the change in flux and the change in conductance.

**Temporal Resolution of the Light-stimulated cGMP Metabolic and Electrical Responses** - An indirect assessment of the time required to initiate and terminate the cGMP metabolic flux response was made by examining the resolution of the responses to 10-ms flashes of relatively high intensity illumination (8.5 x 10⁴ photons·μm⁻²·s⁻¹) presented at increasing frequencies. It was reasoned that if the temporal resolution of the response is as fast or faster than the rate at which 10-ms light flashes are delivered, the sum of the responses measured over the 20-s labeling period will increase in proportion to flash frequency. If, on the other hand, the flashing frequency exceeds the temporal resolution, responses will overlap and the summed responses will not increase in proportion to the frequency. A relatively high intensity light flash was used to eliminate summing of overlapping low intensity responses. The electrical response, which can be studied with a high degree of temporal fidelity is an example of this circumstance (Fig. 3B). As the frequency of flash was increased from 0.125 to 0.5 Hz, the increase in the voltage time integral of the response was nearly proportional to the increase in frequency, so that the value at 0.5 Hz was 186% (instead of 200%) of the value at 0.25 Hz. But, as shown in Fig. 3A, at frequencies greater than 0.5 Hz, the voltage time integral showed an obvious, progressive failure to keep pace, so that, although there were some increases in the total response, the voltage time integral per flash became progressively less. This is interpreted to indicate that at frequencies of 0.5 Hz or greater the temporal resolution of the electrical response is slower than the rate at which the flashes are delivered.

The same assessment of the cGMP metabolic response showed that the increases in ¹⁸O labeling of guanine nucleotide α-phosphoryls were very nearly proportional to the flash frequency up to 1.0 Hz; the value at 1.0 Hz was 198% of the value at 0.5 Hz. At greater frequencies the curve plateaus and small increments in cGMP metabolic flux paralleled the small increases measurable in the voltage time integral. These results indicate that frequencies of greater than 1.0 flash/s exceeded the temporal resolution of the metabolic response.

As shown by comparing the two curves in Fig. 3A, changes in cGMP flux in response to the on and off signals of illumination appear to be temporally resolved at least as rapidly as the light-induced photoreceptor electrical potential changes. The steady state levels of retinal cGMP did not exhibit any correspondence with light-flashing frequency since they fluctuated minimally over the 16-fold range (i.e. 0.5–8 Hz) of frequencies employed (Fig. 3). At the low frequency of 0.25 Hz, there was a 25% increase in cGMP concentration while at the highest frequency used, 16 Hz, a maximum decrease of 16% below the dark level was noted.

**DISCUSSION**

All the evidence obtained to date, including the results in this report, has provided confirmation that the rate of ¹⁸O appearance in the α-phosphoryls of cellular guanine nucleotides is a valid measure of the rate of phosphodiesterase-catalyzed hydrolysis of cGMP. No other enzymatic pathway can account for the ¹⁸O found to be incorporated into this guanine nucleotide phosphoryl within the time frame examined; and the quantitative and kinetic characteristics of ¹⁸O labeling of the α-phosphoryls of all species of ⁵-guanine nucleotides observed in the present studies are predictable consequences of the catalysis promoted by this hydrolytic enzyme. Additional evidence (not shown in this report) derives from the demonstration that 3-isobutyl-1-methylxanthine, an inhibitor of cyclic nucleotide phosphodiesterase (28), produces a concentration-dependent inhibition of the rate of incorporation into retinal guanine nucleotide α-phosphoryls to the extent that 80% of the rate of this ¹⁸O labeling is inhibited by 730 μM 3-isobutyl-1-methylxanthine (29). This analysis of phosphoryl labeling with ¹⁸O has made it possible to measure cyclic nucleotide hydrolysis in intact cells or in whole tissue, and, since 90% of retinal phosphodiesterase activity is confined to the ROS layer (23), measurements of cGMP hydrolysis made on the whole retina reflect primarily photoreceptor activity of this enzymic event. This fortunate circumstance has made it possible to monitor cGMP metabolism in photoreceptors that are functioning in a near physiological state. It is well appreciated that the process of transducing a light stimulus into a relatively large change in photoreceptor membrane conductance requires amplification of the photon signal. The cascade of biochemical events leading to the activation of phosphodiesterase has been thought to provide a potential for amplification; with a partially reconstituted rod disc membrane system Liebman et al. (1, 30, 31) estimated that 10⁻⁶⁻¹⁰⁻⁶ molecules of cGMP could be hydrolyzed per photon through this cascade. The presence of ATP required in a smaller number of cGMP molecules α-hydrolyzed (31, 32), and arrestin has also been reported (33, 34) to impose a suppressive influence on phosphodiesterase activation. The extent to which these and possible other factors act in situ has not been established. In the present study which assessed intact photoreceptor metabolism and function, it was possible to determine that, with a relatively low intensity photic stimulus (e.g. 5.6 x 10⁻⁷·s⁻¹ fractional bleach of rhodopsin), a single photoisomerization was amplified to the extent that approximately 10⁶ molecules of cGMP underwent synthesis and hydrolysis. Since a single photoisomerization produces an easily discernible electrical response in the photoreceptor cell (i.e. about 5% of the maximal response, 24–27), a single photoisomerization should also elicit a detectable change over background noise in the chemistry of phototransduction. The light-evoked increase in cGMP metabolic flux appears to meet this criterion, even though there is a relatively high level of background flux in darkness. This can be deduced from the following considerations. The cGMP flux in darkness was 33 pmol·mg protein⁻¹·s⁻¹; since there are 1.77 x 10⁸ rods/mg protein, this corresponds to 1.3 x 10⁹ molecules of cGMP. This concentration is 3.6 fold greater than the concentration of cGMP necessary to half-maximally activate the cyclic nucleotide phosphodiesterase (28).
FIG. 3. Responses of cGMP concentration, cGMP metabolic flux, and PIII to light flashes of increasing frequencies. Retinas were exposed to light flashes during a 20-s period over the range of frequencies shown. Light flashes were 85,400 photons μm^-2·s^-1 and 10 ms in duration. Retinal cGMP concentration, 32P content of guanine nucleotide α-phosphoryls, and measurements of the PIII were determined as described under "Experimental Procedures." A, the light-evoked increment in cGMP metabolic flux (●) determined by subtracting the rate in darkness (34 pmol·mg protein^-1·s^-1) from the rate during photic stimulation. Electrical response (●) expressed as the voltage time integral of the PIII measurement. Letters identify the voltage time integrals that correspond to the PIII tracings in B. The inset shows cGMP concentration as a function of light flashes of increasing frequencies. B, recordings of PIII responses at frequencies of 0.5, 1.0, 2.0, and 4.0 Hz. Vertical lines above tracings indicate light flashes.

metabolized per ROS·s^-1. Thus the approximately 10,000 molecule increase in cGMP flux elicited by a single photon represents a 0.9% increase over the basal flux occurring in an entire outer segment over a second. If it is assumed that the metabolic response to a single photon lasts only 1/2 s (which seems reasonable from the time course of the PIII response to a dim flash) and involves only 20% of the ROS (21), then within this temporal and spatial domain the 10,000-molecule flux elicited by a single photon would represent a 9% increase over the background flux.

The experiments using increasing intensities of continuous illumination as the test stimuli demonstrated a systematic relationship between the amplitude of the cGMP metabolic response and the amplitude of the electrical response of the photoreceptors. Both responded over virtually the same broad range of light intensities, increasing incrementally over the lower intensities and exhibiting saturation at the higher light intensities with the electrical response saturating first. These characteristics of the relationship between the cGMP metabolic flux and the electrical response are consistent with the metabolic event representing a precursor of the reduction in ion conductance of the ROS.

The experiments using increasing flash frequency to obtain information about the on/off time of the biochemical and electrical responses of the photoreceptor indicate that these two responses also exhibit similar temporal characteristics. The light-related increases in cGMP metabolic flux were linearly related to the number of stimuli presented when the frequencies did not exceed one flash per second and the electrical response exhibited a failure to follow proportionality at somewhat lower frequencies. Therefore, with the relatively bright 10-ms flash used in these experiments, the biochemical event can be estimated to be resolved within 1 s and to exhibit temporal resolution that is at least as rapid as that of the electrical response. The results of these experiments with increasing flashing light frequencies also indicate that most of the loss of temporal resolution occurs between rhodopsin photoisomerization and the change in cGMP metabolism with relatively little loss between the change in cGMP metabolism and the electrical event.

An important difference between studies performed on isolated components of photoreceptor cells and studies performed on intact cells with physiological high energy phosphate metabolism is that in the former, the response to light has been manifest as a rapid decrease in photoreceptor cGMP concentration (3, 4), whereas in the latter, the response to light has been manifest as an increase in the number of cGMP molecules rapidly undergoing synthesis and hydrolysis. Although the results of this study and of our previous study (11) indicate that illumination triggers an acceleration in the rate of cGMP hydrolysis, they do not indicate that the hydrolytic reaction serves the purpose of lowering photoreceptor cGMP concentration. It has not been possible either, in our studies or in others previously reported (35, 36), to demonstrate that photoexcitation within the PIII response range results in decreases in intact photoreceptor cGMP concentration that
correlate with the magnitude or frequency of the illuminating stimuli. In the present study, photic stimuli that covered the entire physiological response range, caused very little change in cGMP concentration, whether they were presented as continuous or as flashing light. In fact, the only significant change observed was an increase in cGMP concentration with low intensities of continuous light. Increases in cGMP concentration at low to moderate light intensities have been observed consistently in all experiments we have conducted thus far (not shown). It seems unlikely that light-evoked reductions in cGMP were missed because of a restoration of the level of cGMP by the time the tissue was harvested; the tissues were quenched for analysis while still being illuminated, under circumstances in which a large reduction in ROS conductance would be expected from electrophysiological studies. It also seems unlikely that a light-evoked reduction in cGMP was obscured because it was occurring only in restricted regions of the ROS; the flux of photons was dense enough to have activated all regions. Though binding of cGMP is likely, it would act to diminish rather than abolish a light-evoked reduction in cGMP concentration and only if there is a slow equilibrium between bound and free. Thus, these results do not eliminate the possibility of cGMP acting as an allosteric effector of a component influencing some photoreceptor function, they reduce the likelihood of such an influence representing a primary event in phototransduction.

The near constancy of cGMP concentration, in the face of the marked increases in cGMP metabolic flux, could only be achieved if the activities of the synthetic and hydrolytic enzymes are increased to nearly the same extent by the photic stimulation. The shift from darkness to 7540 photons \( \cdot \mu m^2 \cdot s^{-1} \) caused the amount of cGMP being hydrolyzed per second to increase by more than 120 pmol \( \cdot mg \) protein \( ^{\cdot} \) and would have hydrolyzed all the photoreceptor cGMP (i.e. 77.7 pmol \( \cdot mg \) protein \( ^{-1} \)) in less than one second had there not been a nearly equally marked increase in the rate of synthesis within a time frame closely approximating that of the hydrolysis.

The measurements of the rates of \(^{18}O\) labeling of guanine nucleotide \( \alpha \)-phosphoryls combined with measurements of cGMP steady state levels indicate that although the activities of the cyclase and phosphodiesterase exhibit a fundamental characteristic of highly coordinated parallel regulation, there may also be an aspect of control deriving from regulation primarily of guanylate cyclase activity that results in alterations of cGMP steady state levels. For example, continuous light of low intensity (32 and 164 photons \( \cdot \mu m^2 \cdot s^{-1} \)) caused increased hydrolytic rates in association with an approximate 50% elevation in the level of cGMP. As pointed out above, increases of this magnitude have been consistently observed in all other previously reported studies. It has been shown that without purification relatively small increases in tissue cGMP concentration are not detectable by radioimmune assay (37).

One of the striking features of photoreceptor cGMP metabolism, uncovered by monitoring the dynamics of cyclic nucleotide metabolism in the intact cell, is the level of expenditure of high energy phosphate required to support the cGMP metabolic flux. From the measurements of \(^{18}O\) labeling of guanine nucleotide \( \alpha \)-phosphoryls, it can be calculated that the molar flux of cGMP in the ROS is 0.86 mM/min in the dark and almost 4 mM/min with light stimulation. Since the equivalent of two molecules of ATP are consumed in the resynthesis of one molecule of cGMP that undergoes hydrolysis, the rate of high energy phosphate utilization to support cGMP metabolism is equivalent to 1.7 mM/min in the dark and almost 8 mM/min with illumination. The photoreceptors constitute about half the volume of the rabbit retina; and, because of the energy requirements of phototransduction, they probably account for more than half of its energy metabolism. If they are assumed to account for 75% of the phosphate bond turnover of the entire retina (470 nmol \( \cdot \) min \( ^{-1} \)) \(^{\cdot}, \) the maximum energy requirements of cGMP flux (83 nmol \( \cdot \) min \( ^{-1} \)) would represent about 34% of the entire metabolic of the photoreceptors. High energy phosphate consumption of this magnitude cannot easily be reconciled with cGMP playing only an allosteric role in phototransduction. It was suggested previously (11) that the functional utility of an accelerated rate of cGMP flux may reside in the pyrophosphate generated in cGMP formation, in the protons generated from the combined synthetic and hydrolytic reactions, and/or in the free energy (10 to 12 kcal/mole of cGMP; 38) inherent to the phosphodiesterase-catalyzed cGMP hydrolytic reaction. The generation of pyrophosphate and the production of protons are not unique to the cGMP metabolic system. On the other hand, the thermodynamic and molecular structure are unique. These considerations combined with the recognized near catalytic "perfection" of the phosphodiesterase (1, 3) underscore the distinctive features of the system that are consistent with the concept that it may be of utility through an energy-linked mechanism.

Several features of cGMP metabolism have now been characterized that indicate that it plays a critical role in phototransduction, even though the process(es) in which it may be involved remains undefined. A very high percentage (i.e. 90%; 3, 6) of the non-rhodopsin proteins in the ROS is dedicated to the metabolism of cGMP or its regulation; a high expenditure of biological energy is invested in the support of cGMP metabolic flux, particularly in the light-stimulated increase in flux; the magnitude of the cGMP flux increase in response to a single photoisomerization corresponds with the expectations of amplification in phototransduction; the on/off rate of the light-stimulated cGMP flux is fast enough to accommodate the kinetics of the electrical response; the graded increases in cGMP flux correspond with the intensity of photostimula-
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and the increments in the photoreceptor electrical responses reflect the increases in cGMP metabolic flux and exhibit the saturation expected of a precursor-product relationship. Notably absent, however, in the metabolic response to photic stimulation is a reduction in cGMP concentration, even with light intensities sufficient to elicit a near maximal PIII. These characteristics of cGMP metabolism, when taken together, suggest that the light-promoted increase in the rate of cGMP hydrolysis does not serve primarily as a means for decreasing the photoreceptor concentration of cGMP but is, instead, part of a mechanism by which changes in cGMP flux are utilized in the process of transducing a photon signal.

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