Light-induced Changes in Energy Metabolites, Guanine Nucleotides, and Guanylate Cyclase within Frog Retinal Layers*

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Freeze-dried sections were prepared from retinas of frogs which were dark-adapted or exposed to varying periods of light. Samples of the discrete layers were dissected, weighed, and analyzed for energy metabolites, guanylate compounds, and the enzyme guanylate cyclase. ATP and P-creatine were measured in both dark- and light-adapted retinas. There was a gradient in ATP and P-creatine levels in dark-adapted retinas, with the lower concentrations in the photoreceptors, and increasing concentrations in the inner retina. After light adaptation, concentrations increased, an observation which supports the concept that transmitter release occurs in the dark and ceases in the light. The sum of GTP plus GDP, GDP, and cyclic GMP were analyzed in dark-adapted retinas and after exposure to 2 min or 2 h of room light. GDP was rather uniformly distributed in the retinal layers, was increased by 2 min of light in all layers but the outer nuclear, and remained elevated at 2 h in the inner retina. GTP values showed a marked localization in the outer nuclear layer, which increased after 2 min or 2 h of illumination; in all other layers GTP was decreased by light. Cyclic GMP in the dark was highest in the photoreceptor cells, decreasing to one-third after 2 min of light; there were significant increases in the outer plexiform and inner nuclear layers at this time. Cyclic GMP remained low in the photoreceptor cells even after 2 h of light, while the inner layers returned to dark values. Guanylate cyclase, like cyclic GMP, was largely confined to the photoreceptor cells and showed a maximal increase after 2 min of light exposure.

Quantitative histochemical studies of metabolites in the layers of retinae have shown that most of those measured are not uniformly distributed throughout the layers, or even within portions of a specific cell type (1-4). Profiles of several of the metabolites have been documented in rapidly frozen eyes or after periods of anoxia. The effects of flash illumination in regions of light- and dark-adapted frog retinae have been described (3). However, there has not been a study of energy metabolites in all the retinal layers during light and dark adaptation.

There have, however, been many studies on the effects of light on guanine nucleotides and related enzymes. Cyclic GMP has been postulated to play a role in the phototransduction mechanism in the rod photoreceptor (4-9). Upon exposure to light, either in vivo or in vitro, cyclic GMP decreases in whole frog retina (10), in isolated frog outer segments (11), and in several layers of the photoreceptor cell (12-15).

GTP is the substrate for the formation of cyclic GMP and may also be involved in the activation of the phosphodiesterase (EC 3.1.5.1) in the outer segments upon exposure to light (12-15), as well as a substrate for protein kinase (16) and GTPase (14). We have reported in an earlier manuscript a decrease of GTP in the frog outer segments, concurrent with an increase in GDP, after in vivo exposure to 2 min of room fluorescent light (9).

Guanylate cyclase activity from isolated outer segments has been reported to be inhibited after exposure to light; in one report the inhibition was a delayed response (17), and in another inhibition occurred after long term exposure (18). Other investigators have pointed out that guanylate cyclase from frog outer segments washed previously to remove the membrane-bound phosphodiesterase does not change its activity upon illumination (19). We have found that guanylate cyclase measured in frog retina prepared as described below is activated by brief illumination. In the present study we have used retinas from frogs which have been quick-frozen in liquid nitrogen. While the freezing time is still a factor, such a model avoids the encumulation of the eye and the concomitant ischemia, however brief. After preparation of the freeze-dried sections, the layers can be hand dissected, weighed, and analyzed using the oil-well technique (20).

The present report includes measurements of the high energy phosphate compounds, ATP and P-creatine, in retinal layers from dark-adapted eyes and those following exposure to light. The changes in guanine nucleotides have been investigated following 2 min or 2 h of exposure to light. Finally, the effect of illumination on the activity of guanylate cyclase in the retinal layers has been assessed.

MATERIALS AND METHODS

Frogs (Rana pipiens) were adapted to a light cycle, 12 h of dark and 12 h of light. Selected animals were immersed in liquid nitrogen at the end of the dark period or after periods of exposure to room fluorescent light. The eyes were removed at −20°C and 6- to 8-μm tangential sections were cut through the back of the eye at −22°C and freeze-dried overnight at −45°C. Samples of retinal layers (0.1-1.0 μg) were hand dissected under a stereomicroscope and assayed for ATP and P-creatine (50), for GTP plus GDP (21), and for cyclic GMP (22, 23).

Guanylate Cyclase Assay—A micromodification of the method described by Trosler et al. (24) was used. The reagent consisted of 50 mM Tris-HCl (pH 7.6), 5 mM MnCl2, 0.5 mM GTP, 1 mM isobutylmethylxanthine, 0.06% bovine serum albumin, 5 mM phosphocreatine, and 150 μg/ml of creatine kinase. Dry samples (2.0-5.0 μg) were loaded into 100 nl of 50 mM Tris-HCl, pH 7.6, under mineral oil. The guanylate cyclase reagent (1 μl) was added, and the samples were incubated for 10 min at room temperature. The reaction was stopped by transferring the whole sample to 50 μl of 0.02 N HCl. The solution
was neutralized with Tris-HCl, pH 8, and cyclic GMP was assayed as described above. The addition of the cyclase reagent and transfer of the sample were performed over timed intervals to ensure exactly equivalent incubation periods.

Chemicals—The enzymes for the analytical methods were purchased from Boehringer Mannheim Biochemicals (Indianapolis, IN), except for lactate dehydrogenase which was purchased from Worthington Biochemical (Freehold, NJ). Nucleotides were bought from Sigma and materials for the cyclic GMP assay were from Schwarz-Mann (Orangeburg, NY). All other chemicals were analytical grade.

RESULTS

ATP and P-creatine—ATP and P-creatine were measured in the outer segments of retinas from dark-adapted frogs or after 2 min or 2 h of exposure to light (Table I). The concentration of ATP is like that of average frog brain, while P-creatine stores are considerably lower (25). There was a significant decrease in P-creatine after 2 min of light, followed by an increase to values almost double those of the dark-adapted retinas after 2 h.

When the individual layers were analyzed for P-creatine and ATP, the retinas were from dark-adapted animals or animals exposed to 2-4 h of room light. The concentrations of ATP showed an increasing gradient from the outer segments toward the inner layers (Fig. 1). There is a fairly abrupt increase in ATP in the outer nuclear layer, with the peak values in the inner nuclear layer. The concentrations of ATP in the retinal layers are higher than those found in the monkey retina (2, 26), although there was a gradient in both species. In the frog, however, the ATP values for the inner segment are as low as in the outer segment, while in the monkey there was a steeper gradient, with the minimal value in the outer segment. The ATP concentrations in the frog inner retina are 3-fold higher than those in frog brain (25): the receptor cells and epithelium are about 25% of the peak values. After prolonged light exposure, the ATP increased in the outer nuclear and outer plexiform layer and decreased in the inner nuclear layer (Fig. 1).

The distribution of P-creatine in the layers of the retina was comparable to that of ATP, although the absolute amounts of P-creatine were greater (Fig. 2). The P-creatine levels in the inner retina were comparable to frog brain (25), while those in the photoreceptor cells were about one-third of those of the brain. The gradient differed from that of the monkey retina (2, 26) in the same manner as ATP. After light-adaptation, P-

| Conditions      | ATP (pmol/μg dry weight) | P-creatine (pmol/μg dry weight) |
|-----------------|--------------------------|---------------------------------|
| Dark-adapted    | 1.78 ± 0.24              | 3.69 ± 0.40                     |
| Light-adapted   | 1.30 ± 0.11              | 2.71 ± 0.20                     |
| 2 h             | 1.80 ± 0.06              | 6.45 ± 0.42                     |

*The values are the means ± S.E. for at least 4 determinations on 4 frogs.

Values different from dark-adapted values; p < 0.05.
creatinine increased in all layers except the inner nuclear and the ganglion cells.

If the sum of the changes in ATP and P-creatine are considered, the high energy phosphates increase most strikingly in the layers from the pigment epithelium inward to the outer plexiform, while the inner nuclear, inner plexiform, and ganglion layers are unchanged (Fig. 3).

Cyclic GMP—Preliminary measurements of cyclic GMP were made in the whole retina of quick-frozen tissue. In the whole retina, or the photoreceptor portion, the cyclic GMP decreased after 2 h of light; however, there was no significant change in the inner retina (Table II). After only 2 min of illumination, however, while cyclic GMP was decreased in the whole retina or in the photoreceptor cells, there was an increase in the inner retina. The outer plexiform layer was included in the inner retina; the observed increase must occur within the inner retina or its synaptic contacts with the photoreceptors.

A more detailed study of cyclic GMP distribution in the retinal layers was made (Fig. 4). The concentrations of cyclic GMP in the frog retina layers showed a similar pattern to rabbit retina (4). The highest values for both species were found in the outer segments; 43 and 142 fmol/µg dry weight, in the frog and rabbit, respectively.

After 2 min of light exposure, cyclic GMP was decreased

| Conditions          | Whole retina | Photoreceptors | Inner retina |
|---------------------|--------------|----------------|--------------|
|                     | fmol/µg dry weight |
| Dark-adapted        | 22.5 ± 0.99  | 41.3 ± 2.73    | 0.93 ± 0.49  |
| Light-adapted       |              |                |              |
| 2 min               | 15.7 ± 1.72  | 13.3 ± 0.39    | 23.5 ± 2.40  |
| 2 h                 | 9.8 ± 1.12   | 13.6 ± 0.86    | 0.44 ± 0.13  |

*The outer plexiform layer was included in the inner retina fraction.
*Results are expressed as the mean ± S.E. for 4-8 determinations.
*Values significantly different from the dark-adapted group; p < 0.05.

![FIG. 4. The concentrations of cyclic GMP in layers of frog retina from dark-adapted animals (●) and after 2 min (○) or 2 h (○) exposure to light. Abbreviations and symbols are as in Fig. 1. The dark-adapted values ± S.E. are given below each layer.](image)

![FIG. 5. The concentrations of GDP in layers of frog retina from dark-adapted animals (●) and after 2 min (○) or 2 h (○) exposure to light. Abbreviations and symbols are as for Fig. 1. The dark-adapted values ± S.E. are given below each layer.](image)

![FIG. 6. The concentrations of the sum of GDP and GTP in layers of frog retina from dark-adapted animals (●) or after 2 min (○) or 2 h (○) exposure to light. Abbreviations and symbols are as in Fig. 1. The dark-adapted values ± S.E. are given below each layer.](image)

from dark values to one-third in the outer segments and to one-sixth in the outer nuclear layer. Surprisingly, there was a 5-fold increase in the outer plexiform layer, and a 14-fold increase in the inner nuclear layer. After 2 h of light, cyclic GMP was still decreased below dark values in the photoreceptor cell portions, while the inner layers had returned to dark-adapted values.

GDP and GTP—The concentrations of GDP in the frog retinal layers from dark-adapted eyes were rather uniformly distributed (0.7-1.2 pmol/µg dry weight; Fig. 5). After 2 min of exposure to light, GDP levels increased in all layers but the outer nuclear. After 2 h of illumination, the concentrations of GDP were equal to those of dark-adapted retinas in the photoreceptor cell, while in the inner retinal layers, the GDP increased even further (inner nuclear, inner plexiform, and ganglion cell layer).

GTP levels were calculated from the difference between the...
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**DISCUSSION**

It is generally accepted that the vertebrate photoreceptors behave as though "dark" is a stimulus and "light" is the absence of stimulus. The hyperpolarization of the receptors that is known to occur in the presence of light (27) might then be accompanied by a decrease in energy demands. Following a transient decrease, the elevation of P-creatine in the photoreceptor portion of the frog retina (Figs. 2 and 3; Table I) support the concept that transmitter release occurs in the dark and ceases in light (28). The second order neurons processing the information can respond by different mechanisms. Bipolar cells can hyperpolarize or depolarize, and horizontal cells can hyperpolarize or present a mixed response. The relative stability of the sum of ATP and P-creatine in these layers may be a reflection of the varied responses.

The decreases in cyclic GMP upon exposure to light in the whole retina or the outer segments from a variety of species have been observed by others (4, 6-11). The transient increase in the outer plexiform and inner nuclear layers is a new observation; preliminary data indicate that the increases in the inner nuclear layer are in the portion nearest the outer plexiform layer. This layer is in part composed of receptor cell elements and the increases in cyclic GMP may reflect the response of these cells to light. However, if there is a real increase in the inner nuclear layer, it implies the involvement of second order neurons in the process of light adaptation. The biochemical events reported here in the inner nuclear and outer plexiform layers may also suggest a possible role for the Muller cells, the main glial compartment of the retina. Karwoksi and Proenza (29) demonstrated that Muller cells respond to stimuli with an initial depolarization, followed by a slow hyperpolarization. The changes in cyclic GMP in the inner nuclear and outer plexiform layers could involve the Muller cells. The increases in cyclic GMP in the outer plexiform and inner nuclear layers exceed the measured cyclase activity in these layers. The cyclic GMP increased 42 fmol/µg in 2 min in the outer plexiform layer and maximal enzyme activity measured was 9 fmol/µg/min. In the inner nuclear layer, cyclic GMP increased 13 fmol/µg in 2 min while the enzyme activity was 4 fmol/µg/min. GTP is relatively high in these layers (Fig. 7) so there is no shortage of substrate, and the enzymes to regenerate GTP from GMP are present in much higher amounts than the cyclase in these layers in

**TABLE III**

| Layer            | Dark-adapted | 2 min | 2 h |
|------------------|--------------|-------|-----|
| Outer segments   | 0.49         | 1.64  | 0.60|
| Inner segments   | 0.50         | 0.50  | 0.36|
| Outer nuclear    | 0.22         | 0.19  | 0.16|
| Outer plexiform  | 0.38         | 0.72  | 0.23|
| Inner nuclear    | 0.29         | 1.21  | 1.94|
| Inner plexiform  | 0.80         | 1.80  | 1.93|
| Ganglion cells   | 0.34         | 1.14  | 1.94|

**Fig. 7.** The concentrations of GTP in layers of frog retina from dark-adapted animals (○) and after 2 min (●) or 2 h (★) exposure to light. **Fig. 8.** Guanylate cyclase activity in layers of frog retina from dark-adapted animals (○) and after 2 min (●) or 2 h (★) exposure to light. Abbreviations and symbols are as in Fig. 1. The dark-adapted values ± S.E. are given below each layer; for the epithelium, outer plexiform, and ganglion cell layers there were only 2 samples for the dark values and 4 for the light-adapted values.

concentration of GTP plus GDP and that of GDP alone. Unlike the GDP concentrations, the sum of GTP plus GDP, and consequently GTP, showed a marked localization within the retinal layers, the peak values being in the outer nuclear region (Figs. 6 and 7). The distribution is quite similar to that found in monkey retina (26), although the absolute values are less. The sum of GTP plus GDP did not change significantly after exposure to either 2 min or 2 h of illumination except in the inner plexiform layer. The calculated changes in GTP do show alteration upon illumination. GTP decreases in all of the layers in which GDP increased; this was true after 2 min or 2 hr of illumination.

The calculated GDP:GTP ratio increased almost 2- to 4-fold in all the regions except the outer nuclear layer after 2 min of light exposure (Table III). The ratio was near dark-adapted values after 2 h of light in the outer segments but increased to 6-fold in the inner nuclear and ganglion cell layers.

**Guanylate Cyclase—** Guanylate cyclase is highly compartmentalized in the frog retina, most of it being confined to the outer and inner segments (2.5- and 2-fold). Although the absolute activity was much lower, there was a 3.5-fold increase in cyclase activity in the outer nuclear area and nearly 2-fold in the inner plexiform layer. The activity of the cyclase in the various layers remained elevated after 2 h of illumination.
monkey and rabbit retina (26). However, even if phosphodiesterase is very low in these layers, as is reported for monkey, rabbit, and squirrel retina (26), it is difficult to explain the observed increase with the present data.

The outer plexiform layer in the rabbit also shows special properties. In that species, cyclic AMP is highest in the outer plexiform layer and decreases markedly after 1 h of light from 56 to 14 fmol/μg (4). Preliminary experiments in our laboratory indicate that cyclic AMP does not change in response to 2 min of light in the frog retina and is lower than in the rabbit (about 4 fmol/μg throughout the layers).

Unlike the energy reserves ATP and P-creatine, GTP decreases in most layers of the frog retina after exposure to light. This response implies a different role for GTP other than energy use. In the photoreceptor portion, GTP may be involved in the phosphorylation of rhodopsin (30, 31). It also must serve as substrate for cyclic GMP formation which may be turning over rapidly in the photoreceptors due to the light activation of guanylate cyclase (this paper) and phosphodiesterase (5, 32). The GDP/GTP ratio increases in all layers after 2 min of light exposure and remains elevated in the inner retina after 2 h. The persistent lower level of the GTP suggests that it is being utilized for phosphorylation and/or cyclic GMP formation faster than it can be restored. However, the precise functions of the GDP or GTP in the different portions of the retina are not clear.

Guanylate cyclase, the enzyme responsible for the formation of cyclic GMP, is most abundant in the outer and inner segments of the frog retina. In contrast to some reports with cell fractions isolated from other species, the guanylate cyclase in the intact frog retina is activated by light. There are also significant increases in cyclase activity in the outer internal and inner plexiform layers. The activity of the particulate form of guanylate cyclase in partially purified preparations has been reported to be decreased by light (17, 18). In the intact frog retina, there may be forms of guanylate cyclase which are activated by light. The soluble form of the cyclase from the retina has been reported to be activated by Ca²⁺, while the particulate form is inhibited (24, 33). Ca²⁺ is known to be released in the outer segments after exposure to light (34). The activation of the guanylate cyclase may be due to the effect of Ca²⁺ on the enzyme.

Phosphodiesterase is known to be activated by light in the photoreceptor cells of frog and bovine retinas (5, 32) and is confined almost exclusively to the outer segment in monkey, rabbit, and squirrel retina (26). The decreases in cyclic GMP in the frog photoreceptor layers in response to light is accompanied by increased guanylate cyclase activity, and presumed high phosphodiesterase activity. The combination of events would predict an active cyclic GMP cycle. The postulated enormous turnover rate for cyclic GMP implies that it could function as a chemical transducer in the visual response.

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