

Calcium and Cyclic Nucleotide Regulation in Incubated Mouse Retinas

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ABSTRACT When retinas from dark-adapted C57BL/6 mice were incubated in the dark for 5 min at 37°C in Earle's medium, they contained 80–120 pmol/mg protein of cGMP and about 13 pmol/mg protein of cAMP. When the incubation in darkness was in calcium-deficient Earle's medium with 3 mM EGTA, a 10–20-fold increase occurred in the cGMP level, peaking at 2–3 min, but no change occurred in cAMP. This elevated level fell in 3 min to normal dark levels on return to normal Earle's medium, but was still about three times that of control levels after 15 min in EGTA-containing solution. Bright light after 2 min of dark incubation of dark-adapted retinas resulted in a 40–50% fall in cGMP, and bright light sharply reduced the elevated dark cGMP level of retinas in calcium-deficient media with 3 mM EDTA. However, no depression of normal dark levels of cGMP has thus far been obtained by increasing external calcium levels, even in the presence of the ionophore A23187. All the above phenomena involving dark cGMP levels and calcium are similar in Earle's medium with 100 mM of K⁺ substituted for Na⁺. Congenic rodless (rd/rd) mouse retinas have <5% of control cGMP and show only traces of calcium sensitivity. Thus, the above phenomena in controls are likely to be largely occurring in rods. The data suggest a dependency of the dark cGMP level on the calcium level, but that the light-induced fall in cGMP may largely be calcium insensitive.

INTRODUCTION

Recent studies have suggested that light both bleaches visual pigments of vertebrate retinas, and induces notable changes in the concentration of guanosine 3',5'-monophosphate (cGMP) (Goridis et al., 1974, 1976; Krishna et al., 1974, 1976; Ferrendelli and Cohen, 1976). The latter molecule, in vertebrate retinas, seems highly concentrated in outer segments of photoreceptors (Fletcher and Chader, 1976; Woodruff et al., 1977) and in the photoreceptor containing layers of the retina (Orr et al., 1976; Farber and Lolley, 1974, 1976; Ferrendelli and Cohen, 1976).

One of the most attractive hypotheses (Yoshikami and Hagins, 1971; Hagins, 1972) for the mechanisms by which light modifies the membrane potential of photoreceptors postulates that light increases the amount of free calcium ion in the cytosol within outer segments of photoreceptors, and that calcium ions

reversibly block sodium channels in the plasma membrane of outer segments. They found support for this hypothesis (Hagins and Yoshikami, 1974) in a study of the effect of calcium on the photoreceptor dark current in slices of rat retina. In further accord with this view, Brown and Pinto (1974), recording from single rods of toads, found that raising external calcium hyperpolarized the cells whereas lowering external calcium produced depolarizations. Larger depolarizations permitted larger amplitudes of photoresponse. Brown et al. (1977) subsequently reported that, whereas injecting Ca^{++} into rods gave hyperpolarizations, injecting the calcium chelator EGTA gave depolarizations. Winkler (1973) also reported increased amplitudes of the *a*-wave of the electroretinogram from isolated rat retinas in low calcium medium. This wave reflects responses of photoreceptors.

Calcium effects on the metabolism of cGMP and adenosine 3',5'-monophosphate (cAMP) in nervous tissue are well known (Ferrendelli et al., 1976; Rasmussen et al., 1975; Berridge, 1975). Thus, it is clearly of interest to explore the relations of the cGMP level to calcium levels in light and dark. Indeed, Lipton et al. (1977*a,b*), recording intracellularly from toad rods, have presented evidence that the applying of agents in the dark that would be expected to raise intracellular cGMP levels, or the exposure of these cells to very low levels of external calcium, results in membrane depolarizations. Furthermore, they found that pharmacologic agents which were expected to diminish cGMP in these cells or the elevation of external calcium levels had hyperpolarizing effects in the dark.

Studies by Bitensky et al. (1975) and others (Chader et al., 1974; Fletcher and Chader, 1976; Brodie and Bownds, 1976; Goridis et al., 1974, 1976) suggest that changes in cGMP levels in outer segments of photoreceptors are largely based upon the modulation of a light-sensitive activity observed in a specific cGMP phosphodiesterase (cG-PDE); but Bitensky et al. have stated that this enzyme activity in outer segments of photoreceptors is not sensitive to calcium levels. Bitensky et al. have also shown that the cG-PDE activation is mediated by a factor appearing in illuminated outer segments, but there was no evidence that calcium was required for the appearance or function of this factor. Sitaramayya et al. (1977) report that only bleached opsin plus ATP are required to activate cG-PDE. More recently, Wheeler et al. (1977) found that light activates a GTPase in outer segments of the frog. GTP is the precursor of cGMP.

It would be useful to measure cGMP directly in photoreceptors after varying their environment. Layer-by-layer analyses of quick frozen eyes are ideal in some respects, but it is hard to manipulate the environment of *in situ* retinas in known ways.

Some time ago we noted in preliminary experiments (Ferrendelli and Cohen, 1976) that mouse retinas lacking photoreceptors because of an inherited receptor dystrophy (rd/rd) had but a few percent of the retinal cGMP concentration of controls. Retinal cAMP levels, on the other hand, were of the same order of magnitude in dystrophics and controls. Because reports of cGMP concentrations in isolated outer segments of photoreceptors indicated very substantial levels of cGMP, this suggested to us that the bulk of a retina's cGMP content might be in

the photoreceptors. Goridis et al. (1976), on other indirect grounds, also argued that most of the cGMP of cattle and frog retinas was in the photoreceptors. Such a view was directly confirmed in our laboratory (Orr et al., 1976) by the analysis of known layers from freeze-dried retinas of rabbit eyes isolated under either infrared or visible light. In rabbits, over 90% of the retina's cGMP came from layers including receptors, whereas cAMP was more evenly distributed.

The above considerations led to the idea of using isolated, intact mouse retinas to study cGMP regulation. If over 90% of the cyclic GMP of the retina is in the photoreceptors, substantial alterations in the content of cGMP of the whole normal mouse retina would be likely to preponderantly reflect changes in the cGMP concentrations within the photoreceptors. The current study reports measurements of cGMP and cAMP from normal mouse retinas which were incubated in either the dark or under a strong bleaching light while in Earle's physiological saline. The influence of calcium and other ions and certain pharmacological agents on retinal cyclic nucleotide regulation is also reported.

METHODS

Tissue Preparation

C57BL/6 mice of about 3 mo of age from a colony on a 12-h light cycle were employed in these experiments. Where rd/rd dystrophic mice were employed, they were congenic with C57BL/6. After decapitation of a mouse, retinas were removed from the evulsed eyes while in ice-cold, oxygenated, Earle's saline, using methods previously described (Cohen et al., 1973). Earle's saline contains 115 mM NaCl, 5 mM KCl, 1.8 mM CaCl₂, 0.8 mM MgSO₄, 2.6 mM NaHCO₃, 0.9 mM NaH₂PO₄, and 5.5 mM dextrose. Incubations were for 2 or 5 min. All media employed were brought to 37°C and, unless otherwise noted, were bubbled with 95% O₂, 5% CO₂ for 30 min before use. The pH under this atmosphere at 37°C was 7.4. Incubation vessels had shallow central wells to which were rapidly added 400 μl of the gassed medium. Extra medium was added outside the well. The vessels were then flushed with the same gas mixture and sealed and stored at room temperature. Before placing retinas in the wells of the vessels, flasks were brought to 37°C and attached to flowing moist gas. The isolated retinas from a pair of eyes were then distributed to two tandem vessels which were briefly opened for this purpose. Gas flow was continuous during the incubations.

The majority of "dark" microscope dissections were carried out in dim red light from a B&L microscope lamp (Bausch & Lomb, Scientific Optical Products Div., Rochester, N. Y.) equipped with a Tiffen no. 70 filter (Tiffen Manufacturing Corp., Roslyn Heights, N. Y.) and operated at 2 V, after mouse decapitations under dim red light from selected GE ruby glass bulbs (BAS 115 V, General Electric Co., Lamp Parts & Equipment Sales Operation, Cleveland, Ohio). For a few experiments, dark-adapted animals were decapitated, and their eyes and retinas were removed under infrared illumination with the aid of a binocular dissecting microscope equipped with image-convertor tubes on its eyepieces. Dark incubations were in the absence of any light. White light for "full" bleaching delivered about 2,000 lx at the incubated retinas and for some experiments, retinas were isolated and transferred in moderate levels of white light, and incubated under white "full" bleaching light. When the initial incubation was in the dark, retinas were from dark-adapted animals and isolated under dim red light. Conversely, when the initial incubation was in white light, the retinas were obtained from light-adapted animals and isolated in white light.

In transferring retinas from the isolation bath to flasks, or from flasks containing one type of solution to those containing another, the retinas were dipped enroute in the second solution to minimize carryover of the first solution on the retina or between the legs of the transfer forceps. The legs of the latter were specially ground to further minimize such carryover. When incubations were completed, retinas were removed from wells with forceps and placed in tubes which were dropped into liquid N₂ for freezing. Tubes with frozen retinas were stored at -80°C until the time for chemistry.

Morphological Methods

After incubations, some retinas were fixed for 2 h at room temperature in 2.5% glutaraldehyde in 0.16 M Na cacodylate (pH 7.4), then briefly rinsed in 0.2 M Na cacodylate (pH 7.4), and postfixed for 1 h in 1% OsO₄ in 0.2 M Na cacodylate. Dehydration began with 50% ethanol and embedment was in Araldite (Ciba-Geigy Corp., Resins Dept., Ardsley, N. Y.).

Biochemical Measurements

The frozen retinas were individually transferred to 7 × 70-mm test tubes at -20°C, and 200 μl of ice-cold 10% trichloroacetic acid was added. At 4°C the tissue was finely dispersed with a ground glass pestle, and the mixture was centrifuged at 16,000 g for 15 min. A 175-μl aliquot of the clear supernatant fluid was transferred to a 10 × 75-mm test tube and washed four times with 4 vol of water-saturated ethyl ether. This acid-free solution was dried under a stream of N₂ in a 50°C bath, and the dried residue was dissolved in 250 μl of 50 mM sodium acetate buffer, pH 6.0. To a 100-μl aliquot of this solution 2 μl of triethylamine and 1 μl acetic anhydride were added to acetylate cyclic nucleotides (Harper and Brooker, 1975). Both cyclic AMP and cyclic GMP were measured by radioimmunoassay (Steiner et al., 1972; Harper and Brooker, 1975). The trichloroacetic acid precipitates were dissolved in 1 N NaOH at 60°C, and the protein content of these solutions were measured according to the method of Lowry et al. (1951).

In all experiments described in either text, figure, or tables each value represents an average of results of assays on each of eight retinas unless otherwise noted.

Papaverine, isobutyl methylxanthine (IBMX), and the protonophore, carbonylcyanide chlorophenyl hydrazone (CCCP) were obtained from Sigma Chemical Co. (St. Louis). The ionophore A23187 was a gift kindly provided by Dr. Hamill of Eli Lilly and Company, Indianapolis, Ind. All chemicals were of the highest grade available.

RESULTS

Morphology

As reported elsewhere in more detail (Mitzel et al., 1977), 5 min of incubation in Earle's medium at 37°C produced a slight swelling of Müller cells near the external limiting membrane and possibly some incipient swelling in processes of the inner plexiform layer. Earle's with 3 mM EGTA or 1 mM IBMX produced some signs of swelling in some outer segments. The retinas were otherwise normal in appearance.

Cyclic Nucleotide Levels before Incubation at 37°C

The concentrations of cGMP and cAMP were first assayed in retinas isolated in the cold under dim red light from dark-adapted normal or "receptorless" rd/rd mice, and in retinas isolated in white light from similar light-adapted animals

(Table I). The concentration of cGMP in the dystrophic retinas was <5% of controls. On the other hand, the cAMP concentration of dystrophic retinas were significantly higher than those of control retinas. In normal animals light adaptation brings about a decline of about 45% in both retinal cGMP and cAMP, as we have previously reported (Ferrendelli and Cohen, 1976). Eyes were also removed from dark-adapted animals, and retinas were isolated from them in the cold, all under infrared illumination. The values for the concentration of cyclic GMP (67.6 ± 2.3 pmol/mg protein) did not differ significantly from those of unincubated controls isolated under dim red light (72.7 ± 2.4 pmol/mg protein). However, values for cyclic AMP (23.2 ± 1.3 pmol/mg protein) exceeded those from dim red light controls (17.1 ± 1.0 pmol/mg protein) by 35%.

TABLE I
CYCLIC NUCLEOTIDE LEVELS OF RAPIDLY ISOLATED
NORMAL AND DYSTROPHIC RETINAS

| | | DK | LT | LT/DK |
|------|-------------------------|-----------------|-----------------|-------|
| | | pmol/mg protein | | % |
| cGMP | { Controls | 68.54±1.67 (28) | 38.75±0.58 (13) | 56.5 |
| | { Dystrophics | 2.09±0.10 (27) | 1.71±0.15 (28) | |
| | Dystrophics/controls, % | 3.0 | 4.0 | |
| cAMP | { Controls | 15.51±0.34 (28) | 8.76±0.30 (13) | 56.5 |
| | { Dystrophics | 19.50±1.04 (27) | 15.69±1.23 (27) | |
| | Dystrophics/controls, % | 125.0 | 179.0 | |

Cyclic nucleotide levels in mouse retinas frozen after isolation (~4-6 min) at 0°C. These were from dark-(DK) and light-(LT) adapted animals. The number of retinas is in parentheses; concentrations are in pmol/mg protein ± SEM. Control retinas contain 327 ± 6 µg protein per retina; dystrophics contain 108 ± 1 µg protein per retina.

Effect of Incubation on Retinal Cyclic Nucleotides

Compared to the cGMP levels of retinas isolated in dim red light and frozen immediately thereafter (Table I), the concentration of cGMP showed an increase during the initial 2 min of a dark incubation (Table II). Incubations of 2 min in the dark in Earle's media yielded average values for cGMP with a range of 80-120 pmol/mg in different experiments. 3 additional min of darkness, however, usually produced no significant difference from the 2-min levels in the same experiment, although longer incubations often revealed a slow increase, but rarely as much as 25% between 2 min and 30 min. The 2-min level was therefore employed as a base level in most of our experiments. 3 min of bleaching light after 2 min in the dark always produced a highly significant 35-60% reduction in the cGMP level (Table II). In contrast to the differences in cAMP levels seen in retinas frozen immediately after isolation from dark vs. light-adapted animals, light-induced shifts in cAMP levels were not significant in retinas obtained from dark-adapted animals and incubated for 2 min in the dark before light stimulation.

Retinas incubated under bright white light for 2 min and then in the dark for 3 min usually showed a cGMP increase of 30% but no change in cAMP (Table II).

However, in some groups of retinas, this elevation of cGMP was not significant. This dark rebound of cGMP yielded a level well below that of retinas continuously dark-incubated for 2 or 5 min. Retinas incubated under bright light for 2 min often showed a small but significant rise in cGMP if the incubation was extended to 5 min, but this was well below both the dark rebound level and the level in continuous darkness. The presence of EGTA gave a heightened dark rebound (Table V).

Influence of Calcium

When dark-adapted retinas were incubated in Earle's medium from which calcium salt had been omitted, the levels of cGMP remained at about 90–110 pmol/mg protein. However, this medium was not completely calcium free inasmuch as atomic absorption spectroscopy revealed that it still contained 5–6 μM Ca^{++} . Moreover, transferring a retina to it from Earle's medium (1.8 mM

TABLE II
EFFECT OF ILLUMINATION CONDITIONS ON CYCLIC
NUCLEOTIDE LEVELS OF INCUBATED RETINAS

| | 2' DK | 5' DK | 2' DK-3' LT | % Change |
|------|------------------------|-----------------|------------------|----------|
| | <i>pmol/mg protein</i> | | | |
| cGMP | 81.8 \pm 6.0 | 76.9 \pm 5.1 | 49.3 \pm 4.2 | -36 |
| cAMP | 13.19 \pm 2.0 | 12.94 \pm 2.0 | 12.48 \pm 0.81 | -5 |
| | 2' LT | 5' LT | 2' LT-3' DK | % Change |
| | <i>pmol/mg protein</i> | | | |
| cGMP | 42.5 \pm 3.0 | 51.1 \pm 3.7 | 66.2 \pm 3.3 | +30 |
| cAMP | 13.94 \pm 1.4 | 13.9 \pm 1.3 | 14.24 \pm 1.0 | +2 |

cGMP and cAMP levels of retinas incubated 2 or 5 min at 37°C in the dark (DK), or light (LT), or with shifts in the light situation. Average of eight retinas \pm SEM.

Ca^{++}), despite a quick dip enroute in 5–6 μM Ca^{++} medium, apparently carried along enough calcium to provide ~ 32 μM Ca^{++} in the final 400- μl incubation volume. Therefore, to test the effect of a complete absence of free extracellular calcium, retinas were incubated in the dark in calcium-deficient Earle's media containing 3 mM EGTA. Under these conditions the dark cGMP level rose to $\sim 1,000$ pmol/mg protein (Table III). A similar increase was obtained with 1 mM EGTA but only a fourfold increase with 0.1 mM EGTA. None of these EGTA levels elevated the concentration of cAMP. The cGMP elevations were apparently due to a chelation of Ca^{++} by EGTA rather than to a direct action of this agent, in that 3 mM EGTA in combination with 3 mM excess Ca^{++} did not elevate cyclic GMP levels.

Cyclic GMP levels rose rapidly in dark-adapted retinas in low calcium, EGTA media, reaching a maximum within 2 min (Fig. 1). With longer incubations cyclic GMP levels gradually fell, and after 15 min were only three- or fourfold higher than control levels. The increase of cyclic GMP induced by calcium chelation was reversible. When retinas were first incubated in calcium-deficient media plus EGTA for 2 min and then placed in media containing low Ca^{++} (32

μM) or normal Ca^{++} (1.8 mM), cyclic GMP levels fell in 3 min from 890 to 270 or 90 pmol/mg protein, respectively. However, if the low calcium medium (32 μM) also contained 1 mM IBMX, the decline of cyclic GMP was blocked (Table III).

The striking effect of Ca^{++} chelation on cyclic GMP levels was only observed in dark-adapted retinas incubated in darkness. Low calcium medium with 3 mM EGTA had no effect on cyclic GMP levels in light-adapted retinas incubated under bleaching light. Furthermore, the retinal cyclic GMP levels when sharply

TABLE III
EFFECT OF CALCIUM CHELATION ON THE cGMP LEVEL

| Medium | 0' DK | 2' DK | 5' DK | 2' DK-3' LT |
|---|-----------|------------|------------|-------------|
| cGMP levels | | | | |
| <i>pmol/mg protein</i> | | | | |
| Control | 83.2±10.9 | 99.5±3.4 | 92.0±3.9 | 48.5±5.2 |
| Low Ca^{++} + EGTA | | 1,009±13.4 | 637.1±56.0 | 59.4±2.0 |
| Low Ca^{++} | | 101.8±2.0 | 107.8±5.1 | 69.4±5.0 |
| Low Mg^{++} | | 109.9±6.7 | 114.1±3.7 | 36.0±1.6 |
| cGMP levels | | | | |
| <i>pmol/mg protein</i> | | | | |
| Low Ca^{++} + EGTA | — | 1688±222 | — | — |
| *Low Ca^{++} + EGTA to Low Ca^{++} + EGTA | — | — | 890±99 | — |
| *Low Ca^{++} + EGTA to Low Ca^{++} + IBMX | — | — | 870±16.0 | — |
| *Low Ca^{++} + EGTA to Low Ca^{++} | — | — | 270±20 | — |
| *Low Ca^{++} + EGTA to Earle's | — | — | 89.1±3.9 | — |

Concentration cGMP, average of eight retinas \pm SEM. Retinas isolated in Earle's and incubated for period and lighting condition shown. EGTA, 3 mM; IBMX, 1 mM; low Ca^{++} was ~15-32 μM Ca^{++} .
* Retinas incubated in one solution for 2 min and then switched to a second for 3 min more.

elevated in the dark by EGTA media could rapidly be returned to normal by bright bleaching light (Table III).

Receptorless retinas from dystrophic animals also showed a small but significant increase in their low level of cGMP on incubation in low Ca^{++} EGTA media in the dark, but this heightened level was <1% of that seen with normal retinas in the dark in the same media.

By switching to a HEPES buffering system, using chlorides to replace phosphate, sulfate, and bicarbonate salts, and gassing with pure O_2 , the usual 1.8 mM Ca^{++} of Earle's medium could be replaced with 45 mM Ca^{++} . Retinas incubated in this medium showed no significant decline in cGMP in the dark at

2 or 5 min. In one experiment we compared the cGMP levels of retinas incubated either in HEPES buffered Earle's with 20 mM Ca^{++} plus 0.4% ethanol or in the same solution plus 20 μM A23187, a Ca^{++} ionophore. Again, no effect was seen on the normal dark level of cGMP, and the usual decrease in the cGMP concentration on exposure to light was observed.

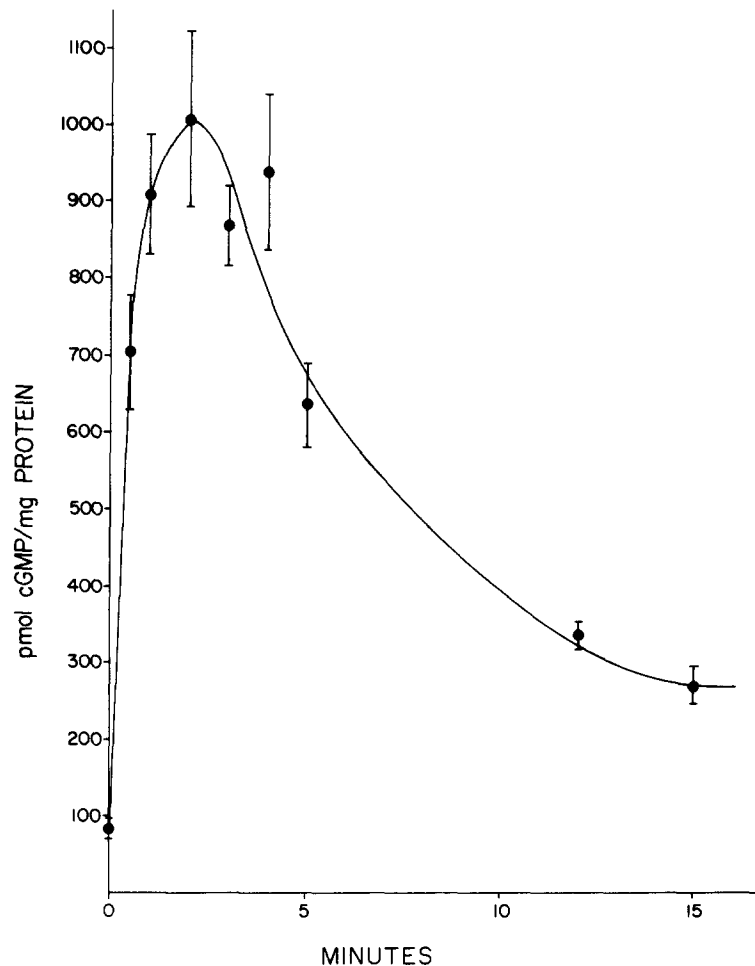


FIGURE 1. The effect of low calcium Earle's medium with 3 mM EGTA on the cyclic GMP level of dark-adapted mouse retinas incubated in the dark at 37°C. Each point represents an average of assays on eight retinas \pm SEM.

Because of the ability of 1 mM IBMX to prevent the decline in the dark of the elevated level of cyclic GMP induced by chelating calcium, we decided to make some additional observations on phosphodiesterase inhibitors. In these experiments, papaverine or IBMX were added to both the retinal isolating and incubating media. The concentration selected for papaverine was that which gave the most marked influence on the electrophysiological response of frog rods in the experiments of Ebrey and Hood (1973) and on the permeance of the

plasma membrane of outer segments in the experiments of Woodruff et al. (1977). Papaverine required adding 1% ethanol to Earle's medium to permit its solution at a concentration of 10^{-4} M. The results (Table IV) showed that neither ethanol nor ethanol plus papaverine influenced the dark cGMP levels or reduced the light response. IBMX (1 mM) also had no effect on the relative decline with exposure to bleaching light; however, it did give a significant increase in both the dark and light levels of cGMP (Table IV). We have not carried out dose-response studies on IBMX. The concentration employed was selected from the range of published IBMX concentrations which were effective for PDE inhibition in other tissues.

One mM IBMX produced about a fivefold increase in both the dark and post-illumination cAMP level (Table IV). Surprisingly, this heightened cAMP level

TABLE IV
EFFECT OF PHOSPHODIESTERASE INHIBITORS ON CYCLIC NUCLEOTIDE LEVELS AND THEIR LIGHT RESPONSE

| Medium | 2' DK | 5' DK | 2' DK-3' LT | 2' LT | 5' LT | 2' LT-3' DK |
|------------------------------|----------|---------|-------------|-----------|-----------|-------------|
| cGMP | | | | | | |
| <i>pmol/mg protein</i> | | | | | | |
| Ethanol | 120±4 | 128±10 | 63±4 | — | — | — |
| Ethanol + papaver- ine | 116±9 | 114±5 | 49±2 | — | — | — |
| IBMX | 147.6±12 | 164.9±9 | 95.7±5 | 107.9±6.0 | 119.8±8.7 | 136.5±6.9 |
| cAMP | | | | | | |
| <i>pmol/mg protein</i> | | | | | | |
| IBMX | 69.8±4 | 77.8±6 | 44.4±2 | 66.2±4.8 | 48.7±5.5 | 71.0±4.6 |

Cyclic GMP or cAMP levels, average of eight retinas ± SEM. Ethanol at 1%, papaverine at 100 μM, isobutyl methylxanthine at 1 mM.

showed the same percent decrease with light as did cAMP in dark-adapted retinas *in vivo*, whereas, as previously noted, in the absence of IBMX, the cAMP of 2-min incubated, dark retinas was essentially irresponsive to light. Papaverine had a similar but much smaller effect on cAMP levels (data not shown).

We also tested the effect of agents which purportedly cause the depolarization of cells or certain cell classes (Sillman et al., 1969). Retinas were isolated in modified Earle's medium made by replacing 100 mM of NaCl by either 100 mM KCl, or 100 mM K aspartate, or 100 mM Na aspartate and then incubated in the same medium. The experiments were then repeated with the same media further modified by the reduction of calcium and the addition of 3 mM EGTA. No change was seen (Table V) in either the dark cGMP or dark cAMP levels or in the light response. The usual cGMP elevation seen on chelating Ca^{++} was again observed. In addition, dark-adapted retinas were incubated in media containing 500 μM ouabain without significant effect on the dark cGMP level or its depression by light.

At the suggestion of J. E. Brown we also considered that the dark elevation in cGMP induced by EGTA might involve proton-calcium exchange. However, the protonophore carbonylcyanide chlorophenylhydrazone (CCCP), at a concentration of 3 μM produced only a slight depression of the cGMP level.

We also studied retinas under 95% nitrogen, 5% CO₂ atmospheres. These were isolated in nitrogen-bubbled media. Only an insignificant decline in cGMP was seen. However, when the experiment was repeated with 1 mM iodoacetate present, in 5 min the dark cGMP level was reduced to that obtainable with bright light (Table VI).

TABLE V
EFFECT OF DEPOLARIZING CONDITIONS ON THE cGMP LEVEL

| Medium | 2' DK | 5' DK | 2' DK-3' LT | 2' LT | 5' LT | 2' LT-3' DK |
|--|------------------------|-------------|-------------|----------|----------|-------------|
| | <i>pmol/mg protein</i> | | | | | |
| High K | 98.3±4.9 | 109.6±4.1 | 47.9±3.0 | 43.1±3.7 | 43.9±1.7 | 38.3±1.1 |
| High K, aspartate | 91.0±3.7 | 99.0±9.1 | 62.7±3.3 | 49.8±3.0 | 47.4±3.9 | 45.0±4.2 |
| High K, low Ca ⁺⁺ , EGTA | 784±59 | 769±105 | 179±13.4 | 58.4±2.9 | 60.0±4.0 | 71.6±3.9 |
| Na aspartate | 115.4±10 | 124.9±7.7 | 54.1±2.6 | 58.3±5.5 | 59.1±4.0 | — |
| Na aspartate low Ca ⁺⁺ , EGTA | 1,539±73.8 | 1,340±111.6 | 84.1±6.1 | 64.3±3.3 | 73.2±3.0 | 176±16.5 |
| Ouabain | 126±4 | 116±10 | 61.8±6.0 | — | — | — |

Cyclic GMP levels, average of eight retinas ± SEM, with Earle's medium modified by replacing 100 mM of NaCl with 100 mM of KCl or K aspartate or Na aspartate, sometimes with low Ca⁺⁺ and 3 mM EGTA in addition. Ouabain was at 500 μM concentration.

TABLE VI
EFFECT OF OXYGEN DEPRIVATION AND IODOACETATE ON THE cGMP LEVEL

| | 2' DK | 5' DK | 2' DK-3' LT |
|--|------------------------|----------|-------------|
| | <i>pmol/mg protein</i> | | |
| Control | 99.5±3.4 | 92.0±3.9 | 48.5±5.2 |
| N ₂ , CO ₂ | 83.2±8.5 | 82.4±5.9 | 35.0±2.1 |
| N ₂ , CO ₂ , iodoacetate | 57.7±5.9 | 42.8±2.7 | 36.0±1.6 |

Cyclic GMP average of eight dark-adapted retinas ± SEM. The control experiment was under 95% O₂, 5% CO₂; the nitrogen experiments were under 95% N₂, 5% CO₂. Iodoacetate was 1 mM.

DISCUSSION

Cyclic GMP Levels in Retina

In our previous studies on quick-frozen rabbit eyes isolated under infrared illumination (Orr et al., 1976), the highest cGMP concentration in the freeze-dried layers of the rabbit retina was in the layer of outer segments. This was 140 μmol cGMP/kg dry wt, which was twice the concentration of the inner segment layer, the retinal layer with the next highest concentration. A mean value for cGMP might be 75 μmol/kg dry wt if referred to the outer retina of the rabbit and perhaps half of this if referred to the full retina. In the current study, after isolation in the cold under infrared, and before any incubation, there was 21 × 10⁻⁶ μmol of cGMP/mouse retina. Inasmuch as a typical retina of this inbred

strain of mice has a dry weight of about 0.61 mg (Ross et al., 1975), this equates to 34 $\mu\text{mol/kg}$ dry weight of the full retina. Thus, the mouse retina, isolated by us in infrared or in dim red light, was probably similar in its dark cGMP concentration to the dark-adapted rabbit retina. This also indicates, as did our microscopic examinations, that the loss of outer segments during isolations and manipulation was minimal.

The level of cGMP in normal, dark-adapted retinas (Ferrendelli and Cohen, 1976 and this report) after isolation in red light was ~ 68 pmol/mg protein before incubation. This is in fair agreement with the recent value of 45–55 pmol/mg protein given by Farber and Lolley (1977) for a normal, dark-adapted mouse retina (DBA/1J). They also confirm the decline in cGMP seen with light and the sharp decline in the level of cGMP in retinas of rd/rd mice after rods degenerate. Their currently reported cGMP levels and its sharp drop after receptor dystrophy are in sharp disagreement with their earlier report (Farber and Lolley, 1974) of ~ 500 pmol cGMP/mg protein for normal mouse retinas (which contain ~ 0.34 mg protein per retina) and of higher cGMP levels in adult rd/rd retinas. Anatomical studies show that rd/rd retinas entirely lack rods from 25 days of age onwards (Karli et al., 1965). However, there are some persistent somata and terminals derived from the original 3% of receptors which were cones,¹ but there are no surviving cone outer segments.

The difference in cAMP levels in infrared vs. red light isolated retinas shows that red light affects the retina. The cold isolating conditions may differentially affect the cGMP and cAMP response to this light, which could be modifying the rod state.

Liebman (1975) gives 3 mM $\pm 10\%$ as a typical rhodopsin concentration in outer segments and Cone (1963) reported 3.2×10^7 rhodopsins per outer segment for rat rods. A mouse retina has about 3.2×10^6 rods whose outer segments are ~ 22 μm in length and 2.5 μm in diameter. It might thus have about 6 or 1 ($\times 10^{14}$) rhodopsin molecules to compare with 0.15×10^{14} cGMP molecules in the dark-adapted state. Woodruff et al. (1977) report a 100 or 50 to 1 ratio for frog ROS. However, not all of a receptor's cGMP may be in its outer segment (Orr et al., 1976).

Effect of Illumination on cGMP of Incubated Retinas

The initial increase in the cGMP concentration on dark incubation after isolation in dim red light is as yet unexplained. It is unlikely to be due to a recovery from dim red light bleaching, because infrared isolated retinas had similar initial cGMP values, nor is it likely to be due to a progressive loss of Ca^{++} to the medium, because once cGMP levels rise they are maintained even at 32 μM levels of external calcium in the absence of EGTA. The decrease in cGMP levels produced by the exposure of dark-adapted retinas to bleaching light most likely results from the activation of cGMP-phosphodiesterase, as suggested by Bitensky et al. (1975). The significant rebound seen in cGMP levels when normal, strongly bleached retinas were returned to the dark is unlikely to reflect the regeneration of rod pigment in these isolated retinas because the pigment

¹ Carter, L. D., and M. M. LaVail. 1977. Personal communication.

epithelium is lacking. Bleached opsin should therefore persist in the dark. It is not clear how the phosphodiesterase activated by light and ATP or GTP would be deactivated under this circumstance unless ATP and GTP levels fall until they become limiting. The few cones might be involved, but it seems possible that a partial recovery of dark cGMP levels does not require significant rod pigment regeneration.

Influence of Calcium

The sharp elevation of cGMP in calcium-chelated modifications of Earle's medium, its return to markedly lower levels in the presence of even low levels of free Ca^{++} (ca 6–32 μM), and the fall to usual dark levels on return to the 1.8 mM Ca^{++} level of unmodified Earle's medium prove that under the above conditions dark retinal cGMP levels can be rapidly modified by manipulating external calcium levels. These data demonstrate that there is a potential for a rapid increase in the cGMP level to one far above that normally found in the dark retina and suggest that the normal dark activity of free intracellular calcium prevents this increase. That it is the photoreceptors that are the preponderant site of the above events, is supported by the virtual absence of these phenomena in "receptorless" retinas. The light or calcium reversals of EGTA-induced elevations are probably rapid, but we have not established these with precision. The removal of EGTA and the supply of calcium greatly accelerate a fall in the cGMP levels which occurs more slowly in the continued presence of EGTA. This slower fall may, in part, relate to the physical deterioration of the receptors in the continued presence of EGTA; depletion of GTP, the precursor of cGMP; and from dark PDE activity. We note that Yoshikami and Hagins (1973), in recording receptor potentials extracellularly, found full electrophysiological recovery from the effects of very low calcium levels on return to normal media. The light-induced fall in cGMP in the presence of external EGTA would either be based on activating a phosphodiesterase indifferent to the calcium level, or on the release of calcium from stores which had shielded it from chelation by EGTA. This fall could easily occur in the presence of activated guanylate cyclase inasmuch as the reported V_{max} of retinal cG-PDE (Farber and Lolley, 1976) is at least an order of magnitude more than that of this cyclase (Troyer et al., 1977).

The dark elevation of cGMP seen with EGTA proceeds from the normal dark level. Thus, in a sense, these phenomena extend beyond the physiological range if an increasing cGMP level is characteristic of progressive dark adaptation. We have not shown that the modest but significant rise in cGMP we see in returning strongly light-adapted retinas to darkness is related to a falling concentration of free calcium. Similarly, we have not been able to diminish the normal cGMP level of dark-adapted retinas to that obtainable with bright light by increasing external calcium in the dark, even with the aid of the ionophore A23187. We have established in interaction of the cGMP system with calcium. The rapidity of the rise in cGMP in the dark in the presence of 3 mM EGTA suggests that in the dark GTP, the precursor of cGMP, is unlikely to be limiting for cGMP production. Inasmuch as it is reported that GTP (or ATP) plus bleached opsin can activate a cyclic GMP phosphodiesterase in rods (Bitensky et al., 1975; Sitaramayya et al., 1977), and inasmuch as ATP is also present, this also makes

it likely that bleaching rhodopsin, rather than generating GTP, controls the light activation of cG-PDE. In addition, the recent report of a light-activated GTPase (but not ATPase) in outer segments of frog rods (Wheeler et al., 1977) provides a second mechanism for reducing the concentration of cGMP in the light, by reducing the level of its precursor. The fact that IBMX can inhibit the slow fall in the elevated cGMP level seen in the dark after exposure to EGTA suggests that there is significant dark activity of cG-PDE as well.

The principal cyclase responsible for cGMP production is a particulate and high affinity enzyme found in outer segments (Bitensky et al., 1975) and in the retina only when receptors are present (Troyer et al., 1977). This cyclase, in the mouse retina, is inhibited by calcium, GTP being nonlimiting (Troyer et al., 1977). Thus, this guanylate cyclase may be the key element in the elevation of cGMP when calcium is chelated, although other mechanisms are possible.

Relation of cGMP Level and Photoreceptor Function

Two hypotheses are under current consideration as to how light might modulate the photoreceptor dark current. The first, that of Hagins and Yoshikami (1974), suggests that light somehow increases the level of free Ca^{++} in the outer segment and that the latter directly blocks sodium channels in the plasma membrane. The second, among others considered by Lipton et al. (1977*a,b*), holds that calcium might act indirectly via controlling the level of cyclic nucleotides. This hypothesis was suggested by their observations that superfusing physiological solutions with either low external calcium or agents which putatively increased cGMP in toad rods produced depolarizations of dark toad rods, whereas perfusing solutions with high external calcium or the ionophore A23187 hyperpolarized the cells. Clearly our observations that very low levels of external calcium do cause major increases in cGMP is required by and is in support of this hypothesis while not proving it. A possible linkage of the cGMP level to the control of sensitivity was also seen by Woodruff et al. (1977). Their studies utilized the rate of swelling of isolated outer segments of frog rods as a response parameter reflecting membrane permeance. The decrease in cGMP and permeance had a similar relation to light intensity. Moreover, beta-gamma methylene ATP enhanced the effect of light in decreasing both permeance and the cGMP level. Evidence obtained by Arden also suggested to him that calcium may modulate the level of an internal transmitter in receptors rather than be the transmitter itself.²

However, our failure to reduce significantly the "normal" dark cGMP level with external calcium levels exceeding 20 mM, although such reduction was easily achieved with light, is not in support of this indirect calcium hypothesis. Further, the main mechanism advanced for light diminishing cGMP, the activation of a specific cGMP phosphodiesterase by bleached opsin plus ATP or GTP (Bitensky et al., 1975; Goridis et al., 1974; Fletcher and Chader, 1976; Sitaramayya et al., 1977) and GDP^3 is said to be insensitive to the calcium level (Bitensky et al., 1975).

There are published data suggesting that light does induce calcium release

² Arden, G. B. 1977. Personal communication.

³ Zimmerman, W. F., and T. Gregg. 1977. Personal communication.

from compartments of outer segments (Liebman, 1974; Bonting and Daemen, 1976; Smith et al., 1977) but it has not been directly demonstrated how the free Ca^{++} level of the intact outer segment is thereby modified. However, Smith et al. suggest that not enough is released to account for the amplification factor in bleaching. Were calcium acting through cGMP in controlling the photoresponse of the membrane potential but released in inadequate amounts, then the release of one calcium would have to reduce the effect of a greater number of cGMP molecules.

GTPase activity potentially influences the cGMP level, but no information has been provided as to whether the light-activated GTPase reported in the outer segments of frogs by Wheeler et al. (1977) is influenced by the calcium level. Sack has reported the presence of a calcium-activated ATPase in outer segments,⁴ although Bonting and Daemen (1976) had failed to find such. Bignetti and Cavaggioni (1977) found no influence of the external calcium level down to 10^{-7} M on a light-induced decline in ATP in outer segments.

Rasmussen and Goodman (1977), and Rasmussen (1977) have suggested that cGMP could control the free Ca^{++} level via regulating its release or uptake by outer segment discs. There have been reports of kinase activity dependent on cyclic nucleotide levels in outer segments (Pannbacker and Schoch, 1973; Lolley et al., 1977). It has also been shown that opsin, in the presence of Mg^{++} and ATP, is phosphorylated via an opsin kinase (Bownds et al., 1972). More calcium binding sites could be created by phosphorylation. However, Weller et al. (1975 *a,b*) have presented evidence that the phosphorylation of disc membrane by kinase activity mainly decreases their permeance to calcium ions, rather than modifying the calcium binding capacity of the discs.

Both papaverine and IBMX failed to inhibit the proportional decline in cGMP levels induced by light although IBMX raised both the dark and minimum level of this molecule in light. Similarly, Brodie and Bownds (1976) report that papaverine increased the level of cGMP in isolated outer segments of frog rods, but had little effect on light-induced changes in cGMP. IBMX produced a proportionally much greater increase in cAMP than in cGMP. This suggests that in the mouse retina, and perhaps in its receptors, when a physiological modulation is seen after the use of IBMX, it might well be due to an elevation of cAMP. There are some puzzling aspects to the IBMX action, such as its failure to inhibit the light response and in its permitting proportionally similar, light-induced, decreases of cGMP and cAMP while dissimilarly elevating their dark levels. Although a light-induced depression of the cAMP level had been observed *in vivo*, in the absence of IBMX, it was not seen in retinas incubated for only 2 min. Light-activated PDE has been said to favor cGMP over cAMP (Bitensky et al., 1975). Clarifying the locations of the cAMP changes in the retina, may help resolve these problems.

Dark cGMP levels and their light-induced decline were intact in the presence of 500 μM ouabain. It is known that the dark current in rat rods can be increased at least fivefold by lowering calcium activity below 10^{-5} M, but that both the dark voltage and photovoltage fall in the presence of 40 μM ouabain

⁴ Sack, R. A. 1977. Personal communication.

(Yoshikami and Hagins, 1973; Hagins and Yoshikami, 1974; Hagins et al., 1975). Our experiments suggest that ouabain-sensitive "pumps" do not indirectly control cGMP levels, at least in the time span of these experiments. Ouabain or high potassium levels are presumably modifying the receptor membrane potential without significantly modifying the cGMP level. The converse, i.e., a direct or indirect effect of the cGMP level on the membrane potential, might still be true, as is indicated by the findings of Lipton et al. (1977*b*).

In studies on brain slices, Ferrendelli et al. (1976) found that cGMP levels rose in the presence of Ca^{++} and high potassium. The entry of Ca^{++} into the cells was an essential part of the potassium induced effect. However, neither Goridis et al. (1977) nor we ourselves found that high potassium levels influenced the dark level of cGMP or its decrease on illumination. Thus, the receptor-dominated retina does not, in net, respond like other brain regions. If the high K^+ permitted a heightened Ca^{++} influx and an increase in intracellular Ca^{++} , it had no effect on the cGMP level.

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