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Distribution of 3′:5′-cyclic AMP and 3′:5′-cyclic GMP in rabbit retina *in vivo:* Selective effects of dark and light adaptation and ischemia

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ABSTRACT **By use of highly sensitive radioimmunoassays, 3′:5′-cyclic AMP (cAMP) and 3′:5′-cyclic GMP (cGMP) were measured in individual layers of light- and dark-adapted rabbit retinas, and the effects of ischemia were determined. In light-adapted retinas, cGMP levels ranged 50-fold, with over 90% of the total concentrated in the photoreceptor cells. The layer of outer segments contained 95 μmol/kg of dry weight, or three times the concentration present in the remainder of the photoreceptor cell layers. By contrast, levels of cAMP varied only 4-fold; the lowest level (6 μmol/kg of dry weight) was found in the outer segment layer and the highest level (22 μmol/kg of dry weight) in the inner segment layer of the photoreceptor cells. Dark adaptation elevated cGMP levels only in retinal layers containing photoreceptor cells, and the greatest proportional increase was observed in the synaptic layer of photoreceptor cells. Dark adaptation also caused increases of cAMP that were restricted to the outer plexiform and outer nuclear layers. Ischemia lowered cGMP levels, but only in retinal layers containing photoreceptor cells, and elevated cAMP levels, primarily in the inner layers of the retina. The effects of ischemia were greater in dark-adapted than in light-adapted retinas. These results indicate that cGMP *and* cAMP levels in retina are influenced by the light adaptational state, that ischemia markedly modifies these processes, and that the effects of both light exposure and ischemia are regionally selective.**

Previous studies have detected the existence of adenosine 3′:5′-cyclic monophosphate (cAMP) and guanosine 3′:5′-cyclic monophosphate (cGMP) in mammalian retina and have demonstrated that this tissue is uniquely rich in cGMP (1, 2). Dark adaptation increases cGMP in outer segments of mammalian photoreceptor cells *in vitro* (3–7), and Fletcher and Chader (8) have shown that cGMP levels are elevated in outer segments isolated from frog retina that has been dark-adapted *in vivo*. Recent preliminary studies in our laboratory have revealed that dark adaptation, *in vivo*, increases cAMP as well as cGMP in mouse retina and that ischemia affects the content of both nucleotides. Although there is indirect evidence that a substantial portion of the cyclic GMP of the retina is contained in the outer segments of the photoreceptor cells, its distribution in the remainder of the retina and the distribution of cAMP throughout the retina have not been well defined. This information is essential for the eventual understanding of the role of cyciic nucleotides in retinal function, as well as for evaluating the physiological relations of cGMP and cAMP systems.

Using highly sensitive radioimmunoassays and histochemical techniques, we have measured the content of both cAMP and cGMP in individual layers of rabbit retinas that were either light- or dark-adapted, *in vivo*. In addition, the effect of ischemia was studied. The findings demonstrate that the cyclic nucleotides are discretely localized in retinas and that both light exposure and ischemia produce regionally selective changes in their levels.

Abbreviations: cAMP, adenosine 3′:5′-cyclic monophosphate; cGMP, guanosine 3′:5′-cyclic monophosphate.

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MATERIALS AND METHODS

Tissue Preparation. Randomly bred, 6-month-old pigmented rabbits were obtained locally. Light adaptation consisted of 1 hr of exposure to two 100-watt tungsten bulbs about 25 cm above the rabbits. After the photopic stimulation, the rabbits were killed by decapitation. The eyes were removed, and after various ischemic periods in room light, were quickly frozen in liquid N_2 cooled to its freezing point by partial evaporation under reduced pressure. Consequently, the liquid N_2 does not boil when the eyes are immersed, and freezing is quicker. Dark adaptation consisted of an overnight period in cages wrapped with black cloth and placed in a completely dark room. After decapitation in this room, the dark-adapted eyes were dissected free with the aid of an infrared light source and an infrared image converter, and after various periods, were frozen in the dark as above. Under the most ideal conditions it required 1 min (46–69 sec) to remove and freeze either the light- or dark-adapted eyes. All eyes were stored at −70° until sectioned. Tangential sections (6 μm) of the retinas were cut at −20° and dried at −40° under reduced pressure. Sections were taken from the paracentral regions of the retina, and care was taken to avoid that portion underlying the myelinated fiber bundle. Paracentral areas of rabbit retina are poorly vacularized (9). Samples (0.03–0.9 μg dry weight) were dissected from the individual retinal layers and weighed on a quartz-fiber balance (10). Stained, undissected retinal sections were used to help identify individual layers.

Assay of cGMP and cAMP. The procedures used for extraction and radioimmunoassay of the cyclic nucleotides are described in detail elsewhere (J. A. Ferrendelli, E. H. Rubin, H. T. Orr, D. A. Kinscherf, and O. H. Lowry, submitted to *Anal. Biochem.*). Briefly, the samples were extracted at room temperature in 1-μl volumes of 10% trichloroacetic acid under mineral oil. Aliquots (0.9 μl) were then removed from the oil wells, placed in 6 × 50 mm culture tubes, and dried on a Virtis Bio-Dryer to remove the acid. The residue was dissolved in 50 μl of H_2O, and the cyclic nucleotides were acetylated by the method of Harper and Brooker (11) with 1.0 μl of triethylamine and 0.5 μl of acetic anhydride. The tubes were dried again and the residues dissolved in 30 μl of Na acetate buffer, 50 mM, pH 6.0. Both cyclic nucleotides were then assayed by the radioimmunoassay described by Steiner *et al.* (12) scaled down to a final volume of 52 μl.

The results shown in Figs. 1 and 2 are the averages from three rabbits at 1 min of ischemia and two rabbits at the other ischemic times. Within each retina, each layer was assayed three to seven times; values for SEM ranged from 5 to 20% of the mean for each case.

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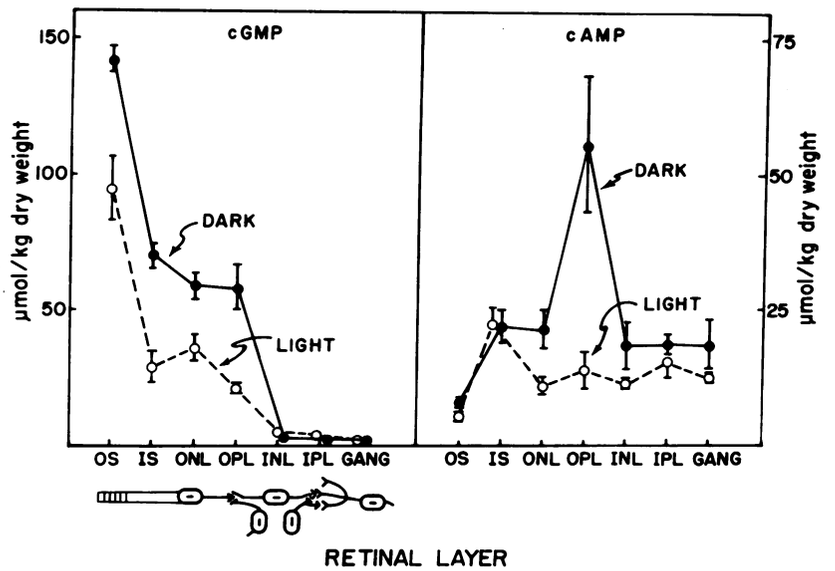

FIG. 1. cGMP and cAMP in the layers of light- and dark-adapted rabbit retina. The eyes were removed and frozen after about 1 min of ischemia. This is the fastest that eyes could be removed from decapitated, unanesthetized rabbits. Each point is the average for three eyes ±SEM, except for the dark-adapted OPL layer (cGMP and cAMP), where the average of two eyes is presented, in which case the bars represent ranges. Abbreviations: OS, outer segment; IS, inner segment; ONL, outer nuclear layer; OPL, outer plexiform layer; INL, inner nuclear layer; IPL, inner plexiform layer; GANG, ganglion cell layer. Lines connecting individual points are placed to assist visualization of the data, but do not imply smooth gradients of cyclic nucleotide content between layers. A schematic representation of the neural connections in the retina is illustrated under the left panel of the figure.

RESULTS

Distribution of cGMP and cAMP in light-adapted retina

In the most rapidly frozen light-adapted retinas, cGMP levels ranged 50-fold. The layers containing the photoreceptor cell had levels (30 μmol/kg of dry weight) at least 10-fold higher than any of the remaining layers of the retina (except the outer plexiform layer, which is partly composed of photoreceptor cell elements) (Fig. 1). Within the photoreceptor layer, cGMP levels were highest at the outer segments, where the concentration (95 μmol/kg of dry weight) was three times greater than layers containing the remainder of the cell. By contrast, the cAMP distribution in these same light-adapted retinas varied only over a 4-fold range (Fig. 1). All but two of the retinal layers had cAMP concentrations between 10 and 15 μmol/kg of dry weight. The two exceptional layers were the outer segments (5.5 μmol/kg of dry weight) and the inner segments (22 μmol/kg of dry weight).

Effect of dark adaptation on retinal cGMP and cAMP

We found that dark adaptation increased cGMP levels in the outer segment layer of the photoreceptors. (The peak level of 142 μmol/kg of dry weight is 300 times the concentration in average brain.) In addition, dark adaptation also increased cGMP levels in the remainder of the photoreceptor cell. Proportionally, the increase in cGMP after dark adaptation was actually higher in layers containing other parts of the photoreceptor cell, especially in the photoreceptor terminals (2.5-fold in the terminal layer compared to 1.5 in the outer segments). The cGMP content of the nonphotoreceptor layers of the retina was not affected by dark adaptation.

Dark adaptation also caused significant increases in cAMP levels in specific retinal layers. The most dramatic increase was seen in the outer plexiform layer, which contains portions of bipolar and horizontal cells and glial cells of Müller, in addition to the photoreceptor terminals. In this layer, cAMP increased from 13.8 μmol/kg of dry weight in light to 55.8 μmol/kg of dry weight in the dark. A significant ($P < 0.05$) rise in cAMP was also found in the outer nuclear layer. The remaining layers of the retina did not show significant ($P > 0.2$) elevations in cAMP after dark adaptation.

Effect of ischemia on cGMP levels

In both light- and dark-adapted rabbit retinas, prolonged ischemia lowered cGMP concentrations only in retinal layers containing the photoreceptors (Fig. 2). The cGMP content of dark-adapted retina changed more with ischemia than that of light-adapted retina. In dark-adapted retina, 3 min of ischemia lowered cGMP levels in every photoreceptor cell layer to levels equal to or somewhat lower than in light-adapted retinas. The cGMP in outer segments decreased 30%, while in the remaining photoreceptor cell layers, it fell 70–75%. In light-adapted retinas with lower cGMP to begin with, only levels in the outer nuclear and outer plexiform layers were altered by ischemia. In these two layers, cGMP decreased 50% during 3 min of ischemia. Ischemic periods of 5 min did not lower concentrations more than those of 3 min. It is significant that the final cGMP levels were identical, whether the retinas had been light- or dark-adapted.

Effect of ischemia on cAMP levels

In contrast to the decreases in cGMP with ischemia, cAMP increased after prolonged ischemia. In light-adapted retinas (Fig. 2), ischemia had no effect on cAMP levels in the outer segment layer. Slight increases in cAMP in the remaining retinal layers were found with 3 and 5 min of ischemia. The most consistent elevations in cAMP were found in the inner layers, with cAMP of the ganglion cell layers showing a 3-fold increase after 5 min of ischemia.

As in the light-adapted retinas, ischemia had no effect on the cAMP levels in the outer segment layer of dark-adapted retinas (Fig. 2). In the other dark-adapted photoreceptor cell layers, ischemia caused only slight elevations in cAMP above the basal levels. However, in the inner layers, dark adaptation clearly increased the susceptibility of cAMP to ischemia. Three minutes

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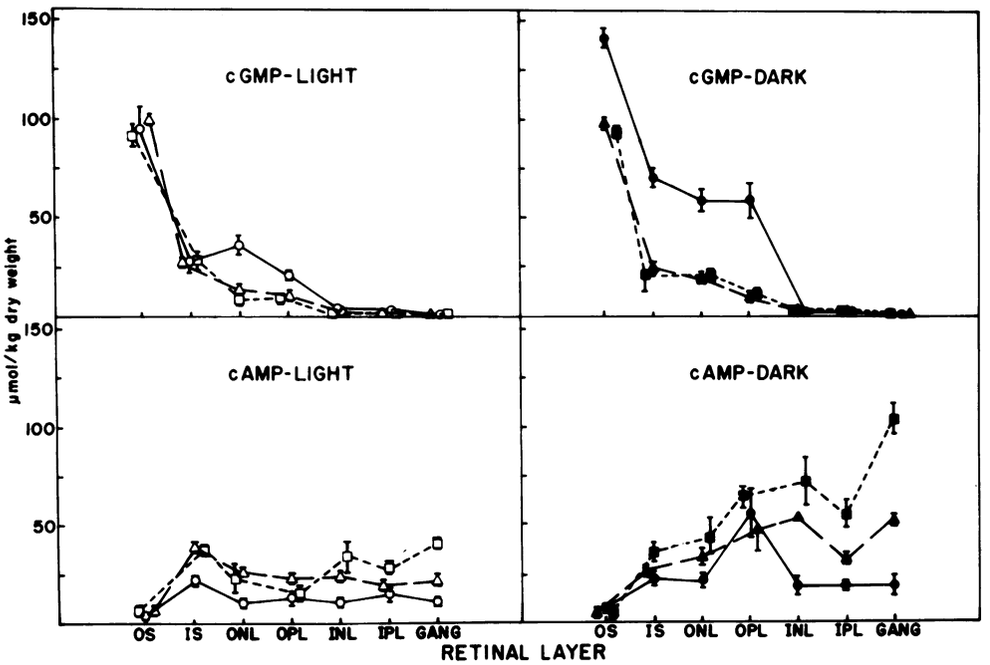

FIG. 2. Effect of ischemia on the cyclic nucleotides in light- and dark-adapted rabbit retina. The eyes were adapted *in vivo*, removed, and allowed to sit either in room light (light-adapted eyes) or dark (dark-adapted eyes) for 3 or 5 min. The 1-min values are from eyes frozen as soon as removed. The 1-min ischemic values are the mean of three eyes ±SEM except for the dark-adapted OPL, where two eyes were used, in which case the range of values is shown by the bar. The 3- and 5-min ischemic values are all the average of two eyes, with the bars representing the range. Where no bars are shown, the range falls within the symbol size. Ischemia: (○, ●) 1 min; (△, ▲) 3 min; (□, ■) 5 min. Abbreviations and presentation are as in the legend of Fig. 1.

of ischemia raised cAMP in the inner nuclear, inner plexiform, and ganglion cell layers to 53, 32, and 52 μmol/kg of dry weight, respectively. After 5 min of ischemia, the cAMP level in each inner layer was elevated further, with cAMP in the ganglion cell layer reaching a concentration of 104 μmol/kg of dry weight. (The average for rabbit brain is only 5 μmol/kg of dry weight.)

DISCUSSION

Normal Distribution of cGMP and cAMP. The results support earlier studies that outer segments of photoreceptor cells contain high levels of cGMP and, in addition, show that the entire photoreceptor cell is rich in this nucleotide. Although high levels of cGMP are present in the outer segment of the photoreceptor, in agreement with data on isolated outer segments, all retinal layers containing photoreceptors are at least 10-fold richer in cGMP than the remainder of the retina. Such a distribution suggests that cGMP has an important role not only in outer segment function, but throughout the photoreceptor cell. This finding is also consistent with an earlier unpublished observation of Cohen and Ferrendelli demonstrating that mouse retinas devoid of photoreceptors have cGmp levels that are less than 10% of normal. In contrast to cGMP, cAMP is much more evenly distributed, and probably has a major function in several cell types of the retina.

Effect of Dark Adaptation. The elevation of cGMP with dark adaptation was strictly confined to layers contining portions of the photoreceptor cell. Proportionally, the largest cGMP increases were found in layers that contain inner parts of the photoreceptor cell; this includes the outer plexiform layer. The cGMP increase in the dark-adapted outer plexiform layer appears even more dramatic when it is considered that in addition to photoreceptor terminals, bipolar and horizontal cell processes and portions of glial cells of Müller are also present. Since the inner nuclear layer, containing horizontal, bipolar, amacrine, and Müller cell bodies, had low levels of cGMP that were not affected by dark adaptation, it is possible that the cGMP increase in the outer plexiform layer takes place in the photoreceptor terminals. Therefore, the actual levels of cGMP within the photoreceptor terminals may be substantially higher than the levels measured for the layer as a whole. It appears likely that a dark-adapted photoreceptor contains extremely high levels of cGMP at both ends.

The elevation of cGMP in all of the dark-adapted photoreceptor layers suggests that the cGMP elevation is related to some event or process that occurs throughout the cell. This might be the depolarization that occurs in the dark (13, 14), since cellular depolarization has been shown to elevate cGMP in other central nervous tissue (15–17).

Dark adaptation also produces localized elevations in retinal cAMP levels in the outer nuclear and outer plexiform layers, especially the latter. It is thought that photoreceptor terminals release more depolarizing transmitter in the dark than in the light (18–21). Thus, the large rise in cAMP in the layer that contains the photoreceptor terminals and postsynaptic elements may be related to the increased amount of photoreceptor synaptic transmission in the dark. Increased cAMP could be associated with neurotransmission either as a second messenger for the photoreceptor transmitter, a role indicated for cAMP in other neuronal systems (22), or cAMP may be an indicator of an increased energy use related to the increased depolarization in the dark (see below).

Effect of Ischemia. As in other neuronal tissue (23), ischemia lowers cGMP and elevates cAMP levels in the retina. Only cGMP located in the photoreceptor cell appears to be susceptible to ischemia, and ischemia appears to have a greater effect in dark-adapted retinas, perhaps only because it is elevated to

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begin with. This indicates that photoreceptor cell metabolism does respond to lack of O_2. Previous studies have suggested that photoreceptor cells are markedly resistant to lack of O_2 (24). It is apparent that a substantial portion of cGMP does not decrease in either light- or dark-adapted retinas even after 5 min of ischemia. This suggests the existence of two "pools" of cGMP in the photoreceptor: (*a*) a labile "pool" elevated by dark adaptation and (*b*) a stable basal "pool."

Except for the outer segments, the cAMP content of the entire retina is susceptible to ischemia to some degree, the inner layers being the most susceptible. Whereas the outer layers were about equally affected by ischemia, whether light- or dark-adapted, the inner layers had a greater cAMP response in the dark-adapted state. We assume that this greater elevation of cAMP in ischemic dark retina is a consequence of the action of released neurotransmitter. However, in response to similar stimuli, certain classes of retinal cells depolarize while others hyperpolarize, so it is hard to predict a net effect of light or darkness beyond the photoreceptor process.

The distribution of cAMP in the dark-adapted retinas after 5 min of ischemia closely parallels the distribution of glycogen in the rabbit retina (25). This elevation of cAMP may be indicative of phosphorylase activation, a process in which cAMP plays an important role (26). The increased release of depolarizing transmitter(s) in the dark by the photoreceptor terminals would be expected to increase the metabolic rate of some cells of the inner retina; this would make them more sensitive to ischemia, i.e., would increase their requirement for glycogenolysis if the blood supply should be shut off. The exaggerated increase in cAMP may be a step in the mechanism to provide more rapid glycogen breakdown.

Under the best conditions, it took 1 min to remove and freeze an eye. The increased susceptibility of cAMP levels to ischemia in dark-adapted retina raises the possibility that the exceptionally high levels of cAMP found in the outer plexiform layer at the earliest time interval reflects a higher-than-average susceptibility of this layer to ischemia. Thus, the increased cAMP levels in the outer plexiform layer could reflect an increased energy demand as in the other layers, rather than indicating a role for cAMP as a second messenger for a released transmitter.

Studies of cyclic nucleotides in retina that are not carefully controlled for ischemia could produce extremely misleading results. For example, in the present study, if retinas were examined only after 5 min of ischemia, one would have concluded that dark adaptation had no effect on cGMP levels and that it elevated cAMP levels in all layers of the retina except the outer two. Obviously, this is not the case.

In conclusion, the present results clearly demonstrate that light affects both cAMP and cGMP levels in retina and also influences the response to ischemia. Moreover, both light and ischemia have regionally selective effects on the two cyclic nucleotides.

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