Guanylate Cyclase: Inhibition by Light in Retinal Photoreceptors

Abstract. *Guanylate cyclase activity of retinal rod outer segments was measured by an assay procedure that minimizes the technical problems caused by the high activity of cyclic nucleotide phosphodiesterase in neural tissue. Cyclase activity in rods is significantly higher than in brain. Moreover, activity is twofold higher in dark-adapted rods than in light-bleached rods, a sensitivity that is lost when the preparation is treated with detergent.*

The visual process involves capture of light energy by rod outer segments (ROS) of the retinal photoreceptor cell. Adenosine 3′,5′-monophosphate (cyclic AMP) has been postulated to be a link between photon capture by rhodopsin and changes in permeability of rod outer segment membranes because the adenylate cyclase enzyme is active in darkness and inactivated by light (*1*).

Significant levels of guanine nucleotides (*2*) and guanylate cyclase (*3*) have also been reported for bovine ROS. However, the presence of extremely high guanosine 3′,5′-monophosphate (cyclic GMP) phosphodiesterase activity and of 5′-nucleotidase activity (*4*) has proved a major obstacle in accurate quantitation of cyclic GMP formation by conventional techniques (*5, 6*). These techniques are also lengthy and cumbersome (*6*) and commonly result in artifactually high recoveries due to collection of major degradation products along with cyclic GMP formed (*7*). We developed a method of column chromatography that isolates cyclic GMP in high yield without contaminating nucleosides or nucleotides (*8*). Using this method, we found that the activity of guanylate cyclase in bovine ROS is strikingly higher than that in rat brain, and that this activity decreases when dark-adapted ROS are bleached by light.

Bovine eyes brought in a lighttight ice chest from a local abattoir were the source of retinas. The ROS were isolated by stepwise or continuous sucrose gradient centrifugation [(*4*) and legend, Table 2]. The purified rods were diluted in 40 m*M* tris(hydroxymethyl)aminomethane (tris) hydrochloride, *p*H 7.5, so that the final assay (200 μl) contained 15 to 30 μg of protein. The reaction was initiated (Table 1) by addition of 4.5 μc of [^{3}H]guanosine triphosphate (GTP) (New England Nuclear, Boston) and 0.25 m*M* unlabeled GTP. Blanks contained boiled tissue samples, and controls for nucleotide recovery were performed by substituting cyclic [^{3}H]GMP for the [^{3}H]GTP in paired reaction tubes. All samples were incubated for 5 minutes at 30°C. The reactions were stopped by boiling for 3 minutes, cyclic GMP was isolated (*8*), and radioactivity was determined in Aquasol (New England Nuclear). Recovery of cyclic GMP was usually 55 to 75 percent. Samples below this range were discarded. All results are corrected for loss in recovery.

The specific activities of the retinal mitochondrial pellet (30,000*g*) and supernatant fractions were lower than the nuclear pellet (1,000*g*) (Table 1). In all fractions, activity was markedly stimulated by Triton X-100 and showed virtually complete dependence on added manganese. In comparison, cyclic GMP formation in rat brain and lung was higher in the 30,000*g* supernatant fraction.

Light-adapted ROS had 100 times higher guanylate cyclase activity than did the retinal nuclear fraction (Table 2). In contrast to a recent report (*3*), guanylate cyclase activity in dark-adapted ROS was consistently twofold higher than that in light-adapted preparations. Addition of 1 percent Triton X-100 to the reaction mixture eliminated this difference. In isolated ROS, guanylate cyclase was also totally dependent on manganese.

The mechanism of light sensitivity and the functional significance of ROS guanylate cyclase remain to be explained. Loss of light sensitivity following detergent treatment is similar to that reported for the light-sensitive adenylate cyclase of ROS (*1, 9*). This may suggest a somewhat more generalized effect of light bleaching on membrane-bound enzymes of ROS (through cation changes and so forth) rather than a direct relation between the cyclase and rhodopsin (*1*); the spectral properties of rhodopsin remain intact in nonionic detergent (*10*).

Changes in cyclic GMP concentration have been noted after perfusion of the mammalian heart and brain with drugs related to the acetylcholine system (*11*). Neural transmission in the retina has

Table 1. Subcellular distribution of guanylate cyclase in retina, brain, and lung. Light-adapted bovine retinas were homogenized in 0.05*M* tris-HCl buffer, *p*H 7.5, and centrifuged at 1,000*g*. The supernatant was centrifuged at 30,000*g*. Whole rat brains were homogenized in 0.05*M* tris-HCl, *p*H 7.5, and centrifuged at 30,000*g*. Assays (final volume, 200 μl) were performed in 0.05*M* tris-HCl, *p*H 7.5, containing 4 m*M* cyclic GMP, 0.1 m*M* papaverine, 10 m*M* theophylline, and 30 m*M* phosphocreatine, with 75 μg of creatine phosphokinase for the GTP generating system; 5 m*M* $MnCl_2$ was included as indicated. An appropriate amount of tissue fractions was added, and the reaction was initiated by addition of 4.5 μc of [^{3}H]GTP plus 0.25 m*M* unlabeled GTP. The tubes were capped, shaken, and incubated at 30°C for 5 minutes. Values represent averages from duplicate experiments which agreed within 5 percent. Protein assay was by a modification of the technique of Lowry *et al.* (*14*); super., supernatant; N.T., not tested.

Preparation	Guanylate cyclase activity (picomoles of cyclic GMP per minute per milligram of protein)		
	Mn^{2+}	Triton, 1%	No Mn^{2+}
Retina (bovine)			
1,000*g* pellet	22	52	0
30,000*g* pellet	12	21	1
30,000*g* super.	3	7	1
Brain (rat)			
30,000*g* pellet	11	N.T.	N.T.
30,000*g* super.	36	N.T.	N.T.
Lung (rat)			
30,000*g* pellet	113	N.T.	N.T.
30,000*g* super.	174	N.T.	N.T.

Table 2. Guanylate cyclase activity in bovine rod outer segments. The ROS were prepared by stepwise sucrose gradient centrifugation (densities of 1.15, 1.13, and 1.11 g/ml; ROS collect at the interface of the two top layers) or continuous sucrose gradient centrifugation (densities 1.05 to 1.18 g/ml) (*7*). Dark-adapted or light-bleached outer segments were incubated for 5 minutes as described in Table 1, with additions as noted above. Values are the mean of duplicate samples from eight separate determinations; duplicate agreement was within 5 percent.

Condition	Guanylate cyclase activity (picomoles of cyclic GMP per minute per milligram of protein)	
	Light-bleached	Dark-adapted
Plus Mn^{2+}	2440	4360
Plus Mn^{2+} and Triton	3180	3210
No Mn^{2+} or Triton	*	*

* No significant activity was detected.

been linked to components of this system (*12*), and changes in levels of acetylcholinesterase and of acetylcholine itself have been reported after exposure of the retina to light (*13*). The establishment of a link between these two light-sensitive enzyme systems in the retina, therefore, would be an obvious subject of further investigation.

RICHARD E. BENSINGER*
R. THEODORE FLETCHER
GERALD J. CHADER†
Laboratory of Vision Research, National Eye Institute, Bethesda, Maryland 20014

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* Present address: Department of Ophthalmology, Washington University School of Medicine, St. Louis, Missouri 63110.
† Send reprint requests to G.J.C.

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In vivo Inhibition of Growth of Two Hormone-Dependent Mammary Tumors by Dibutyryl Cyclic AMP

Abstract. *Growth of hormone-dependent rat mammary tumors was arrested in vivo by* N^6,$O^{2'}$*-dibutyryl cyclic adenosine 3′,5′-monophosphate. Estrogen concentration did not change, but acid ribonuclease activity and synthesis increased during treatment with the dibutyryl cyclic nucleotide, as was shown during tumor regression due to hormonal deprivation. Growth arrest, thus, appears to derive from enhanced tissue catabolism.*

Growth and morphology of a number of cultured cell lines have been reported to be influenced by adenosine 3′,5′-monophosphate (cyclic AMP) or its derivatives. Exposure of various lines of transformed cells to cyclic AMP or the dibutyryl derivative resulted in inhibition of growth rate without affecting cell viability (*1*). Concentrations of cyclic AMP were three- to fourfold higher in confluent 3T3-4 cells than in cells in the logarithmic phase of growth (*2*); higher concentrations of cyclic AMP were found in more slowly growing cells than in rapidly growing cells (*2, 3*); and addition of dibutyryl cyclic AMP decreased the rate of growth (*4*). A specific reduction of the growth rate was also obtained by exposing Reuber H35 hepatoma cells in vitro to dibutyryl cyclic AMP and other cyclic AMP analogs (*5*).

The regulatory effect of cyclic AMP on the growth of neoplastic tissues in vivo has not yet been fully investigated. Gericke and Chandra reported a partial inhibition of growth in vivo by injection of cyclic AMP directly into mouse NKL (Németh-Kellner leukemia) lymphosarcoma (*6*). Webb *et al.* (*7*) showed retarded growth of a mouse tumor in vivo by intraperitoneal injection of theophylline, which competitively inhibits cyclic AMP phosphodiesterase (*8*) and induces the accumulation of cyclic AMP within the cell (*9*).

We have tested the effect of cyclic AMP, dibutyryl cyclic AMP, and closely related substances on the growth in vivo of two hormone-dependent rat mammary tumors. One was a primary tumor induced by 7,12-dimethylbenz[*a*]anthracene (DMBA) in Sprague-Dawley female rats. The other was a transplantable tumor (MTW9) in Wistar female rats. Tumor growth was expressed as the percentage change in volume calculated from the formula, $(4/3)\pi r^3$, where r is one-half the average of the longest and shortest diameters measured by Vernier caliper daily. 3′,5′-AMP, 2′,3′-AMP, dibutyryl cyclic AMP, and 5′-AMP were obtained from Sigma Chemical; sodium butyrate was obtained from Matheson, Coleman and Bell; theophylline was obtained from Schwarz/Mann; and Delestrogen (17β-estradiol) was obtained from E. R. Squibb and Sons. Animals weighing 170 to 200 g each and bearing 2- to 3-g tumors were used at the start of experiments. Acid ribonuclease was assayed in supernatent (105,000*g* for 1 hour) of tumor hemogenates (10 percent by volume) prepared in a mixture of 0.85 percent NaCl and 0.1 percent Triton X-100 by a modification of the method of Kalnitsky *et al.* (*10*) as described (*11*). The enzyme was quantified by single radial immunodiffusion (*11*). Protein was estimated by the method of Lowry *et al.* (*12*). Estrogen levels in tumors were measured by radioimmunoassay (*13*).

Growth of the DMBA tumor was completely arrested in animals treated for 3 weeks with dibutyryl cyclic AMP, while the size of the tumors in control animals increased threefold (Fig. 1). Ovariectomy results in regression of this tumor and 17β-estradiol produces regrowth (*14*). Dibutyryl cyclic AMP also inhibited the growth in the regrowing tumors (Fig. 1). Growth of the MTW9 tumor was arrested during 3 weeks in a similar experiment in which control tumors showed a tenfold increase over their original size (Fig. 1). Cessation of dibutyryl cyclic AMP treatment restored tumor growth (Fig. 1). Although no attempt was made to determine the minimum effective dose of dibutyryl cyclic AMP, the amount injected over a 3-week period was not toxic to the animals, as evaluated by hematocrit levels, body weight, and food intake. Minimum inhibition