Vol. 85, No. 1, 1978 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
November 14, 1978 Pages 293-300

GUANINE NUCLEOTIDE CONCENTRATIONS IN VIVO IN OUTER SEGMENTS OF DARK AND LIGHT ADAPTED FROG RETINA

F.A.M. de Azeredo*, W.D. Lust

and J.V. Passonneau

Laboratory of Neurochemistry
National Institute of Neurological and
Communicative Disorders and Stroke
Bethesda, MD 20014

Received September 25,1978

Summary - Cyclic GMP, GTP, and GDP were measured in outer segments of dark-adapted frog retina, and following exposure to 2 min and 2 hr of light. GTP concentrations decreased after 2 min of light, and there was a concomitant increase in GDP. After 2 hr of light GTP and GDP levels were like those of dark-adapted retinas. Cyclic GMP concentrations decreased to 30% of the values for dark-adapted retinas after 2 min or 2 hr of light exposure. The changes in GTP and GDP were transitory, and apparently associated with light adaptation; the decrease in cyclic GMP occurred within 2 min and remained low at 2 hr, attaining a new steady-state level.

The changes in cyclic GMP in the rod outer segments of retina during light adaptation have focused attention on the cyclic nucleotide as well as on the synthetic and degradative enzymes. A decrease of 85% in cyclic GMP concentrations in isolated outer segments of frog retina following exposure to light in vivo has been observed (2); concomitant changes in guanylate cyclase and phosphodiesterase have also been reported (1,3).

The metabolism of the rod outer segments has been investigated primarily in vitro after isolation of the tissue. Such procedures may be adequate in general for measurement of enzyme activities, but in the case of labile metabolites, the isolation techniques may alter the concentrations in vivo. The decrease in cyclic GMP in vivo following light exposure has been demonstrated by quantitative histochemical techniques (5). However, none of the studies

*Recipient of a postdoctoral fellowship from Conselho Nacional de Desenvolvemento Cientifico e Technologico, Brazil.

0006-291X/78/0851-0293$01.00/0

mentioned included an investigation of possible changes in other guanine nucleotides in the transition from dark to light adaptation.

GTP is not only the substrate for the formation of cyclic GMP, but has also been shown to be an effective activator of phosphodiesterase in the rod outer segments (1,3,7). The relationships among cyclic GMP, GTP and GDP have been a subject of investigation in this laboratory. In the present study, we report an adaptation of the method of Cha (9) for the measurement of as little as 10^{-12} mole of GTP/GDP. In addition a method is described for the measurement of 10^{-13} mole of GDP after removal of ADP from tissues. The changes in cyclic GMP, GTP and GDP in the outer segments of frog retinas exposed to brief (2 minutes) and extended (2 hours) periods of light have been examined.

MATERIALS AND METHODS

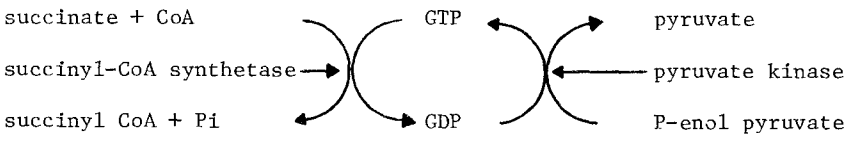
Frogs (Rana pipiens, Oshkosh, WI) were adapted to a cycle of 12 hours dark and 12 hours light for seven days. At the end of the last dark period one group was fixed by immersion in liquid nitrogen under dim red lights. Two other groups were exposed to normal room light for 2 minutes or 2 hours after which the animals were frozen in liquid nitrogen. The tissue was prepared according to Lowry and Passonneau (10). Briefly the eyeballs were dissected in a cryostat (-30°) and mounted on wooden rods. Tangential sections of 8-10 µM thickness were cut through the back of the eyeball at -22°, and the sections dried at -45° under vacuum (0.01 mm Hg) overnight. Outer segments (150-300 ng) were dissected free-hand under a microscope, weighed on a quartz fiber balance (sensitivity $\pm$ 1.8 ng). The samples were assayed for cyclic GMP by the radioimmunoassay of Steiner (11) as modified by Harper and Brooker (14), and for GTP and GDP as described below.

Chemicals. Pyruvate kinase (ATP:pruvate 2-0 phosphotransferase EC 2.7.1.40) from rabbit muscle, creatine kinase (ATP:creatine N-phosphotransferase EC 2.7.3.2) from rabbit muscle, succinyl CoA synthetase (succinate: CoA ligase EC 6.2.1.4) from pig heart, alcohol dehydrogenase (alcohol:NAD oxidoreductase EC 1.1.1.1) from yeast, malate dehydrogenase (L-malate:NAD oxidoreductase EC 1.1.1.37) from pig heart and glutamate oxalacetate transaminase (L-aspartate:2-oxoglutarate aminotransferase EC 2.6.1.1) from pig heart were purchased from Boehringer Mannheim Biochemicals (Indianapolis, IN) and lactate dehydrogenase (L-lactate:NAD oxidoreductase EC 1.1.1.27) was purchased from Worthington Biochemicals (Freehold, NJ). Nucleotides were bought from Sigma (St. Louis, MO) and all other chemicals were analytical grade.

GTP + GDP Assay

Analytic Principle

Step 1.



Step 2.

$$\text{pyruvate} + \text{NADH} + \text{H}^+ \xrightarrow{\text{lactate dehydrogenase}} \text{lactate} + \text{NAD}^+$$

The total amount of guanine di- and triphosphate is assayed in two steps after extraction of the tissue and denaturation of native tissue enzymes by heating in 0.02 N NaOH (Table 1). The commercial preparation of succinyl Coa synthase from pig heart is specific for guanine and inosine nucleotides (12). Since ITP concentration is less than 2.5% of that for GTP in tissue (Dr. Lawrence Rodichok, personal communication) and since ATP does not react, the assay is essentially a measure of guanine di- and triphosphates. The procedures are carried out in oil wells (10) except for the final indicator step. The first analytic step is the GTP-GDP cycle described by Cha and Cha with minor modifications (9). The GTP reacts in the presence of succinyl CoA synthetase, coenzyme A and succinate to produce GDP; the GDP reacts in the presence to P-enol pyruvate and pyruvate kinase to regenerate GTP. Succinate, coenzyme A, and P-enol pyruvate are present in excess and the products of the two reactions accumulate. The cycling reagent is given in Table 1.

The final step is the fluorometric measurement of the accumulated pyruvate. The entire sample is transferred from the oil well to one ml of the indicator reagent (Table 1). The cycling reaction is stopped by the 200-fold dilution in the indicator reagent, and by the EDTA in the reagent which chelates the Mg^{2+} necessary for the succinyl CoA synthetase and pyruvate kinase reactions. A reading is made in the fluorometer at a setting to give maximum deflection with the NADH present, 0.2 µg/ml of beef heart lactate dehydrogenase is added, and the tubes read again after 5 minutes. With the level of enzymes specified, the cycling rate was approximately 2000-fold per hr.

The blank in the cycling step, presumably due to nucleotide contamination, was equivalent to 3×10^{-8} M, while the GDP/GTP concentrations of the sample were $3\text{-}6 \times 10^{-7}$ M. Since pyruvate contamination of P-enol pyruvate has been found to be as much as 1%, the pyruvate was eliminated by treating a stock solution of P-enol pyruvate with a slight molar excess of H_2O_2 at 100° for 5 min.

GDP assay

Analytic Principle

Reactions

Step 1. $\text{ADP} + \text{P-creatine} \xrightarrow{\text{creatine kinase}} \text{ATP} + \text{Creatine}$

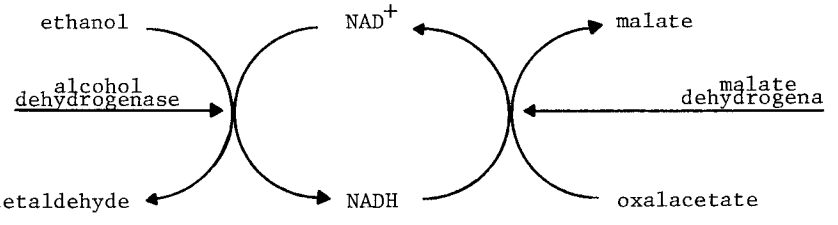
$$\text{pyruvate} + \text{hydrazine} \longrightarrow \text{pyruvate hydrazone}$$

Step 2. $\text{GDP} + \text{P-enol pyruvate} \xrightarrow{\text{pyruvate kinase}} \text{pyruvate} + \text{GTP}$

$$\text{pyruvate} + \text{NADH} + \text{H}^+ \xrightarrow{\text{lactate dehydrogenase}} \text{lactate} + \text{NAD}^+$$

Step 3. Destruction of excess NADH

Step 4.



Step 5. malate + NAD $\xrightarrow{\text{malate dehydrogenase}}$ oxalacetate + NADH + H^+

oxalacetate + glutamate $\xrightarrow{\text{glutamate oxalacetate transaminase}}$

aspartate + α-ketoglutarate

The GDP is measured in 5 steps after extraction of the tissue and destruction of tissue enzymes with 0.02 N NaOH at 80° (Table 1). The first 3 steps are carried out in oil wells as in the previous assay. In the first step, interfering substances are removed: ADP is converted to ATP with creatine kinase in the presence of P-creatine, and pyruvate in the tissue sample or as a contaminant is reacted with hydrazine. The second step is the reaction of the tissue GDP with PK and LDH to form a stoichiometeric amount of NAD^+. In the third step, the excess NADH is destroyed with HCl. The last 2 steps are carried out as described by Kato et al. (13). The NAD^+ is used in a cycling system to generate 15,000-fold increase in malate and acetaldehyde in an hour (see Analytic Principle). After stopping the reaction with heat, the malate is measured in a fifth step to form NADH; the reaction is carried to completion by removal of one of the products, oxalacetate, with glutamate-oxalacetate transaminase. Appropriate standards carried through out the procedure are the basis of calculation.

RESULTS

Two minutes of light exposure was enough to increase the amount of GDP almost two fold and decrease the amount of GTP to less than half of the dark value (Table 2). The increase in the GDP content of 0.73 mmol/kg dry weight could be accounted for by the decrease in the GTP content of 0.92 mmol/kg dry weight. Apparently GTP was converted to GDP during the two minutes of light adaptation. After two hours of exposure to light, the levels of GTP and GDP were the same as the values for the dark-adapted retinas (Table 2). The changes in the nucleotides during light exposure were transitory, and probably associated with the visual adaptation process.

The level of cyclic GMP decreased after 2 min or 2 hr of light exposure. After both time periods, the decrement was to 30% of the dark-adapted retina content. Unlike the GTP and GDP, the cyclic GMP concentrations do not return to those of the dark-adapted retinas, but attain a decreased steady-state level.

DISCUSSION

Much of the biochemical information on the metabolism of guanine nucleotides in the retina comes from experiments performed after the isolation and purification of the rod outer segments. In the isolated preparations light induced a decrease in cyclic GMP and, in combination with GTP, activated

Table 1. Flow Chart

Protocol	GTP + GDP	GDP
Extraction	0.06 µl 0.02 N NaOH 20 min, 80°	0.025 µl 0.02 N NaOH 20 min, 80°
Step 1	4.82 µl cycling reagent I 1 hr, 38°	0.2 µl reagent I 20 min, 25° and 3 min, 80°
Step 2	whole sample transfer to 1 ml indicator reagent I 10 min, 25°	0.2 µl reagent II 30 min, 25°
Step 3		1 µl 0.125 N HCl 10 min, 25°
Step 4		1 µl to 50 µl cycling reagent II 1 hr, 25° and 2 min, 100°
Step 5		whole sample to 1 ml of indicator reagent II 10 min, 25°

Reagents are as follows:

GTP + GDP

Cycling reagent I: 100 mM Tris-HCl pH 7.7, 10 mM succinate, 5 mM P-enol pyruvate, 5 mM coenzyme A, 1 mM $MgCl_2$, 160 µg/ml pyruvate kinase, 80 µg/ml succinyl CoA synthetase.

Indicator reagent I: 50 mM imidazole-HCl pH 7, 5 µM NADH, 1 mM EDTA, 0.2 µg/ml beef heart lactate dehydrogenase.

GDP

Reagent I: 100 mM imidazole-HCl pH 7, 20 µM P-enol pyruvate, 10 mM hydrazine, 4 mM $MgCl_2$, 1.5 mM P-creatine, 0.04% bovine serum albumin, 1 µg/ml creatine kinase

Reagent II: 100 mM imidazole-HCl pH 7, 10 mM hydrazine, 10 mM ascorbate, 3 µM NADH, 40 µg/ml pyruvate kinase, 4 µg/ml beef heart lactate dehydrogenase

Cycling reagent II: 100 mM Tris-HCl pH 8.1, 300 µM ethanol, 2 mM mercaptoethanol, 0.02% bovine serum albumin, 300 µg/ml yeast alcohol dehydrogenase, 30 µg/ml malate dehydrogenase

Indicator reagent II: 50 mM 2-amino-2-methyl-1-propanol-HCl pH 9.9, 10 mM glutamate, 200 µM NAD^+, 5 µg/ml glutamate-oxalacetate transaminase, 5 µg/ml malate dehydrogenase

phosphodiesterase (1,2,3,5). Moreover, it has been shown that the activation of the phosphodiesterase reaches a maximum when only 4% of the rhodopsin bleached and the concentration of GTP is 200 µM (8). However, to evaluate the physiological significance of a biochemical process, it is essential to determine the relevant metabolite concentrations *in vivo*. The GTP concentrations *in vivo*, assuming that the water content is 4 times its dry weight, are 400 µM in dark-adapted

Table 2. In vivo concentrations of GTP, GDP and cyclic GMP in outer segments of dark- and light-adapted frog retina

Nucleotide	Dark	2 minutes of light	2 hours of light
		(mmol/kg dry wgt)	
GTP + GDP	2.41 ± 0.09 (36)	2.24 ± 0.14 (20)	2.34 ± 0.06 (24)
GDP	0.82 ± 0.06 (17)	1.55 ± 0.07(*) (19)	0.82 ± 0.05 (18)
GTP (+)	1.59	0.67	1.52
		(μmol/kg dry wgt)	
Cyclic GMP	43.50 ± 2.26 (17)	12.10 ± 0.98(*) (9)	13.88 ± 0.88(*) (21)

The outer segments from two frogs were used for each experimental group. The results are expressed as the mean ± SEM for (n) determinations.

(*) Values significantly different from the dark-adapted retina ($p < 0.05$).

(+) The GTP levels represent the difference between the values for the combination of GTP and GDP and that for GDP alone.

outer segments of retina, and 170 μM after 2 min exposure to light (Table 2). With these concentrations of GTP and the light intensity used in our experiments, we conclude that phosphodiesterase will be maximally activated throughout the light exposure, and accounts at least in part for the decrease in cyclic GMP. These conclusions of course do not rule out the possibility of situations in which GTP concentrations might be a rate-limiting factor in the activation of phosphodiesterase.

The mechanism of GTP activation of phosphodiesterase has not been elucidated. Some investigators have suggested phosphorylation of proteins and/or intermediates as a possibility (7). Other workers have suggested that phosphodiesterase activity might be modulated by changes in GTP concentrations brought about by light-activated GTP phosphatase activity. Although Wheeler and Bitensky (3) have described a light-sensitive GTP phosphatase, activation occurred only at GTP levels of 5 μM or less, a value far below the concentrations in vivo. Unless

GTP were compartmentalized in such a fashion that 98% was essentially unavailable, activation of phosphatase *in vivo* by light appears unlikely. The utilization of GTP as a substrate for protein kinase and/or energy source appears more probable.

The rapid decrease of cyclic GMP *in vivo* after exposure to light may result from effects on guanylate cyclase as well as phosphodiesterase activity. Guanylate cyclase has been reported to have a Km for GTP at or above the concentrations found *in vivo* (15,16). Consequently, the decrease in GTP which occurred after 2 min of light exposure could result in diminished cyclase activity. It is possible that enhanced phosphodiesterase activity and decreased cyclase activity account for the rapid decrease in cyclic GMP. The maintenance of the low levels of cyclic GMP after 2 hr of light in the face of increased GTP concentrations suggest that the phosphodiesterase activity may be dominant after long exposure to light.

The transitory effect of light on GTP concentration is in contrast with the maintenance of low cyclic GMP concentrations after 2 hours of light. These results suggest that both GTP and cyclic GMP are involved in the initial process of light adaptation, while only cyclic GMP is affected by continued exposure to light.

References

1. Krishna, G., Krishnan, N., Fletcher, R. T. and Chader, G. (1976) J. Neurochem. 27, 717-722.
2. Fletcher, R. T. and Chader, G. J. (1976) Biochem. Biophys. Res. Comm. 70, 1297-1302.
3. Wheeler, G. L. and Bitensky, M. K. (1976) Proc. Natl. Acad. Sci. USA. 74, 4238-4242.
4. Sitaramayya, A., Virmaux, N. and Mandel, P. (1977) Exp. Eye Res. 25, 163-169.
5. Orr, H. T., Lowry, O. H., Cohen, A. I. and Ferrendelli, J. A. (1976) Proc. Natl. Acad. Sci. USA. 73, 4442-4445.
6. Sittaramayya, A., Virmaux, N. and Mandel, P. (1977) Neurochem. Res. 2, 1-10.
7. Liebman, P. A., Yee, R. and Evancuk, A. (1977) 50th Meetings Association for Research in Vision and Ophtalmology, Abstracts p. 191.
8. Yee, R. and Liebman, P. A. (1977) 50th Meetings Association for Research in Vision and Ophtalmology, Abstracts p. 256.
9. Cha, S. and Cha, C. M. (1970) Anal. Biochem. 33, 174-192.
10. Lowry, O. H. and Passonneau, J. V. (1972) A Flexible System of Enzymatic Analysis, Academic Press, New York.
11. Steiner, A. L., Parker, C. W., and Kipnis, D. M. (1972) J. Biol. Chem. 247, 1106-1113.

12. Sanadi, D. R., Gibson, D. M., Ayengar, P. and Jacob, M. (1955) J. Biol. Chem. 218, 505-520.
13. Kato, T., Berger, S. J., Carter, J. A. and Lowry, O. H. (1973) Anal. Biochem. 53, 86-97.
14. Harper, J. F. and Brooker, G. (1975) J. Cyclic Nucl. Res. 1, 207-218.
15. Krishnan, N., Krishna, G., Fletcher, R. and Chader, G. (1978) 50th Meetings Association for Research in Vision and Ophtalmology, Abstracts p. 191.
16. Fleischman, D. and Denisevich, M. (1978) 50th Meetings Association for Reserch in Vision and Ophtalmology, Abstracts p. 255.