PROTEIN PHOSPHORYLATION IN ROD OUTER SEGMENTS FROM BOVINE RETINA: CYCLIC NUCLEOTIDE-ACTIVATED PROTEIN KINASE AND ITS ENDOGENOUS SUBSTRATE

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Received August 8, 1977

SUMMARY

Protein kinase activity of isolated rod outer segments from bovine retinas is activated by cGMP when in a soluble form, and it is cyclic nucleotide independent when associated with the rod outer segment membranes. The soluble protein kinase phosphorylates in a cyclic nucleotide-dependent manner only a single endogenous protein with an apparent molecular weight of 30,000 daltons. The 30,000-dalton phosphoprotein is localized specifically in the visual cells of the retina. It is proposed that the light-induced changes in cGMP levels that occur in rod outer segments in vivo are linked by the cyclic nucleotide-dependent protein kinase to alterations in the content of the 30,000-dalton phosphoprotein.

Rod outer segments[1] of dark-adapted retinas from several species possess high levels of cGMP and the enzymes required for cGMP synthesis and degradation (1-4). Upon bleaching of rhodopsin by light, cGMP phosphodiesterase activity is enhanced (4), with a concomitant fall in cGMP concentration (5). About one-half of the cGMP in frog or bovine retina is hydrolyzed within 3-10 sec of exposure to laboratory illumination (6), and changes are observed within 100 msec in isolated ROS preparations (7). The metabolic or functional role of cGMP in a visual cell or its ROS is still uncertain, but it may be associated with the visual process.

In this report, we show the presence in ROS of a soluble cyclic nucleotide-dependent protein kinase which phosphorylates only one photoreceptor-specific protein in a cyclic nucleotide-dependent manner. It is proposed that cGMP acts in ROS by modulating the level of specific phosphoprotein(s).

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[1] Abbreviation: ROS (rod outer segment).

ISSN 0006-291X

MATERIALS AND METHODS

Bovine eyes were obtained from a local slaughter house and placed immediately on ice in the dark. Thereafter, all procedures were carried out under darkroom conditions with illumination (laboratory fluorescent light) being introduced only where indicated. Retinas were manually dissected and ROS were detached as described in the respective methods (8-10) or as devised in our laboratory for increasing the amount of soluble protein obtained from ROS. For routine preparations, 30 retinas were suspended in 60 ml of 1.13-density sucrose prepared with 5 mM Tris buffer containing 5 mM $MgCl_2$ and 62 mM NaCl (pH 7.6). The tube was shaken vigorously 15 times and then spun at 10,000 x g for 12 min. The supernatant was separated, diluted with 1.5 volumes of 50 mM Tris (pH 7.6) containing 5 mM $MgCl_2$ and centrifuged at 27,000 x g to pellet the ROS. The pellet was resuspended in 4 ml of 50 mM Tris buffer (pH 7.6), and the suspension was frozen and thawed, followed by homogenization and subsequent centrifugation at 100,000 x g for 1 hr. The supernatant and particulate fractions were separated and used immediately or stored at -50°C.

Other methods of ROS preparation were tested in order to assess whether the sample purity or degree of rod structural disorganization influenced the composition of the soluble fraction from bovine ROS. The procedures of Papermaster and Dreyer (8), Miki et al. (9) and Basinger et al. (10) were used without modification.

Rod outer segment particulate material that resulted from the Tris-buffer extraction, above, was extracted (washed) again with 50 mM Tris buffer (pH 7.6) and centrifuged at 100,000 x g for 1 hr. The resulting pellet was then homogenized in 5 ml of 0.1% Lubrol PX detergent in 50 mM Tris buffer (pH 7.6) and centrifuged at 100,000 x g for 1 hr in order to obtain the supernatant and particulate fractions.

The protein kinase reaction was carried out with or without added cGMP in a total volume of 110 ul, containing 50-100 ug of soluble ROS protein (measured by the method of Lowry et al. (11)) in 50 mM Tris-HCl buffer (pH 7.6), 5 mM $MgCl_2$, histone (75 ug/tube) and 0.6 mM [γ-^{32}P]ATP (2-3 x 10^6 c.p.m./tube). All steps were carried out in dim red light, although exposing the soluble protein kinase to controlled laboratory illumination did not affect its activity. After incubation at 30°C for 5 min, the reaction was terminated by adding 10% cold TCA. The TCA-precipitable protein containing incorporated ^{32}P was collected on glass fiber filters, washed with chloroform-methanol and counted. Blanks were prepared by adding TCA to the tube prior to the incubation.

The endogenous proteins from the 100,000 x g Tris-supernatant fraction of bovine ROS that are phosphorylated by soluble protein kinase were separated by electrophoresis. The kinase reaction was carried out as described above, but histone was omitted, and the reaction was terminated with 8% sodium dodecyl sulphate. The mixture was then incubated at 37°C for 2 hr and, subsequently, the proteins were separated on 10% polyacrylamide gels (12). Gels were stained with Coomassie blue and, after scanning at 570 nm, were cut in 1-mm slices and counted in scintillation fluid. The apparent molecular weights of the protein bands from the retinal extract were estimated by interpolation from the mobility of standard proteins.

As a means of identifying photoreceptor-specific proteins in the soluble fraction of retina, we carried out an analogous investigation of the 100,000 x g Tris-supernatant fractions from normal and blind retinas. For this purpose, we used blind mice of the C3H/HeJ strain which have lost post-natally all of their photoreceptors as a result of an inherited retinal disease (13).

Table I: Soluble and particulate protein kinase of bovine ROS: Differential activation by cGMP

Sample	Extractant	Supernatant (pmol/min/supernatant)		Particulate (pmol/min/pellet)	
		Basal	+cGMP	Basal	+cGMP
Purified ROS	50 mM Tris (pH 7.6)	358	863	3097	3406
Pellet from 1st Tris extraction	50 mM Tris (pH 7.6)	99	90	3070	3100
Pellet from 2nd Tris extraction	0.1% Lubrol PX* in Tris buffer	1382	6080	1700	1820

Activities are expressed as total phosphorylated protein/min in the respective fractions and were obtained using histone as substrate. Values are the mean of three separate experiments.
*Corrected for observed Lubrol PX inhibition of protein kinase activity (20-40%).

RESULTS AND DISCUSSION

We reasoned that during the visual process, a link between the bleaching of rhodopsin, which occurs in the ROS disk membranes, and the hyperpolarization of ROS plasmalemma might be found in the ROS cytoplasm where cGMP could serve as a second messenger to light. Therefore, we investigated the soluble, 100,000 x g supernatant fraction of ROS, prepared by conventional methods of purification. Table I shows that protein kinase which is activated by cGMP is present in the soluble fraction of Tris-extracted ROS and that the particulate fraction contains protein kinase activity which is cyclic nucleotide independent. The mild Tris treatment does not solubilize proteins that are intrinsic to the ROS membranes, e.g. rhodopsin. A second Tris-extraction or wash of the ROS particulate material yields very low levels of protein kinase activity. Table I shows further that extraction of the Tris-treated particulate fraction with the detergent, Lubrol PX, yields a soluble protein kinase which is activated also by cGMP. This procedure extracts only a minimal amount of rhodopsin (data not shown).

Table II: Cyclic nucleotide-activated protein kinase: Tris-extraction of ROS prepared by different methods

	Miki Method	Basinger Method	Papermaster Method
Basal Activity	0.24 ± 0.09	0.25 ± 0.08	0.24 ± 0.02
+ cGMP (10^{-4} M)	0.56 ± 0.11	0.48 ± 0.14	0.55 ± 0.03

Activities are expressed as namomoles of phosphorylated protein/mg protein/min and were obtained using histone as substrate. Values represent the mean ± standard error of 5-8 samples.

In order to examine further whether cyclic nucleotide-dependent protein kinase activity in ROS is localized within ROS, three different methods for isolating ROS were evaluated (Table II). The methods yielded ROS preparations that varied in purity, as assessed by rhodopsin content, and in degree of ROS structural disorganization (8-10). The similarity in activity and degree of stimulation by cGMP (Table II) indicates that the soluble protein kinase is localized in bovine ROS. Moreover, independent of the method of preparation or volume of extractant, the soluble fraction contained about 12% of the total basal protein kinase activity of ROS, suggesting that a small percentage of the ROS kinase exists in a soluble or solubilizable form.

The endogenous proteins of Tris-extracted ROS are shown in Fig. 1. The soluble protein kinase phosphorylates proteins with apparent molecular weights of about 70,000, 64,000, 30,000 and 22,500 daltons, respectively. Only the 30,000-dalton protein is phosphorylated in a cyclic nucleotide-dependent manner. In order to verify that these soluble proteins are specific to visual cells, we compared the 100,000 x g supernatant fraction of C3H mice retinas (which lack visual cells) with that of control DBA mice retinas (which possess a full complement of photoreceptors). Soluble protein kinase phosphorylates high-molecular-weight proteins of both C3H and DBA retinas

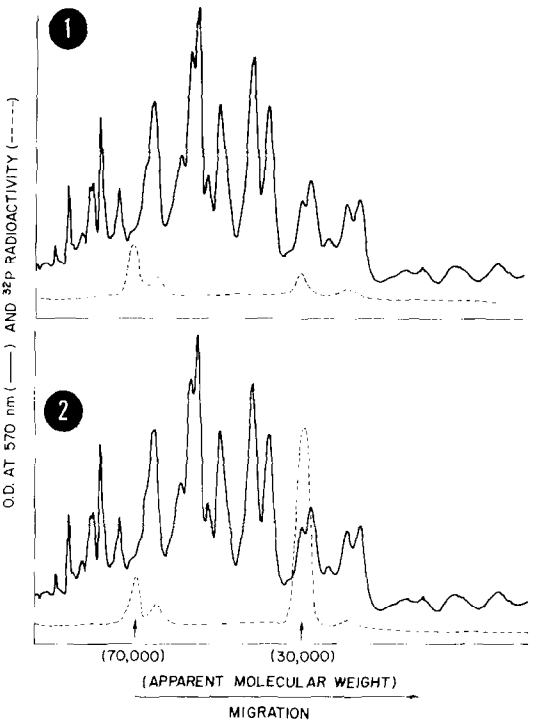

Figure 1: Endogenous proteins from the 100,000 x g Tris-supernatant fraction of bovine rod outer segments which are phosphorylated by soluble protein kinase. (1) Basal phosphorylation; (2) cGMP-activated phosphorylation.

Figure 2: Endogenous proteins from the 100,000 x g Tris-supernatant fraction prepared from 6-8 retinas of adult mice, phosphorylated by soluble protein kinase, and separated on polyacrylamide gels, as described in the Methods. (1) Soluble retinal proteins of blind C3H mice; arrow indicates missing phosphoprotein peak at 30,000 daltons. (2) Soluble retinal proteins of normal DBA mice showing the incorporation of ^{32}P in the absence (-----) or presence (·····) of exogenous cyclic nucleotide.

but the 30,000-dalton phosphoprotein is present only in the normal retina (Fig. 2). The absence of this band from the soluble fraction of blind retina indicates that the 30,000-dalton protein, which is phosphorylated in a cyclic nucleotide-dependent manner, is localized selectively in visual cells.

The idea that cGMP may serve as a second messenger to light in retinal photoreceptor cells has been considered by several investigators (14-17) but the pursuit of this concept has been slow. The reported absence of cyclic

nucleotide-dependent protein kinase activity in isolated ROS (18,19) has been the major obstacle in accepting cGMP as a component of the visual process. Our findings indicate that within a ROS there is a small soluble fraction of protein kinase which is cyclic nucleotide-dependent and a larger membrane-associated fraction which is cyclic nucleotide independent. Moreover, the membrane-associated protein kinase can be solubilized with low concentrations of Lubrol PX. The solubilized protein kinase shows cyclic nucleotide-dependent characteristics. Perhaps the soluble and membrane-associated kinases are interchangeable *in situ*, but further work is needed to clarify such a relationship.

The endogenous proteins that are phosphorylated by the protein kinase of ROS differ also according to whether the enzyme is soluble or membrane-associated. We, and others, find that the membrane-associated protein kinase prefers bleached rhodopsin (a membrane protein) as its endogenous substrate. On the other hand, the soluble kinase phosphorylates soluble proteins and only one of them in a cyclic nucleotide-dependent manner.

Physiological studies indicate that light-induced changes in cGMP may be associated with the visual process (7,16). The biochemical mechanisms involved are still uncertain, but we suggest that in visual cells cGMP modulates the activity of a soluble cyclic nucleotide-dependent protein kinase and the phosphorylation of a specific protein(s) with a molecular weight of about 30,000 daltons.

ACKNOWLEDGEMENTS

We thank Ms. Louise V. Eaton for help with manuscript preparation and Globe Packing Company, San Fernando, California, for their interest and cooperation in this work, which was supported by grants from the NIH (EY00395) and the NSF (BMS 76-81471), and by the Medical Research Service of the Veterans Administration.

REFERENCES

1. Krishna, G., Krishnan, N., Fletcher, R.T. and Chader, G. (1976) J. Neurochem. 27, 717-722.
2. Pannbacker, R.G. (1973) Science 182, 1138-1140.

3. Goridis, C. and Virmaux, N. (1974) Nature 248, 57-58.
4. Miki, N., Keirns, J.J., Marcus, F.R., Freeman, J. and Bitensky, M.W. (1973) Proc. Natl. Acad. Sci. U.S.A. 70(12), 3820-3824.
5. Fletcher, R.T. and Chader, C.J. (1976) Biochem. Biophys. Res. Commun. 70, 1297-1302.
6. Goridis, C., Virmaux, N., Cailla, H.L. and Delaage, M.A. (1974) FEBS Letters 49, 167-169.
7. Woodruff, M.L., Bownds, D., Green, S.H., Morrisey, J.L. and Shedlovsky, A. (1977) J. Gen. Physiol. 69, 667-679.
8. Papermaster, D.S. and Dreyer, W.J. (1974) Biochemistry 13, 2438-2444.
9. Miki, N., Baraban, J.M., Keirns, J.J., Boyce, J.J. and Bitensky, M.W. (1975) J. Biol. Chem. 250, 6320-6327.
10. Basinger, S., Bok, D. and Hall, M. (1976) J. Cell Biol. 69, 29-42.
11. Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951) J. Biol. Chem. 193, 265-275.
12. Farber, D.B. and Lolley, R.N. (1973) J. Neurochem. 21, 817-828.
13. Sidman, R.L. and Green, M.C. (1965) J. Hered. 56, 23-29.
14. Farber, D.B., Brown, B.M. and Lolley, R.N. (1977) Vision Res. (in press).
15. Brodie, A.E. and Bownds, D. (1976) J. Gen. Physiol. 68, 1-11.
16. Lipton, S., Rasmussen, H. and Dowling, J. (1977) J. Gen. Physiol. (in press).
17. Chader, G.J., Fletcher, R.T., O'Brien, P.J. and Krishna, G. (1976) Biochemistry 15, 1615-1620.
18. Weller, M., Virmaux, N. and Mandel, P. (1975) Proc. Natl. Acad. Sci. U.S.A. 72, 381-385.
19. Frank, R.N. and Bensinger, R.E. (1974) Exp. Eye Res. 18, 271-280.