59. The Parallel Study of Protein Phosphorylation and Phosphatidylinositol Metabolism in the Photo-reception of Squid Retina

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(Communicated by Yasuji KATSUKI, M. J. A., Oct. 12, 1981)

Introduction. Light absorption of vertebrate rod visual cell induces a Mg^{2+} -dependent phosphorylation of the visual pigment protein.¹⁾⁻³⁾ This light activated rhodopsin phosphorylation is reversible process,^{4),5)} and it also appears not to be regulated by cyclic nucleotide.^{1),2),6)} In invertebrate photoreceptor membrane, light activated rhodopsin phosphorylation was observed and it was found that the characteristics of phosphorylated protein was similar to that observed in vertebrate rod.⁷⁾

In a variety of tissues, an enhanced metabolism of phosphatidylinositol (PI) was observed when the receptor was activated by hormones or other stimulating materials which utilize Ca^{2+} as a second messenger.^{8),9)} With respect to photoreceptor membrane, the rapid metabolism of polyphosphoinositide (DPI, phosphatidyl-myo-inositol 4-phosphate; TPI, phosphatidyl-myo-inositol 4,5,-diphosphate) played an important role in photoreception.

For these several years, protein phosphorylation and poly-PI metabolism (phosphorylation of PI) have been studied independently. In this report the relationship between the phosphorylation of poly-PI and that of rhodopsin in microvilli of squid retina was studied.

Materials and methods. *Watasenia scintillans*, were collected during the month of April at the Toyama Bay. Eyes were removed and stored at $-80^{\circ}C$. More than 100 eyes were thawed and carefully dissected under dim red light, and the posterior half of the eye was homogenized in 30 ml of 100 mM of phosphate buffer (pH 6.8) containing 4% NaCl and centrifuged for 10 min at $2500\times g$. The supernatant was discarded and the residue was homogenized in 20 ml of 40% sucrose (w/v) in buffer and centrifuged at $16,000\times g$ for 30 min. The floating material was harvested and resuspended in 40% sucrose buffer and centrifuged again at $16,000\times g$ for 30 min. This procedure

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was repeated until no precipitation was recognized. Finally, the floating material was suspended in phosphate buffer containing 4% NaCl and centrifuged at $16,000\times g$ for 10 min. The pellet was dissolved in a reaction mixture. [γ - ^{32}P]ATP was purchased from New England Nuclear. The specific activity of it was 4000 Ci/mmol.

The assay of protein phosphorylation was performed by taking autoradiography of SDS-polyacrylamide gel electrophoresis of ^{32}P incorporated protein. The PI phosphorylation assay was always run in parallel with protein phosphorylation and the lipid extracted by ice cold chloroform/methanol/12HCl (200:100:0.75 v/v) was analysed by one dimensional high performance thin layer chromatography plate impregnated in 1% K-oxalate. The reaction mixture was illuminated for the same period as incubation time of ATP with continuous white light (10^5 lux). The heat from the lamp was cut off by an aquafilter, 5 cm in thickness. All experiments were carried out at 18°C .

Results. Fig. 1 shows protein phosphorylation analysed by autoradiography. The bands of 49 K, 25 K, and 17 K dalton protein were phosphorylated. The 49 K dalton protein may correspond to rhodopsin.¹⁰⁾ The 25 K dalton protein might be retinochrome which is unable to remove from receptor membrane.¹¹⁾ The smallest molecular weight protein with 17 K dalton protein can not be identified at present but a part of it might be calmodulin because the R_f value of it was affected with or without Ca^{2+} in the gel (not shown here).

Fig. 1. Autoradiograph of electrophoretic pattern of ^{32}P -incorporated protein from squid photoreceptor membrane under various incubation time. M.W. is molecular weight whose unit was dalton. The composition of reaction medium was 100 mM NaCl, 10 mM KCl, 10 mM MgCl_2 , 1 mM CaCl_2 , 1 μM ATP, 20 μCi [γ - ^{32}P] ATP and 100 μl of crude membrane suspension (7.5 mg protein/ml) in a total volume of 3.5 ml.

Fig. 2. a: Autoradiograph after thin-layer chromatography of phospholipids on oxalate-impregnated silica gel HPTLC. TPI, triphosphoinositide; DPI, diphosphoinositide; PA, phosphatidic acid; d, experiment in the dark; l, experiment in the light. b: The effect of light on the time course of incorporation of ^{32}P into DPI. Symbols: —●—, control experiments in the dark; --○--, experiments under light irradiation.

As was seen in the figure, the incorporation of ^{32}P into the membrane protein increased with the incubation time. And light dependent ^{32}P incorporation was largely confined to protein part of rhodopsin and retinochrome. This light activated phosphorylation of microvilli membranes prepared from unfrozen retina was ten times less than that from eyes stored at -80°C .

When crude suspension of microvilli was incubated in reaction medium in the presence of $[\gamma\text{-}^{32}\text{P}]\text{ATP}$, incorporation of ^{32}P into DPI seems to be constant as is shown in Fig. 2a. The phosphatidic acid was found to contain a few percent of the incorporated radioactivity. Fig. 2 shows the separation of the labelled phospholipid on oxalate-impregnated HPTLC plate. A quantitative analysis of DPI was achieved by counting the radioactivity of scraped band of the plate.

Effect of illumination on the PI phosphorylation is demonstrated in Fig. 2b for various incubation time. Incorporation of $[\gamma\text{-}^{32}\text{P}]$ of ATP into DPI in the dark showed a monotonous increase with the time. When phosphorylation of DPI is performed under light irradiation, the time course showed a convex curve with its maximum value at 10 min.

Discussion. This paper deals with the comparative study of the phosphorylation of protein (rhodopsin) and phospholipid (DPI) in the presence of $[\gamma\text{-}^{32}\text{P}]\text{ATP}$. From the data it was concluded that

light irradiation increased the amount of radioactive terminal phosphate of ATP incorporated into rhodopsin and DPI within 10 min. For longer incubation, the time course of rhodopsin phosphorylation and PI phosphorylation showed a reverse change. According to the results of Paulsen and Hoppe,⁷⁾ phosphorylation of octopus rhodopsin was relatively slow process and it resembled to the phosphorylation reaction for vertebrate rhodopsin. Our findings of a light induced phosphorylation of squid rhodopsin confirmed their data.

The incorporation of terminal phosphate of ATP into membrane phospholipid is expressed as a summation of several processes such as phosphorylation of DPI by PI kinase, dephosphorylation of TPI by phosphomonoesterase and exhaustion of ATP by ATPase. Therefore it is not possible to draw definite conclusion as to the mechanism by which light influence the PI metabolism.¹²⁾ It is probable, however, that the result reflect enhanced turnover of poly-PI. Because the enhancement is manifested by an increased rate of incorporation of phosphorus into PI or increased breakdown of PI to diacylglycerol within the plasma membrane, which might be accompanied by dephosphorylation of DPI. Recently, Takai *et al.*^{4),5)} demonstrated that the activity of Ca dependent protein kinase was linked to PI and its breakdown product, 1,2-diacylglycerol (DG), suggesting that a functional relation exists between protein phosphorylation and PI metabolism. Since DPI and TPI are very potent chelators of Ca^{2+} and Mg^{2+} , a change in the relative amount of PI/DPI/TPI may affect the conformation of membrane protein and change the amount of Ca^{2+} and Mg^{2+} bound to the membrane.

Because of the postmortem breakdown of poly-PI^{16),17)} a long time preparation should be avoided. In our case, however, it took about 4 hrs to obtain membrane preparations. The shorter preparation time will produce the more apparent photo-irradiation effect on PI metabolism in squid retina.

References

- 1) Kühn, H., and Dreyer, W. J. (1972): FEBS Lett., **20**, 1.
- 2) Bownds, D., Dawes, J., Miller, J., and Stahlmann, M. (1972): Nature, New Biol., **237**, 125.
- 3) Frank, R. N., Cavanagh, H. D., and Kenyon, K. R. (1973): J. Biol. Chem., **248**, 596.
- 4) Kühn, H. (1974): Nature, **250**, 588.
- 5) McDowell, J. H., and Kühn, H. (1977): Biochemistry, **16**, 4054.
- 6) Weller, M., Virmaux, N., and Mandel, P. (1975): Proc. Natl. Acad. Sci. USA, **72**, 381.
- 7) Paulsen, R., and Hoppe, I. (1978): FEBS Lett., **96**, 55.
- 8) Michell, R. H. (1975): Biochim. Biophys. Acta, **415**, 81.

- 9) Michell, R. H., Jafferji, S. S., and Jones, L. M. (1977): Function and Biosynthesis of Lipid (eds. Bazan, N. G. *et al.*). Plenum, New York, pp. 447-464.
- 10) Noshima, K., Mitsudo, M., and Kito, Y. (1979): *Biochim. Biophys. Acta*, **579**, 155.
- 11) Hara, T., and Hara, R. (1973): *Nature*, **242**, 39.
- 12) Jolles, J., Wirtz, K. W. A., Schotman, P., and Gipsen, W. H. (1979): *FEBS Lett.*, **105**, 110.
- 13) Jolles, J., Zwiers, H., van Dongen, C. J., Schotman, P., Wirtz, K. W. A., and Gipsen, W. H. (1980): *Nature*, **286**, 623.
- 14) Takai, Y., Kishimoto, A., Inoue, M., and Nishizuka, Y. (1977): *J. Biol. Chem.*, **252**, 7603.
- 15) Takai, Y., Kishimoto, A., Kikkawa, U., Mori, T., and Nishizuka, Y. (1979): *Biochem. Biophys. Res. Commun.*, **91**, 1218.
- 16) Hayashi, K., Yagihara, Y., Nakamura, I., and Yamazoe, S. (1966): *J. Biochem. (Tokyo)*, **60**, 42.
- 17) Eichberg, J., and Hauser, G. (1973): *Biochim. Biophys. Acta*, **326**, 210.