Vol. 148, No. 1, 1987 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
October 14, 1987 Pages 54-60

PHOSPHATIDYLINOSITOL STIMULATES PHOSPHORYLATION OF PROTEIN COMPONENTS I AND II IN ROD OUTER SEGMENTS OF FROG PHOTORECEPTORS

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Received August 26, 1987

SUMMARY: We studied the effect of phosphoinositides on the phosphorylation of endogenous proteins in the soluble fraction of the frog photoreceptor rod outer segments (ROS). Phosphatidylinositol (PI) stimulated the phosphorylation of two low molecular weight proteins, components I and II (12 and 11 kDa) which are known to be the preferential substrates of the cyclic GMP (cGMP)-dependent protein kinase in the ROS. Polyphosphoinositides (PPI) specifically inhibited the PI-dependent phosphorylation of these two components. On the other hand, PPI stimulated the phosphorylation of 38, 48 and 52 kDa proteins in the absence of PI. These data suggest that PI and PPI may function in the ROS by regulating the phosphorylation of some enzymes or regulator proteins in the transduction mechanism in the ROS. © 1987 Academic Press, Inc.

Phosphoinositides most likely have important roles in the phototransduction mechanism of both vertebrate and invertebrate photoreceptors. The light-mediated hydrolysis of phosphatidylinositol-4,5-bisphosphate (PIP_2) in photoreceptor cells has been demonstrated by biochemical and immunocytochemical studies by many research groups (1-5). However, the true function of the phosphoinositides in the phototransduction mechanism has not been clarified yet. It is important to know the relationship between phosphoinositide metabolism and the cGMP cascade.

Abbreviations: PI, phosphatidylinositol; PIP, phosphatidylinositol-4-phosphate; PIP_2, phosphatidylinositol-4,5-bisphosphate; PPI, polyphosphoinositide; PS, phosphatidylserine; ROS, rod outer segments; G-protein, GTP-binding protein; PDE, phosphodiesterase; EGTA, [ethylenebis(oxyethylenenitrilo)]tetraacetic acid; SDS, sodium dodecyl sulfate.

0006-291X/87 $1.50
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On the other hand, the low molecular weight proteins, components I and II (12 and 11 kDa), in frog ROS are preferential substrates of cGMP-dependent protein kinase in this location (6,7). Hamm and Bownds showed that the phosphorylation of components I and II was inhibited by an antibody raised against the GTP-binding protein (G protein) of the ROS, which could inhibit cGMP phosphodiesterase (PDE) through its inhibitory action on the binding of GTP to the transducin α-subunit (8,9).

This paper reports the effects of phosphatidylinositol (PI) and polyphosphoinositides (PPI) on the phosphorylation of the endogenous proteins including components I and II in the soluble fraction of ROS.

MATERIALS AND METHODS

Materials: [γ-^{32}P]ATP was synthesized from [^{32}P]-orthophosphate (New England Nuclear; NEX-053) by the method of Johnson and Walseth (10) and purified by the method of Hayashi et al.(11). PI was purchased from Serdary Research Laboratory. PIP_2 and phosphatidylinositol-4-phosphate (PIP) were prepared from bovine brain by the method of Hendrickson and Ballou (12).

Preparation of frog ROS and the soluble fraction: Bull frogs (Rana catesbeiana) were fed with vitamin-enriched dog food and kept under cyclic illumination (12 hrs light/12 hrs dark) for at least 2 weeks. Four frogs were decapitated during the dark phase and the heads were chilled in an ice bath. Preparation of the soluble fraction from the ROS was performed in complete darkness using an IR-image converter. Retinas were gently peeled from the pigment epithelium layer in low Ca^{2+}-Ringer's solution (110 mM NaCl, 2.5 mM KCl, 5 mM $MgCl_2$, 0.5 mM EGTA, 18 μM $CaCl_2$ and 10 mM HEPES, pH 7.5). They were carefully transferred into 1.5 ml of the same ice-cold Ringer's solution. The ROS was detached by passing the retinal suspension through a plastic pipet (5 mm in diameter at the mouth) 40 times. ROS suspension was filtered through 4-layers of gauze and the ROS was pelleted by gentle centrifugation (700 x g, 2 min). It was resuspended in 0.2 ml of hypotonic solution (5 mM Tris-HCl, 1 mM EDTA and 1 mM EGTA, pH 7.5 at 0 °C) and left in an ice-bath for 60 min. After incubation, the soluble fraction was obtained by centrifugation (15,000 x g, 40 min). The protein concentration was about 0.7 mg / ml.

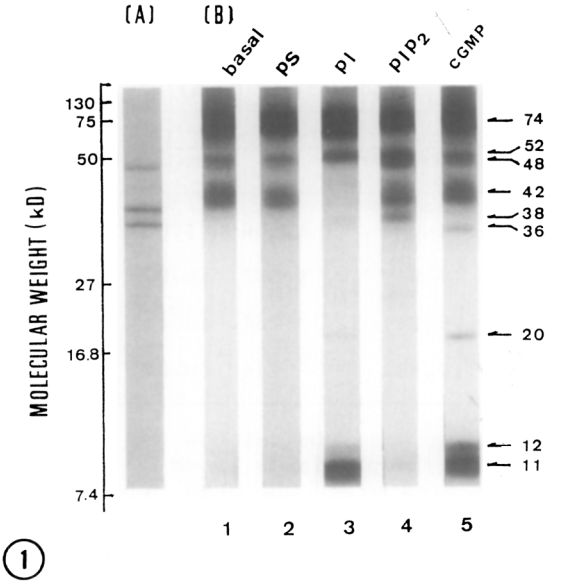
Protein kinase assay: Protein kinase activity was assayed by measuring the incorporation of ^{32}P into endogenous proteins from [γ-^{32}P]ATP. The standard reaction mixture (100μl) contained 5 μg protein, 10 μM [γ-^{32}P]ATP (5μCi), 5 mM $MgCl_2$, 0.5 mM EGTA, 470μM $CaCl_2$ (10^{-6}M free Ca^{2+}) and 20 mM Tris-HCl(pH 7.5). Chloroform solution of phosphatidylinositol or phosphatidylserine was evaporated in vacuo and the dried materials were dissolved in 20 mM Tris-HCl(pH 7.5) by sonication under an argon atmosphere. Each polyphosphoinositide was dispersed in the same buffer by

heat treatment at 80℃ for 2 min under the same atmosphere. The reaction was initiated by the addition of [γ-^{32}P]ATP and the mixture was incubated at 30 ℃ for 10 min. The reaction was terminated by the addition of SDS-sample buffer and the proteins were separated by SDS-gel electrophoresis as described by Laemmli(13). The gels were dried in a gel dryer and the endogenously phosphorylated protein pattern was visualized by exposing the gel to X-ray film (Fuji Photo Film Co.LTD; RX50) at -85℃ for 15 hrs. Radioactive protein bands were cut out and the radioactivity was measured by a liquid scintillation counter (Tri-Carb, Packard).

RESULTS AND DISCUSSION

Fig.1 shows an autoradiograph indicating the effect of phospholipids and cGMP on the endogenous protein phosphorylation in the soluble fraction of frog ROS. In the absence of cGMP or PI, there was no ^{32}P-incorporation into low molecular weight proteins. A significant amount of ^{32}P was incorporated into two low molecular weight proteins (11 and 12 kDa) upon addition of 0.1 mM PI. The effect of PI was also observed on the phosphorylation of 20 and 52 kDa proteins. These two low molecular weight proteins were identical with the substrates of cGMP-dependent protein kinase in the ROS (Fig.1, lanes 3 and 5). It has been reported that these two protein components are preferential substrates of cyclic nucleotide-dependent protein kinase in the frog ROS (6,14). Components I and II reported by Polans et al.(6), and Band 1 and 2 proteins reported by Shinozawa and Yoshizawa (14), seem to be the same components as those phosphorylated by the PI-dependent protein kinase activity. The phosphorylation of the 42 kDa protein was occasionally observed. This phosphorylation was significantly inhibited by the addition of PI.

Fig.2 shows the dependence on the concentration of PI of the ^{32}P-incorporation into components I and II. Maximal stimulation was brought about by 100μM PI. The half maximal activation occurred at about 40μM. PI of greater than 100μM exerted an inhibitory effect on the phosphorylation.



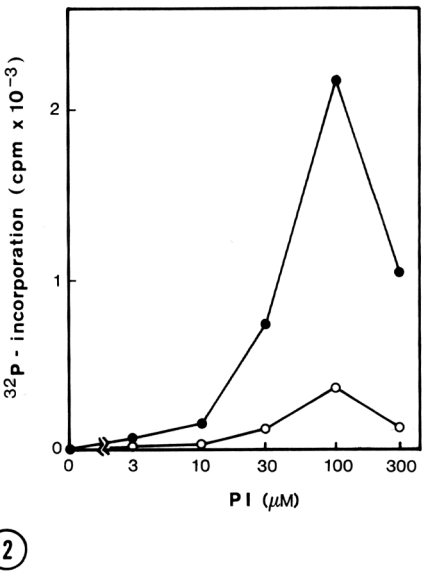


Fig.1. Effect of PI, PIP_2, PS and cGMP on phosphorylation of endogenous proteins in soluble fraction of ROS. Mixtures were incubated for 10 min at 30°C, and reaction products were analyzed by 17.5 % SDS-polyacrylamide gel electrophoresis followed by autoradography. (A) Protein pattern of soluble fraction. (B) Autoradiograms. lane 1, basal; lane 2, 0.1 mM PS; lane 3, 0.1 mM PI; lane 4, 0.1 mM PIP_2; lane 5, 1 mM cGMP.

Fig.2. Dependence of phosphorylation of components I and II on concentration of PI. Protein kinase activity was assayed under standard conditions except that PI was added as indicated. ○ component I, ● component II.

Acidic phospholipids other than PI, i.e. PS, PIP and PIP_2 did not activate the phosphorylation of the components I and II (Fig.1, data not shown for PIP). Therefore, the effect of PI on phosphorylation is not due to the nonspecific action of acidic phospholipid. On the other hand, it may be thought that the effect of PI is mediated by protein kinase C (phospholipid and Ca^{2+}-dependent protein kinase) which is activated by diacylglycerol derived from PI by the action of phospholipase C. However, this hypothesis was contradicted by the fact that PIP and PIP_2, which are good substrates of phospholipase C in the ROS (unpublished results), could not simulate the phosphorylation.

Table 1 indicates that the PI-dependent phosphorylation of components I and II was strongly inhibited by PIP and PIP_2 while

Table 1. Inhibition of PI-dependent phosphorylation of components I and II by polyphosphoinositides

Phospholipids	^{32}P-incorporation (cpm)	
	component I (ratio)	component II (ratio)
PI (100μM)	320 (1.00)	1,850 (1.00)
+ PS (30μM)	300 (0.94)	1,832 (0.99)
+ PIP (30μM)	90 (0.28)	756 (0.41)
+ PIP_2 (30μM)	90 (0.28)	444 (0.24)

The complete reaction mixture contained 10μl ROS soluble fraction (5μg protein), 100μM PI, 30μM phospholipids, 5 mM $MgCl_2$, 0.5 mM EGTA, 0.1 mM EDTA, 10^{-6}M free-Ca^{2+}, and 10μM [γ-^{32}P]ATP (1.1×10^4 cpm/pmol) in 100μl of 20 mM Tris-HCl(pH 7.5). Incubation was carried out at 30°C for 10 min.

PS showed no inhibition. The PI/PPI ratio in the ROS is sensitive to light stimulation (1-5). This ratio also seems to be sensitive to cytosolic Ca^{2+} concentration, since there is Ca^{2+}-sensitive phospholipase C in the ROS (unpublished results). The effect of light or Ca^{2+} on the phosphorylation of protein components I and II *in situ* ROS (6) or detached ROS (7) may be partly due to the changes in PI-dependent protein kinase activity following changes in the PI/PPI ratio in the micro environment of the kinase.

Hamm and Bownds showed that an anti-G protein antibody blocked phosphorylation of components I and II as well as light-induced cGMP-PDE in ROS. The simultaneous blocking suggests close association of the G protein-PDE complex on the disc membrane with the components I and II or their kinase. They also reported that components I and II were phosphorylated even in the absence of cGMP, and such basal phosphorylation of these proteins was susceptible to the antibody. Our results suggest that such a basal and antibody-sensitive phosphorylation was catalyzed by PI-dependent protein kinase which was activated by the PI in the ROS

membranes. Further studies are needed to know the relationship between PI-dependent phosphorylation of these two components and the antibody-sensitive regulatory mechanism of cGMP-PDE.

Fig.1-lane 4 shows that there are several protein components that are phosphorylated in a PPI-dependent manner in the ROS. PIP_2 stimulated the phosphorylation of 38, 48 and 52 kDa proteins, whereas no stimulation was observed on the phosphorylation of the 74 kDa protein. Similar results were obtained by the addition of PIP (data not shown). Such stimulation by PPI was completely arrested by the addition of 100μM PI (data not shown).

In summary, it was revealed that there are both PI-dependent and PPI-dependent protein kinase activities in the soluble fraction of ROS. The preferential substrates of the PI-dependent protein kinase were components I and II which are known to be the substrates of cGMP-dependent protein kinase in the ROS. The PI-dependent phosphorylation was inhibited by PPI. Such a reciprocal regulation of protein kinase by PI and PPI may be a common regulatory mechanism of a certain kind of protein kinase, though the species of the protein kinase in the ROS has not yet been identified.

ACKNOWLEDGMENTS

We thank Dr. Shinozawa, T. and Dr. Fukami, Y. for valuable discussion and encouragement. This investigation was partially supported by research grants No.60304098 and No.60580214 from the Scientific Research Fund of the Ministry of Education, Science and Culture in Japan.

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