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INHIBITION OF PHOSPHATIDYLINOSITOL-4-PHOSPHATE KINASE BY ITS PRODUCT PHOSPHATIDYLINOSITOL-4,5-BISPHOSPHATE

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Summary: Phosphatidylinositol 4,5-bisphosphate (PIP_2) is enzymatically produced when high speed supernatant fraction from bovine retina is incubated with [γ-^{32}P]ATP and phosphatidylinositol 4-phosphate (PIP) as substrates. Exogenously added PIP_2 inhibits PIP kinase activity 50% at equimolar concentrations of product and substrate. Ca^{2+}-dependent phosphodiesteratic activity, resulting in the loss of PIP_2 and PIP and concommitant increase in myo-inositol 1,4,5-trisphosphate and myo-inositol 1,4-bisphosphate, was observed when soluble retinal fractions were incubated with heat-inactivated ^{32}P-prelabeled guinea pig nerve ending membranes as substrate. It is suggested that polyphosphoinositides are under stringent and complex control and that upon receptor activation-mediated stimulation of phosphodiesteratic degradation release of the feedback inhibition shown here may occur and result in the synthesis and replenishment of PIP_2.

Upon specific receptor activation, the phosphodiesteratic degradation of phosphatidylinositol 4,5-bisphosphate (PIP_2) is stimulated (1,2). Both phosphodiesteratic degradation products, inositol 1,4,5-trisphosphate (IP_3) and diacylglycerol (DG), may have secondary messenger functions. IP_3 has been reported to stimulate the release of intracellular Ca^{2+} (3), while DG can modulate the Ca^{2+}sensitivity of the protein kinase C (4). PIP_2 is synthesized from phosphatidylinositol (PI) by two subsequent phosphorylation steps, through the intermediate, phosphatidylinositol

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ABBREVIATIONS USED: DG, diacylglycerol; IP_3, myo-inositol 1,4,5-trisphosphate; IP_2, myo-inositol 1,4-bisphosphate; PI, phosphatidylinositol; PIP, phosphatidylinositol 4-phosphate; PIP_2, phosphatidylinositol 4,5-bisphosphate

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4-phosphate (PIP). The PI kinase is found mostly in the plasma membrane (5,6), indicating the primary location of the polyphosphoinositides; the PIP kinase is found, for the most part, in the supernatant fraction (6). Receptor-mediated stimulation of the phosphodiesteratic degradation of PIP_2 may be expected to rapidly deplete the PIP_2 pool, unless it is replenished through PIP from PI. Little information is available on the regulation of the synthesis and levels of the polyphosphoinositides (7). The observation of Downes and Wusteman (8) that receptor activation resulted in a much smaller generation of inositol 1-phosphate when it was performed under conditions of cellular ATP depletion is suggestive of such a modulation. The mechanism most easily envisioned for such a regulation is an inhibition of PIP kinase by PIP_2. Phosphodiesteratic degradation of PIP_2 would release this feedback inhibition and hence, result in the synthesis and replenishment of PIP_2. We report that, using a PIP kinase containing high speed supernatant preparation of bovine retina, exogenously added PIP_2 has an inhibitory effect on PIP kinase.

MATERIALS AND METHODS

Fresh bovine eyes were obtained from a local abattoir, and retinas were removed under dim red light. After removal of rod outer segments (9), the remaining portion of the retina was suspended in buffer A (30 mM HEPES/NaOH, 0.25 mM EGTA, pH 7.4), frozen in liquid nitrogen and thawed at 37°C. This procedure was repeated twice, after which the material was centrifuged for 60 min at 100,000 x g. The supernatant was collected and dialyzed overnight against buffer A. An aliquot of the high speed supernatant fraction was incubated for 5 min at 37°C in buffer A, which also contained 10 mM $MgCl_2$, 50 M [γ-^{32}P]ATP (ICN Radiochemicals, Irvine, CA; about 10 µCi), and where indicated, about 100 µM PIP and PIP_2. The final volume was 200 µl. Incubation was terminated by the addition of 1.5 ml chloroform-methanol, 1:2, by vol. After the addition of approximately 25 µg of the Folch fraction I from bovine brain containing phosphoinositides (Sigma Chemical Co., St. Louis, MO), lipids were extracted under acidic conditions, separated by thin layer chromatography, visualized autoradiographically and by iodine staining, scraped and quantitated by liquid scintillation counting (10).

^{32}P-prelabeled nerve ending membranes were prepared from guinea pig cortex (10) and used as substrate for the assay of polyphosphoinositide phosphodiesterase activity. To inactivate the endogenous phosphodiesteratic activity in the membranes, the preparation was kept at 100°C for 15 min prior to use (11). An aliquot of the high speed supernatant fraction was incubated for 5 min at 37°C with heat-inactivated ^{32}P-prelabeled nerve ending membranes in buffer A (300 µl final vol), containing 1 mg/ml

deoxycholate and 1 mM ATP (11). Incubations were terminated and lipids extracted as described above and the water-soluble inositol polyphosphates were analyzed by high voltage paper electrophoresis (10).

Stock solutions of the polyphosphoinositides (Sigma Chemical) were made in water and their concentration was determined by spectrophotometry (12). Protein content was determined by the same method using bovine serum albumin as a standard (13).

RESULTS

When a high speed supernatant fraction of bovine retina was incubated with [γ-^{32}P]ATP and PIP as substrates, labeled PIP_2 was produced. The labeling of PIP_2 was linear with the enzyme concentration up to about 0.5 mg/ml of supernatant protein. When PIP_2 was included in the incubation medium, the labeling of PIP_2 was inhibited (Fig. 1). PIP_2 inhibited PIP-kinase activity about 50% at equimolar concentrations of product and substrate.

High speed supernatant fraction was incubated with heat-inactivated ^{32}P-prelabeled guinea pig nerve ending membranes to assay for phosphodiesteratic activity against the polyphosphoinositides. No degradation of the polyphosphoinositides was seen, unless Ca^{2+} was present (Table I). This Ca^{2+}-stimulated loss of label was linear with the supernatant

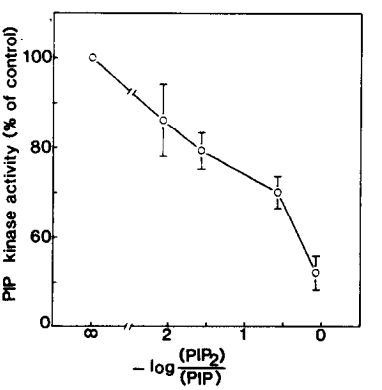

Figure 1. Effect of PIP_2 on the activity of PIP kinase.
An aliquot of the high speed retina supernatant (51.6 mg protein) was incubated as described in Materials and Methods. Various concentrations of PIP_2 also were present, as is indicated by the -log PIP_2/PIP. The concentration of PIP in this particular experiment was 62.5 µm. PIP kinase activity in the absence of added PIP_2 was 27.32 ± 0.13 fmol/µg protein per min. Each value represents the mean ± S.E.M. of the PIP_2-induced inhibition of PIP kinase activity from an incubation performed in triplicate, from an experiment representative of two. Error bars have been ommitted when less than the symbol.

TABLE I: POLYPHOSPHOINOSITIDE PHOSPHODIESTERASE IN RETINA HIGH SPEED SUPERNATANT

	PIP_2	IP_3	PIP	IP_2	PI	PA
Zero Time	32041	n.a.	39962	n.a.	19868	56719
5 Min						
- Ca	32769	3694	41358	17155	20368	57308
+ Ca	24378	20511	26069	39759	19024	57177

An aliquot of high speed supernatant fraction (25.8 mg protein) was incubated with ^{32}P-prelabeled nerve ending membranes, as described in Materials and Methods, in the absence or presence of 1.25 mM free Ca^{2+}. Values are the mean of the radiotracer (cpm) recovered as each of the lipids and inositol phosphates from an incubation in duplicate. n.a. not analyzed.
PA, phosphatidate; see text for abbreviations.

protein concentration under the present conditions employed (data not shown). Deoxycholic acid was added to improve the assay for the use of membranes as substrate (11). The addition of Ca^{2+} to the incubation medium caused a loss of label from PIP_2 and PIP with a concomitant increase in the label recovered as IP_3 and IP_2 (Table I), indicating phosphodiesteratic activity in the retinal high speed supernatant. No decrease in the labeling of PI and phosphatidate was observed.

DISCUSSION

To the best of our knowledge this is the first time that a product feedback inhibition of PIP-kinase has been demonstrated. Prior to this, inhibition has been assumed, for the interpretation of data, from receptor-mediated stimulation of the inositide cycle (1,2,8). Our observations imply a simple mechanism for the regulation and replenishment of the PIP_2 pool. A decreased concentration of PIP_2 caused by its phosphodiesteratic degradation results in a release of the PIP-kinase inhibition, and hence, the rapid replenishment of the PIP_2 pool. Such a mechanism is relevant, inasmuch as upon receptor activation, more inositol phosphates are produced than a static pool of PIP_2 could have generated. One possible explanation for the latter discrepancy is that all three inositides are degraded. However,

analysis of the production of all three inositol phosphates during stimulation indicates a sequential production of inositol 1,4,5-trisphosphate, inositol 1,4-bisphosphate and inositol 1-phosphate (1). This observation suggests that PIP_2 is primarily degraded and that IP_3 is subsequently broken down through IP_2 and IP to inositol (1,14). This model is also consistent with the observations that phosphodiesteratic degradation of endogenous PI in isolated membranes does not occur (Table I, 10). These findings also indicate the presence of phosphodiesteratic activity against the polyphosphoinositides of the retina. It is interesting that only a small amount of PIP kinase and PIP and PIP_2 phosphodiesterase is attached to the membranes (11,15). It is feasible that this location and a possible translocation between soluble and membrane-bound forms of the enzymes comprise a mode of regulation. Another mode of regulation of PIP_2 synthesis has been reported by Gispen and collaborators (15). They showed that the activity of the PIP kinase is under the control of protein phosphorylation-mediated events. Because receptor activation-mediated stimulation of the phosphodiesteratic degradation of the polyphosphoinositides is a widely distributed phenomenon, it would not be surprising if the metabolism of polyphosphoinositides is under stringent and complex control. Our data provide further insight into the mechanism of control for PIP_2 turnover by indicating that this control can be altered by physiological stimulation of a cell.

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