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TWO DISTINCT MEMBRANE-BOUND PHOSPHATIDYLINOSITOL-4-PHOSPHATE PHOSPHATASES IN BOVINE BRAIN

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SUMMARY: Solubilized phosphatidylinositol-4-phosphate 4-phosphatase from bovine brain resolved into two peaks of activity by ion exchange chromatography. Both exhibited substantial detergent binding characteristic of integral membrane proteins, and both appear specific for phosphatidylinositol-4-phosphate, but their pH optima differ: the earlier eluting fraction (peak 1) is optimally active between pH 5.5 and 6, whereas the later eluting fraction (peak 2) is most active around pH 8.5. Detergent inhibition studies suggest that peak 2, but not peak 1, interacts with phosphatidylinositol-4-phosphate in the context of a single mixed micelle. Further characterization of these activities should help shed light on the biological function of polyphosphoinositide phosphatases.

The role of inositol phospholipids in transmembrane signaling and cell stimulation is firmly established. Enzyme activities that interconvert phosphoinositides by phosphorylation and dephosphorylation were known long before the significance of polyphosphoinositides was understood (reviewed in 1,2). Phosphatidylinositol 4-kinase and phosphatidylinositol-4-phosphate 5-kinase are clearly essential for production of phosphatidylinositol-4,5-bisphosphate, the precursor of the phospholipase C-generated second messengers, diacylglycerol and inositol-1,4,5-trisphosphate. The role of phosphomonoesterases that remove phosphate groups from polyphosphoinositides is less clear. These activities have been identified in several tissues by in vitro studies (2), and in isolated membranes, the phosphate monoesters of phosphoinositides have been found to turn over much faster than the phosphodiester linkage (3).

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Abbreviations: MES, 2-(N-morpholino)ethanesulfonic acid; PIP, phosphatidylinositol-4-phosphate; PMSF, phenylmethylsulfonyl fluoride; TAPS, N-tris(hydroxymethyl)methyl-3-aminopropanesulfonic acid.

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Like the hexose phosphate phosphatases of carbohydrate metabolism, phosphoinositide phosphatases may be involved in a deeper level of regulation than is now apparent. Evolved mechanisms not yet appreciated may exist for exerting critical control over the steady state levels or production rate of polyphosphoinositides. On the other hand, kinases and phosphatases may be isolated in distinct cellular compartments acting on different substrate pools. Alternatively, phosphate removal might be catalyzed by poorly-defined phosphatases of broad specificity. Similar questions were raised ten years ago by Downes and Michell (4), and have not yet been resolved.

As an approach to understanding the function of phosphomonoester hydrolysis, we have begun to characterize brain enzymes that can remove the phosphate from phosphatidylinositol-4-phosphate (PIP). Because others have found PIP phosphatase activity enriched in membranes (5), we have focused on the bovine brain particulate fraction. Here we describe separation of two distinct 4-phosphatases and initial studies of their physical and kinetic properties.

MATERIALS AND METHODS

Materials. Bovine brains (Pel-Freez Biologicals, Rogers, AR), bovine liver phosphatidylinositol (Avanti Polar Lipids, Pelham, AL), bovine brain PIP (Sigma, St. Louis, MO), [^{3}H]PIP, and [γ-^{32}P]ATP (Du Pont-NEN, Boston, MA) were obtained from the indicated suppliers. Enzymes were obtained from Sigma. Other materials and reagents were from Aldrich (Milwaukee, WI) or Sigma.

Preparation of [^{32}P]PIP. A mixture containing 50 mM Tris·HCl (pH 7.5), 1 mM EGTA, 20 mM $MgCl_2$, 0.5% (8 mM) Triton X-100, 1 mM phosphatidylinositol, 10 μM [γ-^{32}P]ATP (25-50 μCi), and 50 μl (1.7 μg) rat brain phosphatidylinositol 4-kinase (6) in a final volume of 100 μl was incubated at 30°C for 90 minutes. The reaction was stopped by adding sequentially 3 ml chloroform/methanol, 1:2 (v/v); 0.7 ml 1 M $MgCl_2$, 0.2 M HCl; 1 ml chloroform; and 1 ml 1 M $MgCl_2$, 0.2 M HCl. After mixing and spinning to separate phases, the lower phase was dried and lipids were separated by two-dimensional thin-layer chromatography on Silica Gel 60 plates (E. Merck, Darmstadt), with chloroform/methanol/water/acetic acid, 25:15:4:2 (v/v) in the first dimension, and chloroform/methanol/4 M NH_4OH, 9:7:2 (v/v) in the second dimension. Radioactivity was located by autoradiography, and PIP was eluted from the gel.

PIP phosphatase assay. PIP phosphatase activity was measured in a mixture containing 50 mM Tris·HCl (pH 7.5), 5 mM $MgCl_2$, 0.045% (total) Triton X-100, 10 μM [^{32}P]PIP (40,000 cpm/nmol, 20,000 cpm/assay), and 5-50 μg protein in a final volume of 50 μl. In studies of pH dependence and specificity, buffers other than Tris were used as indicated. After incubation for one hour at 30°C, the reaction was stopped by adding 2 ml chloroform/methanol, 1:1 (v/v) and 0.85 ml of 1 M $MgCl_2$, 0.2 M HCl. After mixing and separating phases in a clinical centrifuge, 0.9 ml of upper phase was removed and mixed with 4 ml scintillation cocktail, and radioactivity was determined. The reaction rate was linear for at least an hour. Parallel assays using [^{3}H]PIP and [^{32}P]PIP confirmed that enzymes being studied were phosphatases rather than phosphodiesterases; ^{3}H release was undetectable (<2% of ^{32}P release). Numerical data reported here are the means from duplicate determinations which generally varied less than 5%.

Preparation of salt-washed particulate fraction. All operations were carried out at 4°C. One brain was weighed, cut into 2-cm pieces, and homogenized in a Waring blender at full speed for one minute with two volumes of buffer A (20 mM Tris·HCl (pH 8), 10 mM 2-mercaptoethanol) containing 1 mM EDTA and 1 mM PMSF. Two volumes more of the same buffer were added, and the suspension was filtered through two layers of cheesecloth. The total particulate fraction was harvested by centrifuging at 13,000 x g for 30 minutes, and resuspended using a Teflon/glass homogenizer in two volumes of buffer A. Solid NaCl was added to a final concentration of 1 M, and the suspension was stirred for 30 minutes. After centrifuging at 125,000 x g for 30 minutes, the pellet was resuspended in buffer A to a protein concentration of 10 mg/ml and stored at -80°C.

Sucrose density gradient centrifugation. Gradients of 4.5 ml, 5-20% (w/v) sucrose were prepared over 0.5 ml 60% sucrose. The buffer was 20 mM Tris·HCl (pH 7.5), 0.05% Triton X-100. Samples (2-5 µl) of standard enzymes (egg white lysozyme, porcine liver malate dehydrogenase, bovine liver catalase) were mixed with 100 µl PIP phosphatase and gently layered on top of the gradients, which were centrifuged in a Beckman SW 50.1 rotor for 19 hours at 45,000 rpm. Fractions were collected from the top by displacement with 80% sucrose. Standard enzymes were assayed as described (7).

Miscellaneous procedures. Protein was assayed as described by Peterson (8) and phosphate was determined by the method of Ames and Dubin (9).

RESULTS AND DISCUSSION

Ion exchange chromatography of solubilized PIP phosphatase. A sample (40 ml) of salt-washed particulate fraction was thawed. After adding 0.4 g Triton X-100, the mixture was stirred for 30 minutes at 0°C. Insoluble matter was removed by centrifugation for one hour at 120,000 x g. PIP phosphatase activity was recovered almost completely (>90%) in the supernatant. The supernatant was applied to a column of Q-Sepharose (2.5 x 10 cm) equilibrated with buffer A containing 0.05% Triton X-100, and PIP phosphatase was eluted with a linear gradient from 0 to 0.6 M NaCl over five column volumes. Two peaks emerged of approximately equal activity, with peak 1 appearing at about 0.16 M and peak 2 at about 0.31 M NaCl (not shown).

Among several preparations, the relative amounts of peak 1 and peak 2 varied. The variability was due to sensitivity of peak 1 to thawing conditions. When the particulate fraction was preincubated at room temperature for one hour either before or after Triton addition, peak 1 was quantitatively lost with no decrease in peak 2. However, if the solubilization mixture was centrifuged first, peak 1 was unaffected by room temperature incubation, indicating that a detergent-insoluble factor in the particulate fraction was responsible for peak 1 instability.

Triton X-100 binding to PIP phosphatases. PIP phosphatase release from the particulate fraction by detergent could indicate either solubilization of a membrane-bound enzyme or release of a soluble, membrane-sequestered enzyme. To determine the nature of the enzymes' membrane association, as

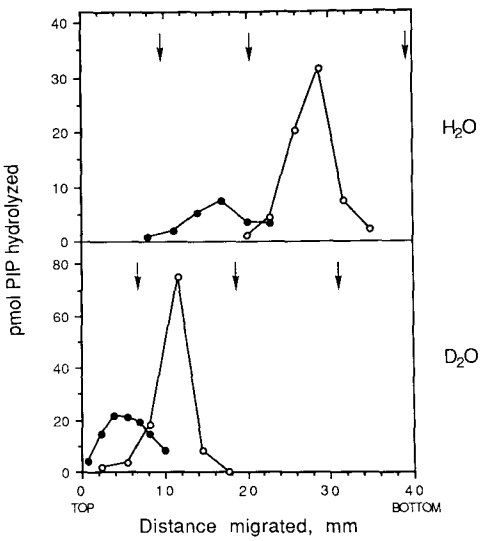

Figure 1. Sucrose density gradient sedimentation of PIP phosphatase activities. Positions of standard proteins are indicated by arrows: (left to right) egg white lysozyme, porcine heart malate dehydrogenase, bovine liver catalase. Peak 1 (–o–) and peak 2 (–●–) were assayed as described in Materials and Methods.

well as to provide some information about their kinetics, we examined their ability to bind nonionic detergents. The sedimentation method used is generally applicable (10) and has been used to study detergent binding by mammalian phosphatidylinositol kinases (11,12). PIP phosphatase migration in sucrose gradients prepared with either water or D_2O was compared with that of soluble protein standards (Figure 1). All proteins migrated more slowly in the denser medium, but the effect was much more pronounced with peak 1 and peak 2 relative to the standards, reflecting the decreased density imparted to the enzymes by substantial detergent binding. Nonionic detergent binding by peaks 1 and 2 indicates that both are probably intrinsic proteins bound to membranes in vivo by hydrophobic interactions and suggests they interact with substrate in vitro in the context of detergent/PIP/enzyme mixed micelles.

Detergent inhibition of PIP phosphatase. PIP phosphatase has been reported to be inhibited by nonionic detergent (13). This would be expected if the reaction occurs in a mixed micelle, since detergent would dilute the effective PIP concentration resulting in apparent rather than true inhibition. The effects of increasing Triton X-100 concentration at a fixed PIP concentration are shown in Figure 2 for peaks 1 and 2, as well as for soluble alkaline and acid phosphatases. Activities of the latter were significantly decreased at 0.07% Triton X-100 but decreased only slightly at higher concentrations. Peak 1 behaved similarly, but peak 2 activity decreased progressively to barely detectable levels as the detergent concentration was increased to 0.52% Triton X-100.

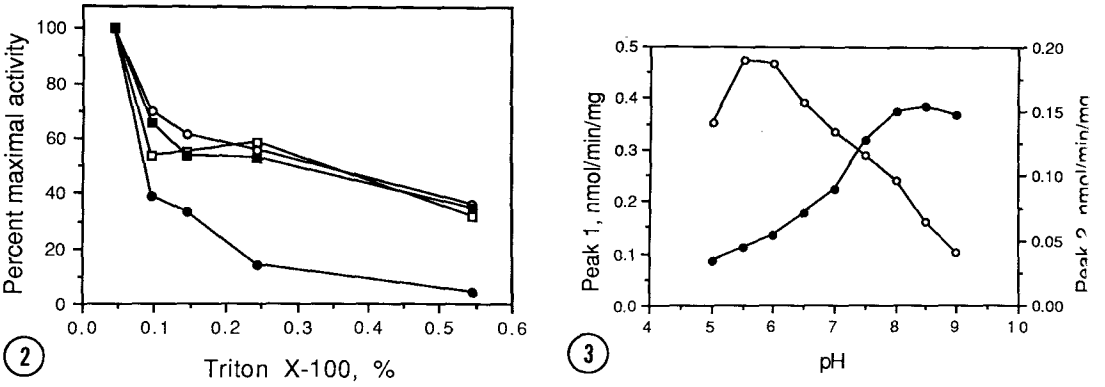

Figure 2. Triton X-100 inhibition of PIP phosphatase hydrolysis by peak 1 (–o–), peak 2 (–●–), E. coli alkaline phosphatase (– □ –), and potato acid phosphatase (–■–). PIP concentration was constant at 10 μM. Amounts of alkaline and acid phosphatases were chosen to give rates of PIP hydrolysis comparable to those shown by peak 1 and peak 2.

Figure 3. pH dependence of PIP phosphatase activity. Peak 1 (–o–) and peak 2 (–●–) were assayed using Na-succinate (pH 5, 5.5, 6), Na-MES (pH 6.5), BisTris-HCl (pH 7), Tris-HCl (pH 7.5, 8, 8.5), and Na-TAPS (pH 9). Results of a representative experiment are shown; similar experiments with other buffers gave similar results.

Dependence of activity on pH. pH profiles of peak 1 and peak 2 activity are shown in Figure 3. Although both showed moderate activity at pH 7.5, peak 2 exhibited a broad optimum centered between pH 8 and 8.5, whereas peak 1 was most active at a more acidic pH between 5.5 and 6.

Substrate specificity. Specificities of peaks 1 and 2 were compared with those of commercial alkaline and acid phosphatases by testing the ability of various unlabeled phosphate esters to inhibit radioactive PIP hydrolysis (Figure 4). PIP hydrolysis by alkaline phosphatase was strongly inhibited by DL-glycerol-3-phosphate, glycerol-2-phosphate, and glucose-6-phosphate at 5 and 20 mM. Acid phosphatase was less sensitive to soluble phosphate esters but was nevertheless inhibited 40-50% by 20 mM concentrations of all three. In contrast, there was no significant inhibition of peak 1 or peak 2 even at 20 mM phosphate esters, and in some cases activity was stimulated.

Conclusions. Finding two peaks of PIP phosphatase activity by ion exchange chromatography was unexpected and prompted comparison of their properties for evidence suggesting that either or both may be specifically involved in phosphoinositide interconversion. Peaks 1 and 2 both behave as integral membrane proteins and are clearly phosphomonoesterases rather than C- or D-type phospholipases. Their pH profiles were distinct. That of peak 1 resembles the pH dependence of hydrolytic enzymes localized in acidic cellular compartments, whereas peak 2 shows a slightly alkaline optimum.

Detergent inhibition of lipid-metabolizing enzymes, as examined in the experiment of Figure 2, is open to several interpretations. Direct inhibition of

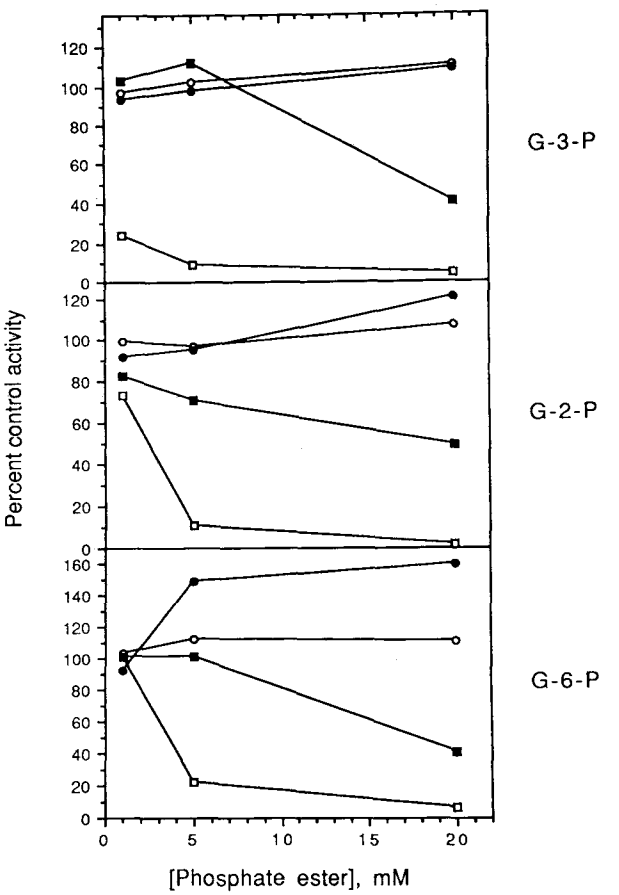

Figure 4. Inhibition of PIP hydrolysis by soluble phosphate esters. Peak 1 (–o–) and potato acid phosphatase (–■–) were assayed using succinate buffer at pH 6, and peak 2 (–●–) and E. coli alkaline phosphatase (–□–) were assayed using Tris buffer at pH 8.5 in the presence of varying concentrations of DL-glycerol-3-phosphate (G-3-P), glycerol-2-phosphate (G-2-P), and D-glucose-6-phosphate (G-6-P).

enzyme by detergent monomers is unlikely because the monomer concentration does not change appreciably over the concentration range used. Neither is it likely that enzymes may be directly inhibited by increased concentrations of detergent micelles, as the data of Figure 1 indicate that both peak 1 and peak 2 are already micelle-associated at a Triton X-100 concentration of 0.05%, and it is hard to envision how further increasing the number of micelles might alter their activities. It is more likely that sensitivity of peak 2 to detergent concentration results from dilution of the micellar PIP concentration almost twenty-fold from an initial value of 2 mol% to 0.1 mol% at the highest detergent concentration used. It is more difficult to explain the kinetic behavior of peak 1. Its similarity to the soluble phosphatases argues that the topography of its interaction with PIP differs from that of peak 2, and could be interpreted to indicate that peak 1 hydrolyzes PIP in a different micelle.

The absence of inhibition of peak 1 or peak 2 by other phosphate esters suggests they are more specific for PIP than are soluble alkaline or acid phosphatases. It is possible that peak 1 or peak 2 could nonspecifically hydrolyze other lipid phosphate esters naturally occurring in cells such as phosphatidic acid, phosphatidylglycerophosphate, or other polyphosphoinositides. Competition studies with unlabeled phospholipids in a micellar system are difficult to interpret, and further clarification of the specificity of these enzymes must await their purification and testing with labeled substrates.

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