Inositol polyphosphate 4-phosphatase is a monomeric 110-kDa protein that hydrolyzes two substrates in the inositol phosphate pathway. Inositol 3,4-bisphosphate is converted to inositol 3-phosphate, and inositol 1,3,4-trisphosphate is converted to inositol 1,3-bisphosphate. We have exploited the fact that inositol hexosulphate inhibits the enzyme to devise an affinity elution scheme from a Mono S cation exchange column that resulted in an 11,300-fold purified preparation of rat brain 4-phosphatase. The resulting 4-phosphatase hydrolyzed phospha	
dositol 3,4-bisphosphate to phosphatidylinositol 3-phosphate with a first order rate constant 120-fold greater than that for inositol 3,4-bisphosphate and 900-fold greater than that for inositol 1,3,4-trisphosphate. This is now the third example wherein the same enzyme hydrolyzes both an inositol lipid and its analogous inositol phosphate.

The agonist-stimulated formation of inositol lipids phosphorylated at the 3-position has recently been demonstrated in many cell types (1,2). These lipids include phosphatidylinositol 3-phosphate (PtdIns(3)P),1 phosphatidylinositol 3,4-bisphosphate (PtdIns(3,4)P2), and phosphatidylinositol 3,4,5-trisphosphate (PtdIns(3,4,5)P3). Stimulation with Met-Leu-Phe leads to a rapid and transient increase in the last two compounds mentioned above in neutrophils (3–5) while responses to growth factors in proliferating cells result in more sustained increases in these compounds (6). In human platelets, PtdIns(3,4)P2 and PtdIns(3,4,5)P3 are synthesized transiently in response to thrombin (4,5). The thrombin-dependent synthesis of PtdIns(3,4,5)P3 in platelets is blocked by the peptide RGDS that inhibits fibrinogen binding to $\alpha_{\text{IIb}}$$\beta_3$ integrin and blocks platelet aggregation. Platelets obtained from patients with thrombasthenia lack $\alpha_{\text{IIb}}$$\beta_3$ integrin and have attenuated synthesis of PtdIns(3,4,5)P3 upon thrombin stimulation suggesting a role for PtdIns(3,4,5)P3 in signaling following platelet aggregation (7). Chinese hamster ovary cells expressing a mutant PDGF receptor do not synthesize 3-phosphate-containing inositol lipids in response to PDGF and do not undergo mitogenesis (8,9). The physiological target(s) for these potential second messengers is not known. However, the $\gamma$ isozyme of protein kinase C has been shown to be stimulated in vitro by PtdIns(3,4)P2 and PtdIns(3,4,5)P3 (10). The quantities of these lipids even at the point of maximal stimulation of cells is small compared with that of other phosphatidylinositols. In a recent measurement of the mass of PtdIns(3,4)P2 in thrombin-stimulated platelets it was found that the level of this lipid was about one-tenth that of PtdIns(4,5)P2 (11).

Several pathways for the metabolism of 3-phosphate-containing inositol lipids have been suggested. In human platelets and NIH 3T3 cells, labeling studies indicate that phosphorylation occurs stepwise beginning with PtdIns(3)P with subsequent phosphorylation of the 4- and 5-positions (12,13). A recent study indicates that this same pathway accounts for the production of these lipids in plants (14). A 4-kinase present in human platelets that phosphorylates PtdIns(3)P has been described (15). However, PtdIns 3-kinase can phosphorylate both PtdIns(4)P and PtdIns(4,5)P2 in vitro (16). Other labeling studies suggest that PtdIns(3,4,5)P3 is formed by the phosphorylation of the 3-position of PtdIns(4,5)P2 in neutrophils (17).

The pathways for degradation of PtdIns(3,4)P2 and PtdIns(3,4,5)P3 have not been characterized. They are not hydrolyzed by any known phospholipase C isoenzymes, and thus it appears most likely that they are degraded by phosphatases (18,19). PtdIns(3)P is degraded by a 3-phosphatase (20).

It was recently reported that the phosphatase that hydrolyzes the 5-phosphate from PtdIns(4,5)P2 is the type II isozyme of inositol polypeptide 5-phosphatase (21). Similarly, the 3-phosphatase that degrades PtdIns(3)P is the same enzyme that degrades the corresponding water-soluble inositol phosphate Ins(1,3)P2 (22). Bansal et al. (23) previously described an inositol polypeptide 4-phosphatase that hydrolyzes the phosphate from the 4-position of both Ins(3,4,5)P3 and Ins(3,4)P2. In this report, we show that a corresponding lipid substrate, PtdIns(3,4,5)P3, is rapidly metabolized by a 4-phosphatase preparation isolated using a novel affinity elution purification method. This is now the third example wherein the same enzyme hydrolyzes both inositol lipid and its analogous inositol phosphate.

**EXPERIMENTAL PROCEDURES**

Materials—[3H]Ins(1,3,4)P3 (17 Ci/mmol) and [γ-32P]ATP were purchased from DuPont NEN. [3H]Ins(3,4)P2 and [3H]Ins(1,3)P2 were synthesized by treating [3H]Ins(1,3,4)P3 with recombinant inositol polypeptide 1-phosphatase (24) and purified inositol polypeptide 4-phosphatase (25), respectively. Dioleoyl phosphatidylethanolamine was purchased from Avanti Polar Lipids. PtdIns(4)P and PtdIns(4,5)P2 were

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1 The abbreviations used are: PtdIns(3)P, phosphatidylinositol 3-phosphate; PtdIns(4)P, phosphatidylinositol 4-phosphate; PtdIns(3,4)P2, phosphatidylinositol 3,4-bisphosphate; PtdIns(3,4,5)P3, phosphatidylinositol 3,4,5-trisphosphate; PtdIns(4,5)P2, phosphatidylinositol 4,5-bisphosphate; PDGF, platelet-derived growth factor; PMSF, phenylmethylsulfonyl fluoride; Mops, 4-morpholinepropanesulfonic acid; HPLC, high pressure liquid chromatography; Tricine, N[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]glycine.
Hydrolysis of Phosphatidylinositol 3,4-Biphosphate

purchased from Boehringer Mannheim. [32P]PtdIns(4)P was the gift of Dr. Linda Pike. Rabbit anti-human PDGF type B receptor polyclonal antibody and PDGF were purchased from Upstate Biotechnology, Inc. Phytic acid (inositol hexakisphosphate), inositol hexasulfate, hexadimethrine bromide, n-octyl glucoside, and PtdIns(4)P were purchased from Sigma. All other chemicals were from Sigma or Fisher. Rat brains were from Pel-Freeze Biologicals (ROGERS, A.).

Assay of Inositol Polyphosphate 4-Phosphatase—The assay for inositol polyphosphate 4-phosphatase using the soluble substrates was performed as previously described (25) in 20 ml of 50 mM Mops and 10 mM EDTA (assay buffer 1). The enzyme fractions obtained following the affinity elution purification step were assayed using assay buffer 1 with the addition of 0.3% hexadimethrine bromide in order to reverse the InsS6 inhibition. First order rate constant assays for soluable inositol phosphates were performed in 20 ml of 50 mM Mops, 10 mM EDTA, 200 mM NaCl, and 0.3% n-octyl glucoside (assay buffer 2) with 3000 cpm of [32P]PtdIns(3,4,5)P3 or [32P]PtdIns(3,4,5)P3 (17 mC/mM) without additional unlabeled substrate. The first order rate constant assay for [32P]PtdIns(3,4,5)P3 was carried out in 20 ml using assay buffer 2, and the reaction was stopped by the addition of 30 ml of chloroform/methanol (1:1), and the fraction of [32P]PtdIns(3,4,5)P3 converted to [32P]PtdIns(3,4)P2 was determined by TLC.

Preparation of [32P]PtdIns(3,4,5)P3—The procedure for preparation of [32P]PtdIns(3,4,5)P3 was adapted from that of Carpenter et al. (16). NIH 3T3 cells were grown to 80% confluence on T75 flasks in Dulbecco’s modified Eagle’s medium containing 10% calf serum and then incubated in 0.5% calf serum for 24 h after which the media were removed and the cells were incubated at 37 °C for 15 min with media containing 0.3% hexadimethrine bromide. The media were decanted, and the cells were washed with 10 mM sodium phosphate, pH 7.0, containing 0.15 mM NaCl (phosphate-buffered saline). Lysis buffer (1.4 ml containing 20 mM Tris, pH 7.4, 50 mM NaCl, 50 mM NaF, 20 mM sodium pyrophosphate, 1% Triton X-100, 200 mM sodium orthovanadate, 1 mM PMSF, and 20 μg of leupeptin/ml) was added to each plate, and the cells were harvested by scraping. The lysate was incubated for 20 min on ice and then centrifuged at 5000 g for 10 min. Polyclonal rabbit anti-human PDGF receptor type B antibody (0.1 ml/mg lystate protein) was added, and samples were rotated for 3 h at 4 °C. Protein A-Sepharose CL-4B (0.2 volume) was added, and after 2 h, the mixture was washed with 100 mM Tris, pH 7.5, 2000 ml LiCl and 200 μM sodium orthovanadate; 100 mM Tris, pH 7.5, containing 200 mM NaCl, 1 mM EDTA, and 1 mM EGTA; 20 mM HEPES, pH 7.4 containing 0.5 mM EDTA and 1 mM MgCl2.

PtdIns(4)P (1 mg) and phosphatidylycerine (0.8 mg) in CHCl3 were dried under N2 and suspended in 2 ml of 20 mM HEPES pH 7.4 containing 0.5 mM EDTA and 1 mM MgCl2. The lipid mixture was then suspended in ice at 100 wattle for 2 min with a probe sonicator, and then 2 ml of an immunoprecipitate (1:1, v/v) was added. [32P]ATP (3 mCi of 3300 Ci/mM) was then added, and the mixture was rotated for 1 h at 37 °C. The reaction mixture was extracted using chloroform/methanol, and the organic layer was washed with an equal volume of 2 x KCl. A typical preparation yielded 5-10 μCi of [32P]PtdIns(4)P. PtdIns(4,5)P2 and PtdIns(4,5,4,5)P2 were prepared identical except that PtdIns(4,5)P2/phosphatidylycerine vesicles (1:1, molar/mol) and PtdIns vesicles were used, respectively.

Thin Layer Chromatography—Silica Gel 60 TLC plates were treated with a solution of 1% potassium oxalate in 50% ethanol. The plates were then placed in a 90 °C oven for at least 30 min prior to use. TLC plates were developed using 20 ml of 20 mM HEPES pH 7.4 containing 0.5 mM EDTA and 1 mM MgCl2. The lipid mixture was then suspended in ice at 100 wattle for 2 min with a probe sonicator, and then 2 ml of an immunoprecipitate (1:1, v/v) was added. [32P]ATP (3 mCi of 3300 Ci/mM) was then added, and the mixture was rotated for 1 h at 37 °C. The reaction mixture was extracted using chloroform/methanol, and the organic layer was washed with an equal volume of 2 x KCl. A typical preparation yielded 5-10 μCi of [32P]PtdIns(4)P. PtdIns(4,5)P2 and PtdIns(4,5,4,5)P2 were prepared identical except that PtdIns(4,5)P2 phosphatidylycerine vesicles (1:1, molar/mol) and PtdIns vesicles were used, respectively.

Proof of Product—Deacylation and deglyceration of inositol lipids were performed as described previously (20). Water-soluble inositol lipids were separated by HPLC on a Whatman Partisil 10 SAX column with a flow rate of 1 ml/min using the following gradients of ammonium formate, pH 3.5: a linear gradient of 40-425 mM over 30 min, a step to 810 mM followed by a linear gradient to 1.3 x over 35 min, and a step to 17 mM followed by a linear gradient to 3 x over 55 min.

Preparation of Rat Brain Homogenate for Phosphocellulose Batch Chromatography, and DEAE-HPLC—Rat brain homogenate was prepared from 3000 frozen unstripped rat brains (wet weight, 445 g). Phospho-

![FIG. 1. Inositol hexasulfate affinity elution of 4-phosphatase from Mono S. 20 μg of the 35% ammonium sulfate pool was loaded on a Mono S 5/5 column. The column was washed with 0.1 mM InsP6 to remove a major contaminating protein. The 4-phosphatase activity was then eluted with 0.5 mM InsS6. Solid circles, protein.](image)
...tases (27, 28).

performed in assay buffer 1.

Ins(1,3,4)P3 in the presence of molecular mass of 160 kDa was then eluted with 0.1 mM InsP6.

ployed to purify various glycolytic enzymes and tRNA synthetases (27, 28).

The partially purified enzyme was bound to a Mono S column at pH 8.5. The column was then washed extensively and re-equilibrated to pH 7.2. The major contaminating protein with a molecular mass of 160 kDa was then eluted with 0.1 mM InsP6. The 4-phosphatase was then eluted with 0.5 mM InsP6 with the addition of the polycationic polymer, hexadimethrine bromide, to the assay buffer, which reversed both InsPs and InsP6.

60-80% recovery in several experiments (Fig. 1). The problem of assaying fractions containing inhibitor ligands was solved by the addition of the polyacrylamide, hexadimethrine bromide, to the assay buffer, which reversed both InsP6 and InsP5 inhibition. Since the activity eluted as a broad peak that did not correspond to the protein profile, the active fractions were combined into two pools. Pool 1 (fractions 15 and 16) contained several contaminating proteins whereas pool 2 (fractions 17-19) had a significantly higher specific activity as shown in Fig. 2 with an apparent Km of 19 µM. The concentration of InsP6 in HL60 cells has been reported to be 50 µM (30). InsP6 may therefore inhibit cytosolic 4-phosphatase suggesting a possible role in regulating enzyme activity.

Inositol polyphosphate 4-phosphatase hydrolyzes the 4-phosphate from both Ins(1,3,4)P3 and Ins(3,4,5)P3 but not Ins(4)P2, Ins(1,4,P, Ins(1,4,5)P3, or Ins(1,3,4,5)P4 (25). In order to determine if the 4-phosphatase also hydrolyzes an analogous lipid, PtdIns(3,4)P2 was synthesized with a 32P label at the 3-position using PtdIns 3-kinase as described under "Experimental Procedures." The 32P-labeled product was deacylated and deglycerated and run on a Partisil SAX HPLC column as described under "Experimental Procedures" and the resulting 32P-labeled product comigrated with PtdIns(3)P on TLC as shown in Fig. 3 (inset). The 32P-labeled product was deacylated and deglycerated and run on a Partisil 10 SAX HPLC column as described under "Experimental Procedures," and the resulting 32P-labeled product comigrated with a standard of [3H]Ins(1,3)P2 consistent with hydrolysis by the 4-phosphatase (Fig. 4). No hydrolysis of [32P]PtdIns(3)P and [32P]PtdIns(4)P on [32P]PtdIns(3,4,5)P3 was detected after treatment with 4-phosphatase.

The rate of hydrolysis of PtdIns(3,4)P2 using 0.3 and 0.6 ng/ml pool 2 is shown in Fig. 3. An interesting characteristic of

![Table I](image)

**Purification of the inositol polyphosphate 4-phosphatase**

| Step            | Volume | Total activity | Protein | Specific activity | Purification | Yield |
|-----------------|--------|----------------|---------|-------------------|--------------|-------|
| Homogenate      | 19,900 | 352            | 9.4     | 0.00189           | 1.0          | 100   |
| Supernatant     | 22,600 | 139            | 4.3     | 0.00142           | 0.75         | 39    |
| Phosphocellulase| 27,100 | 79             | 0.60    | 0.00482           | 2.6          | 22    |
| Ammonium sulfate (55%) | 2,150 | 41             | 2.5     | 0.00770           | 4.0          | 12    |
| DEAE-HPLC + ammonium sulfate (35%) | 17 | 459 | 19.0 | 0.142 | 75.0 | 13    |
| Affinity elution from Mono S load | 1 | 27 | 19.0 | 0.142 | 75.0 | 1   |
| Pool 1          | 2      | 0.72           | 0.056   | 6.43              | 3,400        | 3.5   |
| Pool 2          | 10     | 1.5            | 0.007   | 21.4              | 11,500       | 7     |

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a Assays were carried out using 10 µM [3H]Ins(3,4)P2.

b 6% of the 35% ammonium sulfate cut was loaded on Mono S. Yield figures for this step are normalized to the entire preparation.

c Pool 2 was concentrated to 0.5 ml prior to protein assay.

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![Figure 2](image)

**Fig. 2.** Lineweaver-Burk kinetic analysis of inositol hexakisphosphate inhibition. Lineweaver-Burk plot of the hydrolysis of Ins(3,4,5)P3 in the presence of 0, 20, and 40 µM InsP6. Assays were performed in assay buffer 1.

![Figure 3](image)

**Fig. 3.** Time course of PtdIns(3,4)P2 hydrolysis by purified 4-phosphatase. The fraction of PtdIns(3,4)P2 converted to PtdIns(3)P by 0.3 and 0.6 ng/ml purified 4-phosphatase (pool 2) in assay buffer 2 is plotted as a function of time as determined by the TLC assay. Each point shown is the average of duplicate assays. The inset shows an autoradiogram of a TLC plate indicating time-dependent conversion of PtdIns(3,4)P2 to PtdIns(3)P.

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![Table II](image)

**Table II**

| Tube   | Volume | Total activity | Protein | Specific activity | Purification | Yield |
|--------|--------|----------------|---------|-------------------|--------------|-------|
| Homogenate | 19,900 | 352           | 9.4     | 0.00189           | 1.0          | 100   |
| Supernatant | 22,600 | 139          | 4.3     | 0.00142           | 0.75         | 39    |
| Phosphocellulase | 27,100 | 79          | 0.60    | 0.00482           | 2.6          | 22    |
| Ammonium sulfate (55%) | 2,150 | 41          | 2.5     | 0.00770           | 4.0          | 12    |
| DEAE-HPLC + ammonium sulfate (35%) | 17 | 459          | 19.0    | 0.142             | 75.0         | 13    |
| Affinity elution from Mono S load | 1 | 27          | 19.0    | 0.142             | 75.0         | 1   |
| Pool 1 | 2      | 0.72         | 0.056   | 6.43              | 3,400        | 3.5   |
| Pool 2 | 10     | 1.5          | 0.007   | 21.4              | 11,500       | 7     |

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![Figure 4](image)

**Fig. 4.** TLC diagram of a TLC plate indicating time-dependent conversion of PtdIns(3,4)P2 to PtdIns(3)P.
4-phosphatase hydrolysis of the lipid substrate is that the amount of substrate hydrolyzed was directly proportional to the amount of enzyme added over a wide range of enzyme concentrations. The maximum fraction of PtdIns(3,4)P$_2$ hydrolyzed was approximately 25 and 50% upon the addition of 0.3 or 0.6 ng/ml pool 2, respectively, as shown in Fig. 3. The fraction of PtdIns(3,4)P$_2$ hydrolyzed by 0.3 ng/ml was doubled to 50% when the phospholipid concentration in the assay was halved, and when the enzyme concentration was raised to 5 ng/ml all of the substrate was hydrolyzed. Furthermore, the apparent first order rate constants for the hydrolysis of PtdIns(3,4)P$_2$ by these two enzyme concentrations are essentially identical as shown in Fig. 5, yielding values of 2.6 and 2.7 × 10$^{-2}$ s$^{-1}$ for 0.3 and 0.6 ng/ml, respectively. This deviates from the ideal behavior of first order enzyme kinetics in which the rate constant is directly proportional to enzyme concentration. An explanation for this behavior is that irreversible binding of the 4-phosphatase to the surface of the phospholipid vesicles prevents intervesicle enzyme movement. For the case wherein the number of enzyme molecules is less than the number of phospholipid vesicles, the fraction of PtdIns(3,4)P$_2$ hydrolyzed is proportional to the fraction of vesicles that possess at least one bound enzyme molecule and hence proportional to the amount of enzyme added. The observed rate constant is insensitive to the concentration of enzyme until the number of enzyme molecules is greater than the number of phospholipid vesicles. Consistent with this hypothesis, the preincubation of enzyme with phosphatidylserine-PtdIns(4)P vesicles used in the assays inhibits subsequent PtdIns(3,4)P$_2$ hydrolysis.

The first order rate constants for hydrolysis of Ins(1,3,4)P$_3$ and Ins(3,4)P$_2$ were determined using trace-labeled substrate. The observed rate constants for Ins(1,3,4)P$_3$ and Ins(3,4)P$_2$ in the presence of 0.6 ng/ml pool 2 were 3.0 × 10$^{-5}$ s$^{-1}$ and 2.2 × 10$^{-5}$ s$^{-1}$, respectively, as shown in Fig. 6. The comparison of the lipid versus the soluble substrates by first order rate constants is confounded by the fact that the observed first order rate constant for the lipid substrate is independent of the amount of enzyme added. However, the apparent first order rate constant for hydrolysis of PtdIns(3,4)P$_2$ under these conditions was 900-fold greater than that of Ins(1,3,4)P$_3$ and 120-fold greater than that of Ins(3,4)P$_2$. While the $V_{max}/K_m$ values for the two water-soluble substrates under the conditions used for Michaelis kinetics were similar, the apparent first order rate constant for Ins(3,4)P$_2$ obtained using assay buffer 2 is higher than that of Ins(1,3,4)P$_3$ (Fig. 6) because NaCl stimulates the hydrolysis of Ins(3,4)P$_2$ by 4-phosphatase.

In order to confirm that the activity responsible for the hydrolysis of the soluble and lipid substrates was a result of the same enzyme, a heat inactivation experiment was performed. Since the amount of $^{32}$P-PtdIns(3,4)P$_2$ hydrolyzed is directly proportional to the amount of 4-phosphatase added, the amount of lipid phosphatase activity remaining at various time points could be determined by the maximum $^{32}$P-PtdIns(3,4)P$_2$.
Hydrolized during the assay. This was confirmed in a preliminary experiment where it was shown that the fraction of substrate hydrolyzed was linear from 0.075 to 0.6 ng of enzyme/ml. The rate of activity loss using PtdIns(3,4)P$_2$ ($t_0$ = 10 min) and Ins(3,4)P$_2$ was the same upon heating at 45 °C, indicating that the same enzyme hydrolyzes both substrates as shown in Fig. 7.

Several phosphoinositols have recently been shown to hydrolyze both soluble and lipid substrates. The inositol polyphosphate 4-phosphatase hydrolyzes the lipid substrate, PtdIns(3,4)P$_2$ (21). We have demonstrated now that inositol polyphosphate 5-phosphatase type I hydrolyzes the 5-phosphoester of both Ins(1,4,5)P$_3$ and PtdIns(3,4)P$_2$ (22). The same enzyme hydrolyzes both substrates as shown in Fig. 8.

Several phosphoinositol phosphatases have recently been shown to hydrolyze both soluble and lipid substrates. The inositol polyphosphate 3-phosphatase has been shown to hydrolyze the 3-phosphoester of Ins(1,3)P$_2$ and PtdIns(3)P (22). The rate of activity loss using PtdIns(3,4)P$_2$ (21). We have demonstrated now that inositol polyphosphate 4-phosphatase hydrolyzes the lipid substrate, PtdIns(3,4)P$_2$, and therefore may play a role in controlling cellular levels of 3-phosphate-containing phosphatidylinositols.

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