# Enzymatic Synthesis of Pyrene-Labeled Polyphosphoinositides and Their Behavior in Organic Solvents and Phosphatidylcholine Bilayers

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ABSTRACT: A method is reported for the synthesis of pyrene-labeled analogues of phosphatidylinositol 4-phosphate (Pyr-PIP) and phosphatidylinositol 4,5-bisphosphate (Pyr-$PIP_2$) from *sn*-2-(pyrenyldecanoyl)phosphatidylinositol (Pyr-PI) using partially purified PI and PIP kinase preparations. Phosphorylation of Pyr-PI and Pyr-PIP was extensive (more than 50%) provided that the ATP concentration was high and that stabilizing agents such as sucrose and polyethylene glycol were present in the incubation medium. Pyr-PIP and Pyr-$PIP_2$ were isolated by chromatography on immobilized neomycin. The identity of the products was established by thin-layer chromatography, UV-absorption spectroscopy, and spectrofluorometry. The pyrene excimer/monomer fluorescence technique revealed that, in contrast to Pyr-PI, Pyr-PIP and Pyr-$PIP_2$ formed clusters in organic solvents. By use of the same technique for model membranes, it was shown that in phosphatidylcholine bilayers the collision frequency of the three fluorescent phosphoinositides decreased in the order PI > PIP > $PIP_2$. Addition of $Ca^{2+}$ at concentrations above 0.1 mM increased the collision frequency of Pyr-$PIP_2$ and, to a much lesser extent, Pyr-PIP; $Ca^{2+}$ had no effect on Pyr-PI.

Phosphatidylinositol 4,5-bisphosphate ($PIP_2$)[1] is a key molecule in signal transduction processes (Berridge, 1984). Upon binding of agonists to cell membrane receptors, specific phospholipases C are activated which degrade $PIP_2$, yielding inositol 1,4,5-trisphosphate ($IP_3$) and diacylglycerol as second messengers (Berridge & Irvine, 1984; Volpe et al., 1988; Nishizuka, 1984, 1986). This process is probably under control of guanine triphosphate binding proteins (G proteins) (Crockroft, 1987).

Although many studies have focused on the stimulus-induced breakdown of $PIP_2$, very little is known about the behavior of this phosphoinositide in membranes. At physiological pH, $PIP_2$ has a charge of −4.2 and PIP a charge of −3 (Van Paridon et al., 1986). As a result of this high negative charge, $PIP_2$ in water forms micelles (Hendrickson, 1969). Studies with cations (Toner et al., 1988) and neomycin (Wang et al., 1984; Reid & Gajjar, 1987; Gabev et al., 1989) have provided evidence that, at physiological concentrations, magnesium and polyamines are the predominant counterions of $PIP_2$. $PIP_2$ has also been shown to strongly interact with proteins like protein 4.1 and glycophorin (Anderson & Marchesi, 1985), prophilactin and prophilin (Lassing & Lindberg, 1985, 1988), fibrin (Vickers et al., 1987), and gelsolin (Janmey et al., 1987; Hiyosi et al., 1989) and the phosphatidylinositol-transfer protein (Van Paridon et al., 1988). Apart from some NMR studies (Van Paridon et al., 1986; Reid & Gajjar, 1987; Toner et al., 1988), detailed spectroscopic studies on $PIP_2$ have not been performed because of the lack of $PIP_2$ analogues carrying reporter groups.

One of the reporter groups commonly used in membrane research has been the fluorescent pyrene moiety coupled to fatty acyl chains. Pyrene-labeled phospholipids show two kinds of fluorescence emission spectra: a monomer spectrum and an excimer spectrum originating from excited dimers. The ratio of excimer to monomer fluorescence ($E/M$) is proportional to the collision frequency of the pyrene moieties, which is dependent on concentration and diffusion (Förster, 1969). This special property has been used to obtain information about the distribution and dynamics of (pyrene)phospholipids in membranes, i.e., membrane-phase transitions (Galla & Hartmann, 1980; Somerharju et al., 1985; Mustonen et al., 1987; Ollmann et al., 1987; Viani et al., 1988), phase separation phenomena (Galla & Hartman, 1980), lateral and interbilayer distribution (Galla & Hartman, 1980; Somerharju et al., 1985; Homan & Pownall, 1988), and critical micelle concentration (cmc) (Ollmann et al., 1987). Apart from excimer/monomer studies, pyrene-labeled lipids can be used to obtain information about lipid–protein interaction (Mustonen et al., 1987), lipid movement and distribution in cells (Radom et al., 1987; Gatt et al., 1988), lipid movement between model membranes (Massey et al., 1984; Jones & Thompson, 1989), protein-mediated lipid transfer (Van Paridon et al., 1987a, 1988), and membrane fusion processes (Pal et al., 1988).

In a previous study (Somerharju et al., 1985) the behavior of pyrenylacyl-labeled PI (Pyr-PI) in membranes was investigated. Here we describe the synthesis of Pyr-PIP and Pyr-$PIP_2$ using partially purified PI and PIP kinase preparations. From the fluorescent properties of the pyrene moiety (i.e., $E/M$) we were able to assess the aggregation state of

[1] Abbreviations: Pyr-PI, *sn*-2-(pyrenyldecanoyl)phosphatidylinositol; Pyr-PIP, *sn*-2-(pyrenyldecanoyl)phosphatidylinositol 4-phosphate; Pyr-$PIP_2$, *sn*-2-(pyrenyldecanoyl)phosphatidylinositol 4,5-bisphosphate; Pyr-PC, *sn*-2-(pyrenyldecanoyl)phosphatidylcholine; PI, phosphatidylinositol; PIP, phosphatidylinositol 4-phosphate; $PIP_2$, phosphatidylinositol 4,5-bisphosphate; PC, phosphatidylcholine; PA, phosphatidic acid; PS, phosphatidylserine; $IP_3$, inositol 1,4,5-trisphosphate; PI-TP, phosphatidylinositol-transfer protein; cmc, critical micelle concentration; ATP, adenosine triphosphate; DEAE, diethylaminoethyl; TLC, thin-layer chromatography, DMSO, dimethyl sulfoxide; Tris, tris(hydroxymethyl)aminomethane; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; EDTA, ethylenediaminetetraacetic acid; PEG 20 000, polyethylene glycol 20 000; PMSF, phenylmethanesulfonyl fluoride; BSA, bovine serum albumin; $E/M$, ratio of excimer to monomer fluorescence; $\lambda_{ex}$, excitation wavelength; $\lambda_{em}$, emission wavelength.

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these phosphoinositides in organic solvents as well as their lateral mobility in vesicle membranes.

## Experimental Procedures

### *Materials*

Egg PC, soybean and lima bean trypsin inhibitors, and leupeptin were from Sigma. [$\gamma$-$^{32}$P]ATP (specific activity 3 Ci/$\mu$mol) was from Amersham. PIP was purified from bovine brain according to the method of Hendrickson and Ballou (1964). A carrier–lipid mixture containing PS, PI, PIP, and $PIP_2$ was prepared from bovine brain as described by Kiselev (1982). ATP and neomycin sulfate were obtained from Boeringer; DEAE-cellulose (DE-52), CM-cellulose (CM-52), and phosphocellulose (P11) from Whatman and controlled-pore glass CPG/200 glycophase G (pore diameter 200 Å, particle size 37–39 $\mu$m) were from Pierce. Thin-layer chromatography plates (Kieselgel 60, 0.25-mm thickness) were purchased from Merck. Triton X-100 was from Serva, and dimethyl sulfoxide and ethanol (uvasol) were from Merck. Other solvents were freshly distilled before use. All other chemicals were of reagent grade.

### *Methods*

*Preparation of PI from Yeast.* PI was extracted from yeast as described by Trevelyan (1966) and purified by acetone precipitation (Kates, 1972) and borate-impregnated silica gel column chromatography (Somerharju & Wirtz, 1982). The resulting PI preparation contained PC as a major contaminant. Further purification on a CM-cellulose column yielded pure PI in the chloroform/methanol (1:1 v/v) fraction (Comfurius & Zwaal, 1977).

*Synthesis of Pyr-PI and Pyr-PC. sn*-2-(Pyrenyldecanoyl)-PI (Pyr-PI) was synthesized from yeast PI as described by Somerharju and Wirtz (1982) and Somerharju et al. (1985). Degradation with phospholipase $A_2$ (*Crotalus adamanteus*) and with PI-specific phospholipase C (generous gift of Dr. Somerharju) showed that Pyr-PI was more than 98% pure. *sn*-2-(Pyrenyldecanoyl)-PC (Pyr-PC) was synthesized from egg PC as described (Somerharju et al., 1985).

*Extraction of PI and PIP Kinase from Bovine Brain.* All procedures were carried out at 4 °C. A 25% (w/v) homogenate of three bovine brains (1034 g) was prepared in 50 mM Tris-HCl, pH 7.4, 1 mM EDTA, 0.3% $\beta$-mercaptoethanol, and 1 mM PMSF containing 1 mg/L soybean and lima bean trypsin inhibitor and 0.5 mg/L leupeptin. The brains were homogenized within 2 h after slaughter and, prior to homogenization, stripped from their cerebral membranes. After centrifugation (10000*g*, 1 h), the supernatant was discarded and the pellet was washed once (25% w/v) by homogenization in the same buffer. PIP kinase was released from the washed membrane pellet by making a suspension (25% w/v) in 50 mM Tris-HCl, pH 7.4, 1 mM EDTA, 0.3% $\beta$-mercaptoethanol, 150 mM NaCl, and 0.1 mM PMSF followed by stirring for 30 min. After sedimentation of the membranes (10000*g*, 1 h), the supernatant containing PIP kinase was stored at 4 °C. Prior to storage, PIP kinase activity was stabilized by adding to the supernatant fraction sucrose (0.25 M), Triton X-100 (0.1%), PEG 20000 (0.1%), and ATP (50 $\mu$M).

The ensuing membrane pellet contained PI kinase. Storage at −20 °C after freezing in $CO_2$/acetone resulted in a 10% loss of activity per month. To release PI kinase, the membrane pellet (an aliquot of 55 g) was suspended by use of a Waring blender, in a buffer (165 mL) containing 50 mM Tris-HCl, pH 7.4, 1 mM EDTA, 0.3% $\beta$-mercaptoethanol, 0.1% PEG 20000, 75 mM KCl, 1% Triton X-100, and 0.1 mM PMSF. After stirring for 30 min, the suspension was centrifuged (16000*g*, 1.5 h), and the ensuing supernatant containing PI kinase was collected. Additional stabilizing components were added to the supernatant to yield the composition of buffer A: 50 mM Tris-HCl, pH 7.4, 1 mM EDTA, 0.25 M sucrose, 0.3% $\beta$-mercaptoethanol, 1% Triton X-100, 0.1% PEG 20000, 30 mM KCl, 50 $\mu$M ATP, and 0.1 mM PMSF.

*Partial Purification of PI and PIP Kinase.* The membrane extract containing PI kinase (200 mL) was applied onto a DEAE-cellulose column (2.2 × 25 cm) that was equilibrated with buffer A. The column was rinsed with 200 mL of buffer A (flow rate of 2 mL/min, fractions of 20 mL). The bulk of PI kinase activity was recovered from the run-through. The active fractions were pooled (250 mL) and applied on a phosphocellulose column (1.5 × 6 cm) that was also equilibrated with buffer A. After the column was rinsed with 150 mL of buffer A (flow rate 12.5 mL/h), PI kinase was eluted with 40 mL of buffer A containing 1.2 M NaCl (flow rate 10 mL/h, fractions of 2 mL). The eluent containing PI kinase activity (8 mL) was pink.

PIP kinase released from bovine brain membranes was partially purified by a procedure described elsewhere (Moritz et al., 1990).

*PI and PIP Kinase Assays.* PI kinase and PIP kinase activity were assayed essentially as described by Van Dongen et al. (1984). The incubation medium (volume of 50 $\mu$L) consisted of 100 $\mu$M PI, 15 $\mu$M [$\gamma$-$^{32}$P]ATP (1.5 $\mu$Ci), 50 mM Tris-HCl, pH 7.4, 1 mM EGTA, 10 mM $MgCl_2$, 120 mM NaCl, 0.3% $\beta$-mercaptoethanol, 0.4 mg/mL BSA, and 1% Triton X-100. After preincubation for 5 min at 30 °C, phosphorylation was started by addition of PI kinase (aliquot of 10 $\mu$L) and continued for 10 min at 30 °C. The reaction was terminated by addition of 2 mL of chloroform/methanol/12 M HCl (200:100:0.75 v/v). Then a mixture of carrier lipids consisting of PI, PIP, $PIP_2$, and PS (20–100 nmol each) was added, and phase separation was induced by addition of 0.6 M HCl (0.35 mL). The lower phase was washed once with 1 mL of chloroform/methanol/0.6 M HCl (3:48:47 v/v) (Jolles et al., 1981) and dried by a stream of $N_2$ at 50 °C. Lipids were redissolved in chloroform/methanol/water (75:25:2 v/v), applied on an oxalate-impregnated TLC plate, and separated by chloroform/acetone/methanol/acetic acid/water (40:15:13:12:7.5 v/v). Lipids were identified by iodine staining. After the iodine was sublimed, the area containing [$^{32}$P]PIP was scraped from the plate and counted by liquid scintillation spectrometry (Jolles et al., 1981).

PIP kinase activity was assayed under the same conditions except that PIP was used as a substrate and the Triton X-100 concentration was reduced to 0.02%.

*Synthesis of Pyr-PIP.* Pyr-PI (4 $\mu$mol) was taken to dryness from a chloroform solution by a stream of argon in a small glass-stoppered flask and suspended in 50 mM Tris-HCl, pH 7.4, and 0.3% $\beta$-mercaptoethanol (24 mL, argon saturated). After vigorous vortexing and sonication in a water bath, a homogeneous turbid solution was obtained that was intensely fluorescent (light blue) under UV light. To this suspension was added a solution containing 50 mM Tris-HCl, pH 7.4, 5 mM ATP, 100 mM $MgCl_2$, 5 mM EGTA, 0.3% $\beta$-mercaptoethanol, 4 mM EDTA, 0.4% PEG 20000, and 1 M sucrose (8 mL, argon saturated). The phosphorylation of Pyr-PI was initiated by addition of the PI kinase preparation (8 mL, see above). The composition of the final reaction mixture was 100 $\mu$M Pyr-PI, 1 mM ATP, 0.2% Triton X-100, 50 mM Tris-HCl, pH 7.4, 20 mM $MgCl_2$, 1 mM EGTA, 1 mM EDTA, 0.24 M NaCl, 6 mM KCl, 0.3% $\beta$-mercapto-

ethanol, 250 mM sucrose, 0.1% PEG 20000, and 0.02 mM PMSF. Due to the presence of Triton X-100, the fluorescence of the reaction mixture changed from light blue (excimer) to dark blue (monomer) under UV light. After 1 h at 30 °C, additional ATP (40 μmol) was added and the incubation continued for another 1.5 h. The reaction was terminated by addition of 3 volumes of chloroform/methanol/12 M HCl (100:200:0.75 v/v). Phase separation was induced by addition of 2 volumes of chloroform and 1 volume of 0.6 M HCl. The lower phase was washed twice with 1 volume of chloroform/methanol/0.6 M HCl (3:48:47 v/v). The organic solvents were removed by rotary evaporator, and the lipid residue was dissolved in 8 mL of chloroform/methanol (1:1 v/v). All solvents used were argon saturated.

Pyr-PIP was purified on a column (1.1 × 2 cm) of neomycin immobilized on controlled-pore glass glycophase (Schacht, 1978). After application of the reaction product, the column was rinsed with 12 mL of chloroform/methanol (1:1 v/v) and 10 mL of chloroform/methanol (1:2 v/v). Pyr-PI and Pyr-PIP were separated by use of a stepwise ammonium formate gradient (0–1 M) in chloroform/methanol/water (5:10:2 v/v, total volume 110 mL) (Palmer, 1981). Additional Pyr-PIP was recovered from the column by elution with chloroform/methanol/15 M $NH_4OH$ (3:6:1 v/v and 3:6:2 v/v, 10 mL each). For further details see the legend to Figure 2. After elution, each fraction was acidified at once with HCl and extracted as described by Palmer (1981). Solvents used were argon saturated. Pyr-PIP was stored in chloroform under argon atmosphere at −80 °C. Purity was checked by TLC under UV light (Jolles et al., 1981).

*Synthesis of Pyr-$PIP_2$.* Pure Pyr-PIP (512 nmol) was taken to dryness from chloroform and converted into Pyr-$PIP_2$ by the action of PIP kinase [1 mL, total activity of 1.13 nmol/min; specific activity of 904 nmol/$min^{-1}$ (mg of protein)$^{-1}$ under standard radioactive assay conditions]. The reaction was carried out under conditions similar to those used for the synthesis of Pyr-PIP (in a total volume of 5.1 mL). The reaction mixture consisted of 100 μM Pyr-PIP, 1 mM ATP, 0.02% Triton X-100, 50 mM Tris-HCl, pH 7.4, 20 mM $MgCl_2$, 1 mM EGTA, 1 mM EDTA, 0.20 M NaCl, 0.3% β-mercaptoethanol, 250 mM sucrose, 0.1% PEG 20000, and 0.02 mM PMSF. The extraction was similar to that described for Pyr-PIP.

Pyr-$PIP_2$ was purified on an immobilized neomycin column (1.1 × 2 cm). After the column was rinsed with chloroform/methanol (1:1 v/v and 1:2 v/v, 10 mL each), Pyr-$PIP_2$ was separated from Pyr-PIP by elution with a stepwise gradient of ammonium formate (0–600 mM) in chloroform/methanol/water (5:10:2 v/v, total volume 90 mL). A small additional amount of Pyr-$PIP_2$ was recovered from the column by elution with chloroform/methanol/15 M $NH_4OH$ (3:6:1 v/v, 20 mL). Each fraction was acidified at once and extracted as described by Palmer (1981). Separation was checked by TLC (see above). Pyr-$PIP_2$ was stored in chloroform/methanol (7:1 v/v) under argon at −80 °C.

*Analysis of Pyr-PIP and Pyr-$PIP_2$.* The concentration of Pyr-PIP and Pyr-$PIP_2$ was estimated by measuring the absorption at 342 nm in ethanol/DMSO (75:25 v/v) ($\epsilon$ = 39700 $M^{-1}$ $cm^{-1}$) and by phosphorus determination (Rouser et al., 1970). To investigate the intactness of the pyrene moiety after synthesis, absorption spectra of Pyr-PIP and -$PIP_2$ were recorded on a Hitachi U-3200 spectrophotometer. Fluorescence excitation and emission spectra were recorded on a SLM-Aminco SPF-500 C spectrofluorometer equipped with a thermostated cuvette holder (25 °C).

Table I: Reaction Conditions for PI and PIP Kinase[a]

| component | reaction mixture I | II | III | IV | V |
|---|---|---|---|---|---|
| ATP (μM) | 15 | 50 | 50 | 2000[b] | 2000[b] |
| Triton X-100 (% v/v) | 1 | 1 | 0.2 | 0.2[c] | 0.2[c] |
| $MgCl_2$ (mM) | 10 | 10 | 50 | 50 | 20 |
| NaCl (M) | 0.36 | 0.36 | 0.24 | 0.24 | 0.24 |
| EDTA (mM) | 0.2 | 0.2 | 0.2 | 0.2 | 1 |
| sucrose (mM) | 50 | 50 | 50 | 50 | 250 |
| PEG 20000 (% w/v) | 0.02 | 0.02 | 0.02 | 0.02 | 0.1 |
| KCl (mM) | 6 | 6 | 6 | 6[d] | 6[d] |
| BSA (mg/mL) | 0.4 | 0.4 | | | |

[a] The lipid–substrate concentration was 100 μM. Each incubation was carried out at pH 7.4, 50 mM Tris-HCl, 1 mM EGTA, 20 μM PMSF, and 0.3% β-percaptoethanol; final volume was 50 μL (including 10 μL of PI or PIP kinase preparation). Temperature was 30 °C; incubation time was 2.5 h. [b] 1 mM during the first incubation h, then increased to 2 mM. [c] 0.02% for PIP kinase incubation. [d] Omitted for PIP kinase incubation.

*Lipid Vesicle Preparation.* Single bilayer vesicles consisting of pyrenylacyl-containing phospholipids and egg PC (9:91 mol %) were prepared by ultrasonication. A lipid suspension (44 μM total phospholipid) in 20 mM Tris-HCl, pH 7.4, and 100 mM NaCl was sonified with a Branson probe sonifier (output 50 W) under $N_2$ atmosphere at 0 °C for 9 min (5 s on, 10 s off). Interruption of sonication was necessary to prevent degradation of $PIP_2$ (Toner et al., 1988).

*Fluorescent Measurements.* The experiments were carried out at 25 °C. Single bilayer vesicles (aliquot of 25 μL) were pipetted into a cuvette containing 20 mM Tris-HCl, pH 7.4, and 100 mM NaCl (final volume of 2 mL). Fluorescence emission spectra were recorded ($\lambda_{ex}$ = 346 nm, $\lambda_{em}$ = 360–560 nm, slits 2.5 and 4 nm, respectively) under continuous stirring of the sample. Spectra were corrected for background fluorescence (Raman peak). The excimer to monomer fluorescent ratio ($E/M$) was calculated by dividing the fluorescence intensity at 475 nm by the intensity at 377 nm. Since at these wavelengths the contribution of the excimer to the monomer fluorescence and vice versa is very low (less than 1.5%), no further corrections were required (Somerharju et al., 1985). The buffers used for the fluorescent experiments were routinely filtered through a Millipore filter (0.45 mm).

## Results

*Optimal Incubation Conditions.* To obtain an optimal phosphorylation by PI kinase and PIP kinase, several incubation conditions were tested (Table I). Under the conditions of the standard radioactive assay (condition I) using Pyr-PI and cold ATP (incubation time 2.5 h), no formation of Pyr-PIP was detectable (lane I, Figure 1). Moreover, no fluorescent degradation products of Pyr-PI (Pyr–fatty acid or Pyr–diglyceride) were observed, indicating that the PI kinase preparation used did not contain phospholipase $A_2$ or C activity. Increasing the ATP concentration to 50 μM (condition II) did not give any detectable Pyr-PIP formation either (lane II, Figure 1). However, a decrease of the Triton X-100 concentration to 0.2%, omission of BSA, and an increase of the $MgCl_2$ concentration to 50 mM (condition III) yielded the first evidence that Pyr-PI can be a substrate for PI kinase (lane III, Figure 1). A subsequent increase of the ATP concentration to 2 mM (i.e., a 20-fold excess over the Pyr-PI concentration) (condition IV) led to an approximate 50% conversion of Pyr-PI into Pyr-PIP (lane IV, Figure 1). This conversion was increased even further (up to approximately 80%, see lane V of Figure 1) by raising the sucrose, EDTA, and PEG 20000 concentrations by a factor of 5 (condition V).

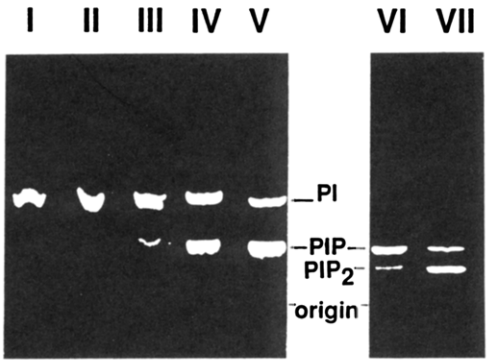


FIGURE 1: Thin-layer chromatogram of Pyr-PI, -PIP, and -$PIP_2$ after various conditions of phosphorylation by PI and PIP kinase. Lanes I–V correspond with conditions I–V in Table I for PI kinase; lanes VI and VII correspond with conditions IV and V in Table I for PIP kinase. After 2.5 h, the reaction mixtures were extracted and applied on TLC as described under Experimental Procedures. The fluorescent lipids as visualized by UV light are shown. The identity of the lipids was confirmed by lipid standards containing PI, PIP, and $PIP_2$, after $I_2$ staining and detection by phosphorus spray (Dittmer & Lester, 1964).

The phosphorylation reaction was carried out for 2.5 h as a time course study indicated that the reaction was complete after 2–3 h (data not shown).

The conditions tested for the conversion of Pyr-PIP into Pyr-$PIP_2$ by PIP kinase were similar to conditions IV and V used for PI kinase, except that the Triton X-100 concentration was decreased to 0.02% and KCl was omitted. Under condition IV (Table I) PIP kinase was capable of phosphorylating Pyr-PIP into Pyr-$PIP_2$, indicating that PIP kinase is also able to phosphorylate a pyrene-labeled phosphoinositide (lane VI, Figure 1). Under conditions where the concentrations of the stabilizing agents were raised (condition V, Table I), conversion of Pyr-PIP was drastically increased to approximately 80% (lane VII, Figure 1). Under all conditions tested there was no evidence for the presence of a PIP phosphomonoesterase or phospholipases $A_2$ or C.

*Synthesis of Pyr-PIP and -$PIP_2$.* In a large-scale synthesis of Pyr-PIP, 4 μmol of Pyr-PI was used as substrate (condition V, Table I). The reaction product was purified on immobilized neomycin (for elution conditions see the legend to Figure 2). As shown in Figure 2, Pyr-PIP (fractions 11–17) was completely separated from unreacted Pyr-PI (fractions 3–6) and Triton X-100 (fraction 1). The elution of Pyr-PIP began at 400 mM ammonium formate as also observed for unlabeled PIP (Palmer, 1981). Total yield of Pyr-PIP was 543 nmol on the basis of pyrene absorption. The product had a phosphorus to pyrene mole ratio of 2.66 (Rouser et al., 1970). This ratio is higher than expected due to the presence of a small amount of PI present in the PI kinase preparation, which also became phosphorylated. The yield was lower than observed in the small-scale synthesis partly due to the fact that the PI kinase preparation used had lost activity as a result of long storage (6 months). The total recovery of fluorescent lipids (including Pyr-PI) was 84%.

Pyr-PIP (512 nmol, on the basis of pyrene absorption) was converted into Pyr-$PIP_2$ by PIP kinase under condition V (Table I). Pyr-$PIP_2$ was purified on an immobilized neomycin column as described under Experimental Procedures. As shown in Figure 3 unreacted Pyr-PIP eluted at 400 mM ammonium formate (fraction 6), whereas the bulk of Pyr-$PIP_2$ eluted at 600 mM ammonium formate (fractions 10 and 11). It was previously observed that unlabeled $PIP_2$ eluted at 1 M ammonium formate (Palmer, 1981). The yield of Pyr-$PIP_2$ was 237 nmol on the basis of pyrene absorption; the total

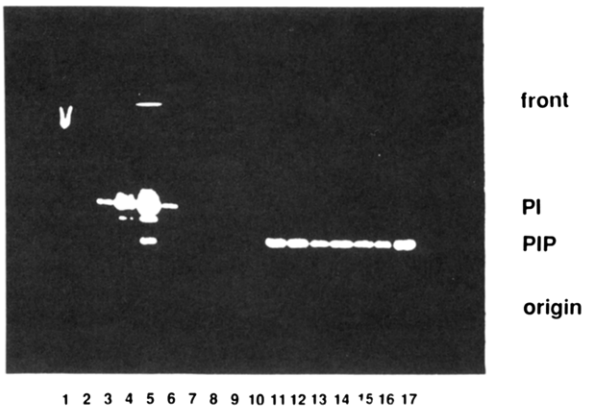


FIGURE 2: Purification of Pyr-PIP on immobilized neomycin as checked by TLC. The mixture of Pyr-PI and Pyr-PIP, resulting from the large-scale synthesis, was applied on immobilized neomycin as described under Experimental Procedures. The column was rinsed with 20 mL of chloroform/methanol (1:1 v/v) and with 10 mL of chloroform/methanol (1:2 v/v) (fractions 1 and 2, respectively). Pyr-PIP was eluted in a stepwise gradient of ammonium formate in chloroforma/methanol/water (5:10:2 v/v). Fractions 3–14 (10 mL each) were obtained from eluting with 0, 20, 20, 20, 40, 40, 100, 200, 400, 400, 600, and 1000 mM ammonium formate, respectively. An additional amount of Pyr-PIP eluted in chloroform/methanol/15 M $NH_4OH$ (3:6:1 v/v) (fractions 15 and 16, 10 mL each), and in 10 mL of chloroform/methanol/15 M $NH_4OH$ (3:6:2 v/v) (fraction 17). After acidification, the lipids were extracted and small aliquots were applied on TLC (see Experimental Procedures). The lipids as detected by UV light are shown.

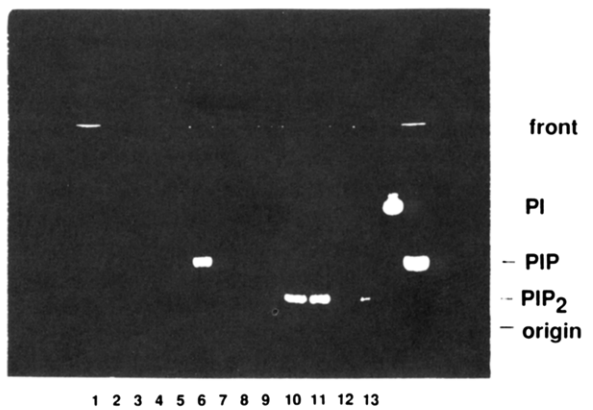


FIGURE 3: Purification of Pyr-$PIP_2$ on immobilized neomycin as checked by TLC. The lipid extract resulting from the Pyr-$PIP_2$ synthesis was applied on immobilized neomycin as described under Experimental Procedures. The column was rinsed subsequently with 10 mL of chloroform/methanol (1:1 v/v) and with 10 mL of chloroform/methanol (1:2 v/v) (fractions 1 and 2, respectively). Then a gradient of ammonium formate in chloroform/methanol/water (5:10:2 v/v) was applied to elute Pyr-$PIP_2$. The ammonium formate concentrations in fractions 3–11 were 0, 40, 200, 400, 400, 400, 400, 600, and 600 mM, respectively. Rinsing with chloroform/methanol/15 M $NH_4OH$ (3:6:1 v/v) (fractions 12 and 13, 10 mL each) eluted a small additional amount of Pyr-$PIP_2$. After acidification, the lipids were extracted and small aliquots were applied on TLC (see Experimental Procedures). The fluorescence of the lipids upon irradiating with UV light is shown. The last two lanes represent Pyr-PI and Pyr-PIP standards.

recovery of fluorescent lipids (including Pyr-PIP) was 60%. This implies that the extent of conversion was in the order of 80%, in agreement with the data in Figure 1, lane VII. The pyrene to phosphorus mole ratio was found to be 3.97, in agreement with the fact that Pyr-PIP contained some unlabeled PIP as substrate.

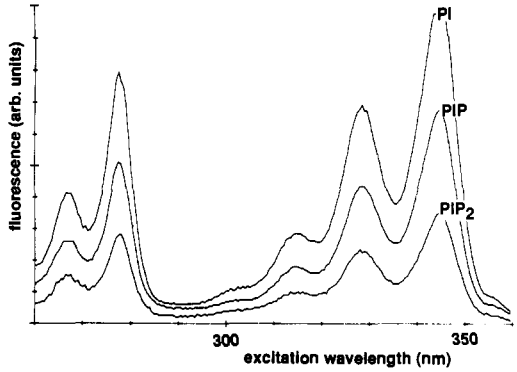


FIGURE 4: Fluorescence excitation spectra of Pyr-PI, -PIP, and -$PIP_2$ in chloroform/methanol/0.6 M HCl (1:2:0.8 v/v). The concentrations of Pyr-PI, -PIP, and -$PIP_2$ were 0.15, 0.1, and 0.05 $\mu$M, respectively. The emission wavelength was 376.5 nm (excitation and emission slits of 2 nm). The spectra were corrected for background fluorescence.

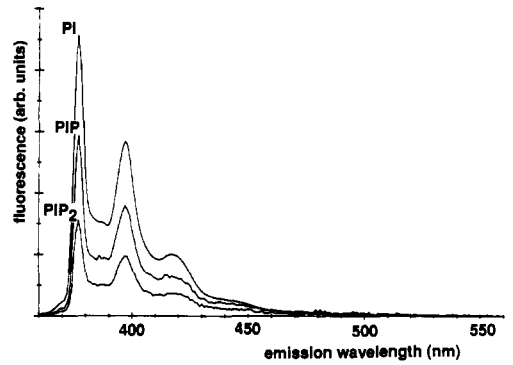


FIGURE 5: Fluorescence emission spectra of Pyr-PI, -PIP, and -$PIP_2$ in chloroform/methanol/0.6 M HCl (1:2:0.8 v/v). Lipid concentrations were identical with those in Figure 4. The excitation wavelength was 344.6 nm (excitation and emission slits of 4 and 2 nm, respectively). The spectra were also corrected for background fluorescence.

*Fluorescent Properties in Organic Solvents.* Fluorescence excitation and emission spectra of Pyr-PI, Pyr-PIP, and Pyr-$PIP_2$ dissolved in chloroform/methanol/0.6 M HCl (1:2:0.8 v/v) are shown in Figures 4 and 5. The spectra were very similar for all three phospholipids, indicating that the pyrene moiety in Pyr-PIP and Pyr-$PIP_2$ has not been affected by the synthesis and purification procedures. Since the emission spectrum (Figure 5) is characteristic for monomers, it is evident that in this solvent at low pH all three inositides occur as single free molecules. Dissolution of Pyr-PC, Pyr-PI, Pyr-PIP, or Pyr-$PIP_2$ (0.2 $\mu$M each) in chloroform/methanol (1:1 v/v) yielded the emission spectra as shown in Figure 6. In contrast to Pyr-PC and Pyr-PI (spectra 1 and 2), Pyr-PIP and Pyr-$PIP_2$ (spectra 3 and 4) displayed a distinct excimer fluorescence ($\lambda_{max}$ = 475 nm) and, consequently, a clearly decreased monomer fluorescence ($\lambda_{max}$ = 378 nm). This indicates that in the absence of acid Pyr-PIP and Pyr-$PIP_2$ behave anomalously in chloroform/methanol (1:1 v/v). Apparently, small clusters of lipids are formed in which the pyrene moieties form excited dimers.

*Pyr-PIP and -$PIP_2$ in PC Bilayers.* To investigate the phosphoinositides in bilayer structures, we have tried to prepare vesicles by the ethanol injection method as described by Batzri and Korn (1973). In a previous study Pyr-PI/egg PC vesicles were obtained by this method using as solvent ethanol/DMSO (75:25 v/v) (Van Paridon et al., 1988). DMSO was required for the complete solubilization of Pyr-PI. Solubilization of Pyr-PIP/PC or Pyr-$PIP_2$/PC mixtures in ethanol/DMSO (75:25 v/v) yielded solutions that still exhibited a small amount

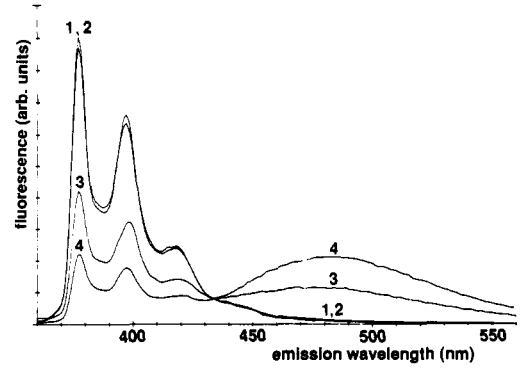


FIGURE 6: Fluorescence emission spectra of Pyr-PC (1), Pyr-PI (2), Pyr-PIP (3), and Pyr-$PIP_2$ (4) in chloroform/methanol (1:1 v/v). The lipid concentration was 0.2 $\mu$M. The excitation wavelength was 344.6 nm (excitation and emission slits of 4 nm). The spectra were corrected for background fluorescence.

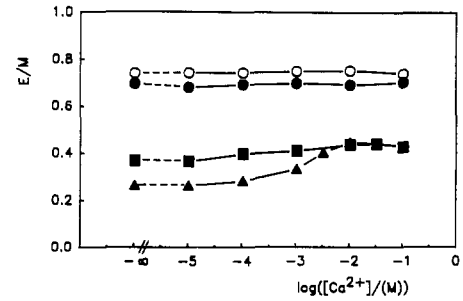


FIGURE 7: Effect of $Ca^{2+}$ on the fluorescent excimer to monomer ratio ($E/M$) of Pyr-PC (○), Pyr-PI (●), Pyr-PIP (□), and Pyr-$PIP_2$ (▲) in egg PC vesicles. The PC vesicles contained 9 mol % pyrene phospholipid. The total lipid concentration was 0.55 $\mu$M. For details see Experimental Procedures.

of excimer fluorescence. This solution proved to be unsatisfactory for the preparation of injection vesicles. It was observed that such vesicles were not stable as the fluorescence yield decreased with time. Despite many efforts we have not been able to find a good solvent from which vesicles containing Pyr-PIP and Pyr-$PIP_2$ could be prepared by injection. In the studies described below vesicles were prepared by ultrasonication (see Experimental Procedures).

Excitation of Pyr-PI, Pyr-PIP, and Pyr-$PIP_2$ (0.5 mol %) in PC vesicles yielded monomer emission spectra with bands identical with the ones observed in organic solvents (cf. Figure 5). As also observed for other pyrene-labeled phospholipids (Levade et al., 1987), the fluorescence intensities were increased in phospholipid vesicles as compared to organic solvents. At a higher concentration of pyrene-labeled phospholipids in PC bilayers (9 mol %) a distinct excimer fluorescence band appears in addition to the monomer bands.

The lateral mobility of the pyrene-labeled phosphoinositides (9 mol %) in PC vesicles was investigated by determining the excimer to monomer fluorescence ratio ($E/M$) as function of the $Ca^{2+}$ concentration (see Figure 7). $E/M$ is a good measure for lateral diffusion as it reflects the collision frequency of pyrene-labeled molecules in a bilayer (Förster, 1969; Galla & Hartmann, 1980). In the absence of $Ca^{2+}$, $E/M$ decreased in the order Pyr-PC ≈ Pyr-PI > Pyr-PIP > Pyr-$PIP_2$ with values of 0.71, 0.69, 0.37, and 0.26, respectively. These observations are in agreement with the high negative charges of Pyr-PIP and Pyr-$PIP_2$ (i.e., −3 and −4.2, respectively, at pH 7.4; Van Paridon et al., 1986), which will greatly reduce the collision frequency of these molecules due to electrostatic repulsion. It is of great interest that the excess of one negative charge in PI as compared to PC has only a marginal effect

on $E/M$ [as also observed by Somerharju et al. (1985)]. $Ca^{2+}$ up to a concentration of 0.1 M had no effect on $E/M$ of bilayers containing Pyr-PC and Pyr-PI (Figure 7). Despite the higher negative charge, the effect of $Ca^{2+}$ on the mobility of Pyr-PIP was also small. $E/M$ increased from 0.37 in the absence of $Ca^{2+}$ to 0.42 at 0.1 M $Ca^{2+}$. Similarly, $Ca^{2+}$ up to 0.1 mM had no effect on $E/M$ of Pyr-$PIP_2$. However, from 0.1 mM to 0.1 M, $Ca^{2+}$ increased $E/M$ from 0.26 to 0.42, indicating that the shielding of the negative charge by $Ca^{2+}$ increased the collision frequency of Pyr-$PIP_2$. The latter effect is in good agreement with the electrophoretic mobility experiments carried out by the McLaughlin group (Toner et al., 1988).

## Discussion

In this study we have synthesized pyrenylacyl-labeled analogues of PIP and $PIP_2$ by use of highly active PI and PIP kinase preparations. To our knowledge this is the first time that polyphosphoinositides with a reporter group have been synthesized. Previously, the semisynthesis of pyrenylacyl-labeled PI from yeast PI was described (Somerharju & Wirtz, 1982; Somerharju et al., 1985). Briefly, this synthesis involved the protection of the hydroxyl groups on the inositol ring by acetylation, removal of the *sn*-2-fatty acyl chain by phospholipase $A_2$, and reacylation with a fluorescent fatty acid followed by deacetylation of the inositol ring. A similar semisynthesis of Pyr-$PIP_2$ has been unsuccessful because acetylated $PIP_2$ was no substrate for phospholipase $A_2$. Here we show that enzymatic synthesis by use of PI and PIP kinase is a good alternative. The products were identified as Pyr-PIP and Pyr-$PIP_2$ from their behavior on TLC and immobilized neomycin columns (Figures 1–3). Absorption spectra of the products demonstrated that the pyrene moiety was unaltered in the course of synthesis and isolation (Figure 4). In the past, studies involving PIP kinase were hampered by the instability of the enzyme (Porter et al., 1988; Ling et al., 1989). Recently we have found conditions at which PIP kinase activity remained stable during purification (Moritz et al., 1990). These stabilizing conditions were also shown to be essential for the high yield synthesis of Pyr-PIP and Pyr-$PIP_2$ (Figure 1, lanes IV–VII).

It is remarkable that under optimal conditions of synthesis more than 50% of Pyr-PI and Pyr-PIP could be phosphorylated. Apparently, the activity of PI and PIP kinase is not adversely affected by the presence of a pyrene-labeled fatty acid in the substrate. This makes one wonder whether the fatty acyl chain composition of PI and PIP has an effect on the activity of PI and PIP kinase. In this respect it is of interest that, particularly in plasma membranes, the fatty acyl composition of naturally occurring PIP and $PIP_2$ is different from that of PI (White, 1973; Augert et al., 1989). It has been suggested that this difference reflects the preference of PI kinase for certain molecular species of PI (Augert et al., 1989). Our data do not necessarily exclude this possibility. However, given the fact that Pyr-PI is a good substrate for PI kinase, we think it more likely that the observed difference results from a PI pool with a distinct fatty acid composition being available for PI kinase (Augert et al., 1989). On the other hand, enzymes involved in the metabolism of PIP and $PIP_2$ (e.g., phosphomonoesterases or phospholipase $A_2$ or C) may have a preference for particular molecular species of these inositides.

The high degree of Pyr-PI phosphorylation (lane V, Figure 1) cannot be explained by the equilibrium constant of 0.003 for the reaction PI + ATP → PIP + ADP as reported by Belunis et al. (1988). This constant predicts a maximal conversion of Pyr-PI into Pyr-PIP of about 20% when applied to our reaction conditions (Table I, condition V). The discrepancy between the calculated (20%) and the observed (>50%) conversion of Pyr-PI indicates that the equilibrium constant of 0.003 is too low.

The extent of phosphorylation reported in this study provided no evidence for a feedback regulation of PI and PIP kinase by PIP and $PIP_2$, respectively. This is in agreement with the observations of Walker et al. (1988) that at PIP/PI ratios of 5 PI kinase is still active. Similarly, PIP kinase is able to phosphorylate PIP at a $PIP_2$/PIP ratio above 1 (Ling et al., 1989). Given the actual PI:PIP:$PIP_2$ ratios in cells, e.g., 7:1:1.5 in rat brain (Palmer, 1981), 9:1:3.5 in chicken brain (Saikh & Palmer, 1976), and 4.2:1:3.3 in bovine brain (Brockerhoff & Ballou, 1962), it is evident that PI kinase must be regulated to give a maximal 10–20% conversion of PI. Since feedback regulation of PI kinase by PIP does not seem important, other mechanisms have to be invoked such as the restriction of PI kinase to certain subcellular organelles (Lundberg & Jergil, 1988), a high rate of PIP metabolism, and/or the limited availability of PI due to the occurrence of metabolically nonactive PI pools (Brockerhoff & Ballou, 1962; Monaco, 1987; King et al., 1987). PIP/$PIP_2$ ratios between 1.5 and 3.5 in whole brain up to as high as 9 in the case of hepatocyte plasma membranes (Augert et al., 1989) are in agreement with our observations that PIP was very effectively converted into $PIP_2$ by PIP kinase.

We have synthesized Pyr-PIP and Pyr-$PIP_2$ to investigate the properties of these phospholipids. In organic solvents, Pyr-PIP and Pyr-$PIP_2$ behaved quite differently from Pyr-PI and Pyr-PC. As shown in Figure 6 both Pyr-PIP and Pyr-$PIP_2$ yielded an excimer spectrum in chloroform/methanol in contrast to Pyr-PI and Pyr-PC, indicating that these phospholipids formed small clusters (micelles) in this solvent. Protonation of Pyr-PIP and Pyr-$PIP_2$ by acidification of the solvent appeared to disrupt these clusters as only a monomer spectrum was observed (Figure 5). This observation clearly demonstrates the usefulness of the pyrene label as the aggregation state of the inositides even at concentrations as low as 0.2 $\mu$M can be assessed by the excimer/monomer technique.

In PC bilayers, Pyr-PI, Pyr-PIP, and Pyr-$PIP_2$ (9 mol %) have different lateral mobilities as reflected by the difference in $E/M$ (Figure 7). In the absence of $Ca^{2+}$ the collision frequency of the inositides decreased in the order Pyr-PI > Pyr-PIP > Pyr-$PIP_2$, parallel to the increase of negative charge on the respective inositides. We explain the increase in $E/M$ for Pyr-$PIP_2$ and Pyr-PIP in the presence of $Ca^{2+}$ (Figure 7) by a partial neutralization of the charge. Due to the decreased electrostatic repulsion, the collision frequency is increased. This is in agreement with the fact that $Ca^{2+}$ affected the electrophoretic mobility of vesicles containing $PIP_2$ (Toner et al., 1988). In this study $Ca^{2+}$ was also reported to have an effect on vesicles containing PI. This was not observed in our experiments, possibly due to the fact that the PI concentration in the vesicles was 3 times lower.

In view of the high repulsion between $PIP_2$ molecules it is unlikely that $PIP_2$ will cluster in certain pools in natural membranes. Strong interactions with positively charged sites on proteins seem more likely. Some authors have reported that a large $PIP_2$ pool is inactive toward stimulus-induced breakdown (Korẽh & Monaco, 1986; Monaco et al., 1987; King et al., 1987). It could be that these molecules are closely attached to proteins or shielded by certain polyamines. Fluorescent derivatives of the inositides may be useful in getting more insight into the nature of protein–lipid or polyamine–lipid interactions, as has been reported for other fluorescent lipids

(Mustonen et al., 1987; Van Paridon et al., 1987b; Rodrigues-Paris et al., 1989). Since pyrenylacyl-labeled PI and PIP are substrates for very specialized and crucial enzymes in cell biology (i.e., the kinases), we believe that these probe lipids are also suitable for studying other membrane-related processes.

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