Mass and Fatty Acid Composition of the 3-Phosphorylated Phosphatidylinositol Bisphosphate Isomer in Stimulated Human Platelets*

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By high pressure liquid chromatography (HPLC) analysis, the occurrence of radiolabeled 3-phosphorylated phosphoinositides has been well documented in several cell systems, including agonist-stimulated platelets. The actual mass amounts and fatty acid composition of these unique lipids, however, have not been reported to date. In the present study, we report the mass and fatty acid composition of phosphatidylinositol (PI) 3,4-P2 from U46619-stimulated platelets using a thin-layer chromatographic system for the separation of PI 3,4-P2 from PI 4,5-P2. The mass of PI 3,4-P2 in the stimulated platelet was 180 ± 0.7 pmol/10^6 platelets (mean ± S.E., n = 4), representing 9.5% of total phosphatidylinositol bisphosphate (PIP2). Based on HPLC analysis, PI 3,4-P2 in unstimulated platelets represented <0.5% of total PIP2 (which corresponds to <7.0 pmol/10^6 platelets). Fatty acid analysis of this lipid revealed a composition very similar to the conventional polyphosphoinositides (stearic and arachidonic acids accounting for 44.2 and 40.4 mol %, respectively, of the fatty acids). Since PI 3,4-P2 also did not appear to be distinct from the other polyphosphoinositides, in regard to radiolabeling properties, it was concluded that this lipid is unlikely to originate from a unique precursor pool. This conclusion validates the use of HPLC analysis of radiolabeled phosphoinositides for the estimation of PI 3,4-P2 mass in agonist-stimulated platelets. The chromatographic procedure described should prove useful for the mass and fatty acid analysis of PI 3,4-P2 from other cell systems.

The formation of 3-phosphorylated polyphosphoinositides is stimulated, by appropriate ligands, in a variety of cell types (1, 2). Phosphatidylinositol (PI) 3-kinase, the enzyme responsible for the formation of these lipids, was first observed in pp60-syre immunoprecipitates from Rous sarcoma virus-transformed cells (3) and has been subsequently shown to be physically associated with a number of growth factor receptors possessing tyrosine kinase activity (1, 2). Site-directed mutational studies of growth factor receptors suggest that PI 3-kinase plays a critical role in mitogenesis (1, 4, 5). PI 3-kinase is also present in terminally differentiated cells such as neutrophils and platelets where it is presumed to play a role in signal transduction (1, 2).

Resting human platelets contain very low levels of PI 3-monophosphate, PI 3,4-bisphosphate (PI 3,4-P2), and PI 3,4,5-trisphosphate (PI 3,4,5-P3) (6, 7). Upon stimulation with thrombin or the endoperoxide analogue, U46619, a dramatic increase in PI 3,4-P2 occurs (6-9). A much less pronounced, but more rapid, increase in PI 3,4,5-P3 has also been observed in response to thrombin stimulation (10).

The biochemical roles played by the 3-phosphorylated phosphoinositides have been the subject of considerable speculation (1, 2, 11). Until very recently, the only experimental evidence for a specific function of these lipids was obtained in formylpeptide-stimulated neutrophils in which a close temporal relationship between the formation of PI 3,4,5-P3 and actin polymerization was observed (12). Nakashishi et al. (13), however, have now presented direct experimental evidence for the selective in vitro activation of the 3,4,5-isomeric of protein kinase C by PI 3,4,5-P3. Whether this isomeric of protein kinase C is also regulated by PI 3,4,5-P3 in vivo remains to be established.

To date, all analyses of 3-phosphorylated phosphoinositides have relied upon radiolabeling cells with either [3P]orthophosphate or [H]inositol and subsequent quantification of the decylation (and in some cases) the deglyceration products by HPLC. The actual mass or fatty acid composition of these lipids has not been reported. We report herein the thin layer chromatographic (TLC) separation of PI 3,4-P2 extracted from stimulated human platelets. This methodology has allowed us to analyze, for the first time, the mass and fatty acid composition of this lipid. We also report the mass and fatty acid compositions of the conventional polyphosphoinositides (PI 4-P and PI 4,5-P2) in resting and U46619-stimulated platelets.

EXPERIMENTAL PROCEDURES

Materials—U46619, HEPES, apyrase (grade V), ANS, PI, and PIP2 standards were purchased from Sigma. [H]inositol (3.33 TBq/mmol) was obtained from Amersham Corp. ENHANCE spray was purchased from Du Pont. Partisol 5 strong anion exchange (SAX) HPLC column was obtained from Whatman. Precoated Silica Gel 60 TLC plates were obtained from Merck. Coulter counter, model ZM, was from Coulter Electronics. Platelet aggregometer, model 800c, was from Payton Scientific.

Platelet Preparation—Blood (400 ml) was collected from healthy human volunteers by antecubital venipuncture into ¼ volume of acid/citrate/dextrose. Platelets were isolated and washed according to the method of Mustard et al. (14) using Tyrodes buffer containing 10 mM HEPES. [H]inositol-prelabeled platelets were prepared as previously described.

doperoxide analogue 9,11-dideoxyc-11a,9a-epoxymethano-prostaglandin F2α; PIP, phosphatidylinositol monophosphate; PI 4-P, phosphatidylinositol 4-monophosphate; PI 4-P2, phosphatidylinositol 4,5-monophosphate; PI 3,4-P2, phosphatidylinositol 3,4-bisphosphate; PI 3,4,5-P2, phosphatidylinositol 3,4,5-trisphosphate; PI 4,5-P2, phosphatidylinositol 4,5-bisphosphate; PI 3,4,5-P3, phosphatidylinositol 3,4,5-trisphosphate; ANS, 8-anilino-1-naphthalenesulfonic acid; SAX HPLC, strong anion exchange high pressure liquid chromatography; BHT, butylated hydroxytoluene (antioxidant).
described (15). Following quantification of platelet yield by particle counting, platelet concentration was adjusted to 1 x 10^9 cells/ml.

**Platelet Incubations**—One ml aliquots of platelet suspension were heated and stirred (37°C and 900 rpm, respectively) in aggerometer cuvettes for 1 min prior to stimulation by 2 μM U46619 for 3 min (HPLC analysis suggested that PI 3,4-P2 mass was maximal at this time point, not shown). Unstimulated (resting) platelets were also subjected to heating and stirring but were not exposed to U46619. Platelet incubations were stopped by the addition of 3.75 ml of chloroform/methanol containing 0.1% BHT (extraction solvent) and the lipids extracted as previously described (15).

**TLC Separation of Phosphoinositides**—For the analysis of PI, the lipids from 1 ml of platelet suspension (i.e. 1 x 10^9 cells) were separated by two-dimensional TLC, using chloroform/methanol/concentrated NH4OH, 65:35:5.5 (v/v/v) in the first dimension and chloroform/methanol/formic acid/H2O, 55:26:5:1 (v/v/v) in the second dimension. For the analysis of the polyphosphoinositides, lipid extracts from four replicate 1-ml aliquots of platelet suspension were pooled. Several (6-12, depending on platelet yield) 2-cm-wide bands were applied to the TLC plates and the lipids resolved in the solvent system chloroform/methanol/concentrated NH4OH, 65:35:5.5 (v/v/v). Lipids were visualized under ultraviolet light following spraying the TLC plates with ANS.

Although PI 4,5-bisphosphate (PI 4,5-P2) appeared to be resolved from the other lipids, PI 3,4-P2 (see Figs. 1 and 2), HPLC analysis of [3H]inositol-labeled glycerophosphoinositides revealed incomplete purification of PI 3,4-P2 by this single TLC procedure (see Fig. 3). For further purification, the PI 3,4-P2 band from all of the U46619-stimulated platelet extract lanes was scraped into a single test tube and the lipid eluted from the gel by the procedure outlined below.

**Elution of PI 3,4-P2 and PI 4,5-P2**—To gel scrapings, 3.75 ml of extraction solvent (chloroform/methanol/concentrated HCl, 20:40:1 (v/v/v) containing 0.1% BHT) was added and the sample sonicated for 20 s using a probe sonicator. Tubes were then refrigerated for 2 h and the supernatant removed after brief centrifugation. A second 3.75 ml of solvent was added to the gel scrapings, and the supernatant from this was pooled with the first supernatant after vortexing (2 min, low speed) and centrifugation to pellet the gel. Phases were obtained by the addition of 2.5 ml of chloroform and 4.5 ml of H2O. The chloroform phase was removed, evaporated under nitrogen, applied to a second TLC plate, and developed using the above described solvent system. To quantify the recovery during elution and rechromatography, 2 lanes of platelet extract PI 4,5-P2 were eluted as described, while a second 2 lanes were scraped and directly transmethylated as outlined below.

**Analysis of Fatty Acids**—Phosphoinositides were transmethyalted for 2.5 h at 85°C using 2 ml of acetyl chloride/methanol, 5:50 (v/v) in the presence of an appropriate quantity of internal standard (17:0, heptadecanoic acid) and the fatty acids analyzed by gas chromatography as described previously (16). Appropriate areas of blank silica gel were scraped from the TLC plates and treated exactly as were the lipid samples (i.e. either directly transmethylated or eluted and rechromatographed, as described above, before transmethylation). This allowed for the subtraction of minor background contamination from the corresponding sample peaks.

**HPLC Analysis of [3H]Inositides**—To verify the purity of the PI 3,4-P2 obtained, samples were deacylated in the presence of silica gel and analyzed by SAX HPLC as previously described (17).

**RESULTS**

As shown in Figs. 1 and 2, primary TLC resulted in the partial separation of PI 3,4-P2 from PI 4,5-P2 (purity 60%, as assessed by HPLC, Fig. 3). The contamination of the PI 3,4-P2 band by PI 4,5-P2 following this single TLC development, represents approximately 4% of the total PI 4,5-P2 applied to the TLC plate. Although no [3H]PI 3,4-P2 was found in the [3H]PI 4,5-P2 band following primary TLC, a low level of contamination (below our limit of detection) may have been present (Fig. 3). Eluting the PI 3,4-P2 from the silica gel and rechromatographing using the same solvent system increased the purity of PI 3,4-P2 obtained to essentially 100% (Fig. 3).

To ascertain the recovery of PI 3,4-P2 through these procedures, we have compared the mass (and composition) of platelet PI 4,5-P2 obtained following elution and rechromatography, with that obtained by primary TLC. The average recovery was approximately 50% based on three separate experiments. This value is similar to that obtained using commercial PIP2 standard (60% recovery). Since it is reasonable to assume that the recovery of PI 3,4-P2 should be very similar to PI 4,5-P2, we have used a factor of 2.06 in calculating the mass of PI 3,4-P2. From this analysis, a value of 160 pmol/1 x 10^5 U46619-stimulated platelets was obtained (Table I). Based on HPLC analysis, unstimulated platelets contained <0.5% of total PIP2 as PI 3,4-P2 (not shown). Although we have not directly measured...
Mass of Platelet PI 3,4-P₂

Table I

<table>
<thead>
<tr>
<th>Polyphosphoinositide</th>
<th>Unstimulated</th>
<th>U46619-stimulated</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>Mass (nmol/10⁹) ± S.E.</td>
<td>Mass (nmol/10⁹) ± S.E.</td>
</tr>
<tr>
<td>PIP</td>
<td>1.10 ± 0.17</td>
<td>1.33 ± 0.12</td>
</tr>
<tr>
<td>PIP₂</td>
<td>2.91 ± 0.31</td>
<td>1.75 ± 0.17</td>
</tr>
<tr>
<td>PI 4,5-P₂</td>
<td>0.18 ± 0.01</td>
<td></td>
</tr>
</tbody>
</table>

* >99% PI 4-P based on HPLC analysis from radiolabeling experiments.

** No observable (mass) amount of PI 3,4-P₂ was present in any unstimulated platelet preparation.

Table II

Fatty acid composition of phosphoinositides in unstimulated and U46619-stimulated human platelets

<table>
<thead>
<tr>
<th>Fatty acid composition</th>
<th>PI 3,4-P₂</th>
<th>PI 4,5-P₂</th>
<th>PI 4,5-P₂</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Unstimulated</td>
<td>U46819-stimulated</td>
<td></td>
</tr>
<tr>
<td>Palmitic acid (16:0)</td>
<td>1.2 ± 0.1</td>
<td>2.0 ± 0.3</td>
<td></td>
</tr>
<tr>
<td>Stearic acid (18:0)</td>
<td>4.4 ± 0.4</td>
<td>4.3 ± 0.6</td>
<td></td>
</tr>
<tr>
<td>Oleic acid (18:1)</td>
<td>5.9 ± 0.2</td>
<td>6.7 ± 0.9</td>
<td></td>
</tr>
<tr>
<td>Arachidonic acid (20:4n-6)</td>
<td>46.3 ± 0.8</td>
<td>46.7 ± 0.8</td>
<td></td>
</tr>
<tr>
<td>Othersab</td>
<td>2.0 ± 0.2</td>
<td>2.7 ± 0.5</td>
<td>3.1 ± 0.5</td>
</tr>
</tbody>
</table>

a Eluted and rechromatographed, three determinations using two separate platelet preparations.
b Represents unidentified fatty acids <1.0 mol % (e.g. linoleic acid) as well as unidentified fatty acids.

PI 3,4-P₂ mass (no PI 3,4-P₂ band was detectable by TLC even when several lanes of unstimulated platelet extracts were pooled, eluted, and rechromatographed), this corresponds to <7.0 pmol/1 × 10⁹ platelets. From Table II, it can be seen that the elution process did not significantly alter the mol % of the various fatty acids of PI 4,5-P₂.

In addition to the formation of PI 3,4-P₂, U46619 stimulation increased the mass of platelet PI 4-P and PI 4,5-P₂ by 164 and 32%, respectively (Table I).

Discussion

The major conclusion that can be drawn from the present results is that PI 3,4-P₂ is unlikely to be derived from a unique phosphoinositide precursor pool. Both the mass and the fatty acid composition data support this conclusion.

The mass of PI 3,4-P₂ in U46619-stimulated platelets (180 pmol/10⁹ cells or 9.3% of total PIP₂) obtained using the present methodology is in good agreement with our previous results using [³H]inositol-labeled platelets and HPLC analysis of the deacylated phosphoinositides. This suggests that PI 3,4-P₂ does not arise from a precursor pool that is either poorly or highly labeled with [³H]inositol. Similarly, Sultan et al. (9), using [³H]PIP₂-labeled platelets, have reported that PI 3,4-P₂ represents approximately 11% of the total PIP₂ following thrombin stimulation. It would appear, therefore, that HPLC analysis of either [³H]inositol or [³H]PIP₂-labeled PI 3,4-P₂ is an accurate reflection of the mass of this lipid, at least in the case of agonist-stimulated human platelets. Although this may be true for other cell systems, this will need to be confirmed by mass analysis. Further research will also be required to determine if this same conclusion is reached for the other platelet 3-phosphorylated phosphoinositides, PI 3-monophosphate and PI 3,4,5-P₃.

As can be seen from Table II, the fatty acid composition of PI 3,4-P₂ is very similar to the conventional platelet phosphoinositides and reflects the well known preponderance of 1-stearoyl 2-arachidonyl species in the phosphoinositides from other mammalian cells and tissues (18). The undistinguishing nature of the fatty acid composition adds additional evidence for the conclusion that the precursor(s) for PI 3,4-P₂ formation is (are) not radically different from that for PI 4,5-P₂. This does not rule out, of course, a spatial compartmentalization of PI 3,4-P₂ precursors that share with PI 4,5-P₂ precursors a very similar fatty acid profile and specific activity.

The increased mass of PI 4-P and PI 4,5-P₂ following U46619 stimulation is in agreement with our previous results in which platelets were labeled with [³H]inositol and directly counted following TLC (15) or deacylated and analyzed by HPLC. The mol % of arachidonic acid in the polyphosphoinositides (Table II) is moderately higher than previously reported (19). This may reflect differences in the concentration of BHT used in the extraction solvents (we have used a considerably higher concentration) but more likely reflects differences in the transmethylation reagents used. Although we have not compared sodium methoxide, transmethylation of commercial PIP₂ standard with acetyl chloride/methanol, 5:50 (v/v) resulted in consistently higher values for arachidonic acid than transmethylation with sulfuric acid/methanol, 6:94 (v/v). Surprisingly, no differences between these two methylation reagents were seen when PI standard was used. We have found the mass of PI 4-P and PI 4,5-P₂ to be roughly equivalent in unstimulated platelets (Table I). This result does not agree with those of Mauco et al. (19), but is in agreement with those of Verhoeven et al. (20), who have previously reported that PI 4-P comigrates with an unidentified phospholipid in many TLC systems (21).

In summary, PI 3,4-P₂ from stimulated human platelets shares with the conventional phosphoinositides a very similar fatty acid composition; this band would be more likely to account for the discrepancy in the results between Mauco et al. (19) with those of Verhoeven et al. (20), as well as the present analysis. We have not yet analyzed the fatty acid composition of this band.

fatty acid profile and radiolabeling properties. This finding supports the continued use of HPLC analysis for estimating the mass of this lipid. The chromatographic procedure described herein should be useful for the analysis of PI $3,4$-$P_2$ from other cell systems.

REFERENCES