A method is described for the preparation of radioactive inositol lipids for studies of their enzymic degradation. Kidney cytosol fractions have been used to produce diesteratic cleavage. High voltage electrophoresis at pH 4.3 is used to separate D-myoinositol 1:2-cyclic phosphate and D-myoinositol 1-phosphate from hydrolysis of phosphatidylinositol. Radioactivity co-migrating with myoinositol diphosphate and triphosphate is separated by electrophoresis at pH 1.5 following enzymatic hydrolysis of phosphatidylinositol phosphate and phosphatidylinositol diphosphate. Relative activities for hydrolysis of the various inositides suggest the presence of more than one phosphodiesterase.

Introduction

The inositol lipids are of considerable interest in membrane phenomena because of their increased turnover under a number of physiological conditions [1,2]. Interest in phosphatidylinositol has recently been increased by the demonstration that D-myoinositol 1:2-cyclic phosphate is a hydrolysis product in a phospholipase C type cleavage reaction [3,4]. The higher analogs, phosphatidylinositol phosphate and phosphatidylinositol diphosphate are also of interest because of their high concentration in excitable tissues [2,5]. A problem in studying the hydrolysis reactions is the difficulty in obtaining suitable amounts of substrate in the case of the polyphosphoinositides. Radioactive substrates may thus constitute a useful tool for further study of enzymes of inositide metabolism.

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Methods

For labeling of inositides, a 10% homogenate of guinea pig brain in 0.32 M sucrose was prepared and a 1000 x g supernatant fraction obtained as described previously [6]. For degradation of purified lipids, kidney supernatant fractions were prepared as described by Tou et al. [7]. Phosphate [8] and protein [9] were determined spectrophotometrically. A mixture of inositol phosphates for use as electrophoresis markers was produced by phytic acid hydrolysis using a modification of a previously reported method [10]. Samples (2 ml) of phytic acid (Nutritional Biochemical Corp., Cleveland, Ohio) were brought to pH 5.2 with 2M NaOH, and heated for 2 h at 100°C. The mixture was treated with 5 ml of Dowex-50 (H+) and centrifuged. The supernatant and 1 ml wash of the resin were combined and brought to 15 ml with water. Then 30 ml of ethanol were added and the mixture was centrifuged. This removed most of the P₁ and inositol monophosphate. The resultant precipitate was centrifuged and washed with ethanol/water (2 : 1, v/v), and finally dissolved in 10 ml of water. This solution of the different inositol phosphates was diluted 1 : 10 and 10 µl were spotted for paper electrophoresis [10]. Carrier-free ³²P in 0.02 M HCl was obtained from New England Nuclear, Unisil from Clarkson Chemical Co., and DEAE-cellulose from Whatman. Phospholipids were chromatographed by modifications of previous methods [11,12] using commercial thin-layer chromatography plates (silica gel G, 0.25 mm, Brinkman Instruments Inc., Westbury, New York).

Results

Preparation of ³²P-labeled inositides

30 ml of 1000 x g supernatant was mixed with 30 ml of medium B as previously reported [6], and 2.5 mCi of ³²P₁ in a 1000-ml Erlenmeyer flask and incubated for 4 h in air at 37°C in a shaking incubator bath. Phosphatidic acid and the inositides were the principle lipids labeled. The reaction was terminated by the addition of 225 ml of chloroform/methanol (1 : 2, v/v) [13]. To ensure complete extraction of polyphosphoinositides, the mixture was acidified by the addition of 5 ml of 0.5 M HCl [5]. A further 75 ml of chloroform and 75 ml of 2 M KCl containing 0.2 M H₃PO₄ were added. After shaking, the lower phase was removed and dried in a rotary evaporator. The dried lipids were dissolved in 64 ml of chloroform/methanol/conc. HCl (2 : 1 : 0.01, v/v) and washed once with 16 ml of 0.05 M H₃PO₄ and once with “theoretical upper phase” [14] containing 0.1 M HCl. The lower phase was dried in a rotary evaporator and the lipid residue dissolved in chloroform/methanol/water (20 : 9 : 1, v/v). The usual yield was 5 · 10⁻⁶—6 · 10⁻⁶ cpm (efficiency >90%).

Lipids were applied to a column (2.5 cm X 16 cm) of DEAE-cellulose-acetate that had been equilibrated with chloroform/methanol/water (20 : 9 : 1, v/v) [15]. Three different fractions were eluted, as shown in Table I. Samples of fractions A, B and C were chromatographed by thin-layer chromatography [11,12] and phospholipid spots identified by radioautography, scraped off, and counted by liquid scintillation spectrometry [16]. Fraction A contains the
TABLE I

CHROMATOGRAPHY OF $^{32}$P-LABELED PHOSPHOLIPIDS ON DEAE-CELLULOSE

Each fraction was eluted with 300 ml of chloroform/methanol/water (20:9:1, v/v) without or with ammonium acetate as indicated. PhA, phosphatidic acid; PhI, phosphatidylinositol; PhIP, phosphatidylinositol phosphate; PhIP$_2$, phosphatidylinositol diphosphate.

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Elution solvent</th>
<th>Added radioactivity recovered (%)</th>
<th>Phospholipid</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>Chloroform/methanol/water (20:9:1, v/v)</td>
<td>1</td>
<td>—</td>
</tr>
<tr>
<td>B</td>
<td>with 0.1 M ammonium acetate</td>
<td>51.2</td>
<td>PhA, PhI, PhIP</td>
</tr>
<tr>
<td>C</td>
<td>with 0.3 M ammonium acetate</td>
<td>37.4</td>
<td>PhIP$_2$</td>
</tr>
</tbody>
</table>

bulk of the other phospholipids which were not labeled in our incubation medium. Fractions B and C were dried separately on the rotary evaporator, redissolved in 32 ml chloroform/methanol (2:1, v/v) and were washed with 8 ml of 0.1 M HCl to remove ammonium acetate. The upper phases contained no radioactivity and were discarded. The lower phases were dried under N$_2$. Initial experiments using a DEAE-cellulose column 25 cm high have a mixture of $^{32}$P-labeled phosphatidylinositol phosphate and $^{32}$P-labeled phosphatidylinositol diphosphate after elution with a third solvent. This polyphosphoinositide fraction was used for enzymatic degradation in several experiments. It was later found that reducing the column height to 16 cm brought the $^{32}$P-labeled phosphatidylinositol phosphate out in fraction B and fraction C contained $^{32}$P-labeled phosphatidylinositol diphosphate with a radiopurity >90%.

Dried fraction B was dissolved in 10 ml of chloroform and applied to a column (0.9 cm internal diameter) of Unisil, 200—395 mesh, packed in chloroform at a ratio of 1 g of Unisil per 30 mg of lipid. Different fractions were collected as shown in Table II. Traces of phosphatidylinositol diphosphate, when present in fraction B, could not be eluted from unisil.

**Assay for phosphatidylinositol-phosphodiesterase activity**

A 3000—5000 cpm (35.9—59.8 nmol) sample of $^{32}$P-labeled phosphatidylinositol in chloroform was placed in the assay tubes and the solvent evapo-

TABLE II

UNISIL CHROMATOGRAPHY OF $^{32}$P-LABELED PHOSPHOLIPIDS

Fraction B (see Table I) was applied to the column and eluted with 30 ml of chloroform/methanol in different proportions as shown. Other details are as in Table I.

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Elution solvent</th>
<th>Added radioactivity recovered (%)</th>
<th>Phospholipid</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>Chloroform</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>II</td>
<td>Chloroform/methanol (95:5, v/v)</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>III</td>
<td>Chloroform/methanol (90:10, v/v)</td>
<td>20.1</td>
<td>PhA</td>
</tr>
<tr>
<td>IV</td>
<td>Chloroform/methanol (85:15, v/v)</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>V</td>
<td>Chloroform/methanol (80:20, v/v)</td>
<td>12.3</td>
<td>PhI</td>
</tr>
<tr>
<td>VI</td>
<td>Chloroform/methanol (34:66, v/v)</td>
<td>42.8</td>
<td>PhIP</td>
</tr>
</tbody>
</table>
The labeled substrate was suspended in the assay mixture and sonicated for 2 min. Assay conditions were as follows in a final volume of 0.25 ml: 20 mM Tris/maleate buffer, pH 5.3, 10 mM NaF and 0.3–0.4 mg protein. Incubations at 37°C, in a shaking water bath were then started by the addition of enzyme protein. Reactions were stopped with chloroform/methanol (1 : 2, v/v) and partitioned [13]. Lower phases were chromatographed on thin-layer chromatography [11] and the upper phases were dried under N₂, redissolved in 25 μl of water and electrophoresed as described [17] but at 4000 V, for 60 min, pH 4.3, pyridine/acetate buffer. In both cases radioactive spots were located by radioautography and counted by liquid scintillation spectrometry.

Rat kidney cortex supernatant was active in degrading phosphatidylinositol with release of D-myoinositol 1:2-cyclic phosphate and D-myoinositol 1-phosphate. Most of this activity (87%) was concentrated in the 0–30% (NH₄)₂SO₄ fraction. A pH curve ranging from pH 5.3 to 7.3 showed that this phosphodiesterase activity is higher at pH 5.3 but the optimum pH could not be determined since lowering the pH caused protein precipitation. With both fractions about 80% of the water-soluble radioactive product was identified as myoinositol 1:2-cyclic phosphate [3]. Fig. 1A shows a time-course of the reaction.

At all times both inositol phosphate esters were produced in about the same proportion, suggesting that both products are produced simultaneously. Activity was proportional to the protein concentration up to 0.3–0.4 mg of protein (Fig. 1B). The sum of the two inositol phosphate esters is virtually equal to phosphatidylinositol degradation (Figs. 1A and 1B).

**Assay for polyphosphoinositide-phosphodiesterase activity**

Samples of ³²P-labeled phosphatidylinositol phosphate (8.9 · 10⁵ cpm/μmol) or phosphatidylinositol diphosphate (4.4 · 10⁶ cpm/μmol) containing 3000–7000 cpm or a mixture of both substrates (53.4% phosphatidylinositol phosphate and 46.6% phosphatidylinositol diphosphate) containing a total

![Fig. 1. Degradation of ³²P-labeled phosphatidylinositol in a subfraction from rat kidney cortex supernatant. A 0–30% (NH₄)₂SO₄ fraction was used. (A) Time-course of reaction, 0.32 mg protein per assay. (B) Protein concentration activity curve, incubation time 60 min. In both experiments 130 nmol phosphatidylinositol was used. Ph₁, phosphatidylinositol; cIP, D-myoinositol 1:2-cyclic phosphate; IP, D-myoinositol 1-phosphate.](image-url)
of 18 000–24 000 cpm were used and assay conditions were as described above for phosphatidylinositol-phosphodiesterase except that 20 mM Tris/maleate buffer, pH 6.8, was used [7]. Incubations were for 15 min (Fig. 2). Lower phases were chromatographed [12] and the redissolved upper phases electrophoresed as previously described [10]. Rat kidney cortex supernatant fraction and the 0–30% and 30–50% (NH₄)₂SO₄ fractions obtained were found to be active in degrading either a mixture of both substrates or the purified inositide. Even in the presence of 10 mM NaF, these fractions have phosphomonoesterase activities, which can degrade phosphatidylinositol diphosphate or the phosphodiesteratic products, D-myoinositol 1,4-diphosphate and D-myoinositol 1,4,5-triphosphate. This was apparent in the rat kidney cortex supernatant, where about 75% of the radioactive-soluble product was P₁ but was only 25% in the 0–30% (NH₄)₂SO₄ fraction and 10–15% in the 30–50% (NH₄)₂SO₄ fraction. The latter was used in most subsequent experiments. Fig. 2 shows the time-course when a mixture of both radioactive substrates was used. Electrophoresis of the upper phases [10] indicated the presence of P₁ and myoinositol mono-, di- and triphosphate. Chromatography of the lower phases showed degradation of both substrates [12] but formation of phosphatidylinositol [11] could not be detected. This result suggests that myoinositol 1-phosphate is not produced from phosphatidylinositol and that phosphomonoesterases mainly attack phosphatidylinositol diphosphate and myoinositol diphosphate and triphosphate. Upper phases from the same experiment were also electrophoresed at pH 4.3, pyridine/acetate buffer, and P₁ and inositol monophosphate were detected, but not the cyclic inositol phosphate ester. Purified substrates were also used. Phosphatidylinositol phosphate showed production of myoinositol diphosphate.

Fig. 2. Degradation of a ³²P-labeled mixture of phosphatidylinositol phosphate and phosphatidylinositol diphosphate in a subfraction from rat kidney cortex supernatant. A 30–50% (NH₄)₂SO₄ fraction was used (0.38 mg protein). Substrate mixture as described in the text (24 000 cpm per assay). IP, D-myoinositol 1-phosphate; IP₂, D-myoinositol 1,4-diphosphate; IP₃, D-myoinositol 1,4,5-triphosphate; P₁, inorganic phosphate.
and some $P_1$ Phosphatidylinositol diphosphate was mainly degraded to myoinositol diphosphate and some myoinositol triphosphate. Some $P_1$ was also present which is indicative of phosphomonoesterase activity attacking phosphatidylinositol diphosphate and/or myoinositol triphosphate.

**Attempts to find a cyclic inositol triphosphate**

By analogy to phosphatidylinositol cleavage, we might expect that diesteratic cleavage of phosphatidylinositol diphosphate would yield diglyceride and $1:2$-cyclic inositol 4,5-triphosphate. By treating D-myoinositol 1,4,5-triphosphate with dicyclohexylcarbodiimide a new electrophoretic spot was found that co-migrated with inositol pentaphosphate of phytic hydrolysates at pH 1.5 [18]. Heating at 100°C with 1 M HCl for 20 min reconverted the material to the myoinositol triphosphate, as evidenced electrophoretically. In the presence of presumptive cyclic myoinositol triphosphate carrier or its absence, diesteratic cleavage of phosphatidylinositol diphosphate did not lead to the formation of a radioactive fast-moving spot. We, therefore, found no evidence for the putative cyclic product.

**Discussion**

Schacht and Agranoff [6] showed that brain homogenate incubated in a specific enriched medium would incorporate $^{32}P_1$ mainly into phosphatidic acid and the inositides. This labeling system was used here in large incubations for the preparation of $^{32}P$-labeled inositides. These inositides were then separated by a combination of DEAE-cellulose and silicic acid chromatography, and provided substrates for the studies of inositide-phosphodiesterases. Lapetina and Michell [19] have reported a radioactive assay for the study of phosphatidylinositol-phosphodiesterase activity, but radioactive substrates for the polyphosphoinositide-phosphodiesterases have not previously been used. Rat kidney cortex fraction actively degraded the three inositol substrates. Phosphatidylinositol-phosphodiesterase activity was mainly concentrated in a 0–30% saturation $(NH_4)_2SO_4$ fraction with production of D-myoinositol 1:2-cyclic phosphate and also some D-myoinositol 1-phosphate. Both inositol phosphate esters were produced in about the same proportion under varying conditions of time and protein concentration, the products probably being produced simultaneously, although different enzymes may be involved.

The presence of phosphomonoesterases in all three fractions studied makes it difficult to determine polyphosphoinositide-phosphodiesterase activities. Tou et al. [7] reported the absence of phosphomonoesterases in the 30–50% $(NH_4)_2SO_4$ fraction, but using the radioactive assay, we found significant activity. This phosphomonoesterase activity may cleave only a specific phosphate bond since most of the degradation product, using either the inositide mixture or purified phosphatidylinositol diphosphate, is myoinositol 1,4-diphosphate.

The 30–50% $(NH_4)_2SO_4$ fraction has practically no phosphatidylinositol-phosphodiesterase activity. This result suggests the possible presence of more than one phosphatidylinositol-phosphodiesterase activity [7,20]; one in fraction 0–30% producing mainly D-myoinositol 1:2-cyclic phosphate from
phosphatidylinositol, and the other, present in all fractions, producing mainly myoinositol 1,4-diphosphate and myoinositol 1,4,5-triphosphate from both polyphosphoinositides.

It is interesting to note that phosphatidylinositol, whose turnover in different tissues may be externally stimulated, is degraded to a cyclic inositol phosphate [1,17], whereas polyphosphoinositides, which have a high turnover but are not stimulated from the exterior [2] appear not to produce cyclic compounds.

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