SPECIFICITY OF OX BRAIN TRIPHOSPHOINOSITIDE PHOSPHOMONOESTERASE

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The ability of brain homogenates to hydrolyze brain phospholipids is well known (1,2) and may be attributed to lipases and phosphatases in the tissue. Thompson and Dawson (3,4) and Dawson and Thompson (5) first described the partial purification of brain phosphatases which act on the polyphosphoinositides, myoinositol-containing phospholipids the exact structures of which have been defined (6). One of the enzyme activities in ox brain homogenates was a phosphomoesterase (5) which dephosphorylated triphosphoinositide (TPI, 1-phosphatidyl-L-myoinositol 4,5-diphosphate) to monophosphoinositide (MPI, 1-phosphatidyl-L-myoinositol). As a measure of enzyme activity, these workers followed the release of inorganic phosphate and the appearance on chromatograms of MPI, and the transient appearance of an intermediate diphosphoinositide (DPI, 1-phosphatidyl-L-myoinositol 4-(or 5)-phosphate). They concluded that one of the pathways for catabolism of triphosphoinositide involves the reaction TPI $\longrightarrow$ DPI $\longrightarrow$ MPI, a suggestion which is consistent with previous studies (7,8) which had indicated that the general metabolism of the polyphosphoinositides in brain slices follows a reversible sequence, MPI $\rightleftharpoons$ DPI $\rightleftharpoons$ TPI.

Although the work of Dawson and Thompson (5) suggests that their solubilized phosphomonoesterase represents a physiologically important enzyme in the above pathway, this conclusion is based largely on the source of the enzyme and its apparent selectivity for the brain polyphosphoinositides as substrates. Any claim for the specificity of the enzyme would be greatly

strengthened if it could be shown that the DPI intermediate formed in the dephosphorylation of TPI was a single isomer; and, moreover, that it was the same isomer as that which is found in the brain itself. On the other hand, if the enzyme activity were an artifact, then the DPI formed would likely be a mixture of the two possible isomers (the 4-phosphate and 5-phosphate). Since a detailed characterization of the DPI formed in the reaction investigated by Dawson and Thompson (5) was not made, their study does not eliminate the possibility that the phosphomonoesterase acted on TPI in a non-specific manner.

In the present paper, pure ox brain triphosphoinositide was incubated with a phosphomonoesterase preparation from the same source. The diphosphoinositide, formed as a transient intermediate in the dephosphorylation to monophosphoinositide was isolated. The diphosphoinositide was degraded by alkali to yield myoinositol 4-phosphate, which established that the lipid was 1-phosphatidyl-L-myoinositol 4-phosphate. Since the soluble phosphomonoesterase produced the same diphosphoinositide as that which has been isolated directly from brain tissue, it seems probable that the same enzyme is involved in the *in vivo* metabolism of the polyphosphoinositides.

EXPERIMENTAL PROCEDURE

Materials. -- Crude ox brain phosphoinositides were isolated by the procedure of Folch (9) and pure MPI, DPI and TPI were obtained from this preparation by column chromatography according to Hendrickson and Ballou (10). The identity of the pure phosphoinositides was confirmed by chromatographic (11) comparison with authentic standards (6).

Preparation of a Soluble Triphosphoinositide Phosphomonoesterase from Ox Brain. -- The procedure of Dawson and Thompson (5) was followed verbatim through the ammonium sulfate fractionation step. This fraction was dialyzed and used without further purification. Ten grams of wet brain tissue yielded about 4 ml of the enzyme solution as used below.

Isolation of Enzymically Formed Diphosphoinositide. -- About 15 mg of chromatographically pure TPI (ammonium salt) (1640 µg P, 17.6 µmoles as TPI) in solution with 6 ml of 6 mM $MgCl_2$, 12 ml of 0.132 M Tris buffer pH 7.2, 3 ml of a histone solution (1 mg per ml) and 4 ml of the enzyme solution was incubated for 11 minutes at 25° (5). The reaction was stopped by freezing the solution in a dry-ice bath, and the mixture was lyophilized to reduce the volume to about 5 ml. This was then extracted with 5 volumes of a chloroform-methanol mixture (1:1) made 0.01 N with concentrated hydrochloric acid. The water layer contained 180 µg of inorganic phosphate and 840 µg of organic phosphate. The organic phase, which contained 624 µg of bound phosphate in the form of recovered phospholipids, was evaporated to a small volume and put on a 1 x 20 cm DEAE-cellulose column (Whatman DE-32). This was eluted with a total volume of 500 ml of the chloroform-methanol-water-ammonium acetate gradient system described previously (10). The elution pattern is given in Fig. 1. The peaks were identified chromatographically as MPI (62 µg P, 2.0 µmoles), DPI (112 µg P, 1.8 µmoles) and TPI (350 µg P, 3.8 µmoles) (Table I).

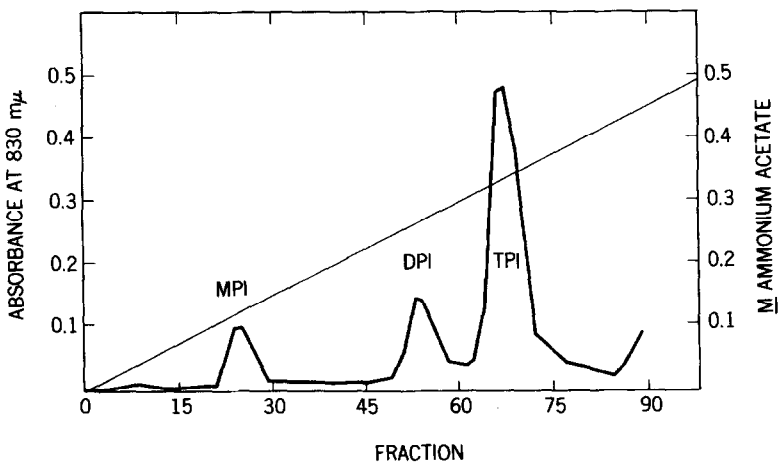

Fig. 1. Elution pattern of phospholipids recovered from the enzymic digestion of pure TPI. The column contained Whatman microgranular DEAE-cellulose and the eluant was a gradient of ammonium acetate in a mixture of chloroform, methanol and water. Fractions of 5 ml were collected and analyzed for total phosphate.

TABLE I

Chromatography of Phosphoinositides

Substance	Distance Migrated (cm)
1-Phosphatidyl L-myoinositol (MPI)	28.6
1-Phosphatidyl L-myoinositol 4-phosphate (DPI)	20.4
1-Phosphatidyl L-myoinositol 4,5-diphosphate (TPI)	16.5
Phosphoinositides recovered from enzymic digestion of TPI	
MPI	28.2
DPI	20.6
TPI	16.5

Determination of the Isomeric Form of Diphosphoinositide by Conversion to Myoinositol 4-Phosphate. -- The phosphatidyl group of DPI was removed selectively by alkaline degradation. About 1 mg of DPI (ammonium salt) was dissolved in 0.5 ml of chloroform to which was added about 0.1 ml of methanolic sodium hydroxide (10 ml of methanol containing 1 ml of 4 N sodium hydroxide). After 30 minutes, 0.5 ml of water was added, the mixture was shaken and the chloroform layer was removed. The water layer was extracted with 1 ml of chloroform to remove the last trace of lipid material. The water phase was then put in boiling water for 30 minutes, cooled and treated with 80 mg of Dowex 50-H (200-400 mesh) to remove the cations. A small amount of cyclohexylamine was added to the solution and the water-soluble products of the alkaline degradation were chromatographed on Whatman No. 1 filter paper with the isopropanol-ammonia system (12) along with synthetic myoinositol 4-phosphate and 5-phosphate standards (13). Pure DPI isolated directly from fresh brain (10) was treated in the same manner and served as a control. Spots were detected on chromatograms by use of the alkaline silver nitrate reagent (14). The results are given in Table II.

TABLE II

Chromatography of Myoinositol Phosphates

Substance	Distance Migrated (cm)
Myoinositol 4-phosphate standard	32.5
Myoinositol 5-phosphate standard	29.7
Myoinositol phosphate from DPI isolated from ox brain	32.5
Myoinositol phosphate from DPI produced by action of phosphomonoesterase on TPI	32.5

RESULTS AND DISCUSSION

The controlled incubation of pure TPI with the soluble phosphatase preparation of Dawson and Thompson (5) resulted in the release of 11% of the phosphate as inorganic phosphate and 51% as water-soluble organic phosphate, and the recovery of 38% of the phosphate in the lipid fraction. The formation of inorganic phosphate must result from the action of the phosphomonoesterase, while the water-soluble organic phosphate has been shown to be myoinositol phosphates produced by the action of a phosphodiesterase also present in the enzyme preparation (4).

Figure 1 shows the elution pattern of the recovered phospholipid, the only phosphate-containing components being MPI, DPI and TPI. The relatively low yield of MPI and DPI resulted partly from the difficulty in selecting the best incubation time, but also reflects the competing reaction with the very active phosphodiesterase.

The water-soluble products from the alkaline degradation of DPI contain free glycerol, glycerol phosphate, myoinositol monophosphate and myoinositol diphosphate, the proportions of which have been determined previously (6). The mechanism of the degradative reaction is such that the myoinositol monophosphate comes exclusively from the part of the molecule containing the mono-

esterified phosphate group. Were the action of the phosphomonoesterase unspecific and the resultant DPI a mixture of phosphatidyl-myoinositol 4-phosphate and 5-phosphate, then the inositol monophosphate produced by alkaline degradation would be a mixture of myoinositol 4- and 5-phosphate. In fact, the inositol monophosphate was shown by paper chromatography to be exclusively the 4-phosphate (Table II). As expected, DPI isolated directly from fresh ox brain also yielded only the 4-phosphate isomer.

Our finding that the dephosphorylation of TPI by the soluble enzyme results in the formation of DPI with the monoesterified phosphate group on the 4-position strengthens the supposition that TPI is the natural substrate of the phosphomonoesterase. TPI in its stable chair conformation has the two monoesterified phosphate groups in equatorial positions, and there should be little steric difference between them. Thus, the selectivity shown by the phosphomonoesterase cannot reasonably be attributed to a nonspecific hydrolysis of a sterically more accessible phosphate group.

The DPI which one isolates from brain must represent material synthesized both as a result of phosphorylation of MPI and *in situ* dephosphorylation of TPI (15,16). TPI in the cell is thought to be bound to protein and this lipoprotein complex could be oriented in such a way as to result in the selective removal of the 5-phosphate group on dephosphorylation *in vivo*. However, since the soluble enzyme gives only one DPI isomer, the sequence of dephosphorylation is accounted for most simply as a function of the substrate specificity of the enzyme.

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