THE BIOSYNTHESIS OF TRIPHOSPHOINOSITIDE BY RAT BRAIN IN VITRO

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The incorporation of ^{32}P orthophosphate into the inositol lipids of rat and rabbit brain *in vivo* (Wagner *et al.*, 1962; Ellis and Hawthorne, 1962) or as tissue slices (Brockerhoff and Ballou, 1962; Rossiter *et al.*, 1964) suggests that triphosphoinositide (TPI) is formed by the stepwise phosphorylation of phosphatidylinositol (1,2). Since this evidence is somewhat

phosphatidylinositol + ATP ⟶ diphosphoinositide + ADP (1)

diphosphoinositide + ATP ⟶ triphosphoinositide + ADP (2)

indirect, we have tried to define the synthetic pathway using rat brain homogenates with purified phosphoinositides as substrates. Using phosphatidylinositol and terminally ^{32}P-labelled ATP under conditions similar to those established with subcellular fractions from rat liver (Michell and Hawthorne, 1965), labelled diphosphoinositide (DPI) was formed according to reaction (1). This work will be reported elsewhere and is similar to that of Colodzin and Kennedy (1965), who used a microsomal fraction from rat brain. It differs, however, in one important respect: our system formed TPI as well as DPI, and in similar quantities. This step has now been studied in greater detail.

The present paper describes the synthesis of TPI by homogenates of rat brain with purified DPI as substrate and provides the first direct evidence that reaction (2) takes place in that tissue.

MATERIALS AND METHODS

DPI was prepared by the method of Hendrickson and Ballou (1964). Chromatography on formaldehyde-treated papers and phosphate determinations on the spots showed that it was 75% pure, TPI accounting for the other 25%. Terminally ^{32}P-labelled ATP was prepared according to Glynn and Chappell (1964).

Brains from male albino rats weighing approximately 250 g. were homogenised at 4^{0} for 1.5 min. in 0.32 M sucrose using a Teflon and glass homogeniser with a radial clearance of 0.04 mm. The pestle rotated at 600 rev./min. and twelve strokes were used. Sucrose solution was added to give a 10% (w/v) homogenate.

The basic incubation system contained the following in a total volume of 1.0 ml : 5 mM labelled sodium ATP (10^{6} counts/100 sec./µmole in a 6% efficient liquid Geiger counter); 30 mM $MgCl_2$; 20 mM Tris/HCl buffer, pH 7.4; 0.9 mM sodium DPI; 2 mM sodium EDTA; 0.2 ml. 10% brain homogenate. Incubation was for 5 min. at 37^{0}.

At the end of the incubation period the mixture was extracted by the method used previously (Galliard et al.,1965). Details of the washing of the lipid extract, chromatography of the TPI on formaldehyde-treated paper and determination of radioactivity are given in the same report.

Assay of 5'-nucleotidase (5'-ribonucleotide phosphohydrolase, E.C.3.1.3.5) was by the method of Michell and Hawthorne (1965) and assay of Na^{+}/K^{+}-stimulated ATPase (ATP phosphohydrolase, E.C. 3.6.1.3) by that of Swanson et al. (1964).

RESULTS AND DISCUSSION

Identification of the labelled product. Authentic brain TPI was added to the washed lipid extract from a 20 ml. incubation and the mixture was hydrolysed under mild alkaline conditions. The resulting phosphate esters were separated by chromatography on Dowex 1 x 10. Details of both procedures are given by Galliard et al. (1965). The radioactive phosphate ester co-

chromatographed exactly with glycerylphosphorylinositol diphosphate, the hydrolysis product of TPI, in two different eluting systems. The labelled product also behaved as TPI on the DEAE column of Hendrickson and Ballou (1964) and on formaldehyde-treated paper chromatograms.

Dependence on DPI, Mg^{++} and ATP. The effect of DPI concentration on production of labelled TPI is shown in Figure 1. A concentration of 0.9 mM gave maximum stimulation. Reaction mixtures were incubated at 37^{0} for 2 minutes.

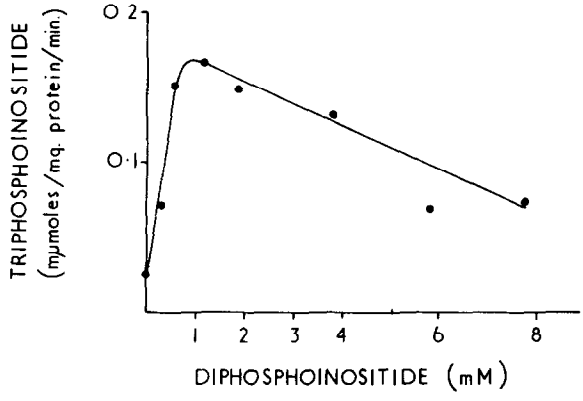

Fig. 1. Effect of DPI concentration on synthesis of TPI.

Using the basic incubation system (0.9 mM DPI) with various amounts of brain homogenate, the synthesis of TPI was proportional to protein concentration up to 4 mg./ml. protein. A time curve under the basic conditions was linear up to 10 minutes. The reaction required magnesium ions, the optimum concentration being 20 mM in the absence of EDTA. With ATP, maximum activity was reached at 5 mM and remained constant up to 12 mM.

Effects of other cations, ouabain and acetylcholine. These effects are summarised in Tables 1 and 2. Addition of sodium ions or sodium and potassium ions reduced the net synthesis of both DPI and TPI. This is not a consequence of increased ATPase activity, since the concentration of ATP in the system would not be sufficiently reduced to affect phosphorylation. Alternatives

TABLE 1. POLYPHOSPHOINOSITIDE SYNTHESIS: EFFECTS OF Na^+, K^+ AND OUABAIN

Basic incubation system with 5 mM tris-ATP and 1 mM phosphatidylinositol* or 0.9 mM DPI**.

NaCl	KCl (mM)	Ouabain	DPI* (mμmole/mg.protein/3 min.)	TPI**	ATPase (μmole P_i/mg. protein/5 min.)
0	0	0	0.21	1.27	1.1
150	0	0	0.08	0.90	1.5
120	30	0	0.06	0.56	2.8
120	30	0.5	0.10	1.19	1.0
120	30	1.0	0.08	0.77	1.1

are a direct action on the phosphorylating systems or activation of the monoesterase attacking the polyphosphoinositides (Dawson and Thompson, 1964). Addition of ouabain (0.5 mM) partly reversed the inhibition by sodium and potassium ions in the case of TPI

TABLE 2. POLYPHOSPHOINOSITIDE SYNTHESIS: EFFECT OF ACETYLCHOLINE

Incubation system as in Table 1, but with 6 mM sodium ATP.

Acetylcholine (M)	Eserine	DPI* (mμmole/mg.protein/3 min.)	TPI**
0	0	0.12	0.60
10^{-5}	10^{-4}	0.21	0.77
10^{-4}	10^{-4}	0.21	0.83

Hayashi *et al.* (1964) studied the effect of sodium ions and ouabain on the incorporation of inorganic ^{32}P into the phosphatidylinositol and DPI of guinea pig brain slices. Sodium ions caused an increased incorporation of phosphate into both lipids which was partly reversed by ouabain. The present results with rat brain homogenate and $AT^{32}P$ differ from those of Hayashi *et al.*, but it is interesting that in both cases the sodium effect is reversed by ouabain.

In the presence of eserine, acetylcholine produced a significant increase in the synthesis of both DPI and TPI (Table 2).

Effect of detergents. Previous experiments showed that the synthesis of DPI from phosphatidylinositol was stimulated by Cutscum (Colodzin and Kennedy, 1965) and by deoxycholate (Kai and Hawthorne, 1966). Synthesis of TPI in the present system, on the contrary, was not stimulated by Triton X-100, which is similar to Cutscum, or deoxycholate. Triton X-100 was used at a range of concentration between 0.5 and 3.0%, deoxycholate between 1 and 15 mM. Triton X-100 caused a slight inhibition.

Subcellular distribution of the phosphorylating enzyme. Rat brain was separated by the method of Nyman and Whittaker (1963) into the following subcellular fractions: nuclear, myelin, nerve-ending, mitochondrial, microsomal and supernatant. The identity of the fractions was confirmed by electron microscopy and by the following estimations: DNA and succinate:INT reductase (E.C. 1.2.99.1) (Michell and Hawthorne, 1965); lactate dehydrogenase (E.C. 1.1.1.27) (Johnson and Wittaker, 1963).

For comparison with TPI synthesis, the synthesis of DPI was also studied in the subcellular fractions. For this, the assay system differed from the basic one in the following respects: no EDTA was used, 1 mM phosphatidylinositol replaced the DPI and the temperature was 30°.

Synthesis of DPI (mμmoles/mg. protein) was most active in the microsomal, nuclear and nerve-ending fractions. The distribution pattern resembled that of 5'-nucleotidase, but the resemblance was less marked than in liver cell fractions (Michell and Hawthorne, 1965). TPI synthesis had a quite different distribution, the supernatant being much more active than any particulate fraction. There was little activity in the myelin fraction.

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