EVIDENCE FOR A NEW INOSITOL PHOSPHOLIPID IN RAT BRAIN MITOCHONDRIA

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Received August 3, 1992

Phosphorylation of phosphatidylinositol (PI), phosphatidylinositol monophosphate (PIP) and diacylglycerol (DAG) was studied in rat brain cortex myelin, synaptosomal and mitochondrial fractions, with ATP as phosphate donor and endogenous phospholipids as substrate. All fractions had PI, PIP and DAG phosphorylating activity with their own characteristic subcellular distribution. However, in the mitochondrial fraction an unidentified lipid was phosphorylated, which had a slower Rf value than PIP₂ on TLC. After hydrolysis of the polar head group of the lipid and separation on anion exchange columns, it appeared to be a phosphoinositide. The elution profile showed that it was not phosphatidylinositol trisphosphate, or a lyso-compound. The available evidence suggests that the unknown inositol phospholipid in rat brain mitochondria is a phosphatidylinositol 4,5-bisphosphate isomer, although the possibility of it being a glycosyl-phosphoinositide cannot be excluded.

Nervous tissue is especially rich in phosphoinositides, a class of phospholipids which are involved in important neurochemical processes related to neurotransmission (1,2). For instance, phosphatidylinositol 4-phosphate (PI-4-P) and phosphatidylinositol 4,5-bisphosphate (PI-4,5-P₂), which are synthesized by sequential phosphorylation of phosphatidylinositol (PI), are implicated in receptor-mediated signal transduction (1). In this process, PI-4,5-P₂ is hydrolyzed by phospholipase C into the second messenger inositol trisphosphate and diacylglycerol (3) followed by rapid phosphorylation of the diglyceride into phosphatidic acid.

Phosphoinositide metabolism has become more complex with the discovery of D-3 phosphorylated inositides (4). The inositides which are phosphorylated at the D-3 position of the inositol ring (PI-3-P, PI-3,4-P₂ and phosphatidylinositol 3,4,5-trisphosphate (PIP₃)) seem to be involved in cell proliferation (5). In addition, it is suggested that these inositides are involved in the cytoskeletal rearrangement that occurs during the mitotic cell cycle and in post mitotic cells during exocytosis of protein tyrosine kinase and growth factor receptors (4,5,6,7,8). PIP₃ has been identified in several cell types (4) and in rat brain (9).

Inositol phospholipids were thought to be primarily located in the plasma membrane (2). Recent subcellular fractionation studies with rat brain, however, have shown that PI kinase, PIP kinase and DG kinase activities are present in all subcellular fractions (10). The common feature in the subcellular distribution of these three lipid kinases appears to be their presence in both membrane and cytosol.
The present paper is concerned with the phosphorylation of brain myelin, synaptosomal and mitochondrial fractions under hypotonic conditions with gamma-labeled ATP as phosphate donor and endogenous phospholipids as substrate. Our assay system has been shown to favor the phosphorylation of inositol phospholipids (11,12). Our results suggest that a new phospholipid, probably an inositol phospholipid, is formed in rat brain mitochondria but not in synaptosomes.

MATERIALS AND METHODS

Animals and brain dissection: Male rats (approx. 165 g) of an inbred Brown-Norway strain were used. After decapitation the head was immediately immersed in liquid nitrogen for 8 seconds. All subsequent operations were performed at 0-4°C. The brain was taken out of the skull, and the cortex was dissected rapidly.

Subcellular fractionation: The tissue was homogenized in 0.32 M sucrose, 1 mM EDTA, 10 mM Tris-HCl, pH 7.4, in a total volume 10 times the brain tissue volume by 10 up-and-down strokes of a Potter-Elvehjem Teflon-glass homogenizer (radial clearance 0.125 mm, 700 rpm). The homogenate was spun at 1000 g for 10 minutes. After centrifugation of the supernatant at 10,000 g for 10 minutes, the resulting crude mitochondrial-synaptosomal pellet was resuspended in homogenization buffer and then applied to a discontinuous Ficoll sucrose gradient as described by Booth and Clark (13). The tubes were centrifuged at 100,000 g for 30 minutes. Myelin and synaptosomes banded at the first and second interphases, respectively, with the free mitochondria being pelleted at the bottom. Membranes from each layer were carefully removed and contaminating synaptosomes appearing on the top of the mitochondrial pellet were eliminated by swirling the pellet with a small amount of 0.32 M sucrose buffer. Myelin, synaptosomes and mitochondria were suspended in 0.9% NaCl, 5 mM Tris-HCl, pH 7.4, and spun down at 10,000 g for 10 minutes to remove the Ficoll. The pellets were subjected to osmotic lysis (the pellets were resuspended in 10 vol. aqua bidest) and stored at -80°C.

Phosphorvlation assay: Endogenous phosphorylating activity was assayed as described earlier (11,12). The lipid phosphorylation reaction was terminated by the addition of 2 ml ice-cold chloroform: methanol: 12 N HCl (200: 100: 0.75, by vol., 14). Protein determination was performed according to the method of Lowry et al. (15).

Lipid extraction, thin-layer chromatography and determination of radioactivity: Lipid extraction and thin-layer chromatography were performed as described previously (11). Two hundred microliters of water was added to the scraped silica proteins followed by 3.8 ml scintillation fluid (Ready Safe, Beckman). Radioactivity was counted in a scintillation spectrometer (Beckman LS 1801) with an efficiency of 100%.

Alkaline hydrolysis of inositol phospholipids and separation of inositol phosphates: The alkaline hydrolysis of PIP, PIP$_2$ and band "X" was performed according to Vadnal and Parthasarathy (9). The hydrolysates were buffered with 200mM Tricine, pH 8.0. Separation of inositol phosphates was performed according to Downes and Michell (16) on Dowex AG1x8 anion exchange columns (formate form), with the exception of the last elution step when 1.5 M HCOONH$_4$/0.1 M HCOOH was used.

RESULTS

The crude mitochondrial/synaptosomal pellet (P$_2$) was further purified by Ficoll discontinuous gradient centrifugation resulting in myelin, synaptosomal and mitochondrial fractions. Fig.1 shows that phosphate incorporation rates into PIP, PIP$_2$ and PA with endogenous lipid substrate had their own unique subcellular distribution, with the highest rate of PIP formation being in the synaptosomal and mitochondrial fractions, the highest rate of PIP$_2$ formation being in the myelin fraction and the highest rate of PA formation being in the synaptosomal fraction.
Surprisingly, a fourth phospholipid was phosphorylated in our assay system which has a short incubation time and use endogenous lipids as substrate. This new phospholipid appeared to be enriched in rat brain mitochondria (fig.1). The phospholipid "X" had a Rf value on TLC of 0.167; PIP2, PIP and PA had Rf values of 0.222, 0.300, and 0.778, respectively.

A time curve of phosphate incorporation, measured in the mitochondrial fraction, showed that phospholipid "X" was formed within seconds and that formation reached a maximum at 60 seconds, whereafter dephosphorylation took place (fig.2).

To identify the new phospholipid, we subjected PIP, PIP2 and "X", extracted and concentrated from the lipid spots on the TLC plate, to alkaline hydrolysis. The hydrolyzed polar head groups of the phospholipids were separated by anion exchange chromatography
Fig. 2. Time curve of phosphate incorporation (pmol phosphate/mg protein) in phosphatidylinositol monophosphate (PIP), phosphatidylinositol bisphosphate (PIP\(_2\)), phosphatidic acid (PA) and the new phospholipid (X) measured in rat brain mitochondria.

(fig.3). The PIP hydrolysate showed one peak eluting in the 0.2 M and one in the 0.5 M ammonium formate fractions, corresponding to IP, and IP\(_2\), respectively. The PIP\(_2\) hydrolysate and the "X" hydrolysate had peaks that eluted in the 0.2 M, 0.5 M and 0.8 M ammonium formate fractions.
ammonium formate fractions, corresponding to IP_1, IP_2 and normally IP_3, respectively. The "X" hydrolysate peak eluting in the 0.8 M fraction was much broader than the corresponding peak in the elution pattern of the PIP_2 hydrolysate. No peak was found in the 1.5 M ammonium formate fraction.

DISCUSSION

The study presented reports the formation of a novel inositol phospholipid in vitro in a mitochondrial fraction of rat brain cortex after incubation with ATP under hypotonic conditions.

Further subcellular differentiation of a crude mitochondrial-synaptosomal pellet by discontinuous Ficoll-sucrose gradient centrifugation resulted in myelin, synaptosomal and mitochondrial fractions. All fractions had PI, PIP and DG phosphorylating activity as previously described by Stubbs et al. (10), who also used endogenous lipid as substrate.

In the mitochondrial fraction, however, we found a fourth radiolabeled phospholipid which had a slower Rf value than PIP_2 on TLC (Rf values were 0.167 and 0.222, respectively). Several investigators have described an inositol phospholipid that migrates slower than PIP_2 does on TLC, and have identified this substance as phosphatidylinositol-trisphosphate (PIP_3) by revealing an inositol tetrakisphosphate after deacylation and deglyceration (9,17,18). In order to identify the polar head group of the new phospholipid found in the mitochondria of rat brain cortex, we subjected this phospholipid to alkaline hydrolysis (9). Instead of an inositol tetrakisphosphate peak eluting in the 1.5 M ammonium formate fraction on Dowex AG1x8 anion exchange columns, we found a peak at the position of inositol trisphosphate (IP_3).

However, the pattern of this peak was clearly different from the inositol trisphosphate after PIP_2 hydrolysis, as the peak showed partial retention on the column (tailing). This new phospholipid found in mitochondria is not PIP_3 but possibly a PIP_2-like substance. Gumber and Lowenstein (19) reported non-enzymic phosphorylation of polyphosphoinositides catalyzed by bivalent metal ions resulting in pyrophosphates. Phosphatidylinositol 4-pyrophosphate had a slower Rf value than the PI-4,5-P_2 standard. However, the new phospholipid described in the present study is probably not PI-pyrophosphate because the phosphorylation of mitochondrial lipids (mitochondria possibly contain higher concentrations of bivalent metal ions than synaptosomes, in analogy to the pyrophosphate hypothesis) would also reveal other pyrophosphates (19), which were not found in our experiments.

The new phospholipid is an inositol phospholipid, because IP_1 and IP_2 were also present in the mixture of products after hydrolysis of the new phospholipid. The unidentified phospholipid is not a lyso-PIP_2 which also migrates slower than PIP_2 (18) because alkaline hydrolysis of a lyso-PIP_2 would produce a normal inositol trisphosphate. The polar head groups of PI_1,3,4,P_2 and PI_1,3,5,P_2 (I_1,3,4-P_3 and I_1,3,5-P_3, respectively) elute at somewhat lower salt concentrations than the polar head group of PI_4,5,P_2 (I_1,4,5-P_2) does (17), but on Dowex anion exchange columns these isomers cannot be separated. Another possibility could be a mitochondrion-specific glycosyl-phosphoinositide, phosphorylated in our assay system.
Glycosyl-phosphoinositides are responsible for anchoring several proteins to the membrane (20). Alkaline hydrolysis of a glycosyl-phosphoinositide could result in diacylglycerol and an inositolphosphate glycan which possibly coelutes with IP$_3$ on Dowex anion exchange columns.

In conclusion, this study reports the in vitro formation of a new inositol phospholipid in purified rat brain mitochondria. The new inositol phospholipid was not identified as phosphatidylinositol trisphosphate. The new inositol phospholipid is probably a phosphatidylinositol 4,5-bisphosphate isomer or a glycosyl-phosphoinositide.

REFERENCES