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Phosphoinositide signal transduction pathway in rat liver mitochondria

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Abstract. Phosphorylation of endogeneous phosholipids of rat liver mitochondrial fractions with γ[^{32}P]ATP revealed formation of all the known inositol phospholipids, such as phosphatidylinositol, phosphatidylinositol phosphate and phosphatidylinositol bisphosphate. Additionally, a new inositol phospholipid was detected. Incorporation of [^{3}H] -labelled insositol followed a similar profile. Enzymatic experiments indicated that the new lipid could possibly be phosphatidylinositol trisphosphate. The presence of phosphoinositides-generated second messengers such as diacylglycerol and inositol trisphosphate was also confirmed. Protein kinase C, which acts as mediator between second messengers and nuclear factors, was also found to be present in mitochondria in significant amount. These results suggest that phosphoinositide signal transduction pathway is operative in rat liver mitochondria.

Keywords. Mitochondria; signal transduction; phosphoinositides; protein kinase C.

1. Introduction

Inositol phospholipids like phosphatidylinositol (PI), phosphatidylinositol-4-phosphate (PIP) and phosphatidylinositol-4, 5-bisphosphate (PIP_2) are thought to be primarily located in the plasma membrane (Abdel-Latif 1986). The phosphoinositides generate active second messengers, such as 1, 2-diacylglycerol (DAG) and D-*myo*-insitol 1, 4, 5-trisphosphate (IP_3) which are involved in the activation of many cellular functions (Berridge 1984,1987; Berridge and Irvine 1984; Eriksson and Anderson 1992; Choudhury *et al* 1995; Mistry *et al* 1995). Phosphoinositide turnover plays a major role in the signal transduction process that controls cell growth and regulation (Nishizuka 1992). Recently, subcellular fractionation studies with rat brain cortex myelin and synaptosome have shown that PI kinase, PIP kinase and DAG kinase activities are present in all subcellular fractions of rat brain (Stubbs *et al* 1988; Bothmer *et al* 1992). In liver, although considerable studies on PI turnover have been carried out in plasma membrane fraction, mitochondria have received little attention. In view of the fact that mitochondria have independent growth and replication mechanism we considered it important to investigate if mitochondria could synthesize their own components of signal transduction pathway. With the discovery of *bcl-2* and its antioxidant properties, it is only likely that mitochondria have some role to play in the signal transduction pathway. It has been established that *bcl-2* is attached to the inner mitochondrial membrane and it is known to be phosphorylated by protein kinase C (PKC), the origin of which is not certain (Hockeribery *et al* 1990; Chang and

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Faller 1995)· If it is from the cytosol then it has to permeate the double membrane envelope of mitochondria. It is reasonable to suppose that mitochondria have their own PKC and if so its endogenous activators also must come from within the organelle· The present paper reports results on the phosphorylation of rat liver mitochondrial fractions with γ- labelled ATP as the phosphate donor and endogenous phospholipids as substrates, and the presence of second messengers and PKC. In addition to the presence of kinases that form polyphosphoinositides, we have detected a new inositol phospholipid that is formed in the mitochondria by a kinase apparently associated with the outer membrane.

2. Materials and methods

2.1 *Chemicals and radiochemicals*

Digitonin, phenyl methyl sulfonyl fluoride (PMSF), dithiothreitol (DTT), ATP, PIP, phosphatidic acid (PA) and DAG were purchased from Sigma Chemical Co. (St. Louis, Mo, USA) . PIP_2 was from Boehringer Mannheim, Germany. DAG assay kit, IP_3 level measurement kit, D-*myo* [^{3}H] inositol (sp. act· 81·9 Ci/mmol) and P [^{3}H] I P_2 (sp. Act. 1 Ci/mmol) were obtained from Amersham International plc (Buckinghamshire, England)· γ[^{32}P]ATP (sp. Act. 3000 Ci/mmol) was from BRIT, Bombay. All other chemicals, reagents and materials were procured locally.

2.2 *Preparation of mitoplast*

Male Wistar rats (120–130g) were sacrificed, the livers were removed, washed and homogenized in 0·25 M sucrose in 0·025 M Tris-HCl (pH 7·4) containing 1 mM PMSF and 1 mM EDTA. The homogenate (10%) was centrifuged at 1000 *g* for 10 min. The post nuclear supernatant was centrifuged at 10,000 g for 10 min to obtain crude mitochondria which were given three washings. Glucose 6-phosphatase assay was conducted to check possible microsomal contamination. Mitochondria were treated with digitonin to remove the outer membrane as described by Schnaitman and Greenawalt (1968). The digitonized mitochondria were centrifuged to sediment mitoplast. The supernatant containing the soluble outer membrane was also preserved.

2.3 *Phosphorylation of endogenous lipids*

Endogenous phospholipids were phosphorylated as described below. A sample containing 400 μg protein (mitoplast, mitochondria or supernatant) was incubated with 10 μCi γ [^{32}P] ATP for 30 min at 37°C. The reaction was terminated by 1·5 ml of $CHC1_3$: CH_2OH (1:2) and the phospholipids were separated by two phase partitioning. Inositol lipids were further purified by thin-layer chromatography (TLC) (Choudhury *et al* 1995; Mistry *et al* 1995). In some experiments similar amount of mitochondrial fraction was reacted with 10 μCi of D-*myo*- [^{3}H] inositol for 30 min at 37°C, after which the phospholipids were extracted and purified as described above. The thin-layer plates were autoradiographed and the spots corresponding to PI, PIP, PIP_2 and the new spot were scrapped of and their radioactivity was determined in an LKB Rackbeta 1217 Liquid Scintillation Spectrometer.

2.4 *PIP_3 kinase assay*

Assay for P I P_3 kinase was carried out essentially according to the method of Remillard *et al* (1991). A sample of 10 µg protein was incubated with either unlabelled PIP_2 and γ [^{32}P] ATP or with [^{3}H]-labelled PIP_2, for different periods after which the inositol phospholipids were purified as described above.

2.5 *PKC assay*

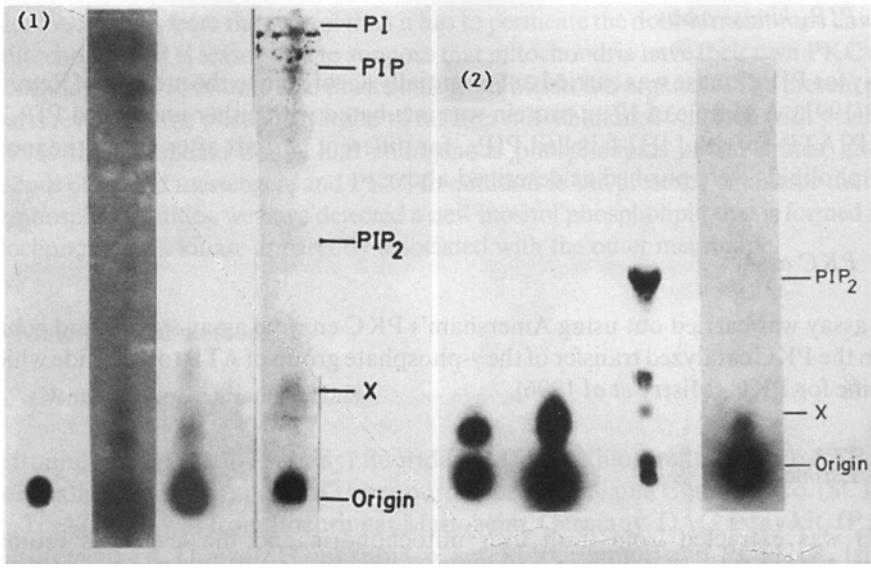
The assay was carried out using Amersham's PKC enzyme assay system and is based upon the PKC catalyzed transfer of the γ-phosphate group of ATP to a peptide which is specific for PKC (Mistry *et al* 1996).

2.6 *Estimations*

DAG was extracted from fresh liver mitochondria and the level was estimated employing DAG kinase (DAG assay kit) which phosphorylates DAG to form [^{32}P] PA in the presence of γ[^{32}P] ATP (Preiss *et al* 1987). IP_3 level was estimated using Amersham's IP_3 level measurement kit which is based on the competition between unlabelled IP_3 and a fixed quantity of tritium labelled IP_3 for a limited number of binding sites on a bovine adrenal binding protein preparation (Chivers *et al* 1991). Protein was estimated by the method of Lowry *et al* (1951).

3. Results and discussion

Intact mitochondria seemed refractory to [^{32}P] ATP labelling as no incorporation into phospholipids could be observed (figure 1, lane 1). Hence, mitochondria were treated with digitonin and mitoplast was separated and tested for phosphorylation of endogenous phospholipids. Figure 1 (lane 2), which is a typical autoradiographic profile, illustrates that all the known predominantly occurring inositol phospholipids viz·, PI, PIP and PIP_2 are produced in the mitoplast. In addition, a more ionic, slow moving band was detected. All the 4 lipids could be detected when the supernatant obtained after centrifuging mitoplast was similarly phosphorylated. The unknown band was, however, more predominant in this fraction (figure 1, lane 3). Experiments with *myo*[^{3}H] inositol also showed a similar pattern thus confirming that the new band is indeed an inositol phospholipid (figure 1, lane 4). Table 1 shows the incorporation of [^{32}P] ATP and [^{3}H] inositol into all the phospholipids in the mitoplast which are so far known to be present only in the plasma membrane. However, the content of the additional phospholipid (designated "X") was found to be much more than that in the plasma membrane where it constitutes a minor fraction (Downes and Carter 1991). This fourth phospholipid ("X") detected in the present study was further investigated· Surprisingly, the major amount of the enzyme that produced this phospholipid was found to be localized in the supernatant fraction left after removal of mitoplast (figure 2, lane 1). The possibility of this enzyme being PIP_3 kinase was checked by using enzymatic assay reported by Remillard *et al* (1991). The assay measures the conversion of exogenous PIP_2 to PIP_3. As can be seen from figure 2 more of unknown band is



Figures 1 and 2. (1) Labelling of phospholipids of mitochondrial fractions. 400 μg protein was incubated with either γ [^{32}P] ATP or D-*myo* -[^{3}H]inositol for 30 min at 37°C and inositol phospholipids were separated on TLC and autoradiographed· Lane 1, Intact mitochondria + labelled ATP; lane 2, mitoplast + labelled ATP; lane 3, supernatant + labelled ATP; lane 4, mitoplast + labelled inositol. **(2)** Autoradiograph of product of PIP_3 assay. Ten mg of enzyme protein (supernatant obtained after pelletting mitoplast) was assayed for PIP3 formation (Remillard *et al* 1991). Lane 1, Enzyme + labelled ATP (endogenous PIP_2 as substrate); lane 2, enzyme + PIP_2 (cold) + lebelled ATP; lane 3, enzyme + [^{3}H] labeled PIP_2; lane 4, heat-inactivated enzyme – PIP_2 (cold) + labelled ATP.

Table 1. Formation of inositol phosopholipids in the mitoplast.

	cpm/h/mg protein in			
Substrate	PI	PIP	PIP_2	X
γ[^{32}P]ATP	5110 ± 570	2355 ± 240	633 ± 92	2413 ± 217
[^{3}H]inositol	3223 ± 545	1265 ± 110	1247 ± 453	1688 ± 255

Mitoplast protein (400 μg) was incubated with either 10 μCi of γ[^{32}P]ATP or 15 μCi of D-*myo*[$_{3}$H] inositol at 37°C for l h. The phospholipids were isolated and inositol phospholipids were separated on TLC as described in the text. Each result is mean ± SD (n = 4).

formed upon incubation with either cold PIP_2 and γ[^{32}P]ATP (figure 2, lane 2) or [^{3}H]PIP $_2$ (figure 2, lane 3) with 10 μg supernatant protein as enzyme source. Appearance of the unknown band was also observed by Bothmer *et al* (1992) when rat brain mitochondria were incubated with [^{32}P] ATP. Results on the PIP_3 kinase activity in the supernatant are presented in table 2, Formation of PIP_3 is much pronounced in both cases, one with cold PIP_2 and [^{32}P] ATP, and the other with PIP_2 in which the inositol is labelled. The time course of formation revealed that the phosphorylation attained the peak at 5 min of reaction after which it decreased, probably due to dephosphorylation

Table 2. PIP_3 kinase activity in the supernatant (outer mitochondrial membrane).

Phospho-inositide	Incorporation of radioactivity from	
	$\gamma[^{32}P]ATP$ (cpm/assay)	$P[^3H]IP_2$ (cpm/assay)
PI	255 ± 27	781 ± 57
PIP	440 ± 53	881 ± 29
PIP_2	925 ± 28	15904 ± 343
PIP_3	19684 ± 592	18052 ± 667

PIP_3 kinase was assayed using 10 μg of the enzyme (supernatant) with either labelled ATP and cold PIP_2 or $P[^3H]$ IP_2. The products were analysed after 5 min as described in the text.

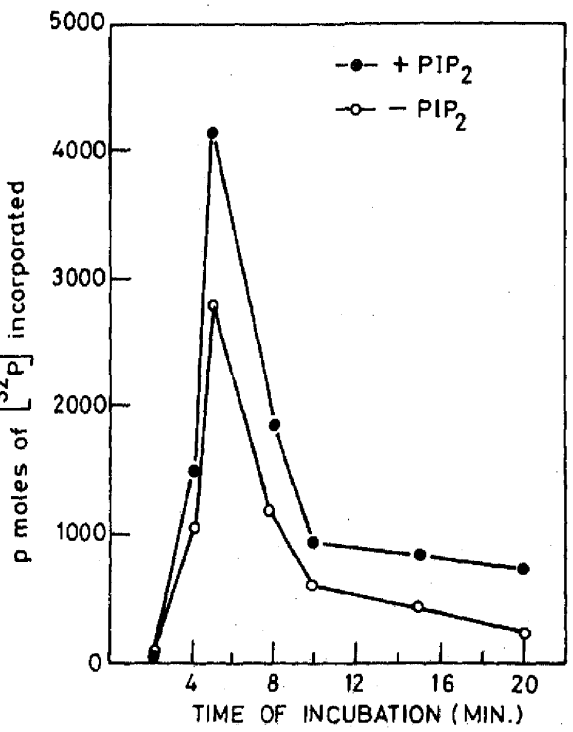


Figure 3. Time course of formation of PIP_3 . Enzyme was incubated with γ [^{32} P]ATP for different periods and the product PIP_3 analysed on TLC·

(figure 3)· Boiling of the sample abolished the activity (figure 2, lane 4) suggesting that it is an enzymatic reaction·

The importance of PIP_2 lies in generating the second messengers DAG and IP_3 Both these were estimated and found to be present in good amounts (DAG: 17nmol/mg protein, I P $_3$:1·2 pmol/mg protein) . Detection of both DAG and IP_3 in rat liver mitochondria established the presence of second messenger generation· DAG has been known to activate PKC (Takai *et al* 1979; Nishizuka 1988,1992) and IP_3 has been

Table 3. PKC activity mitochondrial fractions.

Fraction	[^{32}P] incorporation (pmol/min/mg protein)
Mitochondria	17·82 ± 1·90
Mitoplast	12·06 ± 0·33
Supernatant	11·92 ± 0·93

PKC was assayed using γ [^{32}P] ATP as described in text.

implicated in the release of Ca^{2+} from internal stores (Streb *et al* 1983; Berridge and Irvine 1984)· PKC acts as mediator between second messengers and nuclear factors, and thus plays a central role in the signal transduction process that controls cell growth and regulation (Streb *et al* 1983; Nishizuka 1992). Hence, possible presence of PKC was looked for in mitochondria. The enzyme could be detected in all the fractions studied, in mitochondria, mitoplast and the supernatant by employing the Amersham PKC enzyme assay system (table 3). That the enzyme assayed was only PKC and not any other protein kinases was ascertained by purifying it. The enzyme was partially purified on a DE 52 column, a method standardized earlier in our laboratory (Mistry *et al* 1996) and was found to possess all the properties of PKC.

We have presented evidence for the formation of all the 3 known predominantly occurring inositol phospholipids in rat liver mitochondria. Additionally, mitochondria showed the formation of an inositol phospholipid which has a R_f value lower than that of PIP_2. This fourth lipid is more likely to be PIP_3 (PI 3,4,5-trisphosphate) since exogenously added PIP_2 was found to be phosphorylated further to yield this lipid. This reaction is catalyzed by a PI 3-kinase (Downes and Carter 1991). The present observation clearly indicates that in mitochondria this enzyme is bound to the outer membrane. The presence of PIP_3 has till now been reported to occur in very marginal amount in the plasma membrane (Downes and Carter 1991)· There is an expanding list of cellular responses linked to PI 3-kinase activation (Downes and Carter 1991; Nakanishi *et al* 1995). The importance of PI 3-kinase in cell signaling is emphasized by the fact that 3-phosphorylated inositol phospholipids can enter PI pathway to generate second messengers (Downes and Carter 1991; Stephens *et al* 1991), and their levels are raised in response to the activation of protein tyrosine kinases (Downes and Carter 1991; Cantley *et al* 1991).

It remains to be seen whether PI kinases and PKC which we have detected in mitochondria are the same as found in nuclear/particulate fractions or, as is more likely, other isoenzymes. The presence of inositol phospholipids capable of generating second messengers in mitochondria and the detection of PKC would mean that mitochondria do have the signal transduction pathway. However, its precise role and implication in the regulation of mitochondrial biogenesis/turnover or cellular growth itself is to be ascertained.

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