Activation of intestinal mitochondrial phospholipase D by polyamines and monoamines

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Abstract

Intestinal mitochondria have a phospholipase D (PLD) activity which was stimulated by polyamines and monoamines resulting in the formation of phosphatidic acid (PA) from endogenous phospholipids. When stimulated by polyamines, mitochondrial PLD utilized endogenous phosphatidylethanolamine (PE) as substrate whereas stimulated by monoamines, both PE and phosphatidylcholine (PC) were hydrolysed. Stimulation of PA formation by spermine was enhanced by the presence of calcium. Since polyamines are known to alter the calcium transport by mitochondria and PA is known to possess an ionophore effect, stimulation of PA formation in mitochondria by polyamines suggests that polyamine-induced alteration in calcium homeostasis might involve a PA related mechanism. © 1997 Elsevier Science B.V.

Keywords: Polyamines; Monoamines; Phospholipase D; Phosphatidic acid; Intestinal mitochondria

1. Introduction

Cellular calcium is compartmentalized into separate pools and this helps to maintain the intracellular Ca$^{2+}$ homeostasis. Sarcoplasmic reticulum is an important site of Ca$^{2+}$ storage and although in the normal situation mitochondria store little Ca$^{2+}$, in certain pathological conditions, when the cytosolic Ca$^{2+}$ level increases, mitochondrial influx and efflux of Ca$^{2+}$ are also accelerated which leads to Ca$^{2+}$ cycling by mitochondria [1]. During oxidative stress, mitochondrial Ca$^{2+}$ cycling is increased due to the opening of a membrane pore and this can be prevented by cyclosporin A [2].

Polyamines are non-protein nitrogenous bases which are widely distributed in various cells [3]. They are involved in various cell functions such as protein and nucleic acid synthesis and growth processes [4]. Putrescine and the polyamines spermidine and spermine are polycations which play an important role in cell proliferation and differentiation [5,6]. The molecular mechanism of polyamine action is still poorly understood, but seems to involve specific interactions with cellular DNA [7] or modification of membrane functions [8]. In the small intestinal epithelium the proliferative compartment is limited to the crypts, while the cells differentiate along the villus, acquiring their specific function (i.e., hydrolysis, transport) [9–12] and the functions of polyamines and monoamines in enterocyte mitochondria have not yet been investigated. It has been shown that polyamines also play a...
role in mitochondrial Ca\(^{2+}\) transport [13]. Recently, it was shown that histamine triggered an elevation of intracellular Ca\(^{2+}\) in bovine adrenal chromaffin cells [14].

Our earlier work has shown that enterocyte mitochondria contain a phospholipase D which can be activated by oxygen free radicals and divalent metal ions [15,16]. There is evidence to show rapid PLD activation in response to a wide variety of stimuli in many different cell types [17,18]. There are multiple PLD isoenzymes in mammalian cells, and a membrane-bound PLD activity, first identified in rat brain, [19,20] was recently purified [21]. This enzyme seems to be an integral membrane protein that is highly specific for phosphatidylincholine as substrate and is activated by sodium oleate. A cytosolic PLD was characterized [22–24] which is less strict in its substrate specificity because it can hydrolyse phosphatidylethanolamine (PE), phosphatidylcholine (PC) and phosphatidylserine (PS) in that order of preference [23]. It has been well established that PLD from NIH 3T3 fibroblasts specifically potentiates PE hydrolysis [25]. The increase in ethanolamine release required PKC activation and was therefore probably not an effect directly on the PLD enzyme [26,27]. Since changes in the mitochondrial membrane lipid composition may play a role in the permeability and transport properties of the mitochondria, the present study looks at the effect of polyamines and monoamines on intestinal mitochondrial lipids with special emphasis on PLD activity.

2. Materials and methods

Various lipid standards, polyamines, monoamines, Hepes, fluorescamine and bovine serum albumin (BSA) were all obtained from Sigma Chemical. All other chemicals used were of analytical grade.

2.1. Preparation of mitochondria

Rats weighing 150–200 g, fasted overnight, were decapitated, the small intestine removed and washed with ice cold saline. Mitochondria were isolated from enterocytes as described by Masola and Evered [28]. Isolated mitochondrial fraction was suspended in EGTA free medium containing 250 mM sucrose and 5 mM Hepes (pH 7.4) and stored in ice at a protein concentration of 8–10 mg/ml. Protein was measured using BSA as standard [29].

2.2. Phospholipase D assay

Mitochondrial PLD activity was measured using endogenous mitochondrial phospholipids as substrate. Mitochondria in suspension medium were incubated at 37°C for 15 min with various polyamines separately. Following incubation, total lipids were extracted by Bligh and Dyer’s method [30] and PA formed was separated by thin layer chromatography (TLC) and quantitated by phosphate estimation. Extracted lipids were spotted on silica gel G plates impregnated with 0.5 M oxalic acid and separated using the solvent system chloroform-methanol-Con. HCl (85:134:0.5 v/v) [31]. PA spot corresponding to standard was identified by iodine exposure, scraped and eluted from the plates. PA was quantitated by phosphate estimation after acid digestion [32]. Our earlier studies using intestinal mitochondrial PLD have shown that this enzyme does not catalyse transphosphatidylation in the presence of alcohol [15,16] which is similar to a recent report of a yeast PLD unable to catalyse transphosphatidylation [33].

2.3. Lipid analysis

Neutral lipids were separated on silica gel G plates using the solvent system hexane-diethyl ether-acetic acid (80:20:1 v/v). Spots were identified by iodine exposure, scraped and eluted. Cholesterol [34], diglycerides and triglycerides [35] were estimated as described. Free fatty acids were methylated and quantitated by gas chromatography after separation on a 5% EGSS-X column. Heptadecanoic acid was used as internal standard. Individual phospholipids were separated on silica gel H plates using the solvent system chloroform-methanol-acetic acid-water (25:15:4:2: v/v) [36] and quantitated by phosphate estimation after acid hydrolysis. Individual aminophospholipids were also quantitated after derivatization with fluorescamine and separation on silica gel H plates impregnated with 3% magnesium acetate using the solvent system chloroform-methanol-NH\(_4\)OH-water (60:40:5:2 v/v) [37]. Eluted individual spots were quantitated using Shimadzu SF
5000 spectrofluorometer with excitation at 395 nm and emission at 468 nm.

2.4. Statistical analysis

Three separate estimations were carried out and results are presented giving the mean ± S.E.M. Mann-Whitney \( U \)-test was done to compare the changes.

3. Results

Table 1 shows the effect of various polyamines and monoamines on intestinal mitochondrial phospholipids. All the amines tested altered the mitochondrial phospholipid composition and polyamines decreased the phosphatidylethanolamine content (PE) whereas monoamines decreased the level of both PE and PC. Concomitant with the decrease in these phospholipids, there was an increase in the formation of phosphatidic acid. No alteration in the neutral lipids was observed upon incubation with polyamines or monoamines (data not shown). Fig. 1 shows the level of PA and PE in the mitochondria when incubated in the presence of various polyamines. As can be seen, PA content increased considerably in the presence of various polyamines and increasing the amine concentration from 0.1 mM to 0.5 mM further increased the PA formation. Diamines were equally

![Fig. 1. Effect of various polyamines on phosphatidic acid and phosphatidylethanolamine content in the intestinal mitochondria. Each value represents the mean ± S.E.M. of three separate estimations. \( P < 0.05 \) in polyamine treated samples compared to control.]

Table 1

<table>
<thead>
<tr>
<th>Phospholipids (nmoles/mg protein)</th>
<th>Control (zero time)</th>
<th>Control (15 min incubated)</th>
<th>+ Spermine</th>
<th>+ Spermidine</th>
<th>+ Putrescine</th>
<th>+ Cadaverine</th>
<th>+ Histamine</th>
<th>+ Serotonin</th>
</tr>
</thead>
<tbody>
<tr>
<td>PC</td>
<td>83 ± 3.5</td>
<td>78 ± 3.0</td>
<td>77 ± 2.8</td>
<td>78 ± 2.9</td>
<td>79 ± 2.4</td>
<td>77 ± 2.9</td>
<td>57 ± 2.4</td>
<td>52 ± 2.6</td>
</tr>
<tr>
<td>PE</td>
<td>80 ± 2.5</td>
<td>74 ± 3.0</td>
<td>39 ± 2.0*</td>
<td>40 ± 2.0*</td>
<td>38 ± 2.4*</td>
<td>40 ± 2.0*</td>
<td>58 ± 2.5*</td>
<td>51 ± 2.7*</td>
</tr>
<tr>
<td>Lyso PC</td>
<td>8 ± 0.5</td>
<td>9 ± 0.4</td>
<td>9 ± 0.5</td>
<td>10 ± 0.6</td>
<td>9 ± 0.6</td>
<td>9 ± 0.5</td>
<td>10 ± 1.0</td>
<td>9 ± 0.5</td>
</tr>
<tr>
<td>Lyso PE</td>
<td>10 ± 0.6</td>
<td>12 ± 1.3</td>
<td>13 ± 1.2</td>
<td>13 ± 1.0</td>
<td>12 ± 1.3</td>
<td>12 ± 1.2</td>
<td>13 ± 1.0</td>
<td>12 ± 1.0</td>
</tr>
<tr>
<td>PS</td>
<td>7 ± 0.5</td>
<td>6 ± 0.5</td>
<td>6 ± 0.4</td>
<td>7 ± 0.5</td>
<td>7 ± 0.4</td>
<td>6 ± 0.5</td>
<td>7 ± 0.4</td>
<td>7 ± 0.4</td>
</tr>
<tr>
<td>PI</td>
<td>13 ± 1.0</td>
<td>14 ± 1.2</td>
<td>13 ± 1.0</td>
<td>14 ± 1.3</td>
<td>14 ± 0.8</td>
<td>13 ± 1.0</td>
<td>14 ± 1.0</td>
<td>14 ± 1.0</td>
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<tr>
<td>SM</td>
<td>14 ± 1.2</td>
<td>15 ± 1.3</td>
<td>15 ± 1.3</td>
<td>14 ± 1.3</td>
<td>13 ± 1.0</td>
<td>15 ± 1.1</td>
<td>14 ± 1.3</td>
<td>13 ± 1.2</td>
</tr>
<tr>
<td>CL</td>
<td>24 ± 1.8</td>
<td>25 ± 1.6</td>
<td>25 ± 1.5</td>
<td>25 ± 1.3</td>
<td>23 ± 1.6</td>
<td>25 ± 1.5</td>
<td>24 ± 1.4</td>
<td>25 ± 1.6</td>
</tr>
</tbody>
</table>

PC, phosphatidylcholine; PE, phosphatidylethanolamine; SM, sphingomyelin; PS, phosphatidylserine; PI, phosphatidylinositol; CL, cardiolipin. Each value represents the mean ± S.E.M. of three separate estimations. \( ^* P < 0.05 \) as compared to the incubated control. Amines were used at a final concentration of 0.5 mM.
potent as the polyamines in stimulating PA formation. Concomitant with the formation of PA, there was a decrease in the level of PE. Amino phospholipids were quantitated by fluorescamine derivatization, TLC separation and spectrofluorometric quantitation which is more specific and sensitive. Polyamines did not alter the content of lysophospholipids (data not shown). Fig. 2 shows the effect of various concentrations of spermine in the presence and absence of Cu²⁺ on PA formation and PE degradation in the intestinal mitochondria. In the absence of Ca²⁺ there was a linear increase in PA formation with increasing concentration of spermine. In the presence of Ca²⁺ even with 0.2 mM polyamine nearly maximum PA formation occurred. These results were corroborated with PE decrease in mitochondrial lipids. Table 2 shows the effect of monoamines and basic amino acids on PA formation in the mitochondria. Histamine and serotonin both stimulated the PA formation, and with increasing concentration an increase in PA formation was observed. Basic amino acids, lysine and arginine, did not have any effect on PA formation.

### Table 2

<table>
<thead>
<tr>
<th>Monoamines and Basic Amino Acids</th>
<th>Phosphatidic acid formed (nmol/mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (15 min incubated)</td>
<td>16±1.6</td>
</tr>
<tr>
<td>+ Histamine 0.1 mM</td>
<td>31±1.5 *</td>
</tr>
<tr>
<td>+ Histamine 0.5 mM</td>
<td>47±2.5 *</td>
</tr>
<tr>
<td>+ Serotonin 0.1 mM</td>
<td>39±2.0 *</td>
</tr>
<tr>
<td>+ Serotonin 0.5 mM</td>
<td>55±3.0 *</td>
</tr>
<tr>
<td>+ Lysine 0.5 mM</td>
<td>16±1.8</td>
</tr>
<tr>
<td>+ Arginine 0.5 mM</td>
<td>17±2.0</td>
</tr>
</tbody>
</table>

Each value represents the mean±S.E.M. of three separate estimations. * P < 0.05 as compared to the incubated control.

4. Discussion

The salient observation in this work was that both polyamines and monoamines stimulated PLD activity of intestinal mitochondria, resulting in the formation of PA. Polyamine stimulated PLD preferentially hydrolysed PE whereas when stimulated by monoamines, both PE and PC were hydrolysed. We have also previously observed that intestinal mitochondrial PLD when stimulated by oxygen free radicals or Ca²⁺ preferentially hydrolysed PE whereas stimulated by Mg²⁺ or Ba²⁺ it hydrolysed both PC and PE [15,16]. It is not known whether there is more than one type of PLD in the mitochondria which uses different substrates or the same enzyme uses different substrates when stimulated by polyamines or monoamines. Other studies have indicated the presence of PLD activity in mitochondria. Panagia et al. [38] have identified a PLD activity in myocardial subcellular membranes. Isolated and purified cardiac subcellular organelles showed the PLD activity in sarcotlemma, sarcoplasmic reticulum and mitochondria with 14-, 11- and 5-fold enrichment respectively when compared with the homogenate. Sarcolemmal
PLD showed high specific activity with phosphatidylcholine as substrate although PE and PI were also hydrolysed at low rates. This PLD was found to catalyse transphosphatidylation [38]. PA is a known intermediate in phospholipid biosynthesis. The principal sites for synthesis of complex phospholipids are microsomes. Lyso phosphatidic acid (LPA) and/or PA synthesized in the mitochondrial outer membrane has to be transported to the endoplasmic reticulum for lipid biosynthesis. The mitochondrialy synthesized LPA is transported to microsomes in liver by liver fatty acid binding protein (L-FABP) where it can be converted to PA. L-FABP can also stimulate microsomal conversion of LPA to PA but strongly inhibits this reaction in mitochondria [39]. Thyroid hormone treatment of rat brain mitochondria influences phospholipid metabolism, especially an increase in PE at the expense of PI, PS and PC. The PA content was also increased, suggesting altered phospholipid metabolism and possibly mitochondrial membrane fluidity [40]. A unique mitochondria associated ER-derived membrane that copurifies with mitochondria during isolation has been shown to transfer lipids from ER to mitochondria [41]. It is unlikely that the observed increase in PA on exposure of intestinal mitochondria to polyamines is due to transfer from other subcellular membranes since this study was carried out with isolated mitochondria which were found to be reasonably pure, as judged by the marker enzymes. It is unlikely that polyamines stimulate phospholipase A₂ or C since there was no increase in lysophospholipids, diglycerides or free fatty acids (data not shown). Polyamines are polycationic compounds present in cells and their protonated groups at the physiological pH are known to bind negatively charged cellular components [42]. Polyamines play an important role in control of cell proliferation and differentiation [43]. Polyamines are also known to activate certain phospholipases. Incorporation of polyamines to phospholipase A₂ results in a 2–3-fold increase in its activity [44]. It has been reported that activity of calcium dependent phosphatidylinositol specific phospholipase C of rat brain is influenced by polyamines [45]. Activation of phospholipase C from human amnion by polyamine was enhanced at 1.5 mM concentration and polyamines were stronger activators than diamines [46]. The present study has shown for the first time that polyamines and monoamines stimulate PLD activity even at 0.1 mM concentration, and both polyamines and diamines are equally potent. Our earlier work has shown that mitochondrial PLD is stimulated by divalent cations including calcium [16]. It was seen in this study that the presence of calcium along with spermine increased further activation suggesting that these two compounds do not compete for the same binding site in the enzyme.

Polyamines are also involved in various mitochondrial functions [47,48]. Although in the normal situation mitochondria store very little Ca²⁺, they have the capacity to sequester cytosolic Ca²⁺ when the Ca²⁺ level in the cytosol increases in certain pathological conditions and this leads to an increase in mitochondrial Ca²⁺ cycling [1,2]. It has been shown that spermine stimulates both Ca²⁺ uniporter and ruthenium red insensitive efflux of Ca²⁺ in rat liver mitochondria [13,49]. Polyamines have been shown to serve as messengers to generate Ca²⁺ signal by increasing Ca²⁺ influx. Suppression of polyamine synthesis by difluoromethyl ornithine, a specific inhibitor of ornithine decarboxylase, decreased mitochondrial Ca²⁺ and increased cytosolic Ca²⁺ [50]. PA has been shown to stimulate influx of Ca²⁺ in various cell types through an ionophore effect [51–53]. Oxidative stress is known to alter cellular calcium homeostasis and exposure of mitochondria to oxidants leads to Ca²⁺ efflux [54]. Hypoxia and ischaemia markedly increase putrescine production [55] and it is known that under these conditions Ca²⁺ cycling by mitochondria also occurs. Polyamines are known to bind membrane components, and it has been suggested that isolated mitochondria are likely to have a polyamine content of 2 mM [56] which is far above the concentration required to stimulate PLD activity. In addition to polyamines, monoamines also stimulated the formation of PA by mitochondria. Intestinal epithelial cells are known to contain various polyamines including the bacterially derived polyamine, cadaverine [57].

Polyamines are known to modulate the activity of certain mitochondrial enzymes [47,56,58,59]. It was seen here that polyamines altered the mitochondrial lipid composition by stimulating PLD activity. Decrease in the PE content and increase in the level of acidic phospholipid, PA, on exposure to polyamines may be responsible for the observed changes in activ-
ity of certain mitochondrial enzymes. The increased interaction between polyamines and acidic phospholipids might regulate cell growth and differentiation.

Acknowledgements

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References