Abstract A progressive hydrolysis of phospholipids was observed during the mineralization process mediated by extracellular matrix vesicles. Increasing levels of different hydrolysis products revealed phospholipase A and D activities. The importance of these enzymes for the mineralization process lies in a high rate of hydrolysis of neutral phospholipids and lower rate of degradation of anionic phospholipids, which may favor mineral formation in vesicular membrane and membrane breakdown necessary for the release of mineral deposits into extracellular matrix. In this report, we focus on the phosphorylation-dependent phospholipase D activity during mineral formation initiated by chicken embryo matrix vesicles.

Keywords: Matrix vesicles; Mineralization; Phospholipase C; Phospholipase D; Phosphorylation

1. Introduction

To rapidly mineralize competent cells, osteoblasts, chondrocytes and odontoblasts, produce matrix vesicles (MVs) able to accumulate calcium and inorganic phosphate (P i) and containing nucleation sites for the formation of initial mineral phase [1]. The specific protein and lipid compositions allow MVs to induce the initiation of mineral formation. The morphology and the composition of MVs can facilitate the deposition of hydroxyapatite (HAP) [2,3], whereas ion channels and transporters present in vesicular membrane are responsible for controlling the Ca 2+ and P i uptakes inside MVs [4–6]. The mechanism by which HAP crystals break the membrane and are released into the extracellular matrix is not very well understood. It has been proposed that phospholipases, activated during the mineral formation, could increase the membrane fluidity of the MVs [2,7,8] finally leading to a membrane breakdown. Among these enzymes, phospholipase A (PLA) [2,8,9] was detected in MVs. The PLA class is responsible for a selective degradation of phospholipids during MV-mediated mineralization accompanied by the production of fatty acids [2]. In addition, we observed that during MV-mediated mineralization, the level of phosphatic acid (PA) changed, indicating the activity of phospholipase D (PLD). Indeed, by using a fluo- rescence assay we were able for the first time to identify PLD activity in MVs and to show that this activity is phosphorylation-dependent.

2. Materials and methods

2.1. Isolation of matrix vesicles

MVs were isolated from 17-day-old chicken embryos, as described elsewhere [10]. Long leg bones were cut and extensively washed in ice-cold synthetic cartilage lymph (SCL) containing 2 mM Ca 2+ and 1.42 mM P i. In addition to, 133.5 mM Cl −, 1.83 mM HCO 3 −, 12.70 mM K +, 104.5 mM Na +, 0.57 mM Mg 2+, 0.57 mM SO 4 2−, 63.5 mM sucrose, 5.55 mM D-glucose, 16.5 mM TES pH 7.4 [3]. Tissue was digested with 0.1% trypsin from bovine pancreas (MP Biomedicals) at 37 °C for 5 min at 4 ml of SCL per g tissue, followed by washing in SCL to remove protease. Then, they were digested with collagenase type-I from the Clostridium histolyticum (MP Biomedicals) (200 U of collagenase/ml of tissue) at 37 °C for 3 h in SCL. The digested tissue was filtered through a nylon filter and centrifuged at 13000 × g for 20 min at 4 °C. The pellet was discarded and the supernatant was centrifuged at 80000 × g for 60 min at 4 °C yielding MV pellet which was suspended in 1 mM Ca 2+.

2.2. Functional characterization of MVs

MVs (40 μg of protein/ml) in SCL were incubated at 37 °C. The formation of mineral phase was monitored at 15 min intervals by monitoring light scattering at 340 nm [3] using a 932 Uvikon spectrophotometer (Kontron Instruments). MVs after 24-h incubation in SCL at 37 °C were centrifuged at 3000 × g for 15 min. The pellet was dried, A 100–150 μg aliquot of a dry material was incorporated into 150 mg of solid KBr. Mineral composition was determined using an infrared spectrometer Nicolet 510M FTIR (Nicolet) 32 scans were collected at 4 cm -1 resolution.

2.3. Monitoring of lipid composition changes during MV-mediated mineralization

MVs (15 μg of protein) were incubated at 37 °C in 1 ml of SCL. In time intervals, samples were collected and extracted in chloroform:methanol (2:1, by vol.). The insoluble pellets were treated with 0.5 M EDTA and further extracted in chloroform:methanol:HCl (200:100:1, by vol.) [2]. Lipid composition of the MVs during mineralization was examined using thin layer chromatography. Plates were developed up to 8 cm from the origin in ethyl acetate:1-propanol:chloroform:methanol:0.25% KCl (25:25:25:10:9, by vol.). The solvent was allowed to evaporate for 20 min and plates were developed in the same direction up to 13 cm from the origin in hexane:diethyl ester:acetic acid (75:21:4, by vol.). Lipid spots were visualized by spraying the plate with 10% CuSO 4 in 8% H 3PO 4 and heating at 180 °C for 20 min [11]. Chromatograms were scanned with CAMAG TLC Scanner 3 (Camag). Each experiment was repeated three times on distinct samples. Due to low resolution between certain lipid spots, they were expressed as PS + PI and DAG + cholesterol contents. Changes of relative lipid amounts in % as function of time were determined according to: (density of a lipid spot at a given time/density of all lipid spots at t = 0 h) × 100. This is an approximation since each lipid did not react in the same manner in the presence of 10%
CuSO₄ in 8% H₃PO₄. The relative amount of phospholipids and nonpolar lipids were calculated separately to obtain more accurate estimations.

2.4. Determination of phospholipase D and C activities

Amplex Red Phospholipase D Kit (Molecular Probes) was used to measure the rate of choline production during PC hydrolysis according to the manufacturer’s instructions (Fig. 1A) with slight modifications such as addition of either 2 mM levamisol (Sigma) or 8 U/ml alkaline phosphatase (ALP) from *Escherichia coli* (Sigma) in the reaction buffer. The fluorescence of resorufin induced by the reaction of 10-acetyl-3,7-dihydroxyphenoxazine and H₂O₂ produced by the oxidation of choline to betaine (Fig. 1A), was monitored at 583 nm after sample excitation at 550 nm on the F-4500 Hitachi Fluorescence Spectrophotometer. Slits for excitation and emission were set at 2.5 nm. The experimental procedures suggested by the manufacturer could not distinguish PLC and PLD activities in MVs. Therefore, we modified the experimental procedure as described below (Fig. 1B). MVs (400 μg protein/ml) in 50 mM Tris·HCl, pH 7.4, 140 mM NaCl and 10 mM dimethylsulfoxide, 1% Triton X-100 and 4 mM levamisol were subjected to freeze/thaw cycles carried out within 10–15 min [12]. This was incubated for 30 min at 37 °C, and the reaction was stopped by the addition of 100 μl chloroform and gentle vortexing separating membrane- and aqueous-soluble components. It was centrifuged at 3000 g for 10 min. The second step was to collect 50 μl aliquots of aqueous phase containing choline and phosphocholine, products of phospholipid hydrolysis, and to dilute by a factor of two. This was mixed with 100 μl of reaction buffer containing 400 μM Amplex reagent, 2 U/ml of horseradish peroxidase (HRP) (Molecular Probes), 0.2 U/ml of choline oxidase from *Alcaligenes* sp. (MP Biomedicals). The reactive medium was supplemented with either 8 U/ml of ALP (*Escherichia coli*) or lack ALP to determine the ALP activity. Steady-state fluorescence spectra were recorded as described above after 20 min of incubation at 37 °C. One unit of phospholipase activity is defined as the amount of enzyme which produced 1 μmol of choline or phosphocholine from PC per minute at 37 °C. The phospholipase activities were measured at least seven times on distinct samples.

2.5. Other procedures

Protein concentration was measured using the BioRad Protein Assay system (BioRad). Total phospholipid phosphate content was measured according to Bartlett’s method [14]. Alkaline phosphatase activity was determined by the method of Cyboron and Wuthier [15].

3. Results and discussion

Isolated MVs from chicken embryo bones were characterized by the presence of a marker enzyme, ALP, whose activity amounted to 25.7 ± 1.9 U/mg and by a protein to phospholipid weight ratio of 3.0 ± 0.2. The MVs were able to accumulate rapidly Cu²⁺ from SCL mineralization medium without ALP substrates (Fig. 2A). A typical lag period of 0.5–1 h for the mineral formation was observed, similar to that reported previously for avian MVs [6]. The mineral deposits produced by MVs contained mostly hydroxyapatites (as revealed by the characteristic infrared bands located at 563 cm⁻¹, 603 cm⁻¹, and 1034 cm⁻¹) and phosphate complexes in acidic environment as indicated by the 1123 cm⁻¹ band (Fig. 2B) [16,17]. During mineral formation a progressive hydrolysis of the major classes of phospholipids was observed (Fig. 3). Neutral phospholipids such as sphingomyelin, phosphatidylcholine and phosphatidylethanolamine (SPH, PC, PE) were hydrolyzed to a higher extent than negatively charged phospholipids such as phosphatidylinerine and phosphatidylinositol (PS and

![Fig. 1. Schematic representation of enzymatic reactions used to determine PLD and PLC activities in MVs. (A) One-step enzymatic reaction. Optional presence of exogenous APL in a reaction medium allows dephosphorylation of PLD which resulted in a decrease of its activity. Addition of levamisol inhibited endogenous APL, enabling determination of PLD activity under optimal conditions. (B) Two-step enzymatic reaction. In the first step, levamisol is required to allow dephosphorylation of PLD which resulted in a decrease of its activity. Addition of levamisol inhibited endogenous APL, enabling determination of PLD activity under optimal conditions. In the second step, following enzyme denaturation, exogenous APL transformed phosphocholine to choline allowing the detection of total PLC + PLD phospholipase activity. Asterisks denote the optional presence of levamisol or ALP during experiment.](image1)

![Fig. 2. Functional characterization of MVs. (A) Mineral formation by MVs incubated in SCL at 37 °C in the presence (bold line) and absence of 2 mM Ca²⁺ (thin line). Mineral formation was monitored at 340 nm. (B) Infrared spectrum of mineral formed after 24 h incubation in SCL at 37 °C.](image2)
PS is a major negatively charged phospholipid associated with newly formed mineral phase [18]. PS hydrolysis by PLA₂ can be inhibited when PS forms Ca–PS–P₂ complexes [19], explaining the relative resistance of PS to hydrolysis in comparison to neutral phospholipids [2]. The relative amount of fatty acids doubled during mineralization (Fig. 3 and Table 1), confirming an activation of PLA activity in MVs [2]. Phosphatidic acid (PA) content, which decreased slightly during the initial step of mineralization, finally increased. During mineralization, PA was initially hydrolyzed but later its production due to phospholipid degradations was larger than its hydrolysis (Fig. 3 and Table 1), suggesting the activation of a PLD enzyme. Therefore, we checked PLC and PLD activities in MVs. Their activities were significantly lower than that of PLA. However, a small amount of PA produced in MVs, as from PC hydrolysis, could affect mineral formation. The rate of PC hydrolysis in MVs was measured as a function of time using Amplex Red fluorophore (see Section 2). The fluorescence measurements were performed either with exogenous alkaline phosphatase (ALP) or levamisol, a well-known inhibitor of ALP, and revealed that the apparent total phospholipase activity (PLD + PLC) was lower than PLD activity (Fig. 4A). Addition of ALP decreased the rate of phospholipid hydrolysis suggesting that ALP as a potent dephosphorylating enzyme decreased PLD activity about 4-fold in comparison to the activity measured in the presence of levamisol (Fig. 4B). Since attempts to measure PLD and PLC activities in MVs using standard procedures failed we modified the protocol to distinguish between PLD and PLC activities (Fig. 1B). Therefore, steady-state measurements of resorufin fluorescence (see Section 2) were performed to determine the amount of choline present in samples (Fig. 5). PLD activity amounted to 4.83 ± 0.74 mU/mg of protein (1.61 ± 0.25 mU/mg of phospholipid) while PLC activity was 0.174 ± 0.028 mU/mg of protein (0.058 ± 0.009 mU/mg of phospholipid). Since the standard deviation of the determination of PLD activity is higher than that of PLC activity we considered the level of PLC activity determined in the present report as rather doubtful. This is additionally supported by a relatively stable level of diacylglycerol (DAG) during mineralization while monoacylglycerol (MAG) and free fatty acids (FFA) increased (Fig. 3). Since we observed that up to 70% PI (Fig. 3 and Table 1). PS is a major negatively charged phospholipid associated with newly formed mineral phase

Table 1
Relative changes of lipid amounts in % during mineralization

<table>
<thead>
<tr>
<th>Lipid (%)</th>
<th>Time of mineralization (h)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0</td>
</tr>
<tr>
<td>Neutral phospholipids</td>
<td></td>
</tr>
<tr>
<td>SPH</td>
<td>6.2 ± 0.8</td>
</tr>
<tr>
<td>PC</td>
<td>38.8 ± 1.5</td>
</tr>
<tr>
<td>PE</td>
<td>23.4 ± 3.2</td>
</tr>
<tr>
<td>Anionic phospholipids</td>
<td></td>
</tr>
<tr>
<td>PS + PI</td>
<td>28.2 ± 3.1</td>
</tr>
<tr>
<td>PA</td>
<td>1.6 ± 0.3</td>
</tr>
<tr>
<td>Nonpolar lipids</td>
<td></td>
</tr>
<tr>
<td>MAG</td>
<td>22.1 ± 1.5</td>
</tr>
<tr>
<td>DAG + Ch</td>
<td>31.1 ± 13.6</td>
</tr>
<tr>
<td>FFA</td>
<td>18.5 ± 2.1</td>
</tr>
<tr>
<td>TAG</td>
<td>28.3 ± 3.1</td>
</tr>
</tbody>
</table>

Results are the mean of the ratio of a given lipid spot density at certain time to all lipid spot densities at time 0 h × 100 ± S.D. Phospholipids and nonpolar lipids were calculated separately. The total amount of phospholipid decreased by a factor of two during incubation time of 40 h. The experiments were repeated three times. The abbreviations are the same as in the legend of Fig. 3.
the protein machinery in MVs provides a regulatory mechanism based on phosphorylation–dephosphorylation of PLD to modulate lipid composition and membrane breakdown. In addition, the growth of hydroxyapatite crystals in MV lumen may induce changes of membrane curvature and surface pressure modulating phospholipase activity and accessibility of particular phospholipids.

Acknowledgements: This work was supported by Polish Ministry of Science and Education (Grant No. 2 PO4A 029 29). We thank Dr. John Carew for correcting the English prior to submission.

References


4. Concluding remarks

Phospholipase activities in MVs are important for the mineral nucleation, membrane breakdown and removal of lipid constituents. In 1992, Fedde showed that MVs derived from SaOS-2 cell culture contain at least three protein kinases, likely to play a role in PLD phosphorylation [21]. We suggest that

Fig. 4. PLD present in MVs is a phosphorylation regulated enzyme. (A) Rate of the PC hydrolysis in the presence of 2 mM levamisol or 8 U/ml of Escherichia coli alkaline phosphatase (ALP). (B) Difference of phospholipase activity measured in the same samples in conditions avoiding (+levamisol) and allowing (+ALP) hydrolysis of the phoshoenzyme. Results are means ± S.D. from seven determinations.

Fig. 5. Determination of PLD and PLC activities in MVs using two-step coupled enzymatic reaction assay. Resorufin fluorescence allowed detection of total phospholipase activity (PLD + PLC) (bold line) and PLD activity (thin line). Representative spectra are shown.

of the PLD activity was sensitive to the dephosphorylation, we propose that two PLD isoforms regulated in a different manner were present in MVs, as in the case of c-Src kinase regulated mammalian isoforms PLD1 and PLD2 [20].


