Changes in Phospholipid Extractability and Composition Accompany Mineralization of Chicken Growth Plate Cartilage Matrix Vesicles*

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Matrix vesicles are lipid bilayer-enclosed structures that initiate extracellular mineral formation. Little attention has been given to how newly formed mineral interacts with the lipid constituents and then emerges from the lumen. To explore whether specific lipids bind to the incipient mineral and if breakdown of the membrane is involved, we analyzed changes in lipid composition and extractability during vesicle-induced calcification. Isolated matrix vesicles were incubated in synthetic cartilage lymph to induce mineral formation. At various times, samples of the lipids were taken for analysis, extracted both before and after demineralization to remove deposited mineral. Phosphatidylserine and phosphatidylinositol both rapidly disappeared from extracts made before decalcification, indicating rapid degradation. However, extracts made after demineralization revealed that phosphatidylserine had become complexed with newly forming mineral. Concomitantly, its levels actually increased, apparently by base-exchange with phosphatidylethanolamine. Though partially complexed with the mineral, phosphatidylinositol was nevertheless rapidly broken down. Sphingomyelin and phosphatidylethanolamine also underwent rapid breakdown, but phosphatidylcholine was degraded more slowly, all accompanied by a buildup of free fatty acids. The data indicate that phosphatidylserine forms complexes that accompany mineral formation, while degradation of other membrane phospholipids apparently enables egress of crystalline mineral from the vesicle lumen.

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¶ The abbreviations used are: MV, matrix vesicles; PS, phosphatidylserine; TEM, transmission electron microscopy; HPTLC, high performance thin-layer chromatography; HPLC, high performance liquid chromatography; ELS, evaporative light scattering; FFA, free fatty acids; MG, monoaoylglycerols; CRMV, collagenase-released MV; SCL, synthetic cartilage lymph; PI, phosphatidylinositol; CH, free cholesterol; DG, diacylglycerols; PE, phosphatidylethanolamine; PC, phosphatidylcholine; SPH, sphingomyelin; LPS, lysophosphatidylserine; LPI, lysophosphatidylinositol; LPE, lysophosphatidylethanolamine; LPC, lysophosphatidylcholine.

membrane that is enriched in selected phospholipids, especially phosphatidylserine (PS) (5–6), a lipid with known high affinity for Ca$^{2+}$ (7–8). Initially, PS is largely confined to the inner leaflet of the MV membrane (9). Previous transmission electron microscopic (TEM) studies have shown that the first mineral formed in MV is associated with the inner aspect of the membrane (10). Later, the crystals appear to emerge through the membrane and trigger formation of radial clusters of mineral centered on the remnant of the original vesicle. How the crystals penetrate the MV membrane is currently unknown. While it is possible that simple physical force mediates this process (11), there is indirect evidence that latent lipolytic enzymes become activated during Ca$^{2+}$ accumulation and facilitate breakdown of the membrane. To explore this latter possibility, the composition of lipids in MV was analyzed during the course of MV-mediated mineralization in vitro.

Since previous work had shown that not all lipids were readily extracted from mineralizing tissues (12–13), resident MV lipids were extracted both before and after demineralization using both neutral and acidic lipid solvents. Lipid composition was analyzed qualitatively by high performance thin layer chromatography (HPTLC) and quantitatively by high performance liquid chromatography (HPLC) using an evaporative light scattering (ELS) detector, which enabled accurate quantitation of the various lipids.

Our findings reveal that extensive phospholipid degradation occurred during MV calcification, and this was accompanied by a concomitant rise in the amount of free fatty acids (FFA) apparently released by phospholipases present in the vesicles. The breakdown of MV phospholipids was accompanied by a substantial reduction in the extractability of certain phospholipids, and the composition of MV lipids changed significantly during the process of mineralization. In particular, PS, which became progressively more tightly complexed with nascent mineral, could only be fully extracted after demineralization. It was not only protected from degradation, but was actually synthesized, apparently by a base-exchange mechanism.

EXPERIMENTAL PROCEDURES

Isolation of Matrix Vesicles—Large batches of collagenase-released matrix vesicles (CRMV) were isolated from the metatarsal growth plate cartilage of 6- to 8-week-old broiler strain chickens using previously described methods (14). In brief, cartilage shavings from −300 chicken feet (−160 g) were digested with 0.1% trypsin (type III, Sigma) at 37 °C for 30 min in a synthetic cartilage lymph (SCL) (14) with an ionic composition similar to that found to be present in native cartilage (15). The trypsin solution was removed; tissue slices were rinsed twice with SCL and digested with collagenase (200 units/g of tissue, type IA, Sigma) at 37 °C for 3−5 h. The partially digested tissue was vortexed, and the suspension centrifuged as previously reported to sediment the CRMV (14). The pellet was resuspended as a stock containing 5.0 mg of vesicle protein/ml in SCL modified to have one-half the normal level of...
Ca\textsuperscript{2+} to prevent dissolution of labile Ca\textsuperscript{2+} and P (16) and also to minimize Ca\textsuperscript{2+} uptake by the CRMV during storage. Protein levels were determined by the Lowry et al. method (17).

Mineralization Experiments—To have sufficient material for accurate lipid analyses, the large-scale preparations of CRMV (~20 mg of protein) were allowed to mineralize by incubation in 600 ml of SCL at 37 °C. At each time point (0, 2, 4, 6, and 24 h) for lipid analysis a 110-ml sample was centrifuged at 100,000 \times g for 60 min to sediment the CRMV. The resulting pellets were transferred to individual glass tubes for lipid extraction after reuspension in ~150 \mu l of SCL. Mineral formation in samples of the incubation mixture was monitored by light scattering essentially as described previously (14).

Lipid Extraction and Purification—Lipids were extracted from the CRMV pellets, essentially as previously described (18). For qualitative HPTLC analyses, the vesicle pellets were extracted with chloroform, methanol (2:1) (v/v) (~20 ml/ml aqueous medium), followed by sonication for 1–2 min (extract 1). The tubes were then centrifuged at 3,000 rpm for 12 min to sediment the insoluble residue. Initially, after collecting the lipid-containing supernatant, a second extraction with chloroform, methanol, HCl (200:100:1) (v/v/v) was performed, assuming that any mineral-complexed would be readily extractable. However, subsequent work revealed that significant amounts of the acidic phospholipids remained in the residue. Therefore, for all studies reported here, after the initial lipid extraction, the CRMV pellets were then demineralized with 0.5 M sodium salt EDTA for 20 min at room temperature and sedimented by centrifugation at 3,000 rpm for 12 min. After removal of the supernatant, the decalcified residue was reextracted using chloroform, methanol, HCl (200:100:1) (v/v/v) (extract 2), which was found to quantitatively remove the remaining lipids. The crude extracts were dried under N\textsubscript{2} and partitioned through a Sephadex G-25 column to remove non-lipid contaminants (19).

HPTLC and HPLC Analysis of Lipids—For qualitative analysis, the pure lipids from the preceding steps were analyzed by HPTLC on Whatman LHP-K silica gel plates as described previously (20, 21). Mixtures containing 10 \mu g each of various lipid standards were applied to separate lanes on the same plate as the MV lipid samples. Lipids were visualized by spraying with cupric-phosphoric acid charring reagent (10% CuSO\textsubscript{4}, in 8% H\textsubscript{3}PO\textsubscript{4}) and heating at 180 °C for 10 min in an oven (20). HPTLC plates were photographed and digitized for semiquantitative estimation of lipid levels.

For accurate quantitative analysis, the lipids were analyzed using a Shimadzu HPLC (SCL-10A Systems Controller, SIL-10A Auto Injector, Dual LC-10AT Liquid Chromatographs to provide the gradients). The HPLC system was equipped with an Alltech Varex MR III ELS detector system. Lipids were separated on a Lichrosorb SI-100 4.6 \times 250 mm, 10 \mu m particle size, HPLC column supplied by Alltech Inc. Injection volume was 30–50 \mu l (autosampler). The two mobile phases used were as follows: A, methanol, water (80:20) (v/v); B, chloroform, methanol, 0.1% formic acid (80:20:0.1) (v/v/v). The following gradient program was used: 1) 92% B, 8% A for 6 min; 2) 92% B, 8% A to 56% B, 44% A in 21 min; 3) 56% B, 44% A to 20% B, 80% A in 2 min, 4) 20% B, 80% A to 100% B in 1 min, 5) 100% B to 92% B, 8% A in 2 min. Total run time was 33 min, with a solvent flow rate of 1 ml/min. Nitrogen gas, 11.6 pounds per square inch, was used as the carrier at a flow rate of 2.02 standard liters per min in the ELS detector, which was operated at 72 °C.

RESULTS

Calcification of Isolated CRMV—Mineralization induced by the isolated CRMV was monitored by formation of mineral from the SCL during the incubation period. As had been previously observed, there was a progressive induction of mineral formation during incubation of the CRMV with SCL (Fig. 1A). As is typical of MV mineralization, there was a short lag period (~1 h) during which minimal Ca\textsuperscript{2+} accumulation occurred; thereafter, the rate of mineral formation increased rapidly and by 6 h mineral formation was ~80% of maximum. Fig. 1B shows an electron micrograph of early MV mineralization made after 2 h incubation in SCL.

Lipid Composition of CRMV—Three different methods of analysis were used to assess the lipid composition of CRMV, both before and after incubation with SCL, which induced mineral formation. Initially, for qualitative analysis, HPTLC was used to analyze the various polar and nonpolar lipid classes present in extracts of CRMV made before and after demineralization. From these studies it was evident that significant changes in lipid composition were occurring during MV calcification. Most notable was the dramatic disappearance of PS and phosphatidyl inositol (PI) in extracts made before decalcification after only ~2 h of incubation (Fig. 2A). Also there were progressive increases in the levels of FFA and in the band corresponding to PC and PE (DG). On the other hand, there was a strikingly different lipid composition in extracts made after decalcification (Fig. 2B). Here there was a dramatic increase in the levels of PS at time points when MV calcification was in rapid progress.

While densitometric scans of the HPTLC plates afforded reasonable estimation of the levels of individual lipids present, we used HPLC to more accurately measure the quantitative changes in lipid composition. Initially, we employed a highly sensitive UV detection system at 205 nm to quantitate lipids changes in SCL. However, because of the variability in sensitivity to fatty acids, we found this HPLC technique of limited utility. With careful calibration, some useful information was ob-
Fig. 2. HPTLC analysis of lipids present in CRMV after various times of incubation with SCL. A (extract 1), lipid composition of the CRMV extracts made with chloroform, methanol (2:1 (v/v)) before demineralization. B (extract 2), lipid composition of CRMV extracts subsequently made with chloroform, methanol, concentrated hydrochloric acid (200:100:1) (v/v/v) after demineralization with EDTA.

Values are the mean ± S.E. of replicate analyses, the number shown in parentheses before each. These are the sum of extracts 1 and 2 made before and after demineralization, respectively, and are the composite of data from both HPLC and HPTLC analyses of the lipids. The reason there are more replicates shown here than in studies where lipid composition was analyzed separately in extracts made before and after demineralization is because in a number of initial experiments the lipids present in the two extracts were combined before analysis.

| Lipid          | Incubation time | % of the total phospholipid |
|----------------|-----------------|-----------------------------|
|                | 0   | 2   | 4   | 6   | 24  |
| PE             | (16) | 16.9 ± 1.3 | (9) | 15.8 ± 1.7 | (9) | 12.4 ± 1.1 | (9) | 13.3 ± 1.9 | (8) | 15.7 ± 3.6 |
| LPE            | (16) | 6.0 ± 1.0  | (9) | 6.2 ± 1.5  | (9) | 6.1 ± 1.5  | (9) | 6.6 ± 1.8  | (8) | 10.8 ± 2.3  |
| PS             | (16) | 9.2 ± 1.7  | (9) | 10.8 ± 2.9 | (9) | 12.6 ± 3.1 | (9) | 15.0 ± 4.3 | (8) | 19.2 ± 4.5  |
| LPS            | (13) | 0.05 ± 0.04| (6) | 0.8 ± 0.5  | (6) | 2.4 ± 1.1  | (6) | 1.5 ± 0.6  | (5) | 3.3 ± 1.8   |
| PC             | (16) | 32.1 ± 2.8 | (9) | 57.6 ± 5.9 | (9) | 57.6 ± 0.8 | (9) | 56.1 ± 8.3 | (8) | 58.0 ± 8.3  |
| LPC            | (13) | 1.2 ± 0.6  | (6) | 0.6 ± 0.5  | (6) | 0.9 ± 0.6  | (6) | 0.7 ± 0.4  | (5) | 2.7 ± 1.8   |
| PI             | (13) | 4.0 ± 0.6  | (6) | 2.8 ± 0.7  | (6) | 2.1 ± 0.9  | (6) | 1.9 ± 0.8  | (5) | 2.6 ± 0.9   |
| LPI            | (12) | 1.4 ± 0.4  | (6) | 0.1 ± 0.0  | (6) | 0.2 ± 0.1  | (6) | 0.2 ± 0.2  | (5) | 1.0 ± 1.0   |
| SPH            | (13) | 8.4 ± 0.8  | (6) | 5.1 ± 0.7  | (6) | 5.0 ± 1.2  | (6) | 4.2 ± 1.2  | (5) | 2.6 ± 1.4   |

Change in Lipids during Matrix Vesicle Mineralization

In general agreement with previous analyses (23–24), at $T_0$, phosphatidylcholine (PC) was the major phospholipid in MV, representing about 50% of the total. Phosphatidylethanolamine (PE), —17%, PS —9%, sphingomyelin (SPH), —8%, monoacyl (i.e. lyso)phosphatidylethanolamine (LPE), —6%, and PI, —4% were the principal lipids. Low levels of lysophosphatidylcholine (LPC), 1.4%, phosphatidylethanolamine (LPC), 1.2%, and lysophosphatidylserine (LPS), 0.05% were the only other phospholipids consistently detected. With time, in each successive set of extracts, the lipid composition progressively changed. The most notable changes were the significant increases in the levels of PS, LPS, and LPE and the progressive disappearance of both PI and SPH (Table I).

However, these values were based on the analyses of lipids present in extracts taken at the successive points during MV calcification. During this period, changes in lipid composition were occurring due to both variable lipid degradation and to specific effects on extractability caused by the selective binding of certain lipids to the newly forming mineral. Because of this, an attempt was made to normalize the progressive changes occurring in total lipid composition by combining data on total polar and nonpolar lipids from extracts taken before and after decalcification at $T_0$ to serve as a baseline for changes in individual lipid classes occurring at subsequent time points.
These changes were then expressed as a percentage of the original amount of each lipid class at successive time points.

Changes in Total Lipid Content—Combining data on lipids extractable before and after demineralization at each time point caused only modest (∼10%) overall change in the total lipid (phospholipid + nonpolar lipid) content to occur during MV-induced calcification. However, when the fate of the total phospholipid was separated from that of the total nonpolar lipid, major progressive changes in each type of lipid were evident (Fig. 3A). Whereas the amount of total phospholipid underwent rapid and progressive decline, the opposite occurred with the nonpolar lipid. After 6 h of incubation, only about 50% of the original phospholipid remained, whereas a nearly 33% increase over the original amount of nonpolar lipid was seen. By 24 h, only about 20% of the total phospholipid remained, whereas the amount of nonpolar lipid had increased 66% above its original level.

Examination of the combined data on individual lipids extractable before or after demineralization revealed major differences in the stability of each class of phospholipid (Table II). These revealed that rapid degradation of the acidic phospholipids PS and PI occurred; for both lipids only about 50% of the original remained after 2 h of incubation. However, surprisingly, PS “recovered” and returned to nearly 75% of the original by 24 h. In contrast, levels of PI continued their rapid decline and by 24 h, less than 10% of the original lipid remained. SPH also showed very rapid and continuous degradation, whereas breakdown of PE and PC was significantly slower. The changes in the content of lysophospholipids were complex and will be elaborated on later. However, overall the HPLC findings corroborate and clarify the qualitative impression observed upon HPTLC analyses.

Changes in Extractability of Total Lipids—As MV calcification progressed with time, the percentage of the total phospholipid extractable before demineralization decreased concomitantly, and correspondingly, the proportion extractable only

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**Table II**

Recovery of matrix vesicle lipids during in vitro calcification

Values are the mean ± S.E. of replicate analyses, the number shown in parentheses before each. These are combined data from extract 1 plus extract 2 made before and then after demineralization, respectively. Values are a composite of data from both HPLC and HPTLC analyses of the lipids.

| Lipid | Incubation time | % of the original phospholipid class |
|-------|----------------|-------------------------------------|
|       | 0              | 2        | 4        | 6        | 24       |
| PE    | (6) 100 ± 0    | (6) 84 ± 17 | (6) 61 ± 11 | (6) 46 ± 8 | (6) 25 ± 4 |
| LPE   | (6) 100 ± 0    | (6) 62 ± 22 | (6) 55 ± 21 | (6) 53 ± 25 | (6) 51 ± 30 |
| PS    | (6) 100 ± 0    | (6) 56 ± 14 | (6) 72 ± 8  | (6) 73 ± 9  | (6) 77 ± 12 |
| LPS   | (3) 100 ± 0    | (3) 108 ± 10 | (3) 193 ± 12 | (3) 119 ± 13 | (3) 199 ± 56 |
| PC    | (6) 100 ± 0    | (6) 87 ± 16 | (6) 79 ± 16 | (6) 70 ± 15 | (6) 24 ± 11 |
| PI    | (6) 100 ± 0    | (6) 65 ± 20 | (6) 19 ± 10 | (6) 16 ± 10 | (6) 8 ± 8  |
| SPH   | (6) 100 ± 0    | (6) 63 ± 15 | (6) 34 ± 11 | (6) 22 ± 7  | (5) 7 ± 5  |
| Total | (6) 100 ± 0    | (6) 70 ± 12 | (6) 61 ± 1  | (6) 52 ± 9  | (5) 22 ± 4 |

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**Fig. 3.** Changes in content and extractability of total phospholipid and nonpolar lipid fractions of CRMV during in vitro calcification. Collagenase-released MV were incubated in SCL as described under “Experimental Procedures,” and the total lipids extracted before (extract 1) and then after demineralization with EDTA (extract 2) at each stage of incubation. A, changes in the total lipid content, phospholipids (closed symbols) and nonpolar lipids (open symbols) from combined extracts 1 and 2, expressed as a percentage of the total original lipid present in the vesicles. B, changes in the extractability of total phospholipids extractable before (extract 1, closed symbols) and after (extract 2, open symbols), expressed as a percentage of the total phospholipid present. Values are the mean ± S.E. of six separate analyses.
after demineralization increased (Fig. 3B). At $T_0$, nearly 95% of the total phospholipid present was extractable before demineralization; however, after 4 h of incubation, by which time mineralization was well in progress, less than 80% was extractable. By 24 h, only about 70% of the remaining phospholipid could be extracted prior to demineralization. This indicated that with calcification, progressively more of the surviving phospholipid was bound to the mineral and could not be extracted until the vesicles were demineralized. In contrast, the extractability of the nonpolar lipid was much less affected by mineralization. At $T_0$, about 90% of the nonpolar lipid was extractable before demineralization. With time this increased to about 93–94% (data not shown).

Changes in Extractability and Survival of Individual MV Lipids—Corroborating the impression gained by HPTLC, analysis by HPLC revealed that MV mineralization had a profound effect on the extractability and survival of the two principal acidic phospholipids, PS and PI. At $T_0$ before exposure to the
mineralizing solution, about 90% of the PS was extractable before demineralization; however, after only 2 h of incubation, only 5% of the original PS was extractable (Fig. 4A). There was a corresponding major increase in the proportion of the original PS that survived and could be extracted only after demineralization. This increased from ~10% at $T_0$ to about 75% at $T_4$ and remained at this level thereafter.

A similar pattern of extractability was seen with PI, but as noted in Table II, there was major degradation of this lipid during MV mineralization. At $T_0$, again about 90% of the PI was extractable, but after 2 h of incubation, in contrast to PS, almost one-third of the original PI could still be extracted (Fig. 4B). However, by $T_4$ and thereafter, no PI was extractable before demineralization. Again, in contrast to PS, there was much less of the original PI that could be extracted after demineralization. This value rose from ~7% at $T_0$ to a maximum of only about 18–20% of the original at $T_4$, decreasing gradually thereafter. This indicated that much less of the original PI became complexed and stabilized by the newly forming mineral and thus was susceptible to degradation.

In contrast to the acidic phospholipids, mineralization had only a minor effect on the extractability of the neutral phospholipids. For PE, about 90% of the original was extractable before demineralization at $T_0$. The remaining 10% was apparently complexed with the mineral and not extractable (Fig. 4C) and was protected from degradation, persisting throughout the 24 h of incubation. However, the amount of PE that was not complexed rapidly decreased, indicating that it was rapidly degraded.
Essentially all (>95%) of the choline-containing phospholipids, PC and SPH, were extractable before demineralization, regardless of the length of incubation or extent of mineralization. Fig. 4D reveals that PC was readily extractable before demineralization; however, its rate of degradation was slower than most other phospholipids, remaining essentially linear throughout the incubation. By 24 h, only about 20% of the original PC remained. Fig. 4E shows that SPH, also unprotected by complexation with the mineral, was rapidly degraded. By 4 h, only about one-third of the original SPH remained, and by 24 h there was only about 5% of the original left. Thus, in contrast to all of the other diacyl phospholipids, PS largely survived being protected by the newly forming mineral and resynthesized from other lipids (see later).

Changes in Lysophospholipids—Evident in all lipid extracts of MV were significant amounts of lysophospholipids. LPE, the monoacyl derivative of PE, was the most abundant form representing ~6% of the total phospholipid for most of the incubation (Table I). As evident from Fig. 4F, almost 95% of the LPE present initially was extractable before demineralization. However this rapidly declined; by 4 h only about one-third, and by 24 h less than 20% of the original LPE could be so extracted. On the other hand, the amount of LPE not extractable until after demineralization increased progressively during MV calcification, rising from ~5% at T0 to ~35% at T24. The other lysoderivatives detected during MV calcification were LPI, LPC, and LPS. Small amounts of LPI and LPC (1.4 and 1.2% of the total PL, respectively) were present initially, but there were only trace amounts of LPS (Table I). While levels of LPI and LPC generally decreased thereafter, levels of LPS increased. At 4 h, in lipids extractable after decalcification, levels of LPS were dramatically increased (Fig. 4G). This corresponds to the time when the diacyl form, PS, was no longer detectable in extracts made before demineralization (Fig. 4A). Expressed as a percentage of the original LPS present, it is evident that net synthesis of this lipid had occurred, probably in part from phospholipase A action on PS, but also from base-exchange of serine with LPE (see “Discussion”).

Changes in Nonpolar Lipids—Further insight into the lipid degradation process came from analyses of the nonpolar lipids. Initially at T0, CH was the dominant nonpolar lipid accounting for ~40% of the total; its esterified form, CH esters represented some 28–30%, with free fatty acids FFA and triacylglycerols each accounting for ~9% of the total nonpolar lipid. DG accounted for only 1.3% and MG accounted for ~0.5% of the total. Three uncharacterized, more polar nonpolar lipids accounted for the remaining 10%. Based on HPLC analyses, the levels of total nonpolar lipids in the successive extracts of MV during mineralization rose significantly (Fig. 3A). HPTLC analyses (Fig. 2A) indicated that the levels of FFA and to a lesser extent, 1,2-DG (+CH) increased, whereas levels of 1,3-DG decreased with time. Analyses of these data confirmed that FFA levels do indeed rise during MV incubation (Fig. 5). While levels of 1,2-DG (+CH) were relatively unchanged, levels of 1,3-DG declined.

**DISCUSSION**

Lipids are integral components of the MV membrane that provide the barrier to confine the contents of the vesicles. MV contain significant levels of internal Ca$^{2+}$ and Pi (15), which comprise key components of the nucleational core previously demonstrated to be critical for mineral formation (14, 21). Studies by Eanes et al. with synthetic models of MV (11) have revealed that PS-rich liposomes with lipid composition similar to that of MV block the emergence of mineral (25). On the other hand, TEM of mineralizing MV has shown that the first crystals form in association with the vesicle membrane and thereafter appear to penetrate through the membrane (10). Since the primary function of MV appears to be the induction of mineral formation, which membrane lipids bind to the mineral? Also, what mechanism enables egress of the mineral to propagate extravascular mineralization?

While there is substantial evidence for the binding of mineral to acidic phospholipids in MV (13, 23), for the presence of phospholipase activity in growth plate cartilage (26), and in MV in particular (5, 27), there have been no studies of the changes in lipid extractability and composition that occur when MV are allowed to mineralize under controlled conditions in vitro. Such studies should provide insight into how these aspects of MV mineralization are regulated. Kinetic studies of MV-induced mineralization typically reveal a lag period during which minimal Ca$^{2+}$ accumulation occurs (28, 29), followed by a rapid uptake period, and finally a slower plateau phase during which apatitic crystalline mineral forms (29, 30). Studies on nascent MV reveal the presence of a nucleational core that contains PS complexed with non-crystalline calcium phosphate (14). Fourier-transform infrared studies indicate that the rapid uptake period coincides with the formation of an octacalcium phosphate-like crystalline phase (31). It is speculated that breakdown of the MV membrane is what triggers this critical event.

To address these issues, we performed detailed analysis of the changes in lipid composition that accompany the onset and progression of MV mineral deposition. Our initial studies using HPTLC (Fig. 2) revealed two major features: first, a marked change in the extractability of the certain phospholipids, and second, the progressive breakdown of most MV phospholipids accompanied by the accumulation of FFA. These studies were confirmed and extended with HPLC analyses.

The decline in phospholipids appears to be due to the action of various phospholipases that degraded these lipids releasing FFA and 1,2-DG, thereby increasing the overall nonpolar lipid level.
Which enzymes caused the degradation of the phospholipids that occurred during the mineralization process? Insight has come from consideration of the partial degradation products of these lipids. Action of phospholipase A₁ or A₂ on diacyl phospholipids would be the corresponding monoacyl (i.e. lyso) forms; action of phospholipase C would yield 1,2-DG and the corresponding phosphoryl base (e.g. phosphoryl choline). Subsequent action of other lipases on lysophospholipids and DG would yield FFA and their corresponding water-soluble products.

Since LPE could be generated from the action of phospholipase A₁ or A₂ on PE, and the levels of PE rapidly declined during MV mineralization, it is evident that both formation and degradation of LPE must have been occurring concomitantly at a significant rate. The presence of substantial amounts of LPE in the lipid extracts indicates that there must be substantial phospholipase A₁ or A₂ activity in MV. Comparison of Fig. 4, B and F indicates that the rate of LPE degradation was even greater than that of PE. One might assume that lysophospholipase A activity in MV was even greater than phospholipase A activity, which is in agreement with the lack of accumulation of LPC, despite the fact that PC levels steadily declined. A more likely possibility is that LPE was being converted to LPS by a base-exchange pathway with serine (see later). Alternatively, PC may have been broken down by action of PLC (32), which would yield 1,2-DG. Indeed, there appeared to be some accumulation of 1,2-DG (Fig. 2A), but evidently it was also degraded by a lipase to MG, which appeared to increase during MV incubation. PC also may have been degraded by phospholipase D (33), but there was no indication of an accumulation of phosphatidate.

Another interesting feature of lipid breakdown during MV calcification was the rapid and almost complete loss of SPI. Classical sphingomyelinases cleave SPH, releasing ceramide and phosphoryl choline (34). Recent studies indicate that ceramide enhances apoptosis and breakdown of rabbit articular cartilage (35). Thus, this pathway of MV lipid breakdown could contribute to vascular invasion of the growth plate. Unfortunately, we did not analyze for the presence of ceramide in our samples.

Comment needs to be made concerning the marked changes in extractability of certain phospholipids, particularly PS, during MV mineralization. PS is known to complex with Ca²⁺ (7, 8), particularly in the presence of P₁, which favors the formation of PS-Ca₃P₁ complexes (36). Indeed, after 2 h of incubation, PS could be extracted only after demineralization. What was the state of PS in MV before exposure to SCL and the onset of calcification? At T₀ before demineralization PS was almost fully extractable. This would seem to indicate that it was not complexed with Ca²⁺ and P₁. However, looking back to the original discovery of the PS-Ca₃P₁ complexes, it is evident that PS complexed with amorphous calcium phosphate is quite soluble in chloroform/methanol (36). Indeed, the data indicate that only after the Ca₃P₁ mineral becomes crystalline does the associated PS become non-extractable prior to demineralization.

Another phenomenon that at first seemed improbable was the discovery that total PS levels in the MV actually rose between T₂ and T₄₋₂₄ (Table II). How can this be, knowing that MV lack the ability to produce ATP needed to drive classical phospholipid synthesis? The answer comes from the demonstrated presence of the base-exchange pathway in MV (24). This energy-independent pathway depends on an enzyme that exchanges ethanolamine for serine in both monoacyl and diacyl forms of PE. Our past studies with growth plate chondrocytes have shown that LPE is the primary recipient of serine and forms LPS. This would explain the striking rise in levels of LPS at T₄ during MV mineralization (Table II, Fig. 4G). It is apparent that PE itself was also subject to this base-exchange pathway. This would contribute to the major loss of this lipid and the net synthesis of PS during MV mineralization. PS, thus, is not only protected from degradation by the newly forming mineral, but its level becomes augmented by conversion of PE to PS. Since the PS-Ca₃P₁ complex is known to stimulate mineral formation (37), the conversion of PE to PS itself may facilitate the mineralization process.

What type of phospholipase A activity is predominant in MV, A₁, or A₂? A clue comes from studies in which we attempted to quantitate the levels of MV lipids by HPLC using UV absorption at 205 nm. Absorbivity at that wavelength is dependent on the presence of double bonds characteristic of fatty acids present at position 2 of glycerol in phospholipids. In our analyses of MV phospholipids, LPE showed strong absorbivity at 205 nm indicative of the presence of unsaturated fatty acids. If these reside primarily on C-2 of the glycerol moiety of this phospholipid, then the LPE must have arisen from action of phospholipase A₁ and not phospholipase A₂. Obviously, this needs to be explored by direct analyses of the constituent fatty acids of LPE, but this is beyond the scope of this study.

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