Roles of Alkaline Phosphatase and Labile Internal Mineral in Matrix Vesicle-mediated Calcification

EFFECT OF SELECTIVE RELEASE OF MEMBRANE-BOUND ALKALINE PHOSPHATASE AND TREATMENT WITH ISOOSMOTIC pH 6 BUFFER*

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The roles of alkaline phosphatase and labile internal mineral in matrix vesicle-mediated mineralization have been studied by selectively releasing the enzyme from a wide variety of matrix vesicle preparations using treatment with a bacterial phosphatidylinositol-specific phospholipase C and by demineralization of the vesicles using an isosmotic pH 6 buffer. Following depletion of 50–90% of the alkaline phosphatase activity or treatment with citrate buffer, the vesicles were tested for their ability to accumulate 45Ca2+ and 32P,

with the vesicles (12, 14). In addition, elevated levels of acidic phospholipids (8, 15) are present in the inner vesicle membrane (16). These can combine with calcium and P, to form calcium-P,-acidic phospholipid complexes (17, 18) that possess nucleation ability of their own (19). Matrix vesicles are also markedly enriched in alkaline phosphatase (7, 20–22), an enzyme long associated with the mineralization process (23). This enzyme is notably increased at the calcification front in endochondral calcification (20, 24–27); however, both its function and physiological substrate remain elusive. Some of the roles that have been postulated for alkaline phosphatase in the mineralization process are: 1) hydrolysis of organic phosphate esters which elevates local P, concentration facilitating precipitation of calcium phosphate (7, 23, 28); 2) destruction of physiological crystal growth inhibitors such as P, and ATP through its hydrolytic activity (29, 30); 3) action as a P, transporter (14, 31–36); and 4) active transport of Ca2+ and/or P, through its ATPase activity (7).

We recently reported evidence that alkaline phosphatase may play a role in the transport of phosphate (and perhaps secondarily of calcium) during mineral ion uptake by matrix vesicles (33–36). In those studies we examined in vitro the effects of inhibitors of alkaline phosphatase hydrolytic activity on P, and Ca2+ uptake by matrix vesicle-enriched microsomes (MVEM1) prepared from chicken epiphyseal cartilage. We found that vanadate (36), a potent competitive inhibitor, and L-tetramisole (33–35), an uncompetitive inhibitor, were inhibitory to calcium and especially to phosphate uptake by MVEM. However, these findings were weakened, because tetramisole (which is not inhibitory to alkaline phosphatase activity) was nearly as inhibitory to ion uptakes as L-tetramisole and because vanadate inhibition is not specific for alkaline phosphatase.

To further clarify the role of alkaline phosphatase in matrix vesicle-mediated mineralization, we utilized a bacterial (Staphylococcus aureus) phosphatidylinositol-specific phospholipase C to selectively release alkaline phosphatase from a variety of matrix vesicle preparations and tested the ability of alkaline phosphatase-depleted and control fractions to accumulate Ca2+ and P, in vitro.

EXPERIMENTAL PROCEDURES

Preparation of Matrix Vesicle-enriched Microsomes—MVEM were obtained by fractionation of homogenates by epiphyseal cartilage slices from rapidly growing 8–10-week-old broiler strain chickens, as

1 The abbreviations used are: MVEM, matrix vesicle-enriched microsomes; phospholipase C, bacterial (S. aureus) phosphatidylinositol-specific phospholipase C; TBS, N-tris(hydroxymethyl)methyl-2-aminoethanesulfonate; SCL, synthetic cartilage lymph.

Matrix vesicles are small (100–300-nm diameter) extracellular membrane-bound structures associated with the earliest sites of mineral formation in several types of calcification (1–6). These vesicles appear to derive by blebbing from filopodia on the plasma membrane of nearby cells (4, 7–9). Matrix vesicles isolated from calcifying tissues are capable of accumulating 45Ca2+ from metastable solutions in vitro (10–13); however, the mechanism by which this occurs is unclear. Much of the nucleating ability of these preparations appears to result from the presence of preformed mineral associated

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Matrix Vesicle Calcification

RESULTS

Treatment of MVEM with phospholipase C resulted in a concentration-dependent release of alkaline phosphatase from the MVEM pellet into the supernatant (Fig. 1). While half-maximal release occurred at a phospholipase C concentration of about 2.5 μg/ml, subsequent experiments were carried out with phospholipase C concentrations of 15–25 μg/ml in the incubation mixture to maximize alkaline phosphatase depletion.

Early experiments examining the effects of phospholipase C treatment on uptake of Ca²⁺ and P, by MVEM were hampered by a difficulty in finding suitable controls. Incubation of MVEM with phospholipase C-free buffer as a control gave unexpectedly low ⁴⁰Ca²⁺ uptake, less than phospholipase-treated MVEM, which in turn was less than the nonincubated controls. To eliminate this nonspecific effect, controls were prepared by heat killing the enzyme and then incubating the vesicle fraction with either the heat-killed (control) or the active phospholipase C.

As has been observed previously (36), MVEM were variable in their ability to induce mineralization. This variability appears to result from differences in the amount of preformed mineral contained within the vesicles. Active preparations (Fig. 2, A and B) were characterized by a relative short lag phase (1–2 h), followed by a period (2–6 h) of rapid uptake of substantial levels of P, followed by Ca²⁺ (Fig. 2A) and Ca²⁺ (Fig. 2B), the rate of uptake gradually slowing thereafter. Phospholipase C treatment of such active preparations caused very little effect on ion uptake, except that accumulation was slightly greater in the alkaline phosphatase-depleted MVEM. When the active MVEM preparations were studied the following day, mineralizing activity of these fractions was significantly reduced; however, slight increases above the control in ion uptake of Ca²⁺ (Fig. 2A) and P (Fig. 2B) were again observed in the alkaline phosphatase-depleted MVEM. In less active preparations (Fig. 2, C and D) uptake of mineral ion was slower, with a longer lag phase. In these MVEM, phospholipase C treatment resulted in a significant reduction in the accumu-
phospholipase C-treated vesicles (Table I, B, right). Addition of phospholipase C treatment on both control and demineralized preparations, the effect of the release of alkaline phosphatase by the preformed mineral and alkaline phosphatase in the ion uptake preparations. Addition of ATP to the incubation medium of AMP enabled significant Ca2+ uptake by the citrate-treated phosphatase and its release by phospholipase C (Table I). This release of alkaline phosphatase during the control incubation nearly abolished all Ca2+ uptake in both the control and demineralized phospholipase C-treated MVEM preparations (Fig. 3). While, in the absence of AMP, release of alkaline phosphatase by phospholipase C caused significant reduction in the rate of Ca2+ uptake, citrate treatment slightly enhanced both the activity of alkaline phosphatase and its release by phospholipase C (Table I, A). Addition of AMP again stimulated Ca2+ uptake by all MVEM preparations (Table I, B). While, in the absence of AMP, release of alkaline phosphatase by phospholipase C caused significant reduction in the rate of Ca2+ uptake, citrate treatment nearly abolished all Ca2+ uptake in both the control and phospholipase C-treated vesicles (Table I, B, right). Addition of AMP enabled significant Ca2+ uptake by the citrate-treated vesicles, but rapid ion uptake was delayed to 48 h. Phospholipase C treatment reduced the AMP-dependent accumulation of Ca2+ in these MVEM preparations.

The effect of phospholipase C treatment on traditional alkaline phosphatase substrates on the time course of 45Ca2+ uptake by MVEM. Addition of AMP again stimulated Ca2+ uptake by two MVEM preparations with differing mineralizing activity. A, 45Ca2+ uptake by an inactive MVEM preparation; C and D, ion uptake by less active MVEM. A and C, 32P; uptake; B and D, 45Ca2+ uptake. B, control MVEM (treated with heat-killed phospholipase C); O---O, phospholipase C-treated MVEM (alkaline phosphatase depleted). □ -- □, control MVEM after 1 day storage; ■ --- ■ phospholipase C-treated MVEM after 1-day storage. 45Ca2+ and 32P; uptake assays were carried out in SCL at pH 7.5 (see “Experimental Procedures”). Values are means ± S.E. of duplicate samples. Phospholipase C-treated MVEM contained 35% (active MVEM) and 21% (less active MVEM) of control level of alkaline phosphatase activity. Note the significant decrease in the rate of 45Ca2+ and 32P; uptake after 1 day of storage (active MVEM, dashed lines).

In an effort to examine the relative importance to labile preformed mineral and alkaline phosphatase in the ion uptake process, the effect of the release of alkaline phosphatase by phospholipase C treatment on both control and demineralized (citrate treated) MVEM was studied. First, citrate treatment of MVEM slightly enhanced both the activity of alkaline phosphatase and its release by phospholipase C (Table I, A). Addition of AMP again stimulated Ca2+ uptake by all MVEM preparations (Table I, B). While, in the absence of AMP, release of alkaline phosphatase by phospholipase C caused significant reduction in the rate of Ca2+ uptake, citrate treatment nearly abolished all Ca2+ uptake in both the control and phospholipase C-treated vesicles (Table I, B, right). Addition of AMP enabled significant Ca2+ uptake by the citrate-treated vesicles, but rapid ion uptake was delayed to 48 h. Phospholipase C treatment reduced the AMP-dependent accumulation of both P, (Fig. 2C) and Ca2+ (Fig. 2D).
phosphatase activity, had little ability to accumulate Ca\(^{2+}\) and vesicles (Table 41, bottom left). In subsequent studies, sucrose fractions A and B were combined (AB) and studied, along with the C fraction, to determine the effect of phospholipase C treatment on AMP-stimulated mineral ion uptake. The AB (Fig. 5A) and C (Fig. 5B) fractions accumulated substantial amounts of Ca\(^{2+}\) in the presence of 2 mM AMP; and alkaline phosphatase depletion by phospholipase C treatment significantly reduced this activity.

Thus, these studies showed that addition of AMP could stimulate, and release of alkaline phosphatase significantly reduce, accumulation of Ca\(^{2+}\) and Pi, by the various matrix vesicle fractions. The mechanism by which AMP and alkaline phosphatase together stimulated ion uptake was unclear, but a previous postulate was that hydrolysis of AMP by alkaline phosphatase was coupled with Pi uptake. To determine the relationship between AMP hydrolysis and uptake of Pi, exper-
TABLE III
Effect of nucleotide phosphate esters on \(^{45}\text{Ca}^2\)+ uptake by matrix vesicle-enriched fractions obtained by discontinuous sucrose gradient fractionation of MVEM

<table>
<thead>
<tr>
<th>Vesicle fraction</th>
<th>Incubation time (h)</th>
<th>(^{45}\text{Ca}^2)+ uptake*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
<td>+2 mM AMP</td>
</tr>
<tr>
<td>A</td>
<td>5</td>
<td>0.14 ± 0.18</td>
</tr>
<tr>
<td></td>
<td>20</td>
<td>0.26 ± 0.14</td>
</tr>
<tr>
<td></td>
<td>24</td>
<td>0.16 ± 0.16</td>
</tr>
<tr>
<td></td>
<td>48</td>
<td>0.79 ± 0.33</td>
</tr>
<tr>
<td>B</td>
<td>5</td>
<td>0.07 ± 0.11</td>
</tr>
<tr>
<td></td>
<td>20</td>
<td>0.13 ± 0.17</td>
</tr>
<tr>
<td></td>
<td>24</td>
<td>0.12 ± 0.12</td>
</tr>
<tr>
<td></td>
<td>48</td>
<td>0.18 ± 0.12</td>
</tr>
<tr>
<td>C</td>
<td>5</td>
<td>0.05 ± 0.08</td>
</tr>
<tr>
<td></td>
<td>20</td>
<td>0.31 ± 0.07</td>
</tr>
<tr>
<td></td>
<td>24</td>
<td>0.27 ± 0.08</td>
</tr>
<tr>
<td></td>
<td>48</td>
<td>0.05 ± 0.18</td>
</tr>
<tr>
<td>D</td>
<td>5</td>
<td>0.07 ± 0.09</td>
</tr>
<tr>
<td></td>
<td>20</td>
<td>0.20 ± 0.19</td>
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<tr>
<td></td>
<td>24</td>
<td>0.17 ± 0.12</td>
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<tr>
<td></td>
<td>48</td>
<td>0.18 ± 0.16</td>
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</tbody>
</table>

*Values are the mean ± S.E. of duplicate incubations from a representative experiment. Note the inability of the sucrose gradient fractions to accumulate \(^{45}\text{Ca}^2\)+ in the absence of the nucleotide phosphate esters.

DISCUSSION

This study represents the first time that the three most commonly used types of matrix vesicle preparations have been directly compared with respect to their abilities and requirements for accumulation of \(^{45}\text{Ca}^2\)+ and \(^{32}\text{P}\)+ ions from metastable synthetic lymphs in vitro. We examined conventional collagenase-released matrix vesicles (7), MVEM prepared by homogenization of fresh cartilage tissue (35), and MVEM further purified by sucrose step gradient fractionation (37). Our studies show that there are indeed certain similarities and significant differences between these preparations. All matrix vesicle preparations were rich in alkaline phosphatase. MVEM were the most active preparations with respect to \(^{45}\text{Ca}^2\)+ and \(^{32}\text{P}\)+ uptake, but both they and the collagenase-released matrix vesicles were capable of inducing mineral formation from SCL in the absence of alkaline phosphatase substrates. This is the first time collagenase-released matrix vesicles have been shown to be capable of inducing significant mineral ion uptake in the absence of alkaline phosphatase substrates. This higher activity may have resulted from the more gentle digestion procedure employed here (i.e. the use of lower collagenase concentrations in the presence of fetal

FIG. 5. Effect of phospholipase C treatment on AMP-stimulated \(^{45}\text{Ca}^2\)+ uptake by sucrose gradient fractions of MVEM.

A, combined AB fraction from the 10/30% and 30/40% sucrose gradient interfaces; B, fraction C from the 40/50% sucrose gradient interface. ●—●, controls; ○—○, phospholipase C treated. Incubations were carried out in SCL containing 2.0 mM AMP (see "Experimental Procedures"). Values are the mean ± S.E. of duplicate incubations from a representative experiment. Phospholipase-treated fractions contained 45.6% (AB fraction) and 33% (fraction C) of the control levels of alkaline phosphatase activities.

FIG. 6. Comparison of the effect of phospholipase C on \(^{32}\text{P}\)+ AMP hydrolysis by control AB vesicles; ○—○, AMP hydrolysis by phospholipase C-treated AB vesicles; ●—●, 32P uptake by control AB vesicles; ○—○, 32P uptake by phospholipase C-treated AB vesicles. Values are means ± S.E. for 4 samples for hydrolysis and 2 samples for uptake. AMP hydrolysis is expressed as a percentage of the total AMP present (2 mM), and 32P uptake as a percentage of the total 32P present in the incubation medium. Phospholipase C-treated AB vesicles contained 34.6% of the control levels of alkaline phosphatase activity (asayed at pH 10.3 with p-nitrophenyl phosphate as substrate) and 34.1% of the control AMPase activity (determined from initial rates of AMP hydrolysis above). 32P uptake values were not corrected for backgrounds in this figure in order to better observe any differences in the low level uptake which occurred early in the incubation period.

was rapid, \((t_\alpha = 2 \text{ h})\), but substantial uptake of \(^{32}\text{P}\)+ by the vesicles did not occur until 13 h, well after most of the AMP was hydrolyzed. Although treatment with phospholipase C significantly reduced the level of AMPase activity and the rate of uptake of \(^{32}\text{P}\)+, AMP hydrolysis again was essentially completed before significant uptakes of \(^{32}\text{P}\)+ occurred.

Lower concentrations of AMP (200, 20, and 2 \(\mu\text{M}\)) were also tested for their ability to stimulate mineral ion uptake by the AB fractions, but all were inactive (data not shown). The \(K_m\) for AMP hydrolysis under these conditions was found to be 276 \(\mu\text{M}\) by Lineweaver-Burk analysis.
bovine serum which contains protease inhibitors) but was nevertheless very easily destroyed simply by further incubation at 37°C with the control heat-killed phospholipase C buffer (Table II, B). On the other hand, sucrose gradient fractions of MVEM, MVEM from noncalcifying (polyarticular) regions of the growth plate (34), or acidic buffer-treated matrix vesicle preparations were all incapable of mineral ion uptake in the absence of alkaline phosphatase substrates (e.g. AMP). AMP stimulated ion uptake by all preparations.

These findings stress the critical importance of two major factors in matrix vesicle mineral ion uptake: 1) the presence of labile preformed mineral within the vesicles; and 2) the presence of alkaline phosphatase and sufficient levels of a suitable substrate for its activity.

Amorphous calcium phosphate, a kinetically unstable relatively soluble form of mineral, has been shown to be the initial form of mineral in matrix vesicles (34, 41). The key importance of this labile preformed mineral in the further uptake of mineral ions by matrix vesicles is stressed by several facets of this study. First, simple brief treatment of active MVEM with isosmotic pH 6.0 citrate buffer which removed over 80% of the Ca²⁺ (44) almost totally destroyed all alkaline phosphatase-independent accumulation. This is in agreement with several earlier studies (12-14). Second, the very lability of this mineral is demonstrated by the fact that the simple steps involved in processing the vesicles (e.g., storing the MVEM overnight at 4°C in Ca²⁺-free TMS buffer or incubating the crude collagenase-released matrix vesicles for 40 min at 37°C in the Ca²⁺-free TMS buffer) significantly reduced this activity. The most logical explanation for both these observations is that incubation of MVEM in Ca²⁺-free buffer led to loss of the labile amorphous calcium phosphate and subsequent inability to accumulate mineral ions in the absence of alkaline phosphatase and a suitable substrate. Third, fractionation of active MVEM on sucrose step gradients destroyed essentially all alkaline phosphatase-independent mineral ion uptake ability. While sucrose gradient fractionation causes decreased heterogeneity of the vesicles and significant enrichment in alkaline phosphatase activity, previous studies have shown that this procedure also leads to significant loss of Ca²⁺ and especially P, from the vesicles (37), presumably due to the osmotic shock of the hyperosmotic Ca²⁺- and P₂⁺-free sucrose solutions. Thus, these findings suggest that labile amorphous calcium phosphate "sink" conditions inside matrix vesicles are responsible for the rapid mineral ion uptake. Recent experiments using a Ca²⁺-chelating ion-exchange column assay system (45) have shown that with freshly isolated MVEM, Ca²⁺ is nonexchangeable and co-elutes with the alkaline phosphatase activity (44). This clearly indicates that the initial labile mineral is within the vesicles.

The importance of alkaline phosphatase and a suitable substrate in vesicle ion uptake is revealed from the ability of added AMP to restore Ca²⁺ and P, uptake to mineral-depleted vesicles. Further when the level of alkaline phosphatase is reduced by treatment of the vesicles with phospholipase C, the ability of AMP to restore ion uptake is, as expected, compromised. The mechanism of this effect, however, was not by coupling AMP hydrolysis with P, ion uptake as might have been expected. This was clearly shown from the studies with [³²P]AMP which revealed that AMP hydrolysis proceeded at a much more rapid rate than P, uptake. If hydrolysis of AMP had been coupled with P, uptake, the two processes should have occurred concomitantly. This finding is in basic agreement with earlier data of Heu and Anderson (11), although they did not comment on it. Thus, while alkaline phosphatase clearly is important to matrix vesicle ion uptake, it must not be acting as a P, porter. Similar conclusions regarding the lack of direct involvement of alkaline phosphatase in P, transport in brush border membrane vesicles have been reported by Yusufi et al. (46). In fact, more recent studies with such brush border vesicles suggest that the P, transporter is a membrane proteolipid with cation-dependent P₂⁺-binding affinity (47, 48). It is of further interest here that a proteolipid has been found in matrix vesicles by Boyan-Salyers (49). Whether this proteolipid is the matrix vesicle P, carrier and what its relationship may be to alkaline phosphatase remain to be elucidated.

While our previous studies using alkaline phosphatase inhibitors suggested that matrix vesicle alkaline phosphatase might be acting as a P, transporter, the results were not definitive (35, 36). These earlier studies were done with MVEM incubated in ⁴⁰Ca²⁺ and ³²P, labeled SCL without the addition of alkaline phosphatase substrates. Studies with L-tetrastimole showed that MVEM ³²P, uptake was selectively inhibited at early time points, but paradoxically both the alkaline phosphatase insensitive and the active L-forms of the drug were found to be inhibitory to ⁴⁰Ca²⁺ and ³²P, uptake at intermediate time points (25). Similarly, studies with vanadate on the uptake of Ca²⁺ and P, by MVEM showed early selective inhibition of ³²P, uptake, but not all of the effects could be attributed to alkaline phosphatase (36). Vanadate is known to have inhibitory effects on other systems. This emphasizes the importance of interpreting studies with inhibitors with caution. In light of recent findings, it is possible that levamisole and vanadate were acting on the proteolipid P, transporter; however, this obviously needs to be carefully studied.

The physiological significance of the use of 2 mM AMP in matrix vesicle incubations also must be questioned, inasmuch as lower levels of AMP (200 μM and less) failed to stimulate uptake. Recent studies have shown that the total nucleoside monophosphate level in chicken epiphyseal cartilage extracellular fluid is about 100 nM and the total nucleotide value 350 μM (50). Complete hydrolysis of these compounds would increase the local P, concentration by about 400-500 μM. Whether this amount would be sufficient in itself to induce mineralization is doubtful inasmuch as more recent studies have shown that 1 mM AMP is only weakly stimulatory to ion uptake in mineral-depleted vesicles. Although the flux of phosphorylated compounds into the extracellular fluid is unknown, it is of interest that in vivo extracellular cartilage fluid contains significantly higher levels of P, than does blood plasma (41).

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REFERENCES