Evidence of the Presence of a Specific ATPase Responsible for ATP-initiated Calcification by Matrix Vesicles Isolated from Cartilage and Bone*

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Accumulating evidence indicates that calcification by isolated mammalian matrix vesicles (MVs) can be initiated by ATP. Since ATP can be hydrolyzed by either a specific ATPase or by nonspecific alkaline phosphatase (ALP), it remains to be established whether ATPase or ALP mediates ATP-initiated Ca and Pi deposition. To support the hypothesis that specific ATPase is responsible for ATP-initiated calcification by MVs isolated from mammalian cartilage and bone, the effects of ATP analogs, ALP substrates, and specific inhibitors on ATP hydrolysis and ATP-initiated calcification were compared between intact MVs and monoclonal antibody affinity-purified MV ALP. ATP analogs such as ADP and AMP exerted marked inhibitory effects on both [$\gamma$-32P]ATP hydrolysis and ATP-initiated calcification by intact MVs, whereas phosphomonoesters such as β-glycerophosphate or phosphoethanolamine had no effect. In contrast to intact MVs, purified MV ALP failed to calcify, and its [$\gamma$-32P]ATP hydrolytic activity was readily inhibited by phosphomonoesters. Additionally, [$\gamma$-32P]ATP hydrolysis by purified ALP in contrast to that by intact vesicles was completely inhibited by l-tetramisole, a specific inhibitor of ALP, suggesting a loss of specific ATPase activity responsible for ATP-initiated calcification by MVs isolated from bone or cartilage.

The sequence of steps that lead to the initiation of calcification in skeletal tissues has yet to be elucidated. Since alkaline phosphatase (ALP) was proposed by Robison (1) in 1923 as an initiator of calcification in skeletal tissues, several potential initiators, including type I collagen (2), proteoglycans (3), osteocalcin (4), and phosphoproteins (2), have subsequently been suggested. However, none of these putative agents are firmly established, and indeed many prevent in vitro calcification (4–6). Although matrix vesicles (MVs) have been implicated as the sites of calcification initiation, evidenced through morphological, biochemical, and pathological studies (for review, see Ref. 7), the detailed mechanisms of initiation of calcification by MVs have yet to be defined (for review, see Ref. 8). Despite numerous studies that showed that ATP can initiate calcification mediated by mammalian MVs (9–11), it is not known whether a specific ATPase or ALP is responsible for ATP-dependent calcification, because both enzymes can hydrolyze ATP.

To support the hypothesis that specific ATPase rather than ALP plays a role in ATP-initiated calcification by MVs isolated from mammalian cartilage or bone, the effects of ATP analogs, ALP substrates, and specific inhibitors on ATP hydrolysis and ATP-initiated calcification were compared between intact MVs and monoclonal antibody affinity-purified MV ALP. The results of the experimentation support the hypothesis that specific ATPase plays a role in ATP-initiated calcification by MVs.

EXPERIMENTAL PROCEDURES

Matrix Vesicle Preparation

Epiphysial Cartilage MVs—The extracellular MV fraction was prepared from pooled epiphysial cartilage slices of 35 rachitic rats by the method of Hsu (12). A rachitic growth plate was used as a source of MVs because it is precalcified and readily calcifiable in the presence of exogenous P, (13, 14). Thus, it can be used as an ideal model for studying the initiation of calcification by MVs without complication from proliferation of preexisting mineral. Epiphysial growth plates were removed aseptically, minced into 3–5-mm pieces, and then digested in a solution (10 ml/g of tissue) containing 1,000 units/ml crude collagenase (type I, Sigma), 0.12 M NaCl, 0.01 M KCl, 1,000 units/ml penicillin, 1 mg/ml streptomycin, and 0.02 M Tes buffer, pH 7.45. The digestion was carried out at 37 °C for 3 h. The digest was centrifuged at 30,000 × g for 10 min, and the resulting precipitate of cells and cell debris was discarded. The supernatant was spun at 300,000 × g for 20 min, and the resulting MV-enriched precipitates were then suspended in 10 mM EGTA and 10 mM Tris-buffered saline (TBS), pH 7.6. This final MV precipitate was resuspended in a small volume of TBS to provide a protein concentration of 0.5–1.5 mg/ml. The above-mentioned EGTA treatment was attempted to minimize endogenous mineral content in MVs to study the initial rather than proliferative stage of calcification.

Calvaria MVs—Pooled fetal calvaria were obtained from three near-term pregnant rats. Calvaria were treated with crude collagenase at various time intervals to obtain different bone cell populations, as described by Luben et al. (15). MVs were then isolated from the collagenase digest using the same centrifugation procedure for the cartilage MV preparation.

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1 The abbreviations used are: ALP, alkaline phosphatase; MV, matrix vesicle; TBS, Tris-buffered saline; LT, l-tetramisole; Tes, N-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid.

2 The abbreviations used are: ALP, alkaline phosphatase; MV, matrix vesicle; TBS, Tris-buffered saline; LT, l-tetramisole; Tes, N-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid.
Calcium and Phosphate Deposition

The term "Ca and Pi deposition" is used in this article to mean the process by which Ca and Pi were deposited either as undefined forms of calcium phosphate or as Ca$^{2+}$ and Pi that was taken up by or bound to MVs. To measure calcium and Pi deposition, the method of Hsu (12) was used. Unless otherwise stated, the standard reaction medium (100 ml) consisted of 50 mM Tes, pH 7.6 (it should be stressed here that pH must be adjusted to 7.6 at 37°C, as pH changes with temperature), 85 mM NaCl, 15 mM KCl, 1 mM MgCl$_2$, 10 mM NaHCO$_3$, 1.35 mM CaCl$_2$, 1 mM ATP, 1.97 mM Pi, and MVs (5 mg of protein/ml). It should be noted that although 0.2 mM ATP is sufficient for calcification, the degree of calcification was much less than that obtained with optimal 1 mM ATP. Therefore, to study effects of various agents on calcification, 1 mM ATP was used for calcification study. For Ca$^{2+}$ uptake, $^{45}$Ca ($1 \times 10^6$ cpm) was used as the radioactive tracer. Likewise, $^{32}$Pi was used as the tracer for Pi uptake. The reaction was incubated for 5 h at 37°C in a water vapor-saturated incubation chamber to minimize vaporization of the reaction mixture during incubation. At the end of incubation, the reaction mixture was filtered through 0.1-$\mu$m pore size Durapore membranes (Millipore Inc.). The membranes were washed twice each with 1 ml of TBS and then transferred to vials containing scintillation fluid for radioactivity counting. The nonspecific Ca or phosphate binding to filter papers was calculated from radioactivity bound to the filters under the identical conditions, except that MVs were omitted. The nonspecific binding radioactivity was then subtracted from the radioactivity obtained in the presence of MVs. Ca or Pi deposition is expressed as nmol of Ca or Pi/ml of reaction mixture/5 h, calculated as: [cpm retained on the filter $-$ nonspecific cpm/total cpm] $\times$ concentration of CaCl$_2$ or phosphate (nmol of Ca or Pi/ml).

**Calcium and Phosphate Deposition**

**Specific ATPase Activity**

A putative specific ATPase in MVs is defined as a specific enzyme that solely uses ATP as a substrate and specifically releases the terminal Pi from ATP. Therefore, a subsequent release of $\alpha$- or $\beta$-Pi from ATP by AMPase, ADPase, or ALP was not considered. ATPase activity is expressed as the rate of $^{32}$Pi released from $[^{32}P]ATP$. It should be noted that a part of $[^{32}P]ATP$ hydrolysis by MVs is due to ALP activity, which can be blocked by LT. Unless otherwise stated, the reaction mixture (100 ml) contained 10 mM Tris, pH 7.6, 0.15 M NaCl, 1 mM MgCl$_2$, and 1 mM ATP ($1 \times 10^6$ cpm $[^{32}P]ATP$) and with or without (as a blank) 2.5 mg/ml of MV protein. The reaction mixture was incubated for 30 min at 37°C and terminated by addition of 250 $\mu$l of 0.2 M silicotungstic acid. The $^{32}$Pi yielded from ATP hydrolysis was then extracted into the isobutanol-toluene phase by the method of Martin and Doty (17) and counted in a vial containing scintillation fluid. One unit is expressed as that amount of ATPase required to produce one $\mu$mol of $^{32}$Pi/min from $[^{32}P]ATP$.

Determination of the Relative Strength of ATP Analogs or ALP Substrate in Inhibiting ATP Hydrolysis

The effectiveness of an ATP analog or ALP substrate in inhibiting ATPase is expressed as $I/S_{50}$, which is defined as the required molar ratio of ATP analog or ALP substrate (I):ATP (S) to inhibit 50% of ATPase activity. The higher ratio a phosphoester or ATP analog displays, the less it can inhibit $[^{32}P]ATP$ hydrolysis.

Electron Microscopy

Pellets of MVs after incubation in calcifying solution with or without ATP for 5 h were fixed in 2.5% glutaraldehyde, postosmicated, and embedded in situ in Epon in Beckman Instruments polyallomer centrifuge tubes. Unstained thin sections (approximately 500 A in thickness) were examined and photographed in an electron microscope (model J.O.E.L.).
Determining whether there is direct involvement of a specific ATPase in MV calcification has been hampered by a lack of proof of the presence of a specific ATPase in MVs, as ATP can be hydrolyzed by either specific ATPase or by nonspecific ALP. Therefore, a procedure must be developed to distinguish specific ATPase activity from nonspecific ALP activity, thereby providing evidence that ATPase rather than ALP is responsible for ATP-initiated calcification.

LT, a specific ALP inhibitor (22), has been used as an effective means to demonstrate the presence of ATPase distinguished from ALP in bone homogenates and cartilage slices (23, 24). A cytochemical study at the electron microscopic level also suggests the presence of an LT-resistant ATPase in MVs (25). LT was used in this study to test the correlation between ATPase activity and ATP-initiated calcification. Both bone MVs and cartilage vesicles were selected to assure that specific ATPase is responsible for ATP-initiated calcification in all skeletal tissues. LT at 1 mM inhibited >90% of β-glycerophosphate hydrolysis, whereas LT only partially inhibited ATPase of cartilage MVs or bone vesicles (Table I). Correlatively, LT failed to fully inhibit ATP-initiated calcification by MVs of both cartilage and bone (Table II).

The best approach we used to distinguish a specific ATPase from nonspecific ALP was to determine whether ATP analogs for ATPase were more potent than ALP substrates in inhibiting [γ-32P]ATP hydrolysis by MVs. In the presence of 1 mM LT, various ATP analogs, including AMP and ADP, exerted a markedly inhibitory effect on [γ-32P]ATP hydrolysis by MVs, whereas phosphonoesters, such as β-glycerophosphate and phosphoethanolamine, did not (Table III). Correlatively, ATP-initiated calcification was much more inhibited by ATP analogs than by phosphonoesters (Fig. 2). In contrast, monoclonal antibody affinity-purified MV ALP failed to calcify (data not shown), and its [γ-32P]ATP hydrolytic activity was completely inhibited by either LT or phosphonoesters (Table III), suggesting the loss of ATPase during purification. These observa-

### Table I

| Treatment     | ATP Hydrolysis (nmol/15 h) | ATP Inhibition (%) | β-Glycerophosphate Hydrolysis (nmol/15 h) | β-Glycerophosphate Inhibition (%) |
|---------------|---------------------------|-------------------|------------------------------------------|----------------------------------|
|               | Control                   | LT                | Control                                  | LT                               |
| Cartilage     | 2.09 ± 0.15 (6)           | 2.4 ± 0.2 (3)     | 25.3 ± 3.0 (6)                            | 39.9 ± 8.8 (3)                   |
| Calvaria      | 2.09 ± 0.08 (6)           | 2.5 ± 0.4 (3)*    | 30.2 ± 4.3 (6)*                          | 27.4 ± 7.3 (3)*                  |
| *Significantly different from the respective control (p < 0.01 obtained by Student's unpaired t test).  
†Insignificantly different from the respective control (p > 0.05). |

### Table II

| Treatment     | Ca deposition | ATPase | Pi deposition |
|---------------|---------------|--------|--------------|
|               | No ATP        | 1 mM ATP | No ATP        | 1 mM ATP |
| Cartilage     |               |        |              |          |
| MVs           |               |        |              |          |
| Control       | 2.09 ± 0.15 (6) | 25.3 ± 3.0 (6) | 1.69 ± 0.66 (3) | 11.5 ± 11 (3) |
| 1-Tetramisole | 2.09 ± 0.08 (6) | 30.2 ± 4.3 (6)* | 1.65 ± 0.40 (3)* | 9.4 ± 1.2 (3) |
| Calvaria      | 2.4 ± 0.2 (3)  | 39.9 ± 8.8 (3) | 4.7 ± 1.4 (3) | 11.0 ± 2.4 (3) |
| MVs           | 2.5 ± 0.4 (3)* | 27.4 ± 7.3 (3)* | 4.3 ± 0.8 (3)* | 10.8 ± 0.7 (3)* |
| *Insignificantly different from the respective control (p > 0.05 obtained by Student’s unpaired t test). |

### Other Assays

Inorganic orthophosphate was determined by the method of Martin and Doty (17), and the protein concentration was determined by the method of Lowry et al. (18) using bovine serum albumin as the standard. An adenylyl cyclase assay was performed according to the procedure of Krishna et al. (19).
tions strongly demonstrate that specific ATPase plays an essential role in ATP-initiated calcification.

Since the electron microscopic data show that the electron-dense particles were closely associated with MV membranes (Fig. 1, B and C), a density centrifugation procedure was explored to further purify calcifiable MVs. MVs were exposed to the ATP-containing calcifying medium for 24 h to reach the maximal deposition of mineral by MVs. The calcified MVs were readily sedimented at 800 × g for 10 min due to their higher mineral density than noncalcified vesicles. The resulting sediment was then incubated for 24 h with 10 mM EGTA and 10 mM Tris, pH 6.0, to maximize the removal of MV-associated minerals. The EGTA-treated vesicles were then sedimented at 250,000 × g for 20 min and washed twice with TBS by resuspension and centrifugation. The vesicles were then sedimented at 250,000 × g for 20 min and washed twice with TBS by resuspension and centrifugation. A, electron micrograph of MVs after the EGTA wash. B, electron micrograph of the washed MVs after exposure to the ATP-calcifying medium for 24 h.

### TABLE III

| Phosphomonoesters or ATP analogs | [I/S]$_{50}$ |
|---------------------------------|-------------|
| MVs$^a$                         |             |
| AMP                             | 3.0         |
| ADP                             | 4.0         |
| Ribose-5-phosphate              | 40          |
| PP                              | 11          |
| $\beta$-Glycerophosphate        | >40$^a$     |
| Glycerol                        | >300        |
| $p$-Nitrophenyl phosphate       | >40         |
| Phosphoethanolamine             | >40         |
| Purified ALP                    |             |
| AMP                             | 0.16        |
| $p$-Nitrophenyl phosphate       | 0.17        |

$^a$ [γ-32P]ATP hydrolytic activity was assayed in the presence of 1 mM LT to block ALP activity.

$^b$ $>\gamma$, highest molar ratio tested without inhibition.

$^c$ [γ-32P]ATP hydrolytic activity was assayed in the absence of 1 mM LT.

### FIG. 2. Relative effectiveness of various ATP analogs and phosphomonoesters in blocking ATP-initiated calcification by MVs.

ATP-dependent Ca deposition was performed in the presence of various concentrations of ATP analogs or phosphomonoesters (0.1–3 mM), whereas the original ATP concentration was 1 mM. bGP, $\beta$-glycerophosphate; PEA, phosphoethanolamine.

### FIG. 3. Further purification of calcifiable MVs.

MVs were exposed to ATP-containing media for 24 h to reach the full extent of calcification. The calcified MVs were readily sedimented at 800 × g for 10 min due to their high mineral density. The resulting sediment was then incubated for 24 h in the presence of 10 mM EGTA and 10 mM Tris, pH 6.0, to maximally remove MV-associated minerals. The vesicles were then sedimented at 250,000 × g for 20 min and washed twice with TBS by resuspension and centrifugation. These vesicles were found to contain both ATPase and ATP-dependent calcifying activities, which can be inhibited by AMP but not by $\beta$-glycerophosphate (Fig. 4).
data further support the contention that MVs contain a specific ATPase responsible for ATP-initiated Ca and Pi deposition.

Although vanadate has been shown to inhibit both ATPase (26) and ALP (27), the interpretation of inhibition of MV ATPase by vanadate can be easily obscured by the fact that part of \( ^\gamma\text{P} \text{ATP} \) hydrolysis is due to nonspecific activity of ALP. LT was, therefore, used to completely block \( ^\gamma\text{P} \text{ATP} \) ATP hydrolysis by ALP to study the direct effect of vanadate on specific ATPase in MVs. In the absence of LT, the effect of vanadate on ATP hydrolysis by MVs (ALP and ATPase together) appears to be of a competitive type of inhibition, since an increase of ATP concentration from 0.1 to 1 mM significantly decreased the inhibition from 70 to 50% (\( p < 0.05 \); see Table IV). When ALP was blocked by LT, vanadate inhibition of ATP hydrolysis became noncompetitive, as an increase in the ATP concentration had no effect on the inhibition (Table IV). In contrast to a partial inhibition of ATP hydrolysis, vanadate at 1 mM fully inhibited \( \beta \)-glycerophosphate hydrolysis by MVs or the purified ALP (data not shown). Hence, the kinetic differences between ATPase and ALP in response to vanadate further support the contention that a specific ATPase is present in MVs.

To understand how MVs initiate mineralization, it is necessary to consider the origin of Ca\(^{2+}\) and Pi within MVs. To study the origin of these ions within MVs, it is essential to consider the biogenesis of MVs. The freeze-fracture electron microscopic observation suggests that budding from chondrocyte plasma membranes is a possible mechanism for MV biogenesis (28). If this is indeed the case, then preexisting intracellular P\( _i \) would be likely to provide an initial source of the Pi of MV. Since the cytosolic [Ca\(^{2+}\)] is less than \( \mu \text{M} \), a specific pump system would be necessary to accumulate Ca\(^{2+}\) from surrounding tissue fluids into the vesicles. Ca\(^{2+}\) ATPase could fulfill this role, provided that MVs are an inside-out version of plasma membranes, because the plasma membrane Ca ATPase provides most outwardly directed Ca\(^{2+}\) translocation. Although available evidence indicates that plasma membrane-derived MVs are not endowed during their biogenesis, it must be stressed that all ion pumps are potentially reversible, and Ca\(^{2+}\) tends to flow from an energized compartment to the lower energy side of a membrane, as in the case of isolated plasma membrane vesicles (29, 30). The net flow of Ca\(^{2+}\) is in favor of accumulation despite the possibility of an equal distribution of inside-out and right side-out versions of membrane vesicles. If ATP is provided by hypertrophic chondrocytes, MVs in situ may allow Ca accumulation by the same mechanism as that adapted by isolated liver or kidney plasma membrane vesicles. The most recent data with phosphatidylinositol-specific phospholipase C (31) indicate that MVs are right side-out versions of plasma membranes. This contention has also been supported by the observations that the solubilization of MV membranes by detergents did not further enhance ATPase, ALP, or AMPase (data not shown). To test whether Ca uptake is a prerequisite step before calcification can take place, we tested a batch of inhibitors for Ca uptake and ATPase. We were unable to observe the inhibitory effect of various ATPase-dependent Ca uptake inhibitors, including oligomycin, ouabain, thapsigargin, and vanadate, on calcification. However, we found that cetyltrimethyl bromide, which is a cationic detergent, inhibited both ATPase and calcification. Despite this observation, we feel that the correlation may be coincidental. Thus, it seems that the observed inhibitory effect of ATP analogs provides a more reliable test to see whether a specific ATPase different from other types of ATPase is responsible for ATP-initiated Ca and Pi deposition. Whether ATPase-driven Ca uptake is required for calcification remains to be established.

Although ATP-initiated Ca uptake may serve a role in the mechanism of MV-initiated calcification, sufficient ATP must be provided by chondrocytes or other cells to the vicinity of MVs. The extracellular ATP concentration has yet to be determined in the cartilage matrix, probably due to the instability and trace amounts of ATP. However, it has been shown that various types of cells are capable of exporting ATP (for review, see Ref. 32). A continuous supply of ATP to the vicinity of MVs from chondrocytes could be facilitated by chondrocyte hypertrophy prior to calcification. It has been shown that the hypertrophic zone of cartilage before calcification contains more ATP than the reserve zone (33). The ability of cultured chondrocytes...
to export ATP has been recently reported (34). Several reports also have implicated the role of ATP in calcification: 1) blood ATP levels are reduced in rachitic rats (35, 36); and 2) ATP promotes mineral deposition by chondrocyte culture (34, 37) and cartilage slices (38, 39). In particular, the appearance of hydroxyapatite formed in the presence of ATP was more similar to bone mineral than that obtained with β-glycerophosphate (37). These and our observations thus support the hypothesis that ATP and a specific ATPase are directly involved in the mechanism of MV-initiated calcification.

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