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Further characterization of ATP-initiated calcification by matrix vesicles isolated from rachitic rat cartilage
Membrane perturbation by detergents and deposition of calcium pyrophosphate by rachitic matrix vesicles

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Abstract

Although membrane associated enzymes such as ATPase, alkaline phosphatase, and NTP pyrophosphohydrolase in matrix vesicles (MVs) may underlie the mechanisms of ATP-promoted calcification, prior to the current investigation, the role of the MV membrane in calcification had not been addressed. In this study, various perturbations were introduced to the MV membrane in in vitro calcification systems to determine ideal conditions for ATP-initiated calcification by MVs isolated from rachitic rat epiphyseal cartilage. Membrane integrity appears to be required, since the rupture of the vesicular membrane by vigorously mixing with 10% butanol abolished calcification. In contrast, a mild treatment of MVs with low concentrations (e.g., 0.01%, which is much below the critical concentration for micelle formation) of either neutral Triton X-100 or anionic deoxycholate stimulated calcification by >2-fold, without inducing obvious changes in vesicular appearance. Fourier transform infrared spectroscopic studies were done to identify the mineral phase formed in these experiments. For the first time, rachitic MVs were shown to induce the formation of a calcium pyrophosphate dihydrate-like phase after their exposure to calcifying medium with 1 mM ATP. The integration of spectral areas indicated that calcification was enhanced by Triton X-100. The detergent effect was reversible and appeared to be not mediated through activation of ATPase, alkaline phosphatase, or ATP pyrophosphohydrolase. In contrast to neutral Triton X-100 and anionic deoxycholate, cationic cetyltrimethylammonium bromide inhibited both ATPase activity ($I_{50} = 10$ μM) and ATP-initiated calcification. These observations suggest that membrane perturbations can affect calcification and that the presence of NTP-pyrophosphohydrolase in MVs may play a role in the deposition of $CaPP_i$ in rachitic cartilage.

Keywords: Adenosine triphosphate; Calcification; Rachitic; Matrix vesicle; Calcium pyrophosphate

Abbreviations: MVs, matrix vesicles; ALP, alkaline phosphatase; TBS, 10 mM Tris (pH 7.6) buffered saline; LT, L-tetramisole; Tes, *N*-tris(hydroxmethyl)-methyl-2-amino-ethanesulfonic acid; FT-IR, Fourier transform infrared spectroscopy; ACP, amorphous calcium phosphate; aCPPD, amorphous calcium pyrophosphate

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1. Introduction

The mechanisms of initiation and regulation of calcification associated with normal bone formation, remodeling and regeneration processes, and with many pathological states such as rickets [1], atherosclerosis [2], chondrocalcinosis [3], osteogenesis imperfecta [4], myositis ossificans [5], and various types of calcific diseases [6] have been the subjects of intensive investigation. Currently, however, there are many questions regarding these mechanisms that are still unanswered. In particular, the role of matrix vesicles (MVs) and their components in mineralization remains incompletely understood.

In vitro studies have demonstrated that ATP initiates the deposition of calcium phosphate and calcium pyrophosphate minerals in cartilage slices [7,8] and chondrocyte culture [9,10]. ATP has also been shown in vitro to play a role in calcification by mammalian MVs [11–14]. A previous study with rachitic MVs indicated that ATP-initiated calcification is specific for ATP since various ATP analogs but not phosphomonoesters inhibit ATP-promoted calcification [13]. However, in one study ATP was shown to inhibit $^{45}Ca^{2+}$ uptake by isolated avian MVs [15]. Although ATPase [11–14], alkaline phosphatase (ALP) [16,17], and ATP pyrophosphohydrolase [18,19] are likely to be involved in the mechanisms of ATP-dependent calcification mediated by mammalian MVs, the specific role of MV membranes has yet to be fully defined [16,17,20,21].

The purpose of this paper was to investigate the role of MV membranes in ATP-dependent calcification by studying the effects of various membrane perturbing agents, such as cationic, anionic, and neutral detergents, on mineralization of MVs isolated from rachitic rat epiphyseal cartilage. The diverse effects of detergents reported here suggest that mild membrane perturbations may affect ATP-dependent calcification by isolated rachitic vesicles. Additionally, we have also demonstrated in the course of this study that rachitic MVs contained NTP pyrophosphohydrolase and that MVs can induce $CaPP_i$ formation, suggesting that MVs may play a significant role in $CaPP_i$ formation in cartilage of rachitic patient [22].

2. Experimental procedures

2.1. Induction of rickets

Weanling albino rats of the Charles River strain were housed in a darkened room and fed ad libitum, a high protein, high calcium, low phosphorus and low vitamin D diet. After 3 weeks on the rachitogenic diet the rats were killed, and their proximal tibial growth plates were removed for study.

2.2. MV preparation

2.2.1. Epiphyseal cartilage MVs

The extracellular MV fraction was prepared from rachitic rats according to the method of Hsu and Anderson [13]. Epiphyseal growth plates (3 g) from 35 rachitic rats were removed, minced into 3- to 5-mm pieces, and then digested in a solution (10 ml/g of tissue) containing 0.45% of crude collagenase (Boehringer Mannheim), 0.12 M NaCl, 0.01 M KCl, 1000 units/ml of penicillin, 1 mg/ml of streptomycin, and 0.02 M Tes (*N*-tris(hydroxymethyl)-methyl-2-amino-ethanesulfonic acid) buffer, pH 7.45. The digestion was carried out at 37°C for 3 h. The digest was centrifuged at $30\,000 \times g$ for 10 min, and the resulting precipitate of cells and cell debris was discarded. The supernatant was spun at $300\,000 \times g$ for 20 min and the resulting MV-enriched precipitate was then resuspended in 1 mM EGTA/10 mM Tris-buffered saline, at pH 7.6. The mixture was incubated overnight at 4°C to maximize the removal of the endogenous mineral contaminant. The mixture was then centrifuged at $300\,000 \times g$ for 20 min. After the removal of the supernatant, the precipitate was washed twice by suspension in 10 mM Tris-buffered saline (pH 7.6) (TBS) and centrifugation. The pellet was finally resuspended in 1 ml of TBS to a final protein concentration of 1 mg/ml. The isolated MVs were devoid of membrane leakage since the incubation of MVs with uniformly labeled [^{3}H]ATP or $^{45}CaCl_2$ did not cause nonspecific uptake of radioactive ATP or Ca^{2+}. The EGTA-treatment is effective in removing extraneous preexisting mineral without impediment to calcifiability of MVs [13,23]. The isolated MVs were heterogeneous in size (10–

1000 nm) and shape similar to MVs in situ and devoid of the microsomal marker NADPH-cytochrome reductase activity and mitochondrial succinate dehydrogenase activity [24]. After an exposure to ATP-containing calcifying medium for 5 h, the vesicles are heavily associated with electron dense mineral particles and can be readily sedimented by low centrifugal force of $800 \times g$ [13].

2.2.2. Calvaria MVs

Pooled fetal calvaria were obtained from three near-term pregnant rats. Calvaria were treated with sequential crude collagenase digestion to obtain different bone cell populations as described by Luben et al. [25]. MVs were then prepared from the pooled cell-free collagenase digest by the same procedure for cartilage MV preparations. The uptake of uniformly labeled [^{3}H]ATP or $^{45}CaCl_2$ by MVs was not detected indicating that MV membranes were intact and devoid of observable leakage. The vesicles seen by transmission electron microscopy were similar to cartilage MVs in size and shape, devoid of microsomal and mitochondrial enzyme marker activities, and calcifiable in the presence of ATP.

2.3. Calcification

The term 'calcification' is used in this paper to describe the process by which Ca and P_i was deposited either as undefined forms of calcium phosphate, calcium pyrophosphate, or as Ca^{2+} and P_i that were taken up by or bound to MVs. To measure Ca and P_i deposition, the method of Hsu and Anderson [13] was used. Unless otherwise stated, the standard reaction medium (100 μl) consisted of 50 mM Tes, pH 7.65 (titrated at 37°C), 85 mM NaCl, 15 mM KCl, 1 mM $MgCl_2$, 10 mM $NaHCO_3$, 1.35 mM $CaCl_2$, 1.97 mM P_i, and 1 mM ATP. For Ca and P_i uptake, ^{45}Ca and $^{32}P_i$ (1×10^6 cpm) were used as radioactive tracers, respectively. To determine whether MVs can calcify in the absence of ATP, calcification without ATP was also conducted for each experiment. The reaction was initiated by adding an aliquot of MVs (a final protein concentration of 10 μg/ml) to calcifying media and then incubated for 5 h at 37°C in a water vapor-saturated incubation chamber which was used to minimize vaporization of the reaction mixture during incubation. At the end of incubation, the reaction mixture was filtered through 0.1 μm-pore size Durapore membranes (Millipore). The membranes were washed twice each with 1 ml of TBS and then transferred to vials containing scintillation fluids for radioactivity counting. The nonspecific Ca or phosphate binding is defined as the radioactivity that nonspecifically binds to the filters in the absence of MVs under the identical conditions for each experiment. Since the nonspecific background counts were found to be insignificantly different from Ca and P_i deposition by MVs in the absence of ATP ($P > 0.05$ with 25 sample means), unless otherwise stated, the deposition of $^{45}CaCl_2$ or $^{32}P_i$ by MVs in the absence of ATP was used as the background counts. These background counts were subtracted from the radioactivity in the presence of MVs and ATP under various experimental conditions. Ca or P_i deposition is expressed as nmol Ca or P_i/μg MV protein/5 h, calculated by the following formula: [(cpm with ATP and MVs minus nonspecific binding, i.e., cpm obtained with MVs but without ATP)/total cpm] × concentration of $CaCl_2$ or phosphate (nmol of Ca or P_i/ml) and then divided by concentrations of MV proteins.

2.4. Fourier transform infrared spectroscopy (FT-IR)

Aliquots of MVs (~25 μg protein/aliquot) were exposed to calcifying medium in the presence and absence of 1 mM ATP or 0.01% Triton X-100 for 5 h and then the reaction mixtures were centrifuged at $300\,000 \times g$ for 20 min. The MV precipitates were washed twice with deionized and distilled water (neutralized to pH 7.4 with alkali) and suspended in 10 mg KBr to assure total recovery of the sample. The precipitate/KBr mixtures were freeze-lyophilized and KBr pellets prepared at 12 000 psi (total sample in 200 mg KBr). Pellets were analyzed on a Mattson Cygnus 25 FT-IR spectrometer (Mattson Instruments, Madison, WI) equipped with a DGTS detector. Typically, 64 scans were acquired at 4 cm^{-1} resolution under N_2 purge. GRAMS/32 software (Galactic Industries, Salem, NH) was used for data analysis. Protein, lipid and phosphate species (from the mineral phase) absorb at infrared frequencies characteristic of that particular molecule and thus can be used to identify the components present in a

sample. Spectra of nonmineralized vesicles were subtracted from the mineralized spectra to isolate the mineral phase. Integrated areas of individual absorbance bands are proportional to the quantity of the component present in the sample. Thus, the ratio of the integrated areas of the absorbance in the phosphate region from 800 to 1300 cm^{-1} (mineral) to that of absorbance in the protein amide I region (1585 to 1725 cm^{-1}) were used to obtain a 'mineral to protein' ratio.

Standard calcium phosphate compounds were prepared to aid in identification of the MV mineral phases. Amorphous calcium phosphate (ACP) was prepared according to the method of Gadaleta et al. [26]. Amorphous calcium pyrophosphate dihydrate (aCPPD) was prepared by a modification of the method of Chen and Pritzker [27]: PP_i was added to the calcifying medium (described above) without ATP and MVs to a final concentration of 1 mM. The P_i concentration was adjusted to 2.97 mM. The mixture was incubated at 37°C for 24 h and then precipitated at $300\,000 \times g$ for 20 min and washed twice with neutral deionized water. The washed precipitates were freeze-lyophilized and analyzed as KBr pellets as described above. The amorphous character of the synthetic mineral preparations was confirmed by the X-ray diffraction study.

2.5. Specific ATPase activity

A putative specific ATPase is defined as a specific enzyme that solely uses ATP as a substrate and specifically releases the terminal P_i from ATP. ATPase activity is expressed as the rate of $^{32}P_i$ released from [γ-^{32}P]ATP. Unless otherwise stated, the reaction mixture (100 μl) contained 10 mM Tris (pH 7.6), 0.15 M NaCl, 1 mM $MgCl_2$, 1 mM ATP (1×10^6 cpm [γ-^{32}P]ATP), with or without 5 μg/ml of MV protein [13]. The reaction mixture was initiated by the addition of an aliquot of MVs, incubated for 30 min at 37°C, and then terminated by addition of 250 μl of 0.2 M silicotungstic acid. The $^{32}P_i$ yielded from ATP hydrolysis was then extracted into the isobutanol–toluene phase by the method of Martin and Doty [28] and counted in a vial containing scintillation fluid. One unit (U) is expressed as that amount of ATPase required to produce 1 μmol $^{32}P_i$/min from [γ-^{32}P]ATP.

2.6. ATP pyrophosphohydrolase assay

ATP pyrophosphohydrolase activity was determined using 2 mM p-nitrophenol thymidine pyrophosphate as substrate. The activity was followed by the appearance of nitrophenol spectrophotometrically [19,24].

2.7. Test for damage and leakage in MVs

To determine the damage and leakage of MVs induced by the detergent treatment, the following steps were taken [13]. Untreated MVs and detergent-treated MVs were suspended in TBS containing 1×10^6 cpm of 1 mM [^{14}C]ATP, [^{3}H]ATP, or $^{45}CaCl_2$ for 5 h. The mixtures were then filtered through 0.1 μm pore-size Durapore membranes (Millipore) by using a Millipore vacuum trap device. The membranes were washed twice each with 1 ml of TBS and then transferred to vials containing scintillation fluids

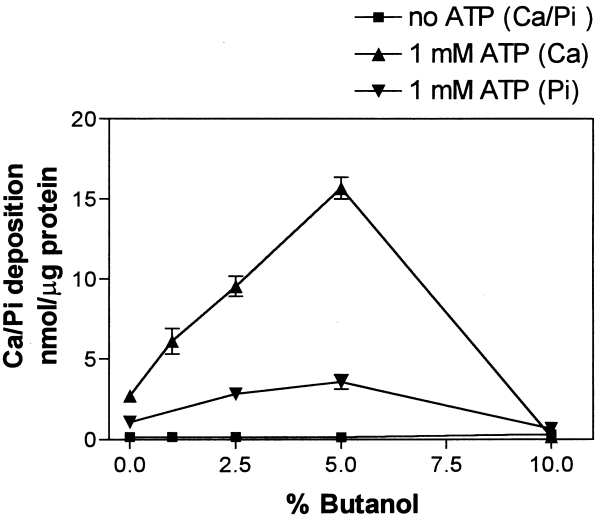

Fig. 1. Effect of butanol concentrations on ATP-dependent Ca and P_i deposition by MVs. The detail of ATP-dependent Ca and P_i deposition is described in Section 2. Various concentrations of butanol ranging from 1% to 10% were included in calcifying media. The letters (Ca) and (P_i) on the upper right side of the graph represent Ca and P_i deposition, respectively. Values are expressed as means ± S.E. calculated from each of duplicate data from three replicate experiments. Note that P_i deposition is calculated on the basis of the initial P_i concentration present in calcifying media. Since 1 mM ATP was completely hydrolyzed after 5 h of incubation yielding 3 mM P_i (data not shown), P_i deposition will be 2.5-fold higher if P_i released from ATP is also considered for calculation. This consideration also applies to Fig. 2.

for radioactivity counting. For the blank control, MVs were omitted to determine radioactivity non-specifically bound to the filters. The degree of damages and leakage is expressed as the ratio: [cpm (treated MVs)−cpm (untreated or fresh MVs)]/cpm (untreated or fresh MVs).

2.8. Determinations of phosphate and protein

Inorganic orthophosphate was determined by the method of Martin and Doty [28]. The protein concentration was measured by the procedure of Lowry et al. [29] using bovine serum albumin as standard.

3. Results

3.1. Effects of membrane perturbing agents on ATP-initiated calcification

To determine whether the vesicular structure was an important factor for ATP-dependent calcification, MVs were vigorously mixed with 10% butanol to rupture the membrane. The treatment not only caused the vesicular structure unrecognizable under the electron microscope (figure not shown) but also abolished the ability of ATP to induce MV-mediated calcification (Fig. 1). However, a milder treatment with lower concentrations of butanol ($<5\%$) did not destroy the apparent vesicular structure (not shown) but markedly enhanced ATP-initiated calcification (Fig. 1). When ATP was not included in calcifying media, neither calcification nor butanol stimulation occurred.

To confirm the observed effects of membrane perturbation by butanol on ATP-dependent calcification, various types of membrane-solubilizing detergents were tested. As indicated by $^{45}CaCl_2$ and $^{32}P_i$ uptake, as low as 0.002% of Triton X-100 was effective in stimulating ATP-initiated calcification (Fig. 2). In contrast, no activation was observed if ATP was not included in calcifying media (data not shown). The addition of 0.01% Triton X-100, which is much below the critical concentration for formation of micelles, to calcifying medium in the absence of ATP neither altered the overall vesicular structure nor induced calcification when examined under the electron microscope (Fig. 3A). After MVs were exposed to ATP-containing calcifying medium without Triton X-100 for 5 h, electron-dense particles were formed in close association with MV membranes (Fig. 3B). However, in the presence of 1 mM ATP and 0.01% Triton X-100, much larger electron-dense particles were evident (Fig. 3C). The vesicular structure became unrecognizable probably as a result of heavy calcification induced by the inclusion of Triton X-100 in calcifying media. Many empty holes seen in Fig. 3C appear to be originally occupied by large mineral deposits which were unable to be retained during electron microscopical processing.

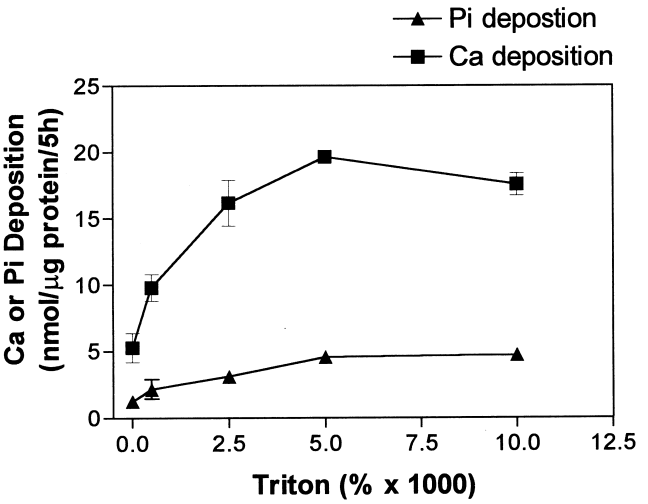

Fig. 2. Effect of Triton X-100 on ATP-dependent Ca and P_i deposition by MVs. The detail of ATP-dependent Ca and P_i deposition is described in Section 2. The concentrations of Triton X-100 used were from 0.001% to 0.01%. Due to low percentages of Triton X-100 in calcifying media, the *x*-axis scale is expressed as the percentage of Triton X-100 multiplied by a factor of 1000. Values are expressed as means ± S.E. calculated from each of duplicate data from three replicate experiments.

Since Triton X-100 is a neutral detergent, it was of interest to determine whether the anionic detergent deoxycholate (DOC) and the cationic detergent cetyltrimethyl-ammonium bromide (CTAB) would also

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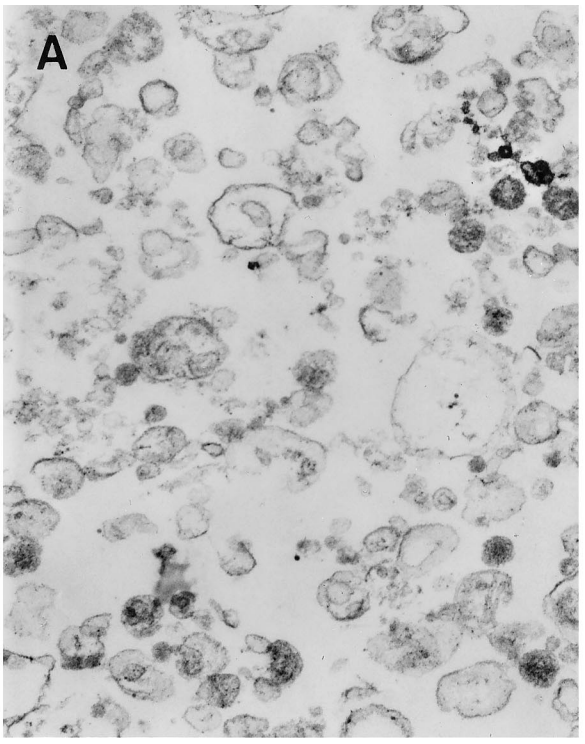
Fig. 3. Electron micrographs of samples obtained after a 5-h exposure of MVs to calcifying medium in the presence and absence of Triton X-100. (A) MVs exposed to calcifying medium with 0.01% Triton X-100. (B) MVs exposed to calcifying medium with 1 mM ATP but without Triton X-100. (C) MVs exposed to calcifying medium with 0.01% Triton X-100 and 1 mM ATP. The detail of ATP-dependent Ca and P_i deposition is described in Section 2.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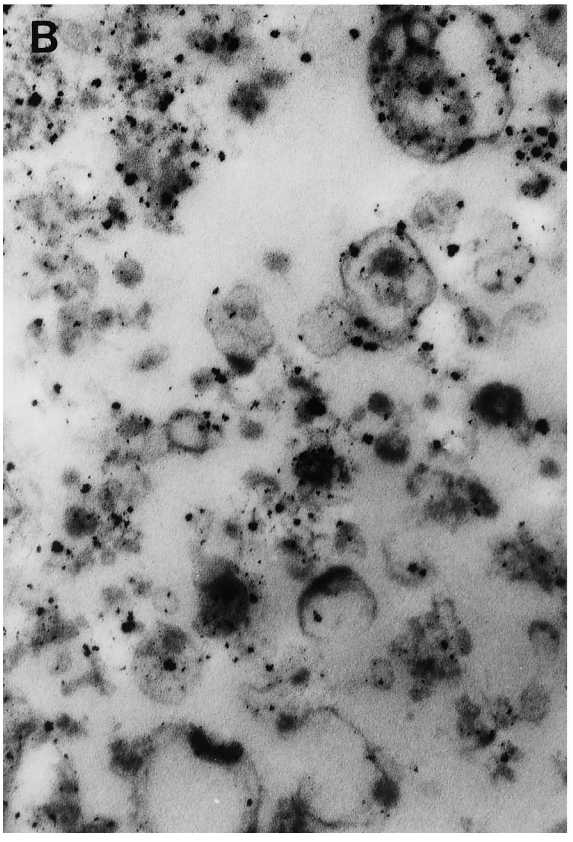
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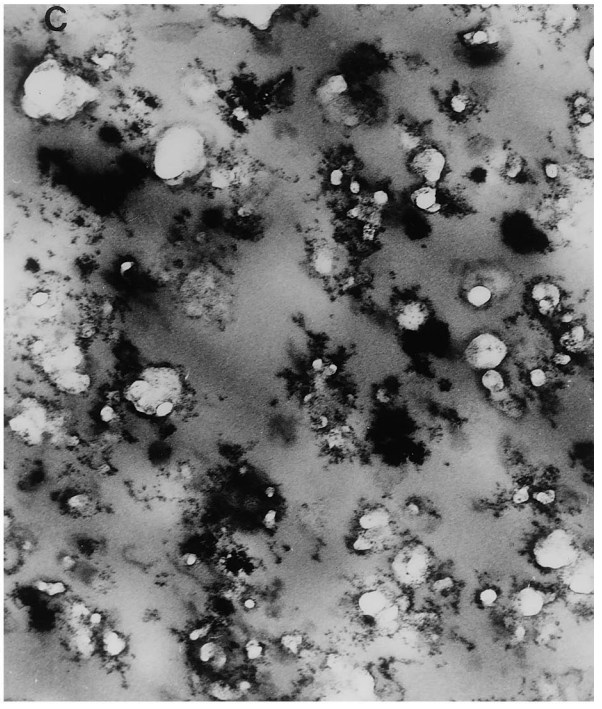
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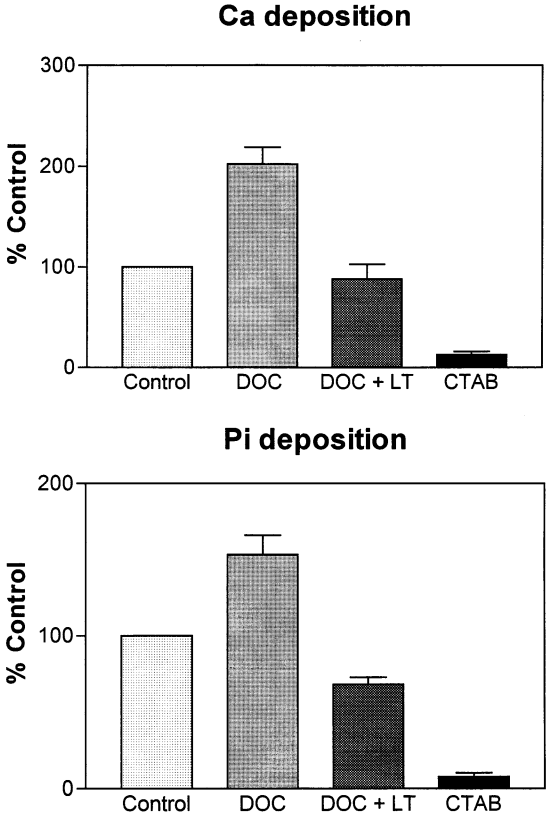

Fig. 4. Effects of deoxycholate (DOC) and cetyltrimethylammonium bromide (CTAB) on of ATP-dependent Ca and P_i deposition by cartilage MVs. The detail of ATP-initiated calcification is described in Section 2. The concentration of the detergents was 0.01%. LT (L-tetramisole) at 1 mM was used to test whether the stimulation by DOC can be abolished by the ALP inhibitor. The effect of the detergents on Ca and P_i deposition is expressed as percentages of control values. Values represent means ± S.E. calculated from each of the duplicate data from three replicate experiments. The control values are 5.2 ± 0.03 nmol Ca/μg protein and 2.1 ± 0.2 nmol P_i/μg protein.

exert a stimulatory effect on ATP-dependent calcification. As shown in Fig. 4, DOC at 0.01% significantly stimulated ATP-dependent Ca and P_i deposition ($P < 0.01$) whereas CTAB at 0.01% inhibited 80% of calcification ($P < 0.01$). The addition of L-tetramisole inhibited stimulation by DOC ($P < 0.01$).

3.2. Demonstration that rachitic MVs are capable of depositing calcium pyrophosphate in the presence of ATP

In an attempt to determine whether MVs play a part in the formation of $CaPP_i$ in rachitic cartilage as reported by Tailor and Hothersall [22], FT-IR spectroscopy was used to identify and obtain relative quantities of the mineral phase deposited by MVs in the presence of ATP. Fig. 5A shows a typical FT-IR spectrum obtained from exposure of MVs to 1mM ATP-calcifying medium for 5 h. A typical nonmineralized MV spectrum is shown in Fig. 5B. Fig. 5C shows the mineral phase isolated by subtraction of the nonmineralized MV from the mineralized MV spectrum. In the presence of 0.01% Triton X-100, the spectrum appears similar to that in Fig. 5A except for the quantity of mineral present. The mineral to protein ratio was calculated from two separate mineralization experiments in the presence and absence of 0.01% Triton X-100. The values of 4.28 vs. 2.26 (with and without Triton, respectively), and 3.26 vs. 2.87 (with and without Triton, respectively), revealed that MV mineralization tended to be greater in the presence of 0.01% Triton X-100 than that in its absence. Identification of the species contributing to the broad absorbance band in the phosphate region was achieved by examination of FT-IR spectra from standard phosphate compounds followed by spectral additions. Fig. 6 shows the spectra of (A) synthetic amorphous calcium phosphate, (B) synthetic amorphous calcium pyrophosphate, and (C) their 1:1 spectral addition. Comparison of spectrum C with spectrum D, the MV mineral phase, revealed that the MV mineral is a mixture of amorphous calcium phosphate and amorphous calcium pyrophosphate. Mixing synthetic calcium phosphate and calcium pyrophosphate at different ratios showed varying degrees of peak magnitude but the specific peak position of the respective minerals remained the same. Additional evidence (data not shown) came from a direct anionic chromatographic elution pattern of P_i and PP_i of re-dissolved radiolabeled mineral deposits using a previous published method [18], revealing the presence of two mineral phases in equal ratio (data not shown). Thus, we have demonstrated for the first time that calcium pyrophosphate can be deposited by rachitic MVs.

3.3. ATP pyrophosphohydrolase activity in rachitic MVs

It has been shown that ATP pyrophosphohydro-

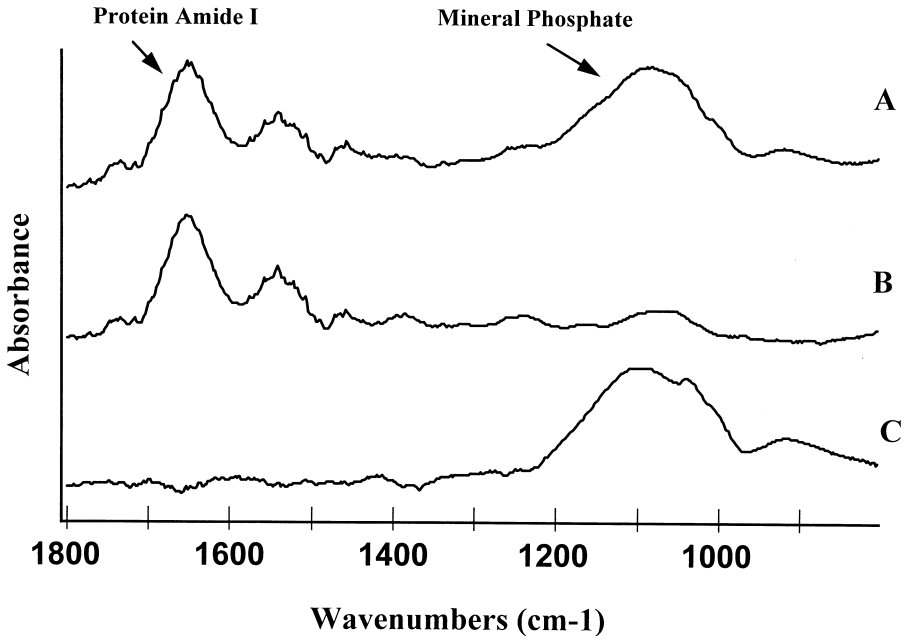

Fig. 5. FT-IR spectra of mineral deposited after a 5 h-exposure of MVs to calcifying medium in the presence of 1 mM ATP with or without 0.01% Triton X-100. (A) The curve represents the spectrum of a sample obtained after a 5-h exposure of MVs to calcifying medium in the presence of 1 mM ATP. The infrared absorbance bands from the protein and mineral components are labeled. (B) An FT-IR spectrum from nonmineralized MVs. (C) The subtraction of spectrum B from spectrum A to isolate the mineral absorbance bands. The detail of ATP-dependent Ca and P_i deposition is described in Section 2.

lase is responsible for ATP-mediated deposition of calcium pyrophosphate by MVs isolated from normal epiphyseal cartilage [18] and from chondrocalcinotic articular cartilage [19]. To test whether the presence of ATP pyrophosphohydrolase in MVs isolated from rachitic cartilage is involved in calcium pyrophosphate deposition in rachitic cartilage [22], the enzyme activity was assayed and calculated from this and two previous batches of MV preparations representing a pool of 105 rachitic rats. The enzyme activity for the first time was demonstrated to be 1.94 ± 0.33 μmol PP_i formed/min per mg MV proteins (mean ± S.E.). The formation of PP_i from ATP by ATP pyrophosphohydrolase was further confirmed by FT-IR spectroscopic demonstration of $CaPP_i$ deposits (Fig. 6D).

3.4. *Effects of detergents on various calcification-related enzymes*

Effects of detergents on various calcification-related enzymes such as ALP, ATPase, and ATP pyrophosphohydrolase were studied to determine whether the detergent effects on calcification was mediated through these enzyme activities. Neither Triton X-100 nor DOC stimulated ATPase, β-glycerophosphate hydrolysis, and ATP pyrophosphohydrolase activities (data not shown). In contrast, CTAB at 8×10^{-5}% (10 μM) inhibited 50% of ATPase but was much less inhibitory to β-glycerophosphate hydrolysis ($I_{50} > 100$ μM, see Fig. 7). CTAB at 1 mM did not affect ATP pyrophosphohydrolase activity (data not shown).

3.5. *Testing whether Triton X-100 stimulation of ATP-initiated calcification was due to the release of activators from MVs*

It is possible that the stimulation of ATP-dependent Ca and P_i deposition by the detergents was due to the release of stimulating factors from the MV membranes, or factors that may be inhibitory if bound to the MV membrane. To rule out Triton X-100 or detergent-solubilized factors as contributing factors, the following steps were taken. MVs were initially incubated with 0.01% Triton X-100 for

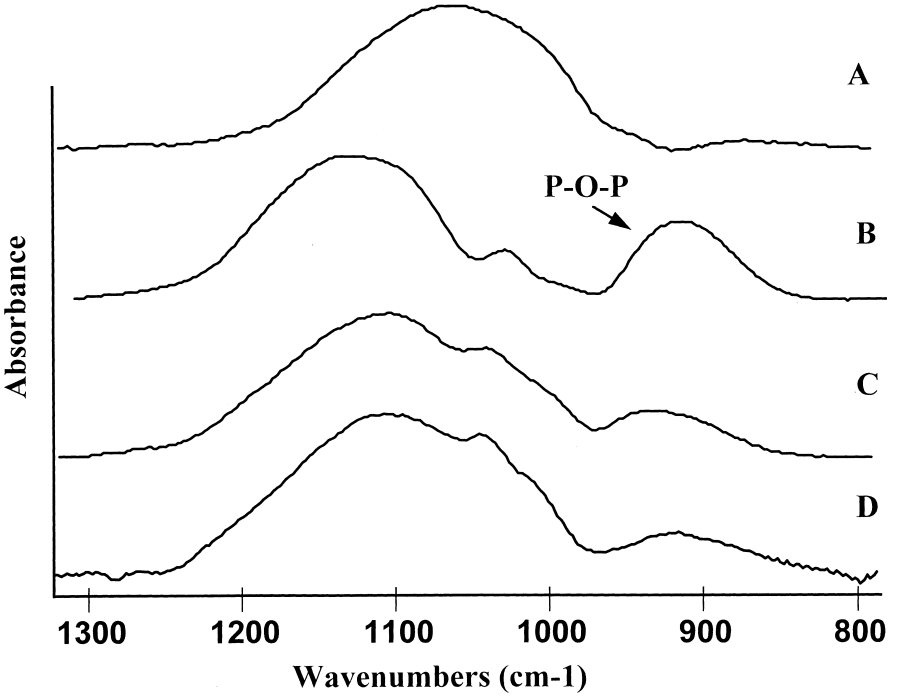

Fig. 6. FT-IR spectroscopic identification of the mineral deposited after exposure of MVs to calcifying medium in the presence of 1 mM ATP and Triton X-100. (A) FT-IR spectrum of synthetic amorphous calcium phosphate (ACP). (B) FT-IR spectrum of synthetic amorphous calcium pyrophosphate dihydrate (aCPPD). The characteristic absorbance at $\sim$920 cm^{-1} arises from the pyrophosphate (P–O–P) bond. (C) FT-IR addition spectrum of ACP and aCPPD in a 1:1 ratio. (D) FT-IR spectrum (after subtraction of MV spectrum) of mineral region from a 5-h exposure of MVs to 1 mM ATP-calcifying medium in the presence of 0.01% Triton X-100. Comparison of spectra C and D indicates that the MV mineral is a mixture of ACP and aCPPD. The detail of ATP-dependent Ca and P_i deposition is described in Section 2.

30 min. The detergent and detergent-solubilized factors were removed by centrifugation. The resulting MVs were then tested to see if the stimulated level of calcification remained unchanged. As shown in Fig. 8, the stimulated level of ATP-dependent Ca and P_i deposition returned to nonstimulated levels after Triton X-100 was removed (not significantly different from the basal level, $P > 0.05$). The addition of Triton X-100 to calcifying media partially restored the stimulation ($P < 0.01$). Electron microscopic examination indicated that the overall structure of MVs was not altered by the treatment with low concentrations of Triton X-100 (Fig. 3A). The detergent did not cause nonspecific uptake of ^{45}Ca or $^{32}P_i$ in the absence of ATP or the uptake of uniformly labeled [^{3}H]ATP by MVs indicating that the detergents did not cause detectable leakage of the membrane to Ca^{2+}, P_i, and ATP (data not shown).

3.6. *Effect of L-tetramisole on stimulation of ATP-dependent calcification by detergents*

To determine whether ALP contributed to detergent stimulation of calcification, 1 mM L-tetramisole was used, which can completely inhibit [γ-^{32}P]ATP hydrolysis by MV ALP but not by MV ATPase [13,30]. L-Tetramisole at 1 mM did not significantly inhibit the basal level of ATP-dependent Ca^{2+} and P_i deposition ($P > 0.05$, Fig. 9), but markedly decreased Triton X-100 ($P < 0.01$, Fig. 9) or DOC ($P < 0.01$, Fig. 4) stimulation of ATP-dependent calcification by cartilage MVs. As depicted in Fig. 9, L-tetramisole was also effective although to a less extent in inhibiting the stimulatory effect of detergents on calcification by bone MVs ($P < 0.05$).

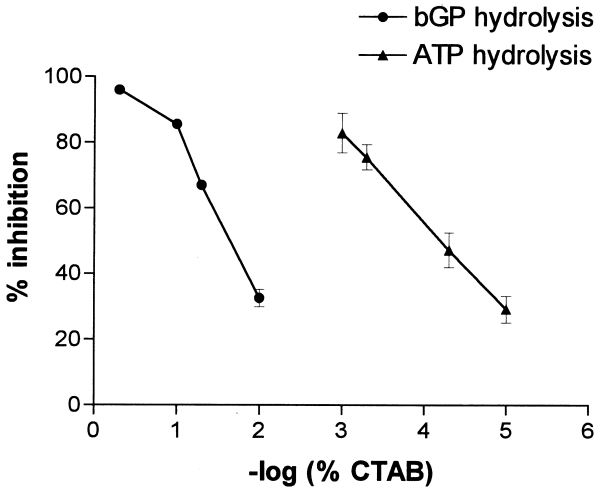

Fig. 7. Effect of cetyltrimethylammonium bromide (CTAB) on ATPase activity and the hydrolysis of β-glycerophosphate. The detail of ATPase is described in Section 2. For β-glycerophosphate hydrolysis, the assay medium consisted of TBS, 1 mM $MgCl_2$ and 1 mM β-glycerophosphate, and the release of P_i from β-glycerophosphate after 30 min of incubation was determined colorimetrically as reported previously [15]. The effect of the detergent on ATPase and β-glycerophosphate hydrolysis is expressed as percentages of the control value, i.e., without detergents. The values are expressed as means ± S.E. calculated from each of the duplicate data from three replicate experiments. The control values for ATPase and β-glycerophosphate hydrolysis are 0.57 ± 0.03 and 0.20 ± 0.02 U/mg protein, respectively.

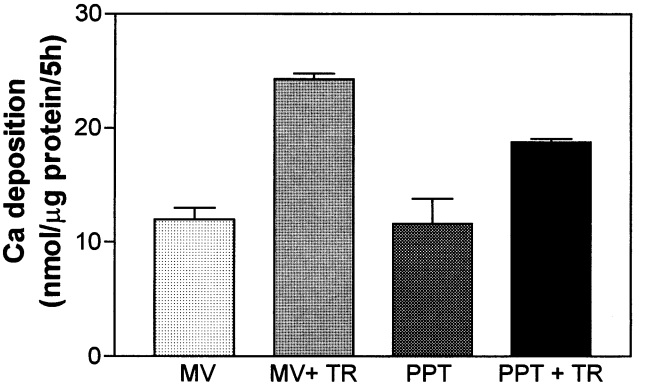

Fig. 8. The reversibility of the stimulatory effect of Triton X-100 on ATP-initiated Ca deposition. MVs were incubated with or without 0.01% Triton X-100 in TBS at 4°C for 30 min and followed by centrifugation at 270 000 × *g* for 30 min. The resulting precipitates from Triton X-100-pretreated MVs were washed twice by TBS and followed by suspension in aliquots of TBS. The washed pellets were then assayed for Ca depositing activity in the presence or absence of 0.01% Triton X-100. MV, Ca deposition by MVs without pretreatment with Triton X-100; MV+TR, Ca deposition by MVs in the presence of Triton X-100; PPT, Ca deposition by 270 000 × *g* pellets obtained from MVs pretreated with Triton X-100; PPT+TR, Ca deposition by 270 000 × *g* pellets in the presence of Triton X-100.

4. Discussion

MVs from rachitic cartilage were used in this report to study their role in the mechanisms that may cause deficient mineralization in rickets. In addition, since rachitic MVs may contain less innate mineral than normal vesicles, the use of MVs from rachitic cartilage can offer an ideal model for studying the initiation of MV-mediated calcification without complications resulting from proliferation of preexisting mineral [13].

The observation that ATP can initiate calcification by rachitic MVs confirms our previous finding that ATP-initiated calcification is ATP-specific since it can be specifically inhibited by ATP analogs but not by phosphomonoesters [13]. The roles of ATPase [11–14], alkaline phosphatase (ALP) [16,17], or ATP pyrophosphohydrolase [18,19] in ATP-mediated calcification have been well established. Although the interest in the role of MV membranes in calcification has been raised by various researchers [16,20,21], its precise function in ATP-dependent calcification by rachitic MVs has not been addressed prior to this investigation. An intact vesicular structure appears to be required for ATP-initiated calcification, since the rupture of MV membrane by butanol either from bovine fetal cartilage [31] or from rachitic cartilage (Fig. 1) destroys ATP-initiated calcification. The requirement of vesicular structure is further supported by the observation that soluble ALP released from MVs by phosphatidylinositol-specific phospholipase C treatment fails to calcify in the presence of β-glycerophosphate [16,17]. Other studies also indicated that membrane perturbation induced by active vitamin D metabolites may have a profound effect on membrane fluidity although its effect on ATP-initiated calcification was not addressed [20]. Recently, a membrane associated nucleation complex [21] was shown to play a role in mineralization. Thus, it is likely that the interplay of various membrane components is required for optimal calcification by MVs.

Perturbation of the membrane by low concentrations of detergents can either markedly stimulate or

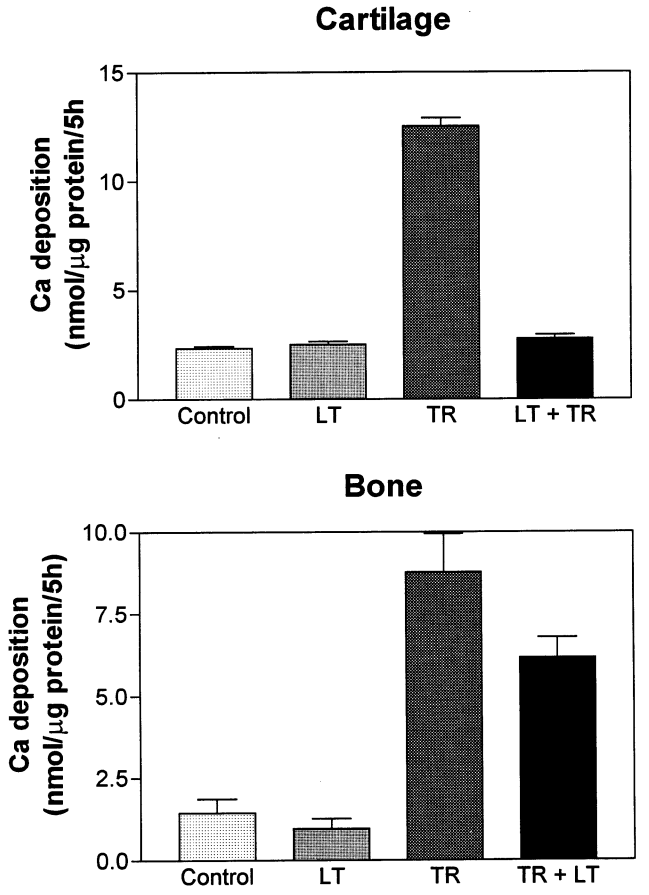

Fig. 9. Effect of a specific ALP inhibitor, L-tetramisole, on Triton X-100 stimulation of ATP-dependent Ca deposition by cartilage and bone MVs. The detail of ATP-dependent Ca and P_i deposition is described in Section 2. The concentrations of L-tetramisole and Triton X-100 were 1 mM and 0.01%, respectively. LT, L-tetramisole; TR, Triton X-100. Values are expressed as means ± S.E. calculated from each of the duplicate data obtained from three experiments.

inhibit ATP-initiated calcification depending on the ionic nature of detergents. These observations suggest that the ionic, hydrophobic, or fluidity nature of the MV membrane in addition to ATP hydrolysis may also play an important role in the mechanism of ATP-mediated calcification. The association of mineral with the membrane as evidenced by electron microscopic observations ([13] and Fig. 3B) further supports this contention. The activation of ATP-dependent calcification by anionic or neutral detergents did not appear to be mediated through enzyme activities because both DOC and Triton X-100 failed to stimulate ALP, ATPase, or ATP pyrophosphohydrolase activity. Nor was the stimulation attributable to the enhancement of membrane permeation to Ca^{2+} or P_i as the detergent treatment did not cause the leakage of MV membrane as measured by nonspecific uptake of [^{14}C]ATP or $^{45}CaCl_2$ (data not shown). The mechanism by which L-tetramisole, an inhibitor of ALP, inhibited detergent-stimulation of ATP-initiated calcification is unknown but may suggest that participation of both ATPase and ALP may be required for optimal mineralization. This assumption may also serve to explain the difference in the L-tetramisole effect on calcification by bone and cartilage vesicles in response to detergents.

The reason for the opposite effect of cationic CTAB and neutral Triton X-100 or anionic DOC on ATP-initiated calcification is unclear. It is plausible that the differences in the net charge among the three detergents may be responsible for the contrasting effect. Since CTAB was much less inhibitory ($I_{50} > 100$ μM) to ALP than to ATPase ($I_{50} <$ 10 μM), CTAB may inhibit calcification through its effect on ATPase or alternatively due to a direct interaction between the detergent and MV membranes. Although these observations seem to suggest the role of ATPase in ATP-dependent calcification, its precise role remains to be established. A recent observation that ATP analogs but not phosphomonoesters can block both ATPase activity and ATP-dependent calcification suggested that ATPase or NTP-pyrophosphohydrolase may have a role [13].

The FT-IR spectroscopic data in this report demonstrate for the first time that amorphous calcium pyrophosphate can be deposited by rachitic MVs. The observed ability of rachitic MVs to deposit $CaPP_i$ may underlie the mechanisms of abnormal deposition of $CaPP_i$ in cartilage of patients with rickets [22]. Despite the reported crystalline nature of calcium pyrophosphate in pseudogout, whether or not the calcium pyrophosphate deposit in rachitic chondrocalcinosis is crystalline or amorphous remains to be established [22].

The ability to deposit $CaPP_i$ was demonstrated using both normal MVs isolated from fetal bovine cartilage [18] and in MVs from articular cartilage of patients with chondrocalcinosis [19]. Since only calcium pyrophosphate dihydrate (CPPD) was found in the diseased articular cartilage but not in normal cartilage, a sufficient amount ATP may need to be present in the extracellular fluid of diseased cartilage

to produce the mineral [19]. The possibility of high levels of ATP in cartilage fluids has been raised by two research groups [32,33]. For example, Howell et al. [32] found that about 25 mg of nucleotides per gram of proteins was present in normal cartilage fluids. Since the concentration of proteins in cartilage fluids was shown to be 32 g/l [32], the nucleotide level can be deduced from these data to be about 1–2 mM. Wuthier [33] also reported the same range of nucleotides. It is likely that these nucleotides may consist of ATP and various nucleotides resulted from ATP degradation. Such levels of nucleotides are likely derived from intracellular ATP which is present in millimolar ranges in chondrocytes [34]. Although the total nucleotide levels in cartilage fluids are about the same for normal and rachitic rats [32], the levels of ATP may not necessarily be equal between the two different cartilage fluids. It has been shown that blood P_i and ATP levels in rachitic rats are significantly reduced [32,35,36]. Since rachitic cartilage slices are readily calcifiable in the presence of exogenous P_i [23,32], this raises the possibility that sufficient levels of ATP may be needed to provide P_i for mineralization in rachitic cartilage. Future studies are needed to address the levels of ATP in normal and rachitic cartilage fluids.

Since amorphous calcium phosphate instead of hydroxyapatite was deposited by isolated rachitic rat MVs in the presence of ATP, these observations suggest that additional factors or other conditions absent in vitro situation may be needed to produce biological apatite. Currently, we are attempting to identify the factors in bone and cartilage that may favor hydroxyapatite formation including collagens [37], phosphoproteins [38], and proteoglycans [39].

A recent study by Wu et al. [21] using chick MVs showed that ^{45}Ca uptake can be stimulated by Triton X-100 in the absence of ATP. We found in this report that neither MV-mediated calcification nor stimulation by Triton X-100 occurred unless ATP was added to calcifying media. Since ATP can initiate calcification by mammalian MVs [11–14] but not by chick MVs [21], the difference in their responses to Triton X-100 treatment between two species may be due to either species variations in calcification or to experimental variations.

As ATP-dependent calcification by normal or rachitic MVs can be markedly influenced by detergents, it would be of clinical importance in future studies to determine whether the same effects can be seen with MVs isolated from chondrocalcinotic cartilage or atherosclerotic aortas known to be associated with dystrophic calcification.

The mechanisms of dystrophic calcification in atherosclerosis and bioprosthetic heart valves have remained unresolved. Accumulating evidence indicates a close link between MVs and calcification in atherosclerosis, i.e., mineral-associated MVs have been identified by electron microscopy at the site of abnormal calcification [40–42]. In one study, 25-hydroxycholesterol, which is structurally similar to DOC, was shown to stimulate calcification by osteoblast-like vascular cells [43]. Thus, the modulation of MV-mediated calcification by lipids and detergents may offer a means by which detrimental calcification can be controlled.

Overall, we have demonstrated the presence of ATP pyrophosphohydrolase in rachitic MVs and the ability of isolated rachitic MVs to deposit $CaPP_i$. These observations suggest that MVs may underlie the mechanisms of $CaPP_i$ deposition in cartilage of rickets [22] provided that ATP levels are abnormally high. Secondly, the marked stimulation of ATP-dependent calcification by neutral and anionic detergents, in contrast to the inhibition by the cationic detergent, suggests the perturbation of MV membrane can have a profound effect on ATP-mediated calcification. Whether the effects on calcification were due to a change in membrane fluidity or ionic microenvironment of the membranes remains to be resolved.

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