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Bone and Mineral 26 (1994) 69–79

BONE AND MINERAL

A facilitative role for carbonic anhydrase activity in matrix vesicle mineralization

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(Received 15 November 1993; revision received 4 February 1994; accepted 7 March 1994)

Abstract

Carbonic anhydrase (CA) which catalyzes the reversible hydrolysis of carbon dioxide is known to be important in osteoclastic bone resorption, however, suggested roles in calcium phosphate mineral formation have not been previously demonstrated. Biochemical evidence is provided for the presence of CA in growth plate matrix vesicles (MV) and the level of activity determined by enzyme assay. Inhibition of CA activity with the specific inhibitor acetazolamide resulted in reduced rates of MV mineralization. Other inhibitor studies showed that MV mineralization was also impaired by 4,4-diisothiocyanatostilbene-2, 2-disulfonic acid (DIDS), a blocker of membrane bicarbonate channels. No evidence was found for the presence of any proton pumps or channels. When acetazolamide and DIDS were combined, their inhibitory effects on MV mineralization were additive. These findings suggest that MV posess a pH regulation system composed of carbonic anhydrase and a putative bicarbonate channel. This system may function in the MV by providing intraluminal buffering capacity. The control of intravesicular pH is important for the stabilization of the acid-labile nucleational core complex and in preventing the build-up of protons during calcium phosphate phase transformations.

Keywords: Carbonic anhydrase; Matrix vesicles; Bicarbonate channel; pH regulation; Growth plate cartilage; Mineralization

1. Introduction

In the epiphyseal growth plate of vertebrate bone de novo mineral formation occurs within matrix vesicles (MV) and subsequently propagates into the extracellu-

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SSDI 0169-6009(94)00701-H

lar matrix [1]. Infrared spectroscopic analysis has shown that in matrix vesicles, the first crystalline mineral phase to appear is octacalcium phosphate (OCP) which then transforms into hydroxyapatite (HAP) [2]. As OCP is converted into the more stable HAP, protons are lost from the acidic phosphate groups present in this mineral phase [3]. In the enclosed microenvironment of the MV lumen, the build-up of H^+ ions as mineral phase transformation occurs could be detrimental to the further growth of the acid-labile calcium phosphate crystals unless the MV posess some mechanism for preventing the concomitant drop in pH.

Carbonic anhydrase (CA), a zinc metalloenzyme which catalyzes the reversible hydration of carbon dioxide, has been localized in both bone and calcified cartilage [4–6]. In bone, carbonic anhydrase has been shown to be necessary for the mineral reorbing activity of osteoclasts [6,7]. The role, if any, of carbonic anhydrase at sites of active mineral deposition is not known although it has been suggested to function in pH regulation [8]. Recently, carbonic anhydrase has been localized in extracellular matrix vesicles by immunochemical methods suggesting that it may be involved in mineral formation by these structures [9]. The present study was initiated to determine the enzymatic activity of carbonic anhydrase in MV and assess its role in the mineralization process.

2. Materials and methods

2.1. Matrix vesicle isolation

Two types of matrix vesicle preparations were used in the present studies. Collagenase-released matrix vesicles (CRMV) were isolated from growth plate cartilage of 6–8-week-old broiler strain chickens as described previously [10]. In brief, tissue slices were digested with 0.1% trypsin (Sigma, Type III) in a synthetic cartilage lymph (SCL) designed to mimic the ionic composition of growth plate extracellular fluid [11] for 20 min at 37°C. SCL contained 2.00 mM Ca^{2+}, 1.42 mM Pi, 105 mM Na^+, 134 mM Cl^-, 12.7 mM K^+, 1.83 mM HCO_3, 0.57 mM Mg^{2+}, 0.57 mM SO_4^{2-}, 5.55 mM D-glucose, and 16.5 mM *N*-tris[hydroxymethyl]methyl-2-aminoethanesulfonic acid (TES; Sigma). After draining the trypsin solution, the tissue slices were rinsed twice with an equal volume of SCL and digested for 3 h at 37°C with collagenase (200 U/g tissue, Sigma, Type IA) in SCL. Red blood cells present in the tissue slices are lysed during the trypsin treatment. To harvest the CRMV, the collagenase-digested tissue was vortexed and the suspension subjected to differential centrifugation at 13 000 *g* for 20 min and 100 000 *g* for 1 h. The CRMV pellet resulting from the final spin was resuspended in a small volume (1–4 ml) of Ca^{2+}-free SCL and the vesicle protein concentration determined by the method of Lowry [12]. In CRMV used for specific activity assays, the pellet was resuspended in a small volume of 0.25 M sucrose, 1 mM $NaHCO_3$ (pH 7.4).

Matrix vesicle-enriched microsomes (MVEM) were obtained by homogenizing growth plate tissue slices in a Waring blender in an equal volume of SCL. The tissue homogenate was filtered through a course nylon mesh to remove large fragments and subjected to differential centrifugation at 20 000 *g* for 20 min and 100 000 *g* for 1 h. The initial centrifugation step removes blood cells and blood cell membrane

fragments. The MVEM pellet was resuspended and assayed for protein as described above.

2.2. Gel electrophoresis and Western blot analysis

SDS-PAGE was used to analyze for proteins in the CRMV and MVEM preparations using 3.0% stacking gels and 7.5–15% gradient resolving gels according to Laemmli [13] followed by staining with coomassie blue. For immunoblot analysis, proteins were transferred from the SDS gels onto nitrocellulose sheets [14]. After blocking with 3% gelatin in 500 mM NaCl, 20 mM Tris, pH 7.5 (TBS) buffer, the sheet was incubated with antibodies diluted 1:500 in 1% gelatin-TBS for 16 h. Rabbit anti-chicken carbonic anhydrase antibodies were provided by Dr. Paul Linser (University of Florida). The blots were developed with horseradish peroxidase-goat antirabbit IgG (Bio-Rad) using 0.02% H_2O_2 and 0.05% 1-chloro-4-napthol as substrate.

2.3. Enzyme assays

To measure carbonic anhydrase activity an electrometric assay was used [15]. Briefly, up to 1 ml of CRMV suspension was added to 3 ml of 10 mM sodium phosphate buffer (pH 7.4) maintained with stirring at 4°C ± 0.1°C. To this mixture was added 2 ml of ice-cold 10% CO_2-saturated deionized water through a substrate delivery tube to initiate the reaction. The change in pH as CO_2 and H_2O was converted to H^+ and HCO_3^- was detected with a pH meter and recorded on a chart recorder as δ mV. Each set of assays was accompanied by a curve of the uncatalyzed hydration of CO_2, the slope of which was substracted from the catalyzed reaction curves prior to calculation of enzyme specific activity. Controls consisted of assays run in the presence of boiled CRMV suspension. In some experiments, 0.2 ml 10% Triton X-100/sucrose buffer was added to the CRMV suspension prior to the assay.

For the enzyme inhibitor studies, a modification of the bromothymol blue colorimetric assay [16] was used. In this assay, 0.1 ml of CRMV suspension was added to 2 ml ice cold 50 mM barbital buffer (Sigma), pH 8.15, containing 5 mg/l bromothymol blue. The reaction was initiated by adding 2 ml ice-cold CO_2-saturated water and the time required for the pH to change from 8.0 (blue) to 6.3 (yellow) was recorded. Inhibitors were added prior to the initiation of the reaction using dimethylsulfoxide (DMSO) as a vehicle to yield final inhibitor concentrations of 10^{-4} M except as noted. Inhibitors tested include acetazolamide, sulfadimethoxine, *o*-phenanthroline, EDTA, DIDS, and 3-bromopyruvic acid (2 mM). DMSO at 0.1% final concentration had no effect on the assay.

2.4. Mineralization assays

MV mineralization was assayed by measuring ^{45}Ca accumulation. The assay was initiated by inoculating aliquots (80 μg protein) of CRMV suspensions into capped polypropylene tubes (15 × 100 mm) containing 2 ml of ^{45}Ca-labeled SCL (1×10^6 counts/min/ml) and incubating at 37°C using a constant temperature shaker bath. At timed intervals, 100 μl aliquots of the SCL incubations were sampled by microfiltration through Millipore HA filters (0.45 μm pore size, 25 mm diameter)

and the filters washed twice with TMS buffer (50 mM Tris, 1.5 mM $MgCl_2$, 10% sucrose, pH 7.5). Filters were counted in 5 ml of Beckman Ready Protein scintillation cocktail. A number of carbonic anhydrase and ion transport inhibitors were tested for their ability to interfere with ^{45}Ca accumulation. These included acetazolamide (10^{-4}–10^{-6} M), sulfanilamide (10^{-4}–10^{-6} M), DIDS (10^{-4} M), ethylisopropyl amiloride (EIPA) (10^{-4} M), and dicyclohexyl-carbodiimide (DCCD) (10^{-4} M) added to the SCL incubations from stock solutions using DMSO as a vehicle. Control treatments recieved vehicle only. Prior to the start of the experiments CRMV aliquots were pre-treated at 25°C for 30 min with the same concentration of inhibitor to be tested. The ^{45}Ca uptake curves were compared statistically by ANOVA for differences overall and at individual time points by the SAS General Linear Models procedure. Differences between treatments were considered significant at $P < 0.05$.

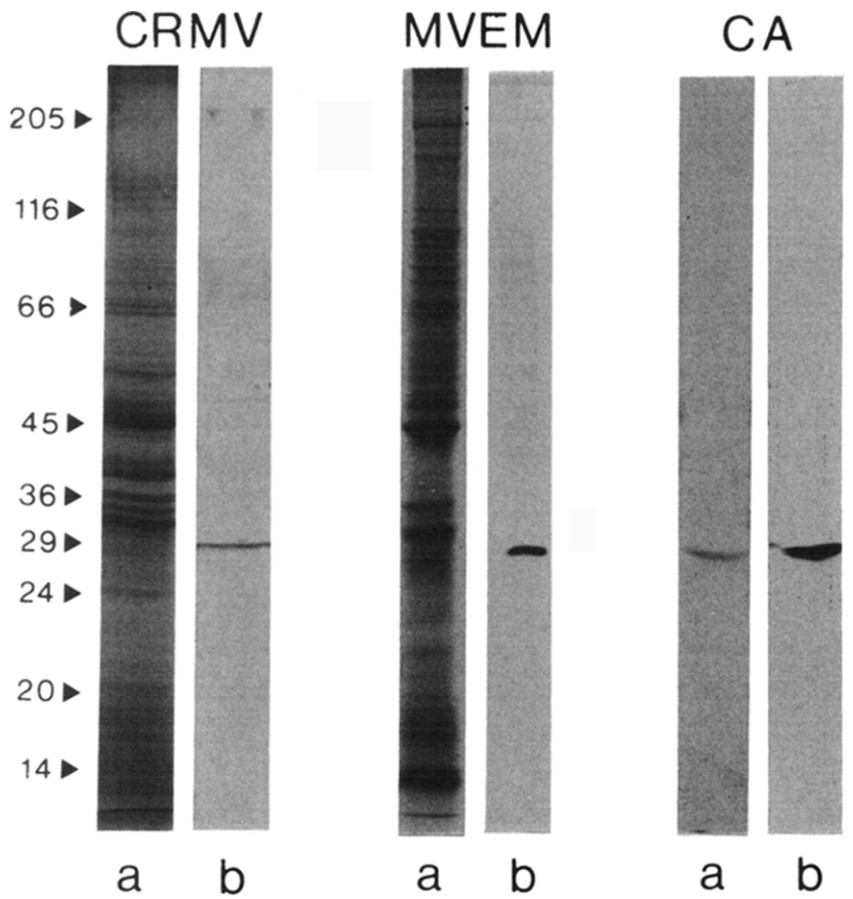

Fig. 1. SDS-PAGE (a) and Western blot analysis with anti-carbonic anhydrase antibody (b) of collagenase-released matrix vesicles (CRMV), matrix vesicle-enriched microsomes (MVEM), and purified carbonic anhydrase (CA). Positions of molecular mass markers (kDa) indicated at left.

3. Results

Western blot analysis demonstrated the presence of carbonic anhydrase in both CRMV and MVEM preparations (Fig. 1). MVEM appeared to contain higher levels of carbonic anhydrase than CRMV. The SDS-PAGE patterns indicate that the enzyme is present in small amounts relative to some of the other vesicle proteins. The SDS-PAGE analysis shows that the matrix vesicle carbonic anhydrase has an approximate molecular mass of 29 kDa.

Carbonic anhydrase activity could be measured in both the cell and CRMV fractions of growth plate tissue digests (Table 1). CA-specific activity in the cell pellet was more than twice that observed in the MV. In both fractions, disruption of the cell or vesicle membranes by treatment with Triton X-100 resulted in an approximate 3-fold increase in measurable activity. Treatment of CRMV with the CA inhibitors acetazolamide or sulfadimethoxine resulted in the complete loss of carbonic anhydrase activity (Table 2). The metal ion chelators, EDTA and *o*-phenanthroline, caused a partial loss of activity while bromopyruvic acid or DIDS treatment did not cause a significant decline in CA activity.

The accumulation of Ca^{2+} by CRMV incubated in SCL is characterized by three different stages (Fig. 2). Initially there is a lag period during which little accumulation of Ca^{2+} occurs. The duration of the lag period varies with different CRMV preparations but is typically 1–3 h in length. The lag period is followed by a period of very rapid mineral ion uptake, and finally a plateau period in which Ca^{2+} accumulation continues at a slower rate. When CRMV were mineralized in the presence of the carbonic anhydrase inhibitor acetazolamide (10^{-4} M), a significant partial inhibition of Ca^{2+} accumulation was observed. Similar results were observed with 10^{-5} M acetazolamide while at 10^{-6} M a weaker inhibition was observed (data not shown). The primary effect of acetazolamide was a lengthening of the lag period. Another sulfonamide drug, sulfanilimide, had a more limited effect on CRMV mineralization. After 24 h there was no significant difference in Ca^{2+} accumulation between the control and inhibitor treatments.

When the cation-dependent H^+ transporter inhibitors ethylisopropyl amiloride (EIPA) or dicyclohexyl carbodiimide (DCCD) were added to the CRMV incuba-

Table 1
Carbonic anhydrase specific activity in fractions of collagenase-digested growth plate tissue. Values are given for each fraction before and after membrane disruption with Triton X-100

Tissue fraction	CA specific activity* (nmol CO_2/min/μg protein)	Number of determinations
Cell pellet	7.38 ± 0.96	3
Cell pellet + Triton	20.82 ± 3.92	4
Matrix vesicle (CRMV)	2.94 ± 0.90	3
Matrix vesicle + Triton	8.58 ± 1.38	4

*Values given are mean ± SEM.

Table 2
Effect of inhibitors on matrix vesicle carbonic anhydrase activity.

Inhibitor[a]	Percent of Control
Acetazolamide	0*
Sulfadimethoxine	0*
o-Phenanthroline	32*
EDTA	46*
Bromopyruvic acid	93
DIDS	87

Values given are the mean percent of control CA activity remaining when CRMV were assayed in the presence of inhibitor ($n = 3$).
[a]All inhibitors were tested at a concentration of 10^{-4} M except bromopyruvic acid which was tested at 2 mM.
*Treatment significantly different than control at $P < 0.05$.

tions, no significant changes in Ca^{2+} accumulation were observed (Fig. 3). DCCD caused only slight increases in Ca^{2+} accumulation while, with EIPA, a slight decrease was observed. In contrast, addition of diisothiocyanostilbene disulfonic acid (DIDS), an inhibitor of HCO_3^- transport, to the CRMV incubations resulted in a significant delay in the onset of rapid Ca^{2+} accumulation (Fig. 4). When DIDS was combined with acetazolamide, the inhibitory effect was more pronounced.

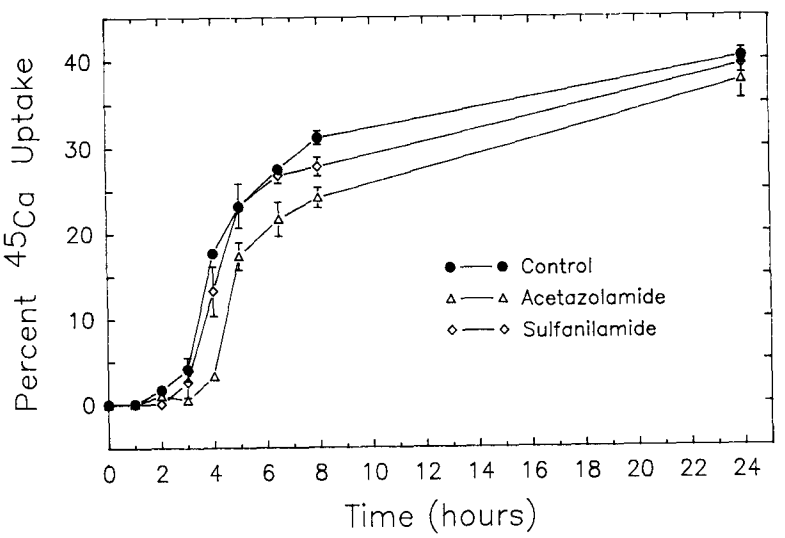

Fig. 2. Effect of the carbonic anhydrase inhibitors acetazolamide (100 μM) and sulfanilimide (100 μM) on calcium accumulation by collagenase released matrix vesicles (CRMV) during incubation in synthetic cartilage lymph (SCL). Each point is mean ± SEM ($n = 3$). By ANOVA, the acetazolamide treatment was significantly different than the control ($P < 0.01$).

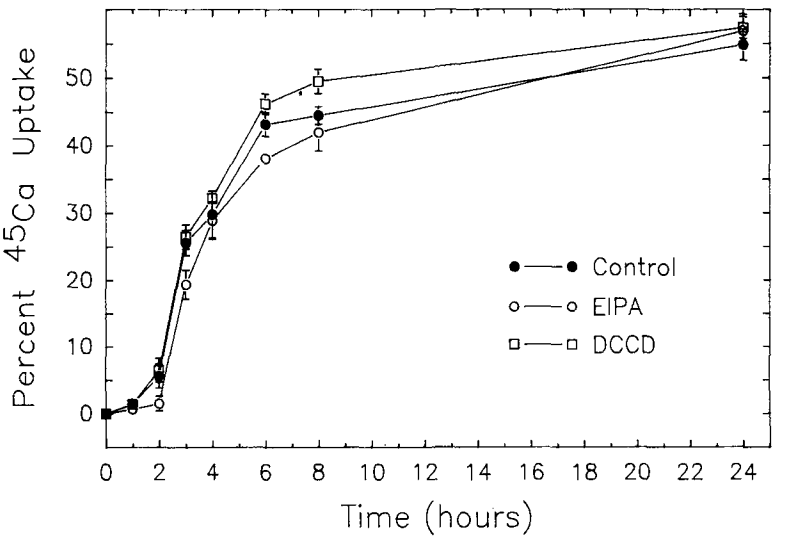

Fig. 3. Effects of two inhibitors of cellular proton export, ethyl isopropyl amiloride (EIPA) and dicyclohexyl carbodiimide (DCCD) on calcium accumulation by collagenase-released matrix vesicles (CRMV) during incubation in SCL. Both inhibitors were tested at 100 μM. Each point is mean ± SEM (n = 3).

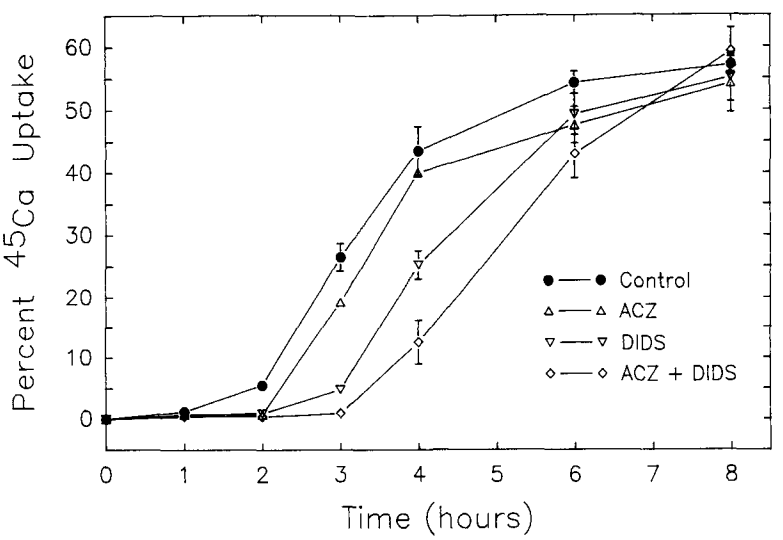

Fig. 4. Effect of acetazolamide (ACZ) and the bicarbonate channel blocker diisothiocyanostilbene disulfonic acid (DIDS), singly and in combination, on early stages of calcium accumulation by collagenase-released matrix vesicles (CRMV) during incubation in SCL. Inhibitor concentrations were 100 μM. Each point is mean ± SEM (n = 3). By ANOVA, all treatments were significantly different than the control ($P < 0.05$).

4. Discussion

The occurrence of carbonic anhydrase (CA) in vertebrate bone is most commonly attributed to its important role in osteoclastic bone resorption [6,17–19]. In this capacity, CA catalyzes the reaction $CO_2 + H_2O \rightleftharpoons H^+ + HCO_3^-$ which provides hydrogen ions for the acidification of the resorption site that is necessary for bone mineral dissolution. At the same time, the identification of this enzyme at extracellular sites in actively mineralizing tissues such as growth plate cartilage [4,5] suggests that CA may also function in mineral formation. Since mineral formation is an acid-sensitive process, CA may assist mineralization by removing H^+ via the reverse hydrolysis reaction.

The Western blot analysis (Fig. 1) and CA activity assays (Table 1) presented here, as well as recent findings with rat tissue [9], conclusively demonstrate the presence of CA in matrix vesicles, the sites of initial mineral formation in growth plate cartilage and other mineralized tissues. The increase in activity observed when the vesicle membranes were disrupted by detergent treatment indicates that the enzyme is localized in the vesicle lumen. The level of CA activity found in CRMV is in good agreement with levels reported in rat growth plate vesicles [9]. In the rat, matrix vesicles were found to be enriched in CA relative to the chondrocytes. In the present study, the cell fraction of growth plate digests had higher activity, however this fraction also contained a small amount of red blood cells which are known to have high CA activity. By SDS-PAGE analysis, matrix vesicle CA appears as a very faint band with molecular mass 29 kDa. Proteins that have previously been identified in CRMV include annexins V (33 kDa), II (36 kDa) and VI (67 kDa) [20,21], proteoglycan link protein (39 kDa), hyaluronic acid region binding protein (46 kDa) [22], and alkaline phosphatase (77 and 82 kDa) [23]. A number of other bands visible in CRMV by SDS-PAGE are due to associated collagen type II and X fragments [24].

The sulfonamide drugs are well known inhibitors of CA activity. Of these, acetazolamide is the most specific and completely blocks CA activity in solution at concentrations as low as 10^{-7} M [25]. Sulfanilamide and its derivatives such as sulfadimethoxine are somewhat weaker inhibitors of CA. In the present study, relatively high concentrations of inhibitors were chosen in order to establish a large concentration gradient across the MV membrane and ensure rapid entry of the inhibitor into the MV lumen where the CA is localized. In this way rapid inhibition of MV CA activity by acetazolamide was accomplished (Table 2). The metal ion chelators EDTA and *o*-phenanthroline which are capable of removing Zn^{2+} ions from the active site of the enzyme caused partial inhibition of CA activity. The enzyme present in matrix vesicles appears comparable to mammalian CA II in activity since it was not inhibited by bromopyruvate as is mammalian CA I [26]. Similarly, immunohistochemical localizations have shown that mammalian growth plate MV contain CA II but not CA I [4,9].

The activity of CA in the matrix vesicle lumen appears to be involved in pH regulation during the early stages of mineralization. Blocking matrix vesicle CA activity with acetazolamide resulted in a significant delay in the onset of rapid Ca^{2+} accumulation (Fig. 2). The weaker inhibitor, sulfanilamide, had a lesser effect. The

production of H^+ ions as the initial octacalcium phosphate crystallites are tranformed into hydroxyapatite [2] would tend to arrest the mineralization process unless a buffering mechanism were present in the vesicle lumen. In order to test whether other mechanisms of pH regulation are operative in matrix vesicles, we tested for the presence of cation channels using specific inhibitors of Na^+/H^+ exchange and a proteolipid H^+ porter. In many contractile cells, intracellular pH is controlled in part by the activity of a Na^+/H^+ exchanger that is sensitive to the amiloride derivative EIPA [27]. In calcifying bacteria, H^+ extrusion has been attributed to a proteolipid ion channel that is blocked by DCCD [28]. The counter ion for this H^+ porter is not known but was suggested to be Ca^{2+}. In the present study, neither of these cation channel blockers had an influence on matrix vesicle mineralization. In the absence of any known mechanism for proton extrusion, the intravesicular buffering of H^+ by CA is the best alternative for preventing acidification of the lumen.

Carbonic anhydrase appears to be part of a two-component system for intravesicular pH regulation. The present data indicate that the second part of the pH regulation system is a vesicle membrane bicarbonate porter which is blocked by DIDS. Stilbene derivatives such as DIDS are known to be strong inhibitors of HCO_3^--dependent anion channels [27]. When DIDS was added to CRMV incubations, mineralization was delayed even more than was observed with acetazolamide (Fig. 4). DIDS did not inhibit vesicle carbonic anhydrase activity (Table 2). The identity of the MV anion channel is not presently known. In red blood cells, a reversible anion channel which transports HCO_3^- in exchange for Cl^- ions has been shown to be a transmembrane glycoprotein with a molecular mass of 106 kDa [29]. The identification and characterization of the MV bicarbonate channel will require further study.

When acetazolamide and DIDS were simultaneously added to the CRMV incubations, their inhibitory effects were additive (Fig. 4). This strongly suggests that both carbonic anhydrase and a HCO_3^- channel are involved in the contol of intravesicular H^+ levels. In this scenario, the elevated levels of HCO_3^- which are known to occur in growth plate extracellular fluid [8] would provide the driving force behind the MV uptake of HCO_3^-. These HCO_3^- ions can then become the substrate for the removal of H^+ ions by the reverse hydrolysis reaction catalyzed by CA.

Although CA and the bicarbonate porter apparently have a role in facilitating MV mineralization, they are not absolutely essential to this process. When the function of both components of the proposed pH regulation system were blocked by inhibitors, the MV were still able to mineralize albeit at a slower rate (Fig. 4). In the growth plates of young animals, however, more rapid rates of mineralization may be required to keep up with the rate of tissue growth. The pH regulation system of MV may therefore contribute to MV mineralization by enhancing the rate at which this process can occur. Very recently, the nucleational core complex responsible for mineral induction in matrix vesicles has been identified [30]. This complex of Ca^{2+}, P_i, phosphatidylserine and protein can promote very rapid mineralization if the vesicle membrane is disrupted by detergent treatment. This nucleational complex is very acid-labile, however, and MV mineralization does not occur at pH 7.2 or lower [31].

Since the stability of this complex is critical to MV function, another role of the MV CA and bicarbonate porter may be to provide pH buffering capacity for the MV lumen during the early stages of matrix vesicle/core complex formation and development.

The identification of a CA-bicarbonate porter pH regulation system in matrix vesicles illustrates the importance of pH regulation in mineralizing systems. The present study does not preclude the possibility that other as yet unidentified pH regulating mechanisms may exist in other mineralizing systems or even in MV themselves. Since biological mineralization results in the production of H^+ ions yet is at the same time an acid-sensitive process, the control of pH at the site of mineral formation is as important as the local Ca^{2+} and P_i concentrations in the induction and proliferation of calcium phosphate crystal growth. Consequently, further studies on mechanisms of pH regulation would greatly contribute to our understanding of normal bone and tooth formation.

Acknowledgements

This study was supported by US Public Health Service grants DE05513 and DE10196 from the National Institute of Dental Research. We kindly thank Dr. Paul Anderson and Merck Sharp & Dohme, West Point, PA for providing ethylisopropyl amiloride. We also thank Dr. Roy Wuthier for his advice and discussions throughout this study.

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