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IDENTIFICATION, CHARACTERIZATION AND LOCALIZATION OF A (Ca^{2+} + Mg^{2+})-ACTIVATED PURINE NUCLEOSIDE TRIPHOSPHATE PHOSPHOHYDROLASE FROM CALCIFYING CARTILAGE

STEPHEN E. KAHN * and CHARALAMPOS ARSENIS **

Department of Biological Chemistry, College of Medicine, University of Illinois, Chicago, IL 60612 (U.S.A.)

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Summary

A purine nucleoside triphosphate phosphohydrolase (unspecified diphosphate phosphohydrolase, EC 3.6.1.15) was chromatographically separated from the bulk of alkaline phosphatase activity by gel filtration chromatography of butanol and EDTA extracts of fracture callus and bovine epiphyseal cartilage. The callus enzyme differed from alkaline phosphatase in a variety of characteristics. The purine nucleoside triphosphate phosphatase hydrolyzed a more specific group of substrates, required Ca^{2+} and Mg^{2+} for optimal activity, remained unaffected by a potent alkaline phosphatase inhibitor, and demonstrated a narrower range of optimal pH for catalytic activity. The enzyme was localized in the microsomal pellet following subcellular fractionation of callus chondrocytes. These characteristics indicate a role for the enzyme in Ca^{2+} transport.

Introduction

The hypothesis that a phosphohydrolytic activity fulfils an important role in the calcification process was introduced by Robison [1]. Subsequently, many investigators have attempted to localize and characterize the phosphohydrolytic activity of various calcifying tissues [2—7]. Recent studies in this

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** To whom reprint requests should be addressed at Biological Chemistry, College of Medicine, University of Illinois, 1853 West Polk Street Chicago, IL 60612, U.S.A.

laboratory have demonstrated the existence of at least three phosphohydrolytic activities in bone and calcifying cartilage. The first activity is a broad specificity alkaline phosphatase, the second activity hydrolyzes only simple phosphate esters, while the third activity appears to be an inorganic pyrophosphatase [7,8]. In earlier studies [9], it was demonstrated that the first activity is associated with the plasma membrane and extracellular matrix vesicles of fracture callus calcifying cartilage. The matrix vesicle is now thought to be ubiquitous in calcifying tissues [10] and is derived from the plasma membrane [9].

Despite the previously summarized studies, the role, if any, of the phosphohydrolytic activities in biological calcification has not been clearly established. It has been suggested that calcification may require movement of calcium by a transport system. Recently, the presence of a $(Ca^{2+} + Mg^{2+})$-ATPase in rat incisor odontoblasts has been demonstrated [6].

This paper reports the existence of a $(Ca^{2+} + Mg^{2+})$-ATPase activity in calcifying cartilage which is different from all the phosphohydrolytic activities previously reported in the same tissue. The isolation, characterization, and partial localization of this activity is also described.

Experimental procedures

Materials

Tissue Source. Male New Zealand white littermate rabbits (2—2.5 kg), were purchased from Scientific Small Animal Laboratories (Chicago, IL). Fracture callus cartilage was obtained as described elsewhere [11]. Calf scapulae were purchased from a local slaughter house.

Chemicals. The following chemicals were purchased from Sigma Chemical Company (St. Louis, MO): bovine serum albumin, ascorbic acid, sodium pyrophosphate, nucleotides (except those listed below), sugar phosphates, and β-glycerophosphate. Adenosine 5′-diphosphate, deoxyadenosine 5′-diphosphate, cytidine 5′-triphosphate, guanosine 5′-triphosphate, inosine 5′-triphosphate, α,β-methylene adenosine 5′-diphosphate and β,γ-methylene adenosine 5′-triphosphate were purchased from P.L. Biochemicals, (Milwaukee, WI). *p*-Nitrophenylphosphate was purchased from Calbiochem (Los Angeles, CA). Sepharose 6B and Blue Dextran were purchased from Pharmacia Fine Chemicals (Piscataway, NJ). R-8231, an analog of tetramisole, was kindly provided by Dr. M. Borgers (Janssen Research Foundation, Beerse, Belgium).

Methods

Extraction of tissue. Fracture callus cartilage or calf scapula cartilage was minced into 1—2 mm^3 pieces and suspended in 0.1 M glycine buffer (pH 9.8). The suspension was homogenized using a blade-equipped Virtis homogenizer. The homogenized tissue suspension was extracted with an equal volume of *n*-butanol at 4°C for approx. 16 h using a magnetic stirrer. EDTA was added to the emulsion at a final concentration of 0.5 mM and the tissue was extracted further for 2 h at 4°C. The suspension was centrifuged at 12 350 × *g* for 20 min in a Sorvall SM-24 rotor at 4°C. The butanol phase was discarded and the aqueous phase collected. The precipitated cell debris was washed twice with

0.1 M glycine buffer (pH 9.8), centrifuged as before and the precipitate was discarded. The combined aqueous phase and washings were dialyzed against the same glycine buffer for 2 h at 4°C and used for further experiments.

Sepharose 6B gel filtration chromatography. To resolve the purine nucleoside triphosphate phosphatase (unspecified triphosphate phosphohydrolase, EC 3.6.1.15) from the major alkaline phosphatase activity, the dialysate following butanol and EDTA extractions was layered onto a Sepharose 6B column (80 cm × 2.5 cm) pre-equilibrated with 0.1 M glycine (pH 9.8). Elution of the phosphohydrolytic activities was accomplished by washing the column with the same buffer. Column flow rates were approx. 16 ml/h. Determination of the void volume (V_0) of the column was made using blue dextran 2000. Determination of the total elution volume was made by adding NaCl to the sample (10 mg/ml) and identifying the eluted NaCl peak with a Radiometer Conductivity Meter (Type CDM2e). Active fractions of the purine nucleoside trisphophate phosphatase were pooled, concentrated by ultrafiltration (Amicon Corporation, Lexington, MA) and kept for further analysis.

Assays. Alkaline phosphatase was assayed at pH 10.5 using 1.0 mM *p*-nitrophenylphosphate as described [12]. Pyrophosphate activity using 1 mM sodium pyrophosphate and phosphatase activity using 2 mM β-glycerophosphate or ATP were assayed as described [12]. For certain experiments, substrate concentration, pH, or divalent cation concentrations were varied to determine optimal assay conditions and to investigate the effects of specific modulators. Cytochrome oxidase was assayed following the oxidation of reduced cytochrome *c* [13]. β-*N*-Acetyl-D-glucosaminidase at pH 5.0 was assayed by following the *p*-nitrophenolate ion released from 6 mM *p*-nitrophenyl-*N*-acetyl-β-D-glucosaminide [14]. NADPH-cytochrome *c* reductase was assayed by following the reduction of cytochrome *c* at pH 7.4 [15]. Protein was assayed according to Lowry et al. [16] using bovine serum albumin as standard.

Chondrocyte isolation and subcellular fractionation. Cartilage was minced into 1—2 mm^3 pieces and digested by trypsin and collagenase as described elsewhere [9]. The cartilaginous digest was filtered through No. 80 monofilament nylon mesh. The digest was centrifuged at 750 × *g* in an IEC-269 head for 10 min at 4°C. The cellular pellet was resuspended in 50 mM Tris-HCl buffer (pH 7.4) and recentrifuged as before. The chondrocyte pellet was suspended in the same buffer and sonicated in 1 ml aliquots for 45 s in 5-s bursts, using the Branson Model W185 Sonifier. Subcellular fractionation of the broken chondrocytes was performed according to a published procedure [17]. The various subcellular pellets were resuspended in 5 mM Tris-HCl (pH 7.4) and analyzed for various organelle-specific enzymes and protein.

Results

Chromatographic separation of the (Ca^{2+} + Mg^{2+})-ATPase activity from the nonspecific alkaline phosphatase activity

Fig. 1 shows the results of the Sepharose 6B gel filtration chromatography when an extract from fracture callus proliferating and hypertrophic cartilage was chromatographed. Two peaks of phosphohydrolytic activity were eluted between the void volume (V_0) and the total eluted volume (V_t) of the column.

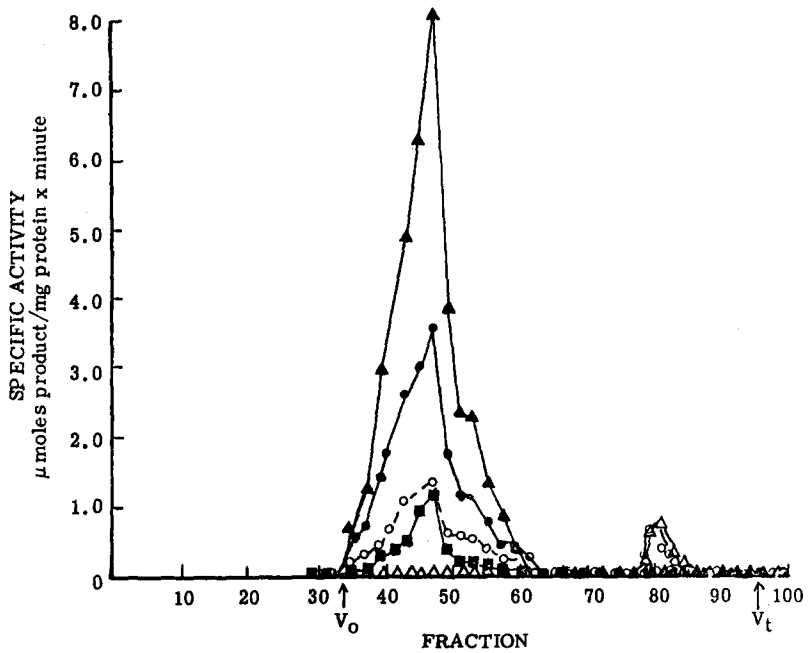

Fig. 1. Sepharose 6B gel filtration of an aqueous extract of callus cartilage following prolonged exposure to butanol and EDTA. 46.8 mg protein (10.3 μmol P_i liberated/min using ATP as substrate) from the aqueous dialysate following butanol and EDTA extraction were layered onto a Sepharose 6B column (80 cm × 2.5 cm) pre-equilibrated with 0.1 M glycine (pH 9.8). The void volume (V_0) was calculated by the exclusion of dextran blue 2000 while the total elution volume (V_t) was calculated by exclusion of NaCl. All assays of fractions were corrected for endogenous phosphate content and for non-enzymatic hydrolysis of substrate. The ordinate is specific activity (μmol product release/mg protein per min) and the abscissa is fraction number. The column volume was approx. 390 ml and was eluted with 0.1 M glycine (pH 9.8); flow rate approx. 16 ml/h. Active fractions from the second peak of activity were pooled, concentrated by ultrafiltration and used for further characterization of the enzyme. ▲———▲, β-glycerophosphate; ●———●, *p*-nitrophenylphosphate; ■———■, pyrosphosphate; ○———○, ATP; △———△, ATP + 0.1 mM inhibitor R8231.

The first peak represents the major alkaline phosphatase activity of callus cartilage and was active towards β-glycerophosphate, *p*nitrophenylphosphate, pyrophosphate or adenosine triphosphate at alkaline pH. This activity, using the same substrates, was completely inhibited by 0.1 mM R8231, a potent inhibitor of cartilage alkaline phosphatase.

The second peak of phosphohydrolytic activity was found in fractions 76—86 in Fig. 1 and was active only towards ATP. Mg^{2+} and Ca^{2+}, in a stoichiometric concentrations, optimized the activity from the second peak. In contrast to the phosphohydrolytic activity of the first peak, the second peak of activity was unaffected by 0.1 mM inhibitor R8231.

Fig. 2 shows the results of the Sepharose 6B column chromatography when an extract from calf scapula proliferating and hypertrophic cartilage was chromatographed under similar conditions. Similar to the callus, a second peak of phosphohydrolytic activity is also observed. This activity was specific for ATP in the presence of Ca^{2+} and Mg^{2+} and was unaffected by 0.1 mM inhibitor R8231. Since fresh cartilage from the fraction callus was more readily available,

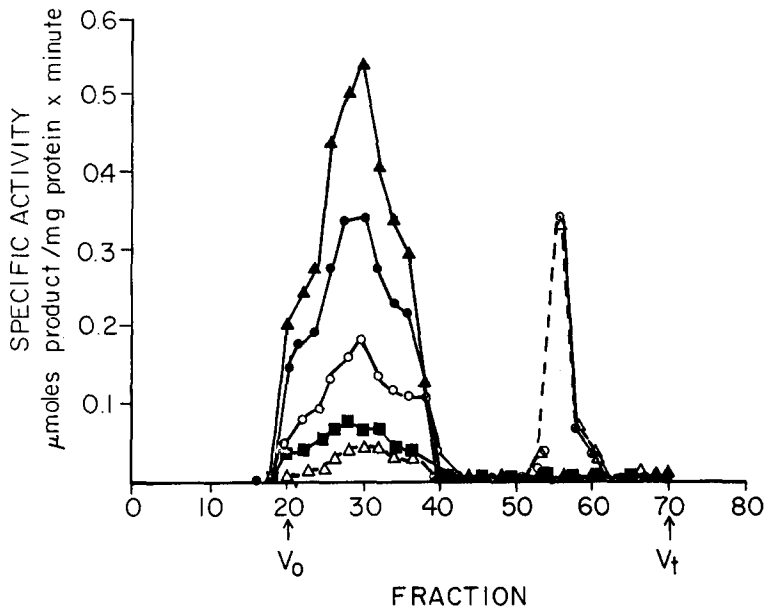

Fig. 2. Sepharose 6B gel filtration of an aqueous extract of bovine epiphyseal cartilage following prolonged exposure to butanol and EDTA. 18.2 mg protein (4.96 μmol P_i liberated/min using ATP as substrate) from the aqueous dialysate following butanol and EDTA extraction were layered onto a Sepharose 6B column (80 X 2.5 cm) pre-equilibrated with 0.1 M glycine (pH 9.8). Elution of the phosphohydrolytic activities was accomplished by washing the column with the same buffer. The void volume (V_0) was calculated by the exclusion of dextran blue 2000 while the total elution volume (V_t) was calculated by exclusion of NaCl. All assays of fractions were corrected for endogenous phosphate content and for non-enzymatic hydrolysis of substrate. The ordinate is specific activity (μmol product released/mg protein per min) and the abscissa is fraction number. The column volume was approx. 390 ml. ▲———▲, β-glycerophosphate; ●———●, *p*-nitrophenylphosphate; ■———■, pyrophosphate; ○———○, ATP; △———△, ATP + 0.1 mM R8231.

the studies on the localization and characterization of the enzyme were carried out using this source of tissue.

Partial localization of the activity in the second peak

Chondrocytes were broken by sonication and the suspension fractionated into nuclear, mitochondrial, lysosomal, microsomal and high speed supernatant fractions. Aliquots of the whole digest, extracellular digest, sonicated cell suspension and each subcellular fraction were assayed for specific enzymes characteristic of certain subcellular particles. The aliquots were also assayed for alkaline phosphatase activity using *p*-nitrophenylphosphate and ATP in the presence of 1 mM Ca^{2+}/Mg^{2+} with and without 0.1 mM inhibitor R8231.

Table I shows the distribution of the different enzymatic activities in all the fractions following digestion and subcellular fractionation. Cytochrome oxidase, a mitochondrial marker, *N*-acetyl-β-D-glucosaminidase, a lysosomal marker and NADPH-cytochrome *c* reductase, a microsomal marker, each showed the highest specific activity in the appropriate fraction. Alkaline phosphatase assayed with *p*-nitrophenylphosphate at pH 10.5 and in the presence of 1 mM Mg^{2+} was more or less evenly distributed among all intracellular

TABLE I

DISTRIBUTION OF ENZYME ACTIVITIES IN THE VARIOUS FRACTIONS AFTER DIGESTION AND SUBCELLULAR FRACTIONATION

All specific activities expressed in μmol product/mg protein per min.

Fraction	Volume (ml)	Protein		Cytochrome oxidase		NADPH-cytochrome *c* reductase		*N*-Acetyl-β-D-glucosaminidase	
		mg/ml	Total mg	Spec. act.	% act.	Spec. act.	% act.	Spec. act.	% act.
1. Whole digest	70.0	2.50	175.00	0.008	100.0	0.005	100.0	0.028	100.0
2. Extracellular digest	61.0	1.45	88.45	0.001	6.3	0	0	0.002	3.6
3. Cell pellet	8.0	6.92	55.36	0.018	71.2	0.012	75.9	0.069	78.0
4. Nuclei	3.0	3.95	11.85	0.018	15.2	0	0	0.007	1.7
5. Mitochondria	3.0	2.86	8.58	0.057	34.9	0.006	5.8	0.065	11.4
6. Lysosomes	3.0	1.94	5.82	0.015	6.2	0.021	14.0	0.340	40.4
7. Microsomes	3.0	1.32	3.96	0	0	0.096	43.4	0.167	13.5
8. Supernatant solution	6.5	1.90	12.35	0	0	0.005	7.1	0.005	1.3

Fraction	Alkaline phosphatase (*p*-nitrophenylphosphate)		Phosphatase (ATP)		Phosphatase (ATP + 0.5 mM R8231)	
	Spec. act.	% act.	Spec. act.	% act.	Spec. act.	% act.
1. Whole digest	0.16	100.0	0.22	100.0	0.04	100.0
2. Extracellular digest	0.22	69.5	0.28	64.3	0.02	25.3
3. Cell pellet	0.14	27.7	0.19	27.3	0.07	55.4
4. Nuclei	0.14	5.9	0.26	8.0	0.04	6.8
5. Mitochondria	0.20	6.1	0.22	4.9	0.03	3.7
6. Lysosomes	0.19	3.9	0.21	3.2	0.08	6.7
7. Microsomes	0.20	2.8	0.44	4.5	0.40	22.6
8. Supernatant solution	0.13	5.7	0.11	3.5	0.05	8.8

fractions. Approx. 2/3 of the total activity recovered and assayed with *p*-nitrophenylphosphate or ATP was extracellular. In the presence of 0.5 mM inhibitor R8231, only 1/3 of the activity was extracellular. These results indicate that the inhibitor R8231-insensitive phosphatase activity is predominantly intracellular, and approx. 2/3 of the inhibitor R8231-sensitive activity assayed using either *p*-nitrophenylphosphate or ATP is extracellular. Cell breakage caused by the enzymatic digestion, assayed by DNA released, and actual cell counts, was limited to a maximum of 10%. When the phosphohydrolytic activity of the different fractions was assayed with ATP in the presence of Mg^{2+}, Ca^{2+} and inhibitor R8231, the activity was concentrated in the microsomal fraction which contains endoplasmic reticulum fragments as well as other membrane fragments. Experiments designed to further resolve the microsomal fraction by centrifugational procedures failed to provide more information on the specific localization of the inhibitor R8231-insensitive phosphatase activity (unpublished data).

Characterization of the inhibitor R8231-insensitive ATPase activity

Fig. 3 shows the dependence of the inhibitor R8231-insensitive ATPase activity on pH. The pH optimum of the activity asssayed in the presence of 0.5 M Tris-HCl buffer, 1 mM ATP, 1 mM $MgCl_2$, 1 mM $CaCl_2$ and 0.5 mM inhibitor R8231 was 9.0. There was activity between pH 7.5 and 10.0.

The dependence of the activity on the concentration of various cations is shown in Table II. Prior to the assays, the enzyme was dialyzed for 30 min against 0.5 M Tris-HCl, 0.1 mM EDTA (pH 9.0). This was followed by a dialysis for 60 min in the same buffer without EDTA. Using 1 mM ATP as the substrate at pH 9.0, optimal activity (100%) was demonstrated in the presence of 1 mM $MgCl_2$ and 1 mM $CaCl_2$. Inclusion of 0.5 or 5.0 mM inhibitor R8231 yielded 95.3% or 97.7%, respectively, of the maximal activity. Mg^{2+} alone was insuffi-

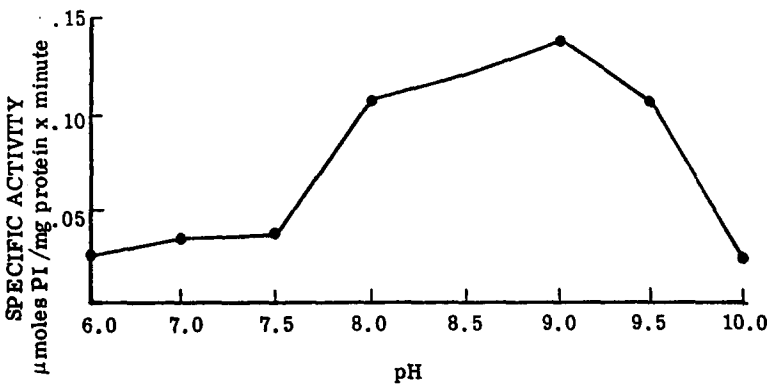

Fig. 3. pH optimum of inhibitor R8231-insensitive phosphatase. The enzyme was assayed using 1 mM final concentrations of ATP, $MgCl_2$ and $CaCl_2$ in 0.5 M Tris-HCl. 0.5 mM inhibitor R8231 was included to inhibit alkaline phosphatase. Results shown are from one experiment. The ordinate is specific activity (μmol inorganic phosphate liberated/mg protein per min). The abscissa is $-\log[H^+]$ or pH. Assays were performed at intervals of 0.5 pH unit from pH 6.0 to 11.0. pH was monitored at the start and the finish of the assay incubation.

TABLE II

MODULATION OF INHIBITOR R8231-INSENSITIVE PHOSPHATASE BY DIVALENT CATIONS

Each assay was run on inhibitor R82310insensitive phosphatase using 1 mM ATP, pH 9.0, in 0.5 M Tris-HCl. 100% was arbitrarily set as the amount of product liberated by the enzyme in the presence of 1 mM $MgCl_2$ and 1 mM $CaCl_2$. (100% = 0.58 μmol P_i/mg protein per min). Substances added are 1 mM unless indicated.

Substance added	Percent activity
No addition	0
$MgCl_2$ + $CaCl_2$	100.0
$MgCl_2$ + $CaCl_2$ + 0.5 mM inhibitor R8231	95.3
$MgCl_2$ + $CaCl_2$ + 5.0 mM inhibitor R8231	97.7
0.5 mM $MgCl_2$ + 0.5 mM $CaCl_2$	95.7
$MgCl_2$	8.3
0.5 mM $MgCl_2$	6.0
$CaCl_2$	41.6
0.5 mM $CaCl_2$	48.6
$ZnCl_2$	1.0
KCl	5.6
NaCl	9.0
KCl + NaCl	8.0
$MnCl_2$	3.0
$MnCl_2$ + $CaCl_2$	15.3
$MgCl_2$ + $CaCl_2$ + EDTA	1.0

cient for a significant catalysis to occur. However, Ca^{2+} alone at 0.5 mM showed 48.6% of the maximal activity; Zn^{2+}, Mn^{2+}, K^+ and Na^+ together at 1 mM were insufficient for any significant catalysis to occur. When EDTA was added in stoichiometric amounts relative to Mg^{2+} and Ca^{2+} the activity of the enzyme was severely inhibited.

TABLE III

SUBSTRATE SPECIFICITY OF INHIBITOR R8231-INSENSITIVE PHOSPHATASE

Each assay was run on inhibitor R8231-insensitive phosphatase using 1 mM $MgCl_2$, 1 mM $CaCl_2$, 0.5 mM R8231 and 1 mM substrate at pH 9.0 in 0.5 M Tris-HCl. 100% was arbitrarily set as the amount of product liberated by the enzyme using ATP as substrate. (100% = 0.54 μmol P_i/mg protein per min). Percentages are experssed as the mean of three experiments ± S.D.

Substrate	Percent activity
ATP	100
ADP	51.0 ± 1.1
dADP	3.1 ± 1.0
AMP	4.6 ± 1.2
dAMP	2.1 ± 0.3
GTP	93.3 ± 2.7
GMP	2.9 ± 0.6
ITP	89.1 ± 1.9
UTP	10.6 ± 1.2
UMP	3.0 ± 0.6
CTP	7.0 ± 1.0
CMP	2.9 ± 1.0
TMP	4.0 ± 0.7
β-Glycerophosphate	11.9 ± 1.7
Glucose 6-phosphate	4.8 ± 0.9
Pyrophosphate	4.1 ± 0.6

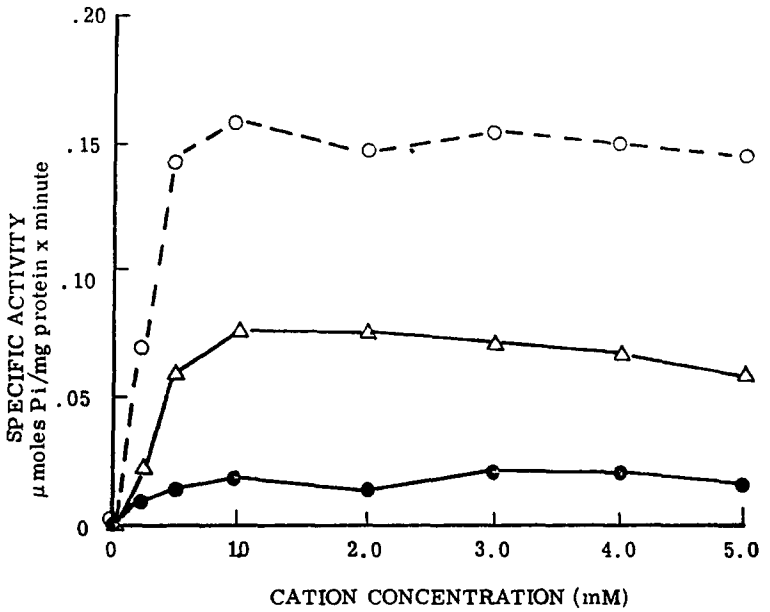

Fig. 4. Requirement of the inhibitor R8231-insensitive phosphatase for Ca^{2+} and Mg^{2+}. The inhibitor R8231-insensitive phosphatase was made dependent on exogenous Ca^{2+} and Mg^{2+} by dialysis against 0.1 mM EDTA for 30 min at 4°C in 0.5 mM Tris-HCl (pH 9.0). EDTA was removed by dialysis against 0.5 mM Tris-HCl (pH 9.0) for 60 min at 4°C. $CaCl_2$, $MgCl_2$, or both were added to the assay mixture at cation concentrations of 0.25, 0.5, 1.0, 2.0, 3.0, 4.0 and 5.0 mM. The ordinate is specific activity (μmol inorganic phosphate liberated/mg protein per min). The abscissa is cation concentration in mM. Data points are the mean of three determinations. Results shown are from one experiment, but are representative of data obtained from two similar experiments. ●———●, Mg^{2+}; △———△, Ca^{2+}; ○———○, Ca^{2+} + Mg^{2+}.

The substrate specificity of the inhibitor R8231-insensitive phosphatase is shown in Table III. Using 1 mM $MgCl_2$, 1 mM $CaCl_2$, 0.5 mM inhibitor R8231 at pH 9.0, ATP showed the highest activity among the substrates tested at 1 mM concentration. Substitution of other purine nucleoside triphosphates for ATP yielded 93.3% of the maximal activity with GTP and 89.1% with ITP. Substituting ADP for ATP yielded 51.0% of the maximal activity. Nucleoside monophosphates, pyrimidine nucleoside monophosphates and triphosphates, β-glycerophosphate, glucose 6-phosphate, pyrophosphate, α,β-adenosine monophospho-methylene phosphate or adenosine diphosphate-β,γ-methylene phosphate failed to show any significant activity (approx. 10% or less). Therefore, the inhibitor R8231-insensitive ATPase activity appears to be specific for purine nucleoside triphosphates.

Fig. 4 illustrates the requirement of the inhibitor R8231-insensitive phosphatase for Ca^{2+} and Mg^{2+}. The enzyme was dialyzed for 30 min against 0.5 M Tris-HCl, 0.1 mM EDTA (pH 9.0) and then for 60 min against 0.5 M Tris-HCl (pH 9.0) to insure dependence of activity on exogenous cations. Addition of Mg^{2+} alone resulted in a small increase (approx. 15% of optimum) in specific activity compared to zero activity without Mg^{2+} or Ca^{2+}. Addition of Ca^{2+} alone resulted in a significant increase (approx. 50% of optimum) in specific activity compared to zero activity without Mg^{2+} or Ca^{2+}. However, optical

specific activity was demonstrated in the presence of Ca^{2+} and Mg^{2+}, twice the activity when Ca^{2+} alone was present. The results correlated well with the data of Table II.

Discussion

The phosphohydrolytic activity of calcifying cartilage has been implicated in the process of calcification [1]. Despite the recent progress in characterizing this activity [3—5,7,8], the exact role it might play in calcification has to be established. Calcification may involve movement of both calcium and phosphate across intracellular and extracellular membranes. Despite extensive search for a calcium transport system, no such system unique to calcifying tissues has been identified.

While there have been reports indicating the existence of two different enzyme activities capable of degrading ATP at alkaline pH in rat incisor ondotoblasts [18], the existence of two activities in calcifying cartilage has not been described. The new activity is present in both bovine epiphyseal cartilage and fracture callus cartilage and therefore, it presumably is present in other types of calcifying cartilage.

The association of the purine nucleoside triphosphate phosphohydrolase with the microsomal fraction is in agreement with the histochemical studies on bovine fetus tooth germ [19]. Granstrom and his coworkers [6] have reported similar localization of the Ca^{2+}-activated inhibitor R8231-insentitive and the nonspecific inhibitor R8231-sensitive ATPase activity. However, extensive histochemical and biochemical studies in our laboratory have demonstrated that the inhibitor R8231-sensitive ATPase activity, i.e. alkaline phosphatase, of calcifying cartilage is localized in microsomes, plasma membranes and extracellular vesicles [9]. It has been reported that 10—15% of the ATPase activity of extracellular vesicles isolated from chick epiphyseal cartilage remained unaffected by levamisole [3]. A preparation of callus cartilage matrix vesicles [9] showed no purine nucleoside triphosphate phosphydrolase activity (unpublished data). Red blood cells and muscle sarcoplasmic reticulum contain enzymatic activities which are activated by Ca^{2+} and Mg^{2+} [20]. The preliminary studies reported in this paper do not allow an extensive comparison between the cartilage and the muscle enzyme [21]. Nevertheless, the two enzymes appear to be different in at least the substrate specificities [22]. The data on substrate specificity and the effects of divalent cations on the inhibitor R8231-insensitive enzyme indicate that it is a (Ca^{2+} + Mg^{2+})-activated purine nucleoside triphosphate phosphatase. Preliminary studies using Sepharose 6B gel filtration with markers of known molecular weight indicate this activity has a molecular weight of 20 000—25 000 (unpublished data). The presence of a (Ca^{2+} + Mg^{2+})-ATPase in calcifying cartilage suggests a role in calcification for the enzyme. Further studies are currently being conducted to confirm this role.

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