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Kinetic characterization of a membrane-specific ATPase from rat osseous plate and its possible significance on endochondral ossification

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Abstract

Treatment with phosphatidylinositol-specific phospholipase C of rat osseous plate membranes released up to 90–95% of alkaline phosphatase, but a specific ATPase activity (optimum pH = 7.5) remained bound to the membrane. The hydrolysis of ATP by this ATPase was negligible in the absence of magnesium or calcium ions. However, at millimolar concentrations of magnesium and calcium ions, the membrane-specific ATPase activity increased to about 560–600 U/mg, exhibiting two classes of ATP-hydrolysing sites, and site–site interactions. GTP, UTP, ITP, and CTP were also hydrolyzed by the membrane-specific ATPase. Oligomycin, ouabain, bafilomycin A_1, thapsigargin, omeprazole, ethacrynic acid and EDTA slightly affected membrane-specific ATPase activity, while vanadate produced a 18% inhibition. The membrane-specific ATPase activity was insensitive to theophylline, but was inhibited 40% by levamisole. These data suggested that the membrane-specific ATPase activity present in osseous plate membranes, and alkaline phosphatase, were different proteins.

Keywords: ATPase; Osseous plate; Phosphatidylinositol-specific phospholipase C; Endochondral ossification; Alkaline phosphatase

1. Introduction

Biological calcification and bone formation are complex processes mediated by the concerted action of physicochemical and biochemical factors which lead to deposition of a mineral phase into a specific organic matrix. According to several authors, matrix vesicles are the focuses where calcification of cartilage and bone starts, but the exact mechanism of initiation of calcification is not clear as yet [1–4].

Alkaline phosphatases from cartilage and bone are ectoproteins anchored to the matrix-vesicle membrane by glycosylphosphatidylinositol [5–7], but probably they are not the only membrane-bound enzymes relevant to the calcification process [1]. An intrinsic ATPase activity present in matrix vesicles from rachitic rats has also been implied in the deposition of calcium and phosphate [1,6,8,9]. However, the progress in characterizing this novel ATPase activity has been a difficulty due to the lack of suitable methods for obtaining alkaline phosphatase-depleted matrix-vesicle membranes [1,4]. The scarce data available show that, regardless of the amount of phosphatidylinositol-specific phospholipase C used, not more than 80% of membrane-bound alkaline phosphatase was released [1,6]. Thus, the high residual alkaline phosphatase activity impaired recognition of whether alkaline phosphatase and ATPase activities were the same or different proteins. An alterna-

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tive method has been the use of alkaline phosphatase-depleted membranes, associated with alkaline phosphatase inhibitors [4,8,9].

A suitable method for isolation of large quantities of alkaline phosphatase from rat osseous plate, which allows monitoring its activity during endochondral ossification, has been reported by us [10]. This multifunctional enzyme [4,11–13], which requires magnesium ions for full activity [14,15], constitutes of two apparently identical subunities of M_r 65 kDa [16], and is attached to the membrane by a glycosylphosphatidylinositol anchor [5]. Treatment of rat osseous plate membranes with phosphatidylinositol-specific phospholipase C from *B. thuringiensis*, resulted in alkaline phosphatase-depleted membranes, containing less than 5% alkaline phosphatase activity [4] and a membrane-specific ATPase [5].

Here, we describe the characterization of a membrane-specific ATPase activity, present in phosphatidylinositol-specific phospholipase C-treated rat osseous plate membranes. Our data suggested that this membrane-specific ATPase activity, and alkaline phosphatase activity, stem from different proteins. The kinetic properties of this membrane-specific ATPase could be of physiological significance, providing new insights into the calcification process.

2. Materials and methods

All solutions were made up by using Millipore MilliQ ultra pure apyrogenic water. Bovine serum albumin, theophylline, levamisole, Tris, 2-amino-2-methyl-propan-1-ol (AMPOL), TCA, 2-*N*-morpholine ethanesulfonic acid (MES), *N*-(2-hydroxyethyl) piperazine-*N*′-ethanesulfonic acid (Hepes), oligomycin, ouabain, bafilomycin A_1, thapsigargin, ethacrynic acid, ATP, GTP, ITP, CTP, UTP, and *p*-nitrophenyl phosphate (PNPP) were from Sigma. Calcium chloride, pyrophosphate (PPi), EDTA, dimethylsulfoxide (DMSO), sodium metavanadate and magnesium chloride were from Merck. Purified phosphatidylinositol-specific phospholipase C (PIPLC) from *B. thuringiensis* was obtained from Oxford University. Omeprazole was a gift from Dr. R. B. Oliveira (Hospital das Clínicas, FMRP/USP). Analytical grade reagents were used without further purification.

2.1. Preparation of rat osseous plate alkaline phosphatase

Membrane-bound alkaline phosphatase was prepared from rat osseous plate according to Curti et al. [10].

2.2. Preparation of alkaline phosphatase-depleted membranes

Alkaline phosphatase-depleted membranes were prepared by incubation of membrane-bound alkaline phosphatase (2 mg/ml) in 50 mM Tris–HCl buffer, pH 7.25 with 0.1 U PIPLC from *B. thuringiensis* for 1 h, under constant rotary shaking, at 37°C, according to Pizauro et al. [5].

2.3. Enzymatic activity measurements

Adenosine-5′-triphosphatase (ATPase), pyrophosphatase and *p*-nitrophenylphosphatase (PNPPase) activities were assayed discontinuously, at 37°C [5]. Standard conditions for PNPPase determination were 50 mM AMPOL buffer, pH 9.4, containing 2 mM $MgCl_2$ and 1 mM PNPP, in a final volume of 1.0 ml. For ATPase determination, standard conditions were 50 mM AMPOL buffer, pH 9.4 (or 50 mM Hepes buffer, pH 7.5) containing 2 mM $MgCl_2$ and 2 mM ATP, in a final volume of 1.0 ml. GTP, ITP, CTP, UTP were also used as substrate in the same conditions described above. Standard conditions for pyrophosphatase activity determination were 50 mM Tris–HCl buffer, pH 8.0, containing 2 mM $MgCl_2$ and 2 mM sodium pyrophosphate, in a final volume of 1.0 ml. Activities were also assayed in the presence of 5 mM theophylline. Determinations were carried out in duplicate and the initial velocities were constant for at least 30 min provided that less than 5% of substrate was hydrolyzed. Controls without added enzyme were included in each experiment to quantify the non-enzymic hydrolysis of substrate.

2.4. Estimation of protein

Protein concentrations were estimated according to Hartree [17] and bovine serum albumin was used as standard.

2.5. pH sensitivity of catalysis

The effect of pH on ATPase and PNPPase activities was carried out in the range between 5.5 and 11.0. MES buffer was used in the range 5.5–7.0; Tris from 7.0 to 8.6 and AMPOL from 8.6 to 11.0. The pH of the reaction mixture was measured both before and after the assay and it did not vary more than 0.05 units.

2.6. Effectiveness of inhibitors on membrane-specific ATPase activity

Initial rates were measured in 50 mM Hepes buffer, pH 7.5, containing 2 mM ATP, 5 mM theophylline, 2 mM $MgCl_2$, and the inhibitor, in a final volume of 1.0 ml. Except for thapsigargin and bafilomycin A_1, which were dissolved in DMSO, all other inhibitors were prepared in 50 mM Hepes buffer, pH 7.5. For EDTA, the enzyme was pre-incubated for 4 h prior to the measurement of the activity.

2.7. Estimation of kinetic parameters

Maximum velocity (V), initial velocity (v), apparent dissociation constant ($K_{0.5}$) and Hill coefficient (n), obtained from substrate hydrolysis were fitted on a 486 IBM microcomputer as described by Leone et al. [15]. Free species in solution were calculated as described elsewhere [13]. Data are reported as the mean of triplicate determinations in which $P < 0.05$ was considered to be statistically significant.

3. Results

Table 1 shows that high levels of ATPase activity (pH 7.5 and 9.4) were still bound to PIPLC-treated membranes, while pyrophosphatase (pH 8.0) and PNPPase (pH 9.4) activities were nearly completely released.

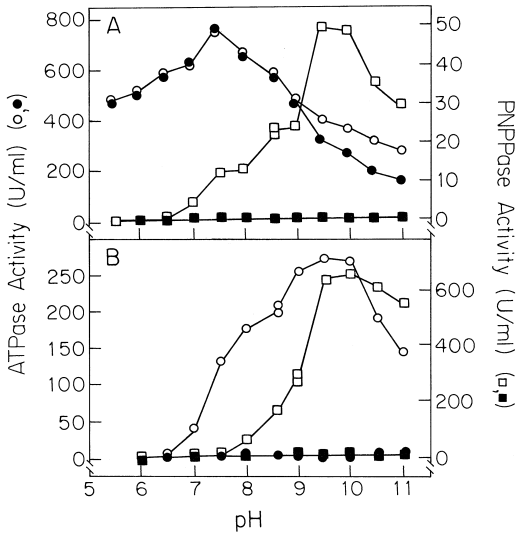

Fig. 1. pH sensitivity of the hydrolysis of ATP and PNPP by: (A) alkaline phosphatase-depleted membranes. (B) PIPLC-released alkaline phosphatase. (○) ATPase activity. (□) PNPPase activity. Assays were buffered with 50 mM buffer containing 2 mM $MgCl_2$ and the substrate (2 mM ATP or 1 mM PNPP). Closed symbols represent the respective activities in the presence of 5 mM theophylline.

Hydrolysis of ATP by alkaline phosphatase-depleted membranes showed an apparent optimum pH of about 7.5, while for PNPP it was 9.4 (Fig. 1(A)). Interestingly, ATPase activity was practically unaffected by 5 mM theophylline, while PNPPase was fully inhibited. On the other hand, PIPLC-solubilized alkaline phosphatase showed an apparent optimum pH value of about 9.4, independent of the substrate used (Fig. 1(B)). Both PNPPase and ATPase activi-

Table 1
Effect of treatment with phospholipase C from *B. thuringiensis* on rat osseous plate alkaline phosphatase activity

Substrate	Activity (U/ml)			
	Pellet		Supernatant	
PNPP (pH 9.4)	48.6 ± 1.7	(710.3 ± 9.8)	663.3 ± 10.3	(11.4 ± 1.3)
ATP (pH 7.5)	864.0 ± 31.1	(1,280.6 ± 65.9)	159.0 ± 5.0	(15.3 ± 0.9)
ATP (pH 9.4)	426.1 ± 14.9	(919.1 ± 31.7)	276.1 ± 6.0	(10.1 ± 0.7)
PPi (pH 8.0)	12.6 ± 2.8	(86.5 ± 9.1)	91.1 ± 4.5	(6.7 ± 3.3)

Values between parentheses represent control values for each activity at given pH.

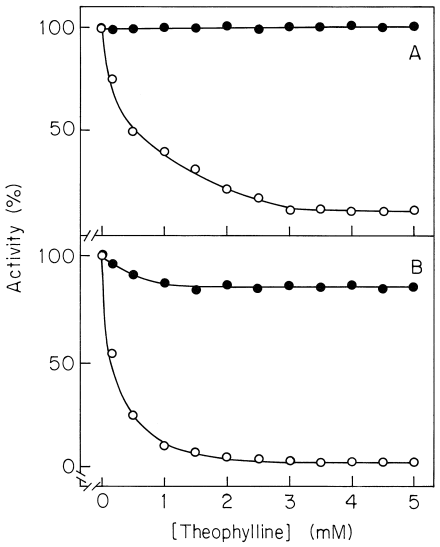

Fig. 2. Inhibition of ATPase and PNPPase activities of alkaline phosphatase-depleted membranes by theophylline at pH: (A) 7.5. (B) 9.4. (○) PNPPase activity. (●) ATPase activity. Assays were buffered with 50 mM buffer containing 2 mM $MgCl_2$, the substrate (2 mM ATP or 1 mM PNPP) and theophylline. The reaction was started by the addition of 40 μg of alkaline phosphatase-depleted membranes.

ties of PIPLC-released alkaline phosphatase were completely inhibited by 5 mM theophylline. The same occurred with pyrophosphatase activity (not shown). Taken together, the data in Fig. 1 suggested that the partial inhibition (less than 8%) by theophylline, of PNPPase activity of alkaline phosphatase-depleted membranes, was due to the alkaline phosphatase still remaining in the membrane.

Fig. 2 shows the effect of theophylline on PNPPase and ATPase activities of alkaline phosphatase-depleted membranes at pH 7.5 and 9.4. PNPPase activity was fully inhibited by 5 mM theophylline at both pH. In contrast, the membrane-specific ATPase activity was not affected by theophylline at pH 7.5 (Fig. 2(A)), and was slightly inhibited (8–15%) at pH 9.4 (Fig. 2(B)). Levamisole inhibited completely PNPPase activity (pH 7.5 and 9.4), and only partially (40%) the membrane-specific ATPase activity (Table 3).

The dependence on ATP concentration of the rate of hydrolysis by membrane-specific ATPase, at pH 7.5, is shown in Fig. 3. Relatively complex results suggested that at least two classes of hydrolyzing sites were involved, independently of the presence of magnesium or calcium ions. The high-affinity sites, appearing in the range of 1.0 μM to 1.0 mM ATP corresponded to about 70% of total activity, while the low-affinity sites, observed above 1.0 mM ATP, represented 30% of total activity. In the absence of metal ions ATP hydrolysis was negligible. Rather than ATP alone, metal–ATP seemed to be the true substrate of the enzyme, since between 10^{-6} and 10^{-2} M ATP, ATPase activity increased progressively with increasing concentrations of metal–ATP, but not free ATP, (inset of the figure). Moreover, the membrane-specific ATPase activity was inhibited by ATP concentrations above 3 mM (not shown). Table 2 summarizes the values for the calculated kinetic parameters of the corresponding ATP hydrolysing sites. It should be stressed that GTP, ITP, UTP and CTP were also hydrolyzed by membrane-specific ATPase (not shown).

The membrane-specific ATPase activity had an absolute requirement for magnesium and calcium ions (Fig. 4). Increasing concentrations of metal ions from 5×10^{-6} to 2×10^{-3} M, increased progressively the membrane-specific ATPase activity, resulting in complex curves in which at least two different ATP-hydrolysing sites were identified. Under these conditions no significant differences were observed

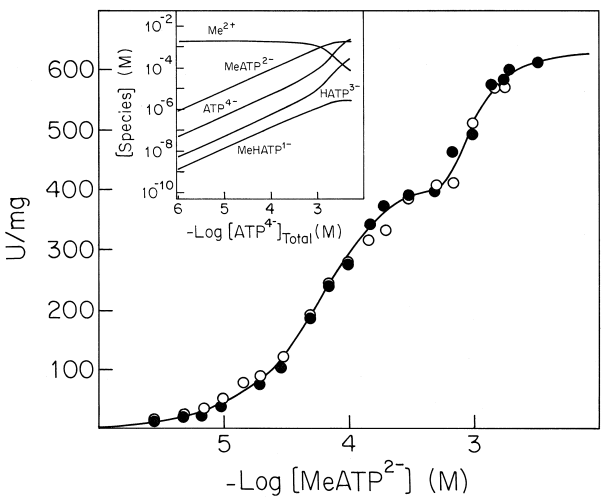

Fig. 3. Effect of ATP concentration on membrane-specific ATPase activity of rat osseous plate membrane in the presence of: (○) 2 mM $MgCl_2$. (●) 2 mM CaCl2. Assays were buffered with 50 mM Hepes buffer, pH 7.5, containing 5 mM theophylline and the metal ion. The reaction was started by the addition of 50 μg of alkaline phosphatase-depleted membranes. Inset: concentration of free species in solution for the above conditions.

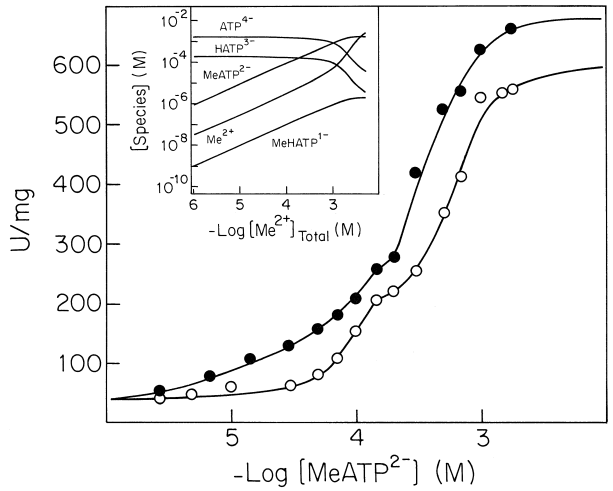

Fig. 4. Effect of magnesium and calcium ions on membrane-specific ATPase activity of rat osseous plate membrane. (○) magnesium ions. (●) calcium ions. Assays were buffered with 50 mM Hepes buffer, pH 7.5, containing 2 mM ATP and 5 mM theophylline. The reaction was started by the addition of 50 μg of alkaline phosphatase-depleted membranes. Inset: concentration of free species in solution for the above conditions.

on the values of the calculated kinetic parameters for the effects of these ions on membrane-specific ATPase activity (Table 2).

Oligomycin, ouabain and omeprazole had no effects on ATP hydrolysis by membrane-specific ATPase (Table 3). Thapsigargin and bafilomycin A_1 had only scarce effects even at concentrations up to 1 μM. Ethacrynic acid and vanadate caused a 10–18% inhibition of membrane-specific ATPase activity, while

Table 2
Kinetic parameters for the hydrolysis of calcium–ATP or magnesium–ATP by specific membrane-bound ATPase of rat osseous plate at pH 7.5

Conditions		Sites [a]	Kinetic parameters		
Variable	2 mM		V (U/mg)	$K_{0.5}$ (μM)	n
ATP	Mg^{2+}	HA	407.3	63.2	1.3
		LA	156.2	910.0	5.0
ATP	Ca^{2+}	HA	409.3	55.9	1.6
		LA	191.0	950.0	3.7
Mg^{2+}	ATP	HA	217.8	94.0	2.3
		LA	305.2	620.0	4.5
Ca^{2+}	ATP	HA	306.1	109.2	1.2
		LA	288.9	510.0	2.7

[a] HA = high-affinity sites; LA = low-affinity sites.

Table 3
Relative effectiveness of several reagents on the activity of ATPase activity of alkaline phosphatase-depleted rat osseous plate membranes

Reagent	% Vm
Oligomycin (1 μg/ml)	100.9 ± 5.0
Ouabain (1 mM)	112.2 ± 5.6
Bafilomycin A_1 (1 μM)	97.2 ± 4.9
Thapsigargin (1 μM)	96.5 ± 6.5
Omeprazole (5 μM)	99.5 ± 1.5
Vanadate (1 mM)	82.1 ± 6.5
Ethacrynic acid (2 mM)	90.0 ± 3.1
EDTA [a] (5 mM)	99.2 ± 3.4
Levamisole (5 mM)	60.1 ± 2.1
DMSO (10% v/v)	96.0 ± 2.4

[a] The enzyme was pre-incubated for 4 h, prior the determination of ATPase activity as described in Section 2.
Initial rates were measured in 50 mM Hepes buffer, pH 7.5, containing 2 mM ATP and 2 mM $MgCl_2$, in a final volume of 1.0 ml, as described in Section 2. Specific activity of 100% ATPase activity corresponded to 616.6 U/mg.

40% inhibition was observed for 5 mM levamisole. The lack of inhibition by EDTA of membrane-specific ATPase activity even after 4 h incubation with alkaline phosphatase-depleted membranes should be noted.

4. Discussion

Alkaline phosphatase has been considered for a long time to be involved in tissue calcification [18]. To date, it is well known that several activities other than alkaline phosphatase exist as intrinsic components of matrix-vesicles membranes, but no experimental data explains whether these activities work in concert in the calcification process [2,4,19]. In addition, the lack of suitable methods to prepare matrix-vesicles fully depleted of alkaline phosphatase activity, makes the understanding of matrix vesicles-mediated mechanisms of initiation of calcification difficult [1,4,6,8,20,21].

Levamisole has been used as a specific inhibitor of alkaline phosphatase [22], to explore the role of a specific ATPase which remains bound to the membrane after PIPLC treatment [1,8]. In spite of inhibition of levamisole nearly completely PNPPase and ATPase activities of PIPLC-released alkaline phos-

phatase from rat osseous plate, a 40% inhibition of the membrane-specific ATPase activity was also observed (Table 3). On the other hand, the use of theophylline allowed an improvement in such studies, since it inhibited specifically PIPLC-released alkaline phosphatase (see Fig. 1(B)), but not the membrane-specific ATPase activity at pH 7.5 (see Fig. 1(A)). In spite of the inhibition data (see Table 3) being in agreement with those reported for the ATPase from rachitic rats [9], the 18%-inhibition observed for vanadate, does not imply unequivocally that this membrane-specific ATPase is a P-type enzyme [23]. More detailed studies remain to be carried on.

Several evidences suggested that the membrane-specific ATPase activity, and alkaline phoshatase of rat osseous plate, were different proteins. The optimum pH value of the membrane-specific ATPase activity present in alkaline phosphatase-depleted membranes, near 7.5 (see Fig. 1(A)), was quite close to that reported for chick femora enzyme [24], but significantly different from that of PIPLC-released alkaline phosphatase (Fig. 1(B) and reference [5]). Optimum pH values for ATPase and PNPPase activities of alkaline phosphatase-depleted membranes differed by almost two units (see Fig. 1(A)). PNPPase and ATPase activities of PIPLC-released alkaline phosphatase were completely inhibited by 5 mM theophylline (see Fig. 1(B)), but the membrane-specific ATPase activity was not (see Fig. 1(A)). PNPPase activity of alkaline phosphatase-depleted membranes represented less than 10% of membrane-specific ATPase activity, on a molar basis. The membrane-specific ATPase activity of alkaline phosphatase-depleted membranes was negligible in the absence of calcium or magnesium ions (see Figs. 3 and 4), while PNPPase activity of osseous plate alkaline phosphatase is stimulated 25% by magnesium ions [15]. EDTA had negligible effect on the membrane-specific ATPase activity, while osseous plate alkaline phosphatase is inactivated irreversibly [25]. Moreover, the presence of a L-tetramisole-insensitive specific ATPase in skeletal tissue cartilage slices [22] and bone homogenates [26] also argues in favor of the existence of a specific ATPase activity, not stemming from alkaline phosphatase.

The requirements for calcium and magnesium ions of membrane-specific ATPase activity from rat osseous plate (Table 2 and Fig. 4), similar to those reported for the specific ATPase from matrix vesicles of rachitic rats [8], also suggested that it did not stem from alkaline phosphatase. Furthermore, taking in account the concentration of magnesium (0.7–0.8 mM) and calcium (1.5–1.7 mM) ions in cartilage extracellular fluid [27], and the apparent affinity ($K_{0.5}$) values of the membrane-specific ATPase for these ions, it appears that magnesium or calcium ions concentration was not a limiting factor for such activity. On the other hand, the $K_{0.5}$ values of about 60 μM for high-affinity ATP sites (see Table 2 and Fig. 3), quite close to ATP concentrations in the extracellular fluid surrounding the vesicles [8], suggest that the availability of ATP is indeed the limiting factor for the membrane-specific ATPase activity. Further, the fact that the hypertrophic zone of cartilage, before calcification occurs, contains ATP concentrations higher than that of the reserve zone, [28], also supports the above suggestion.

The hydrolysis of ATP, by a membrane-specific ATPase, at pH 7.5 is of physiological significance for the calcification process, since it is known that ATP is a more efficient substrate for initiating calcification than phosphomonoesters [8,9]. However, whether a specific ATPase is required to trigger calcification of cartilage and bone have yet to be established [1,9]. In our opinion, the lack of substrate specificity, the multifunctional properties attributed to alkaline phosphatase and the difficulty in obtaining alkaline phosphatase-depleted matrix-vesicles, are aspects of the mineralization process deserving special attention. On the other hand, the PIPLC treatment associated with the use of an inhibitor that blocks the non-specific hydrolysis of ATP (due to alkaline phosphatase activity) without destroying the ability of the matrix to calcify is without doubt a powerful tool to study the calcification process that emerged in the last years [4,9].

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