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CALCIUM-STIMULATED ADENOSINE TRIPHOSPHATASE IN THE MICROSOMAL FRACTION OF TOOTH GERM FROM PORCINE FETUS

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Summary

The characterization and localization of a Ca^{2+}-ATPase (ATP phosphohydrolase, EC 3.6.1.3) in the tooth germ of the porcine fetus are reported. This enzyme, a microsome fraction, is preferentially activated by Ca^{2+}. In the presence of 0.5 mM ATP, maximal enzyme activity is obtained at 0.5—1.0 mM $CaCl_2$. The maximal rate of ATP hydrolysis is approx. 20 μmol per h per mg of protein as the enzyme preparation is used here. At optimal Ca^{2+} concentration, the Mg^{2+} has an inhibitory effect. The enzyme does not require Na^+ or/and K^+ for activation by Ca^{2+}. Other nucleotide triphosphates may serve as the substrate, but V for ATP is the highest. The K_m for ATP is $8.85 \cdot 10^{-5}$ M. The optimal pH for Ca^{2+} activation of the enzyme lies around 9.2. Well known inhibitors of (Na^+ + K^+)-ATPase, mitochondria ATPase and Ca^{2+}-ATPase in the erythrocyte do not inhibit the enzyme. In the subcellular order the enzyme may be assumed to be localized in the smooth endoplasmic reticulum fraction containing cell and Golgi body membrane fragments and in the tissue order in the enamel organ containing an ameloblast layer, stratum intermedium and stellate reticulum.

Introduction

Ca^{2+}-activated ATPase (ATP phosphohydrolase, EC 3.6.1.3) has been shown to be part of the system which transports calcium into sarcoplasmic reticulum [1—4]. Other types of Ca^{2+}- and Mg^{2+}-stimulated ATPase have been known to be distributed widely in various tissues which are involved in calcium transport, reabsorption and excretion [5—9]. These enzymes are believed to play a role in the transport of Ca^{2+} and Mg^{2+}. Divalent cation-dependent ATPase is further known to be detected in the microsome fraction of rat liver

Abbreviation: EGTA, ethyleneglycol-bis-(β-aminoethylether)-*N*,*N*'-tetraacetic acid.

[10] which does not seem to be responsible for active Ca^{2+} and/or Mg^{2+} transport. In an attempt to elucidate biochemical events concerning the tooth calcification, we investigated the Ca^{2+} and Mg^{2+} stimulation of an ATPase in the microsome fraction isolated from calcifying tooth germ. We established that an ATPase which is stimulated by Ca^{2+} is present. This paper describes the characterization and localization of this ATPase.

Materials and Methods

Materials and chemicals. ATP and other nucleotides were purchased from Sigma; ruthenium red from Chroma Gesellschaft Schmid and Co; *p*-nitrophenylphosphate from Nakarai Chemicals, KK; EDTA and ethyleneglycol-bis-(β-aminoethylether)-*N,N'*-tetraacetic acid (EGTA) from Dogin Chemicals Laboratories Inc. Milk tooth germs of porcine fetus (crown-rump length 12—18 cm) were removed and stored at −20°C until the experiments were carried out. [γ-^{32}P]ATP was prepared by the method of Glynn and Chappell [11] and purified by column chromatography on Dowex 1-X8. [2,8-^{3}H]ATP was purchased from New England Nuclear.

Subcellular fractionation and washing the microsome with EDTA. Milk tooth germ was homogenized with 9 volumes of Buffer A (0.25 M sucrose, 10 mM Tris · HCl, pH 7.4) in a Potter homogenizer fitted with a teflon pestle. The calcified portion of the tooth germ sedimented to the bottom and the supernatant was subjected to differential centrifugation by the method used for rat liver subcellular fractionation [12]. After cell debris and nuclei were eliminated by centrifugation at 900 × *g* for 10 min, three successive centrifugations were performed at 5000 × *g* for 10 min, at 8000 × *g* for 20 min and at 100 000 × *g* for 60 min, resulting in the sedimentation of mitochondria, lysosomes and microsomes, respectively. Each subcellular fraction was washed once with Buffer A. Finally the washed fraction were resuspended in the same buffer. The 100 000 × *g* supernatant was utilized as the soluble fraction.

The microsome fraction was washed by suspending Buffer A containing 10 mM EDTA (disodium salt) and precipitated by centrifugation at 100 000 × *g* for 60 min. This procedure was repeated twice. To remove EDTA the washed microsome was suspended with Buffer A and precipitated. This pellet was suspended with a small volume of Buffer A and used in the present experiments as the Ca^{2+}-ATPase enzyme preparation.

Enzyme assay. ATPase activity was assayed by measurement of $^{32}P_i$ released from [γ-^{32}P]ATP or P_i liberated from ATP. The former assay method was performed essentially as described by Conway and Lipmann [13]. The standard reaction mixture contained the following components in 0.1 ml: 50 mM Tris/maleate, pH 7.0, 1 mM $CaCl_2$, 5.5 μg protein of enzyme preparation and 0.5 mM [γ-^{32}P]ATP (3 · 10^4 cpm). The reaction was started by an addition of [γ-^{32}P]ATP. After incubation at 37°C for 5 min, 0.25 ml of 20 mM silicotungstic acid in 0.01 M H_2SO_4 and 0.65 ml of 1 mM KH_2PO_4 were added to stop the reaction. The precipitate was removed by centrifugation. To the supernatant was added 0.25 ml of 5% $(NH_4)_2MoO_4$ in 2 M H_2SO_4. The phosphomolybdate complex formed was extracted with 1 ml of isobutanol/benzene (1 : 1, v/v) saturated with water. The radioactivity in the 0.5 ml

portion of the isobutanol/benzene layer was measured by an Aloka 2 π gas flow counter. ATPase activity by determination of P_i was measured in 0.5 ml of a standard reaction mixture except that 11 μg of protein of enzyme preparation and 1 mM non-radioactive ATP were used. The P_i liberated was determined by the method of Martin and Doty [14]. P_i in the substrate and the enzyme preparation was corrected.

Separation of rough and smooth endoplasmic reticulum fraction from microsome. Separation was performed by Rothschild method [15]. 3 ml of microsome fraction (unwashed microsome with EDTA, 2.34 mg/ml) was layered on a sucrose solution of 1.23 M in 10 mM Tris · HCl, pH 7.4. Centrifugation was carried out at 100 000 × *g* for 15 h using a HITACHI RP 40 rotor. The rough endoplasmic reticulum fraction was precipitated at the bottom and suspended in a small volume of Buffer A. The smooth endoplasmic reticulum fraction was collected by a J pipette since the fraction was dispersed widely over the applied microsome on the 1.23 M sucrose layer. After a 4—5-fold dilution with Buffer A, the fraction was precipitated by centrifugation at 100 000 × *g* for 60 min and suspended in a small volume of Buffer A.

Dissection of enamel organ and dental papilla from tooth germ. The tooth germs were separated into enamel organ, mineralized portion and dental pupilla. The dissection was performed using an stereomicroscope in Buffer A at 4°C.

Analytical methods. ^{3}H-labelled adenine nucleotides were measured by the method of Roisin and Kepes [16]. The protein concentration was determined by the method used by Lowry et al. [17] with bovine serum albumin as the standard. Extraction of RNA from the membrane fraction was performed by the modification of the procedure of Schmidt and Thannhauser [18]. The RNA concentration was determined by the Mejbaum [19] method with yeast RNA as the standard.

Results

Presence of the Ca^{2+}-ATPase in the microsome fraction

In a typical distribution pattern, the microsome fraction possessed maximal specific activity (12.2 μmol/h per mg) and 64% of the sum of enzyme activity of the subcellular fraction. The Ca^{2+}-ATPase activity was present in soluble (28%), lysosomes (6%) and mitochondria (2%) fraction in decreasing order of relative activity. The specific activity in soluble, lysosomes and mitochondria fraction as 0.421, 5.64 and 2.32 μmol/h per mg, respectively. The protein content of the soluble fraction amounted to 90% of the sum of the subcellular fractions. The microsome fraction was washed with EDTA as described in Materials and Methods. The washed microsome was used as enzyme preparation in subsequent experiments unless otherwise described.

Effect of divalent cation on the ATPase activity

Table I (Expt A) indicates that the ATPase was inhibited remarkably by EDTA and EGTA which are well-known as a divalent cation chelator and a specific Ca^{2+} chelator, respectively. By an addition of the Ca^{2+} the highest ATPase activity was obtained. In the absence of added divalent cation this enzyme preparation exhibited 54% of the activity of the Ca^{2+} addition. The

TABLE I

EFFECT OF DIVALENT CATION ON THE ATPase REACTION

The ATPase was measured as described in Materials and Methods. Expt A: $1.29 \cdot 10^6$ cpm per μmol of specific activity of [γ-^{32}P]ATP. 6.4 μg of washed microsomes. Expt B: $2.60 \cdot 10^6$ cpm per μmol of specific activity of [γ-^{32}P]ATP. 5.5 μg of washed microsomes. The values in parentheses represent percentage of maximal activity.

Expt	Cation and chelator	ATPase activity (μmol/h per mg protein)
A		
	None	11.1 (54)
	$CaCl_2$ 1 mM	20.6 (100)
	$MgCl_2$ 1 mM	7.3 (35)
	EDTA 1 mM	1.6 (8)
	EGTA 1 mM	3.8 (18)
B	in the presence of 0.1 mM EGTA	
	None	4.2 (21)
	$CaCl_2$ 0.2 mM	16.5 (84)
	1.0 mM	19.7 (100)
	$MgCl_2$ 0.2 mM	5.0 (25)
	1.0 mM	6.4 (33)
	$MnCl_2$ 0.2 mM	9.0 (46)
	1.0 mM	7.4 (38)
	$SrCl_2$ 0.2 mM	5.2 (27)
	1.0 mM	10.6 (54)
	$CdCl_2$ 0.2 mM	10.0 (51)
	1.0 mM	6.3 (32)
	$ZnCl_2$ 0.2 mM	10.7 (54)
	1.0 mM	10.5 (53)

Mg^{2+} stimulated less activity than that in the absence of added cation. The effect of Ca^{2+}, Mg^{2+}, Mn^{2+}, Sr^{2+}, Cd^{2+} and Zn^{2+} on the ATPase activity in the presence of 0.1 mM EGTA was studied (Expt B). Of all the divalent cations tested Ca^{2+} was the most effective. The other cations stimulated the ATPase activity in half of the cases of Ca^{2+} stimulation. No (Na^+ + K^+)-stimulated, ouabain-sensitive ATPase activity was detectable. Both NaCl and KCl inhibited the Ca^{2+}-ATPase up to about 50% (at 0.35 M) in the same manner by increasing the concentration thereof. No activation was observed when NaCl and KCl were used at the same time.

Effect of $CaCl_2$ and $MgCl_2$ concentrations

The optimum concentrations of $CaCl_2$ and $MgCl_2$ were obtained at 0.5—1.0 mM. Fig. 1 shows that the difference of Ca^{2+}- and Mg^{2+}-activated ATPase activity was remarkable. The effects of $MgCl_2$ concentration in the presence of optimum $CaCl_2$ (0.5 mM) and $CaCl_2$ concentration in the presence of optimum $MgCl_2$ (0.5 mM) were studied. It appears from Fig. 2A that the Mg^{2+} inhibited Ca^{2+}-activated ATPase activity. Addition of 2.0 mM $MgCl_2$ in the presence of 0.5 mM $CaCl_2$ resulted in 57% inhibition of the optimum ATPase activity. Ca^{2+} scarcely stimulated ATPase activity in the presence of Mg^{2+} (Fig. 2B).

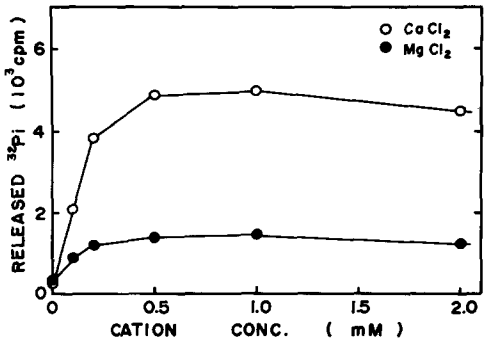


Fig. 1. Effect of $CaCl_2$ and $MgCl_2$ concentration on ATP hydrolysis. The ATPase assay was performed as described in Materials and Methods except that the concentration of $CaCl_2$ and $MgCl_2$, respectively, were varied as shown in the figure and EDTA was added at 0.1 mM. The reaction mixture containing the enzyme preparation (6.4 μg protein) was incubated for 3 min at 37°C. Specific activity of [γ-^{32}P]ATP was $7.94 \cdot 10^5$ cpm per μmol.

Substrate specificity

Substrate specificity was low since the rate of P_i liberation from other nucleotide triphosphates was observed in 73—94% of the rate of hydrolysis of ATP (Table II). ADP, AMP and *p*-nitrophenylphosphate were hydrolyzed with rates equivalent to 23, 21 and 6%, respectively, of the rate of ATP hydrolysis. PP_i was not hydrolyzed under these conditions.

The rate of the Ca^{2+}-ATPase reaction was constant over a period of 20 min under standard conditions and proportional to the concentration of enzyme preparation within the range of 0—12.5 μg protein.

Effect of pH on the Ca^{2+}-stimulated P_i release from ATP, ADP and p-nitrophenylphosphate

As shown in Fig. 3, Ca^{2+}-stimulated P_i release from ATP, ADP and *p*-nitro-

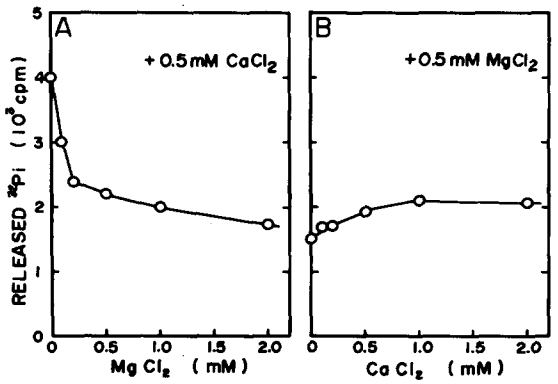


Fig. 2. (A) Effect of $MgCl_2$ concentration on ATP hydrolysis in the presence of 0.5 mM $CaCl_2$. The reaction mixture and assay procedure were as described in Materials and Methods except that $MgCl_2$ and $CaCl_2$ concentrations were varied as shown in the figure. Specific activity of [γ-^{32}P]ATP used in this experiment was $7.65 \cdot 10^5$ cpm per μmol. (B) Effect of $CaCl_2$ concentration on ATP hydrolysis in the presence of 0.5 mM $MgCl_2$. The reaction mixture and assay procedure were as described in the legend of A.

TABLE II

SUBSTRATE SPECIFICITY

The amount of P_i liberation was measured as described in Materials and Methods except that various nucleotides and other phosphate compounds were used as the substrate. The reaction mixture (0.5 ml), containing 10.9 μg protein (Expt A) or 21.8 μg protein (Expt B) of enzyme preparation, 1 mM $CaCl_2$ and 0.1 mM EGTA was incubated for 40 min (Expt A) at 37°C (Expt B: 20 min). The activity was expressed as the relative activity of P_i formation.

Expt	Nucleotides and other compounds (1 mM)	Relative activity of P_i formation (%)
A	ATP	100
	ADP	23
	AMP	21
	ADP + AMP (0.2 mM)	27
	p-nitrophenylphosphate	6
	PP_i	0
B	ATP	100
	GTP	79
	UTP	94
	ITP	73
	CTP	79

phenylphosphate was carried out by changing the pH to discover whether the substrate specificity of the Ca^{2+}-ATPase reaction changed in alkaline pH. Relative activity of P_i released in the percentage of ATP is shown in Fig. 3B. ATP was the most effective compound between pH 5.6 and 10.4 except for *p*-nitrophenylphosphate at pH 10.4. Substrate specificity became low in alkaline pH. Below pH 7.0 P_i release from ADP was observed in amounts less than 25% of those of ATP. AMP was found to be hydrolyzed in almost the same manner as ADP, but the data of AMP were omitted to clarify the figure.

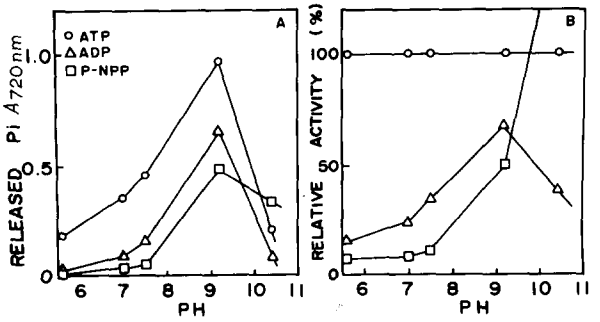


Fig. 3. (A) Effect of pH on Ca^{2+}-stimulated P_i release from ATP, ADP and *p*-nitrophenylphosphate (P-NPP). The reaction was performed as described in Materials and Methods except that ATP, ADP and *p*-nitrophenylphosphate as the substrate and 50 mM buffer were used. The pH of the reaction medium was adjusted to 5.6 with acetate, to 7.0 with Tris/maleate, to 7.4 with Tris · HCl, to 9.2 with glycine/NaOH and 10.4 with carbonate/bicarbonate. The reaction mixture containing the enzyme was incubated for 40 min at 37°C. The activity was expressed as the absorbance of Ca^{2+}-stimulated P_i release which was corrected for that of P_i release in the absence of Ca^{2+}. (B) The plots of the relative activity of data in A.

Product analysis of the Ca^{2+}-ATPase reaction

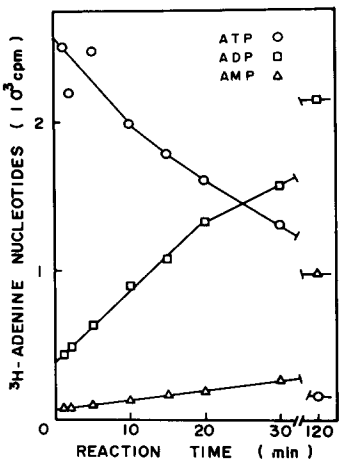
A test was performed to confirm whether ATP was hydrolyzed directly to ADP and P_i. The product of the Ca^{2+}-ATPase reaction was analysed using [2,8-^{3}H]ATP. The result is shown in Fig. 4. The initial rate of decrease of ATP was almost the same as that of the increase of ADP. The slow increase of AMP was observed. These data suggest that P_i or $^{32}P_i$ measured for the Ca^{2+}-ATPase assay came from the reaction of ATP to ADP and P_i, but not from two-step reactions of ATP to AMP and PP_i and PP_i to P_i and P_i.

Effect of ATP concentration

The effect of ATP concentration on the Ca^{2+}-ATPase was examined using an excess of Ca^{2+}. The result is shown in Fig. 5. Double reciprocal plots of the data gave a straight line which crossed on the ordinate. An apparent K_m value for ATP was calculated to be $8.85 \cdot 10^{-5}$ M. Apparent K_m did not change significantly at various free Ca^{2+} concentration. At the molar ratio of $CaCl_2$ to ATP of 1 : 1, K_m and V, respectively, are almost the same as in the case of the excess of $CaCl_2$.

Inhibition of the Ca^{2+}-ATPase activity under various conditions

As shown in Table III, the effect of various compounds on the Ca^{2+}-ATPase was examined. Oligomycin and ouabain, which are inhibitors of mitochondria ATPase and (Na^+ + K^+)-ATPase, respectively, had no effect on the



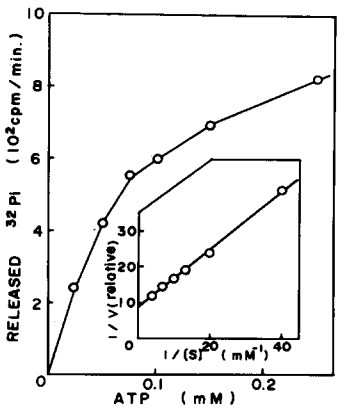


Fig. 4. Product analysis of the Ca^{2+}-ATPase reaction. The reaction was performed as described in Materials and Methods except that [2,8-^{3}H] ATP was used as the substrate. The reaction was started by an addition of 10 μl of [^{3}H]ATP (250 μCi/5 μmol per ml) and a portion of reaction mixture was added to formic acid containing non-radioactive ATP, ADP, AMP and adenosine to terminate the reaction at the time described in the figure. Adenine nucleotides were isolated as described in Materials and Methods and their radioactivity was counted with an Aloka LSC-651 liquid scintillation counter. Adenosine was not shown in the figure because its increase was negligible.

Fig. 5. Effect of ATP concentration on the Ca^{2+}-ATPase reaction. The reaction mixture was as described in Materials and Methods except that ATP concentration was varied as shown in the figure. The reaction mixture containing 0.5 mM $CaCl_2$, 6.4 μg protein of enzyme preparation and $7.95 \cdot 10^5$ cpm per μmol of specific activity of [γ-^{32}P]ATP was used in this experiment. Double reciprocal plots of data in the figure are as shown in the inset.

TABLE III

INHIBITION OF THE Ca^{2+}-ATPase ACTIVITY UNDER VARIOUS CONDITIONS

The reaction was performed as described in Materials and Methods except that the reaction mixture was incubated under the various conditions described in the table. Specific activity of [γ-^{32}P]ATP used in these experiments was $1.09 \cdot 10^6 - 2.18 \cdot 10^6$ cpm per μmol. The enzyme was incubated with trypsin in 20 μl of 50 mM Tris/maleate, pH 7.0. After incubation at 37°C for 120 min, the Ca^{2+}-ATPase activity was measured as described in Materials and Methods.

Expt			Activity as percent of control
A	Control		100
	Oligomycin	5 μg/ml	102
	Ouabain	1.0 mM	99
	Ruthenium red	0.1 mM	97
	N-Ethylmaleimide	5.0 mM	98
	L-Cysteine	5.0 mM	105
B	Control		100
	Triton X-100	1.0%	80
	Sodium lauryl sulfate	0.2%	6
	2,4-Dinitrophenol	0.5 mM	91
	Fusidic acid	1.0 mM	83
	Ethanol	15%	32
	P_i	0.1 mM	87
C	Control		100
	ADP	0.5 mM	69
	AMP	0.5 mM	81
	Freeze-thawing	10 times	60
	KCN	1.0 mM	97
	NaN_3	1.0 mM	103
	Trypsin	0.374 μg/μg	92

Ca^{2+}-ATPase reaction, and 2,4-dinitrophenol, the uncoupler of oxidative phosphorylation did not show any stimulative effect. The hexavalent dye, ruthenium red, inhibitor of the Ca^{2+}-ATPase in the erythrocyte membrane [20] and fusidic acid, a potent inhibitor of the elongation factor G-dependent GTPase of ribosomes [21] showed scarcely any significant effect. Sodium lauryl sulfate (0.2% final concentration) destroyed 94% of the Ca^{2+}-ATPase activity, but the enzyme was resistant to Triton X-100 (1.0% final concentration). Ethanol (15%) reduced the activity by 68% as compared to the control. Inhibition by P_i, AMP and ADP was not remarkable. Freezing and thawing of the enzyme preparation, 10 times within 30 min in solid CO_2/acetone and water bath at 37°C led to a 40% loss of activity. KCN and NaN_3 did not affect the enzyme activity. Trypsin treatment (under conditions shown in the legend of Table III) had no noticeable effect.

Localization of the Ca^{2+}-ATPase in microsome fraction

To determine whether the Ca^{2+}-ATPase was localized in the rough or smooth endoplasmic reticulum fraction, the microsome fraction was separated. A typical summary of the specific activity of the Ca^{2+}- and Mg^{2+}-ATPase activity and RNA content of fractions during the separation is presented in Table IV. RNA content in each fraction was lower than that reported by previous re-

TABLE IV

LOCALIZATION OF THE ATPase IN THE MICROSOME FRACTION

Isolation of smooth and rough endoplasmic reticulum from the microsome fraction (unwashed microsome with EDTA) was performed as described in Materials and Methods. The ATPase activity was measured as described in Materials and Methods except that 3.2—11.7 μg protein of membrane fraction and $2.43 \cdot 10^5$ cpm per μmol of specific activity of [γ-^{32}P] ATP were used in this experiment. The values in parentheses represent percentage of ATPase activity by $CaCl_2$ addition.

Fraction	Volume (ml)	Protein concentration (mg/ml)	ATPase activity (μmol/h per mg)		RNA content (μg/mg protein)
			$CaCl_2$ (1 mM)	$MgCl_2$ (1 mM)	
Unwashed microsome	3.0	2.34	4.61 (100)	2.66 (58)	22.7
Smooth endoplasmic reticulum	1.53	0.648	13.2 (100)	5.00 (38)	8.42
Rough endoplasmic reticulum	2.19	1.77	2.90 (100)	1.74 (60)	33.5

searchers working with liver membrane fractions, but about 80% of the total microsomal RNA was recovered in fraction rough endoplasmic reticulum while the recovery of RNA content in fraction smooth endoplasmic reticulum was only about 5%. The recovery of Ca^{2+}-ATPase activity in fraction smooth endoplasmic reticulum and rough endoplasmic reticulum was about 40 and 35%, respectively. When compared in terms of specific activity, it will be noticed that the specific activity of Ca^{2+}-ATPase was the highest in fraction smooth endoplasmic reticulum and that of the enzyme in fraction rough endoplasmic reticulum was lower than that in the microsome fraction. These results indicate that the Ca^{2+}-ATPase may be assumed to be localized in fraction smooth endoplasmic reticulum containing cell and Golgi body membrane fragments.

Localization of the Ca^{2+}-ATPase in tooth germ

To discover whether the Ca^{2+}-ATPase was localized in the enamel organ or in the dental papilla, tooth germ was dissected. The microsome fraction was isolated by the same method from the enamel organ, dental papilla and undissected tooth germ. Table V shows that isolated enamel organ microsomes ex-

TABLE V

LOCALIZATION OF THE ATPase IN THE TOOTH GERM

The ATPase activity was measured as described in Materials and Methods except that 4.3—6.7 μg protein of the microsome fraction (unwashed microsome with EDTA) and $1.72 \cdot 10^5$ cpm per μmol of specific activity of [γ-^{32}P] ATP were used in this experiment. The values in parentheses represent percentage of ATPase activity by $CaCl_2$ addition.

Microsome fraction	ATPase activity (μmol/h per mg protein)		
	$CaCl_2$ (1 mM)	$MgCl_2$ (1 mM)	No addition
Tooth germ	11.7 (100)	7.44 (64)	10.1 (86)
Enamel organ	29.5 (100)	13.3 (45)	20.8 (71)
Dental papilla	3.15 (100)	3.7 (120)	2.55 (81)

hibited 2.5 times the Ca^{2+}-ATPase activity of tooth germ microsomes, and dental papilla microsomes, about one-fourth that of tooth germ microsomes. Using unwashed microsomes a low dependency of ATP hydrolysis on Ca^{2+} was observed. However, about 80% inhibition was obtained on microsomes of the enamel organ by an addition of 3 mM EGTA. It was noted that Mg^{2+} stimulated higher ATPase activity than that of Ca^{2+} stimulation only in the dental papilla microsomes. These results suggest that the Ca^{2+}-ATPase was localized in the enamel organ containing ameloblast, stratum intermedium and stellate reticulum.

Discussion

The present report described some properties and localization of the Ca^{2+}-ATPase in calcifying tooth germ. With no addition of divalent cation, the enzyme preparation exhibited 54% of ATPase activity in the case of an addition of Ca^{2+} (Table I). This activity, that seemed to exist in the presence of residual Ca^{2+}, varied from 40 to 70% of the Ca^{2+}-stimulated ATPase activity with the use of various lots of enzyme preparation. It was difficult to remove completely the residual Ca^{2+} from enzyme preparation because a large amount of Ca^{2+} seemed to be eluted from the calcifying portion of tooth germ during homogenization and to be confined into membrane vesicles. According to histochemical studies, strong alkaline phosphatase activity has been observed on the cell membrane of the stratum intermedium adjacent to ameloblast elaborating the enamel matrix [22—24]. Moreover, both AMP and *p*-nitrophenylphosphate hydrolysis at alkaline pH suggest that alkaline phosphatase may be present in our enzyme preparation. However, our results show that hydrolysis of ATP, AMP and *p*-nitrophenylphosphate is sensitive to Ca^{2+}. It is unclear whether AMP and *p*-nitrophenylphosphate are hydrolyzed by alkaline phosphatase in alkaline pH (Fig. 3). A low value of 4.61 μmol/h per mg was obtained as the specific activity of the Ca^{2+}-ATPase of the microsome fraction in Table IV. This seemed to be due to the fact that the tooth germ was stored at −20°C for a long term (about 8 months). The Ca^{2+}-ATPase of tooth germ had different properties from the Ca^{2+}-activated ATPase which was a component of the calcium transport system of the sarcoplasmic reticulum. Also the enzyme differs from the ATPase of rat liver microsome fraction with regard to divalent cation dependency. The ATPase of rat liver microsomes is known to be activated by both Ca^{2+} and Mg^{2+} to the same extent and Mg^{2+} does not inhibit the ATPase activity in the presence of Ca^{2+}. Moreover, we found that the microsome fraction of fetal porcine liver possessed ATPase of the same type as rat liver. These results suggest that the Ca^{2+}-ATPase described here is a specific feature of calcifying tooth germ microsomes, especially in the smooth endoplasmic reticulum fraction.

The ATPase showed optimum activity with an addition of 0.5—1.0 mM $CaCl_2$, a concentration which was of the same order as that in body fluid [25] and almost the same as the ATP concentration used. However, higher concentrations of $CaCl_2$ (above 1.0 mM) and ATP (above 1.0 mM) inhibited the ATPase activity. Evidence which interested us very much suggested that the greater part of the Ca^{2+}-ATPase was located in the enamel organ. However, it is

still unclear as to where the Ca^{2+}-ATPase is located in the cell layer of the enamel organ. The attempt to detect ATP-dependent calcium uptake of microsome vesicles remains unsuccessful. The biological significance of the Ca^{2+}-ATPase in tooth germ remains unclarified. Further studies on the nature of the enzyme are needed in order to elucidate its biological role.

Acknowledgments

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