Novel Ca²⁺-ATPase Found in the Human Milk Membrane Fraction

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SUMMARY

Calcium-stimulated ATPase (Ca²⁺-ATPase) which has optimal pH value at 7.0 was found in the membrane fraction of human milk, and its enzymatic properties were studied. The purified Ca²⁺-ATPase required 0.45 mM Ca ion for maximal activity. Among the nucleosides, Ca²⁺-ATPase showed a higher substrate specificity to ATP and UTP than to CTP and GTP. Ca²⁺-ATPase had apparent Km value of 0.065, and Vmax of 7.63 mol ATP hydrolyzed/mg protein per min, respectively. Ca²⁺-ATPase was potently inhibited by lanthanide, vanadate, and p-chloromercuribenzoate, and inactivated by EDTA, and CDTA and EGTA, but were unaffected by N-ethylmaleimide, NaN₃, ouabain, or oligomycin, and was completely inactivated by heating at 60℃ for 10 min. This enzyme activity was concentrated in the membrane fraction of the cream and skim milk membrane, but not founded in bovine milk.

I. INTRODUCTION

The plasma membrane of most mammalian cells contains several ATPases. They can be distinguished by their requirement for monovalent and divalent ions, specificity for nucleotides and susceptibility to such inhibitors as ouabain, vanadate, and N-ethylmaleimide[1]. Among these ATPases, Ca²⁺-ATPase (EC 3.6.1.3) is responsible for ATP-dependent Ca²⁺ transport[2,3].

We have found Ca²⁺-ATPase in human milk, these enzymes being contained in the membrane fraction isolated from the cream of human milk. The membrane fraction, referred as the milk fat globule membrane (MFGM), is derived from the origin of the apical plasma membrane of mammary secretory cells during the secretion of milk lipid[4-7]. As shown in Fig. 1, speculating from the origin of milk fat globule membrane, Ca²⁺-ATPase found in the human milk membrane fraction probably participate in regulating the calcium in concentration reqired for milk secretion in the mammary gland[4]. Since it is very difficult to obtain human lactating mammary cells,
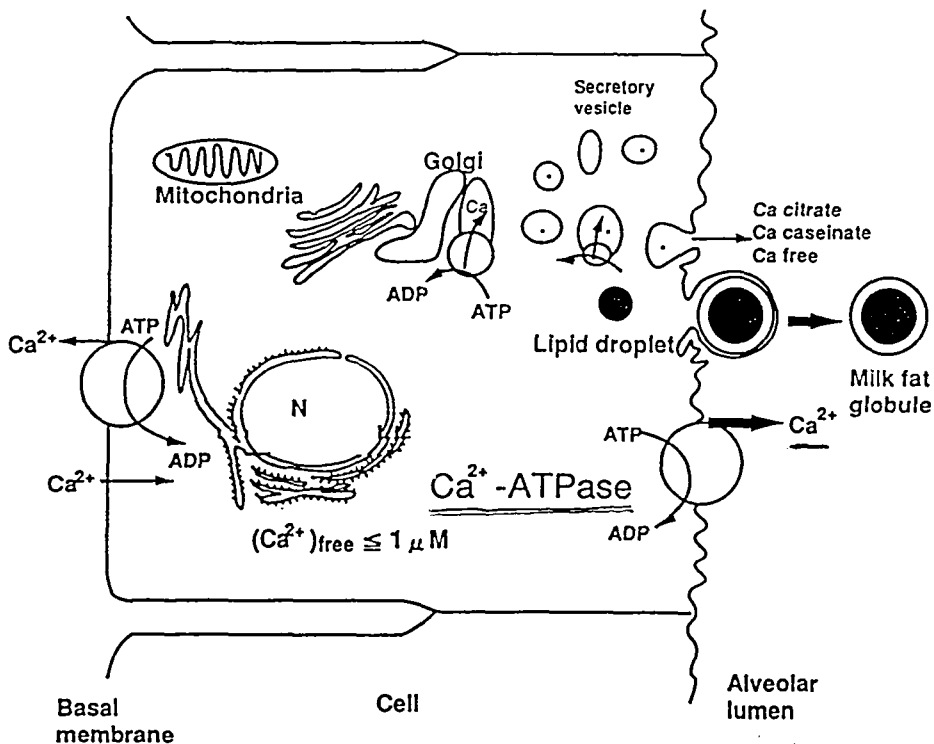
Fig. 1. Hypothetical scheme for Ca²⁺-ATPase in the lactating mammary epitherial cells.

the membrane fraction of human milk has merits as a useful substitute for the membrane of the mammary gland.

This work was undertaken to enzymatically characterize the purified Ca²⁺-ATPase which was found in the membrane fraction of human milk.

II. MATERIALS AND METHODS

1. Materials

Mature human milk samples were obtained from healthy mothers by expressing with a manually operated breast pump, the fresh milk being immediately fractionated. The sodium salts of 5'-AMP, 5'-GTP, 5'-CTP, 5'-UTP, 5'-TTP and 5'-ADP were from Yamasa Shoyu Co., Ltd (Chiba, Japan). Imidazole, L-Histidine, tris(hydroxy)aminomethane, L-ascorbic acid, ethylenediaminetetraacetic acid (EDTA) and ethyleneglycol-bis-(β-aminoethyl ether)-tetraacetic acid (EGTA) were from Wako Junyaku (Osaka, Japan). 5'-ATP disodium and Tris salts (equine muscle), and oligomycin were from Sigma Chemical Co. (MO, U.S.A). Trans-cyclohexane-1, 2-diaminotetra-acetic acid (CDTA) was from Aldrich Chemicals Co. (SI, U.S.A), the other chemicals used being of analytical grade.

2. Fractionation of the milk samples

The milk was centrifuged for 90 min at 100,000×g and at 4℃ on a swing rotor (Hitachi RPS 27-2) and then fractionated into four parts; subnatant (whey fraction), a fluffy layer (skim milk membrane fraction) just over the casein micellar pellet, and the pellet itself. First the skimmilk membrane fraction was immediatly collected by gentle suction, and the packed cream layer was carefully collected by scooping up with a spatula. The casein pellet was dispersed in 3 volumes of deionized water. The average percentage by weight of each milk fraction was 4.4% for the cream, 80.2% for the whey, 6.0% for the skim milk membrane, and 8.0% for the casein pellet. Churning can be accomplished by agitation, with a hand mixer.

3. Preparation of the membrane-enriched fraction

The cream was dispersed in 2.5 volumes of deionized water. chilled in an ice bath and then churned by vigorous hand shaking. The separated buttermilk fraction, rich in milk fat globule membrane, was collected, divided, and stored at −80℃. Unless otherwise specified, the protein concentration being to 2 to 5 mg/ml.

4. Ca^{2+}-ATPase activity assay system

Ca^{2+}-ATPase activity was measured in an assay medium consisting of 0.9 volume of 30 mM imidazole-histidine buffer (pH 7.0), 1 mM NaCl, 3 mM KCl, 0.5 mM $CaCl_2$, 10 to 50 g of protein, 0.1 vol. of 10 mM ATP or other nucleotisides. The purified Ca^{2+}-ATPase fraction was used for enzymatic studies. Inhibitors, chelating agents, and metal ions were included in an assay medium before starting the reaction. Incubation was done for 30 min or the indicated time with shaking in a water bath at 37℃ or the indicated temperature, the reaction being started by adding 0.1 ml of an ATP solution after 5 min of preincubation. The reaction was stopped by adding 1 ml of 9% trichloroacetic acid, and the reaction mixture was immidiately chilled with ice. The mixture was centrifuged for 5 min at 3,500 rpm, and 1 ml of the supernatant was taken for an analysis of the released phosphate by the method of Chen et al[8]. All the samples were corrected for the spontaneous hydrolysis of ATP, and the ATPase activity is expressed as mol/mg of protein/min. Protein content was measured by the method of Markwell et al[9]. using bovine serum albumin as the standard.

5. $(Na^{+}+K^{+})$-ATPase assay

$(Na^{+}+K^{+})$-ATPase (EC 3.6.1.4) was measured in 0.9 ml of 30 mM imidazole-histidine buffer (pH 7.5) containing 120 mM NaCl, 12 mM KCl, 5 mM $MgCl_2$, and 0.5 mM disodium EDTA. The reaction was started by adding 0.1 ml of 10 mM ATP (pH 7.5) and stopped by adding 1 ml of 9% trichloroacetic acid, agter incubation for 30 min at 37℃. The released phosphate was measured as already mentioned.

6. Other enzyme assays

The activities of alkaline phosphatase (EC 3.1.3.1), acid phosphate (EC 3.1.3.2), γ-glutamyltranspeptidase (EC 2.3.2.2), 5'-nucleotidase (EC 3.1.3.5), and phosphodiesterase I (EC 3.1.4.1) were measured by the methods described in our previous paper at an assay temperature of 37°C [11,12].

III. RESULTS

1. Properties of the purified Ca²⁺-ATPase

The optimal pH of the purified Ca^{2+}-ATPases was measured in an assay mixture containing 0.9 mM NaCl, 2.7 mM KCl, and 0.45 mM $CaCl_2$. Fig. 2 shows the pH-activity profiles of Ca^{2+}-ATPases, the optimal pH being 7.0 for Ca^{2+}-ATPase.

The effect of the Ca concentration on the rate of ATP hydrolysis were studied at pH 7.0 (Fig. 3). ATP hydrolysis at pH 7.0 was increased by the increasing Ca concentration. The maximum ATP hydrolysis was reached at 0.45 mM $CaCl_2$.
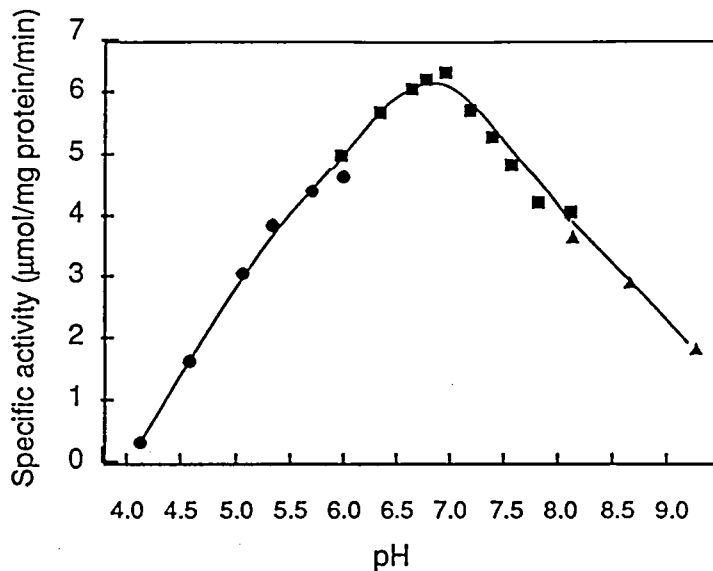

Fig. 2. Effects of pH on the rate of ATP hydrolysis by Ca^{2+}-ATPase purified from human milk membrane fraction. The rate of ATP hydrolysis was measured in 30 mM acetate buffer at pH 4.0~6.0 (●-●), 30 mM Imidazole-Histidine buffer at pH 6.5~8.0 (■-■), and 30 mM Tris-HCl buffer at pH 7.5~9.0 (▲-▲).

The specificity and kinetic parameters of the purified Ca^{2+}-ATPase for a number of nucleotides is shown in Table 1. Ca^{2+}-ATPase hydrolyzed ATP and UTP at a higher rate than
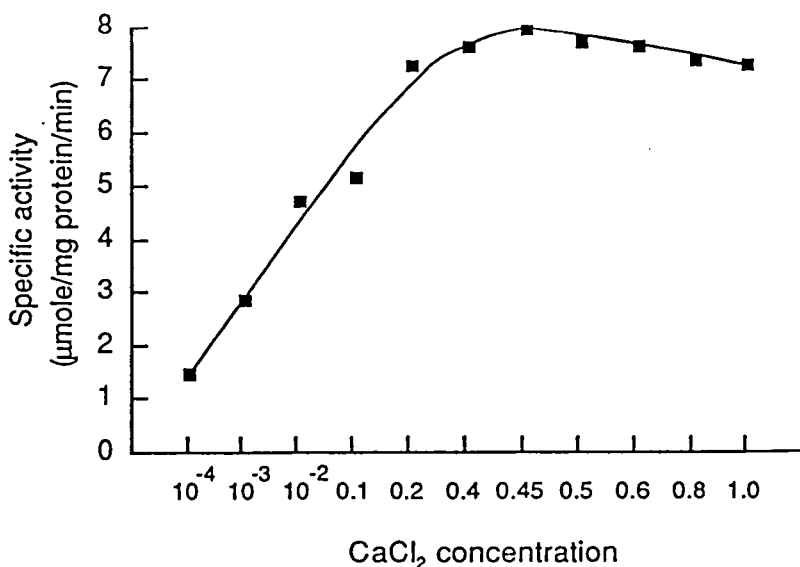
Fig. 3. Effects of calcium concentration on the rate of ATP hydrolysis at pH 7.0 by
Ca^{2+}-ATPase purified from human milk membrane fraction.

Table 1. Substrate specificity and kinetic parameters of the purified Ca^{2+}-ATPase from the
membrane fraction of human milk

Substrate	Sp. act.	Ratio	V_{max}	K_m
ATP	7.605	100	7.633	0.064
UTP	7.485	98.4	7.620	0.108
CTP	3.047	40.1	4.011	0.053
GTP	2.852	37.5	2.985	0.049
ADP	0.482	6.33	0.418	0.027
AMP	0.028	0.368	0.026	0.011
Ap_5A	0	0	0	—
AMP-PNP	0.01	0	0	—
PNPP	0	0	0	—

Sp. act., specific activity as μmol /mg of protein; V_{max}, μmol /mg of protein /min; K_m, mM /liter.
The data show the mean of triplicate assays, SD being less than 0.05 for Sp. act. and 0.01 for V_{max} and K_m
(n=3). Final concentration of the substrate was 1mM, except for 10 mM of AMP, Ap_5A, AMP-PNP, and
PNPP (ρ-nitrophenyl phosphate).

CTP and GTP. The rate of hydrolysis of the other nucleotides corresponded to 38~98% that of
ATP. The hydrolysis of ADP was 6.3% by Ca^{2+}-ATPase. AMP was hydrolyzed by the
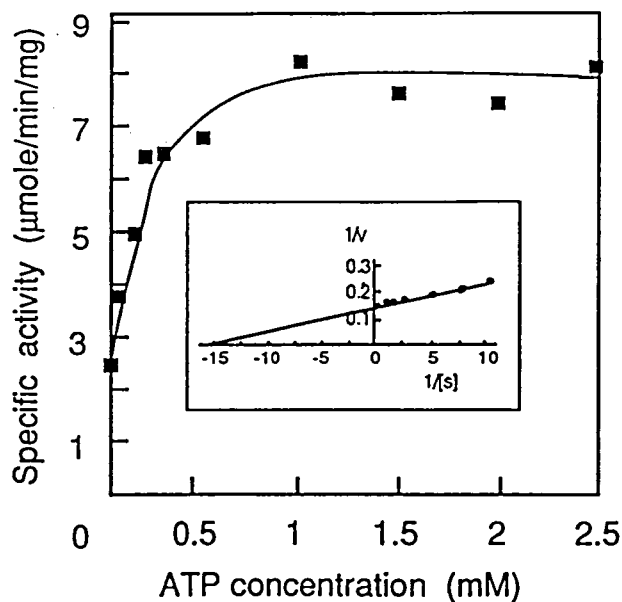Ca^{2+}-ATPases by 0.4% in comparison with that of ATP.

Fig. 4. Dependence of the purified Ca^{2+}-ATPase on ATP concentration. Insert represents a Lineweaver-Burk plot. V_{max} of 7.63 μmol/mg of protein/min, and K_m of 0.065 mM obtained for Ca^{2+}-ATPase with a correlation coefficient of 0.998.

Table 2. Effects of various agents on the specific activity of the purified Ca^{2+}-ATPase in human milk

Inhibitor	Concentration(mM)	Specific activity(μmol/min/mg protein)
Control		100%
LaCl$_3$	0.5	32.7±2
Na$_3$VO$_4$	0.1	68.6±2
Ruthenium Red	0.1	41.2±3
PCMB	0.1	75.3±6
NEM	5	98.1±2
GSSG	5	98.1±7
NBD-Cl	0.1	93.8±1
Valinomycin	0.1	99.7±6
Oligomycin	1	100.2±1
NaN$_3$	10	100.0±1
Ouabain	1	100.9±8

A relationship between the hydrolysis rate and substrate concentration for Ca^{2+}-ATPase is also shown in Fig. 4. The apparent K_m for ATP of Ca^{2+}-ATPase, which was derived from a

Table 3. Effects of chelating reagents and metal ions on the specific activity of the purified Ca^{2+}-ATPase in human milk membrane fraction

	Concentration (mM)	Ca^{2+}-ATPase(Specific activity)
Control		100%
$MnCl_2$	2	10.2±1
$NiCl_2$	2	100.3±6
$CoCl_2$	2	60.4±1
EGTA	2	19.5±7
CDTA	2	0
EDTA	2	0

Control specific activity was 7.76μmole /mg of protein /min.
EGTA, glycoletherdiaminetetraacetic acid
EDTA, ethylendiaminetetraacetic acid
CDTA, diaminocyclohexanetetraacetic acid.

Lineweaver-Burk plot, was approximately 0.065±0.015 mM /liter, and the V_{max} about 7.633±1 μmol /mg of protein /min(n=3).

The effects of inhibitors on the Ca^{2+}-ATPase are shown in Table 2. The activitie of Ca^{2+}-ATPase was inhibited by 50% by fluoride, which is an inhibitor of monophosphatase[13] and Mg^{2+}-ATPase in muscle[14], and by 67% by lanthanide, which is an inhibitor of Ca^{2+}-ATPase in erythrocyte and other systems[15,16] Ouabain, which is an inhibitor of (Na^++K^+)-ATPase had no effect on the Ca^{2+}-ATPase actibity[17]. NaN_3 and oligomycin, which are both inhibitors of mitochondrial ATPase[18,19], had no influence on the Ca^{2+}-ATPase. PCMB, which inhibits Ca^{2+} transport by inhibiting the SH groups, decreased the rate of ATP hydrolysis, but N-ethylmaleimide and oxidized hlutathione did not. These results indicate that the enzyme contained SH groups, and that these SH groups were not required SH groups, and that these SH groups were not required for ATPase activity[19]. The Ca^{2+}-ATPase activity was decreased by 33% by vanadate, which is a potent inhibitor of P-type ATPase systems[19,20].

The effects of chelating reagents and metal ions on the purified Ca^{2+}-ATPase are shown in Table 3. Chelating agents such as EGTA, EDTA and CDTA had an inactivating effect of Ca^{2+}-ATPase at low concentrations (Table 3). At 2 mM, the Ca^{2+}-ATPase activity was more inhibited by CDTA than by EGTA. This result indicates that a low concentration of the Mg ion may be required for Ca^{2+}-ATPase. Ca^{2+}-ATPase activity was not affected by the Ni ion, but was inhibited by the Mn and Co ions.

Ca^{2+}-ATPases was stable at 50℃ for 10 min, but was inactivated at 60℃ (Table 4). At 40℃, the Ca^{2+}-ATPase was stable at least for the 24 hr test period. The enzyme in the membrane fraction was stable for one month at −20℃ and −80℃. However, the activity of enzyme in the

Table 4. Effects of temperature on the specific activity of the purified Ca^{2+}-ATPase from human milk membrane fraction

Temperature and time	Ca^{2+}-ATPase
A. Temperature (10min)	
Control	100%
40°C	100±2
50°C	98±5
60°C	0±2
80°C	0±1
B. Heating at 55°C	
Control	100%
10 min	52±1
20 min	42±6
30 min	36±5
60 min	29±3
120 min	27±2

The data show the mean of triplicate assays.

fraction solubilized with 1% Tween 20 had decreased by 20% at −20°C and by 5% at −80°C after freezing for one month (data not shown).

The Ca^{2+}-ATPases and $Na^+ + K^+$-ATPase were assayed in human milk, in comparison with the activities of marker enzymes, γ-glutamyltranspeptidase and alkaline phosphatase, which are localized in the plasma membrane of lactating bovine mammary cells and in the bovine milk fat globule membrane[11]. The specific activities and distribution in the milk fractions are shown in Table 5. The Ca^{2+}-ATPases and γ-glutamyltranspeptidase had higher activity than the other enzymes assayed. The activities of the enzymes related to phosphate hydrolysis, except for the Ca^{2+}-ATPase, were especially low (data not shown: 0.1 and 0.06 μmol / mg of protein /min for 5'-nucleotidase, 0.6 and 2.1 for phosphodiesterase I, and 2.0 and 14.3 for alkaline phophatase in whole milk and cream, respectively) in agreement with the results for γ-glutamyltranspeptidase by Sobiech and Wieczorek[21] and for alkaline phosphatase by Worth et al[22]. The distributions of these enzymes were higher in the whey fraction (46~58%) than in the cream (25~35%). In contrast, the total activities of the Ca^{2+}-ATPase and γ-glutamyltranspeptidase were higher in the cream fraction (53~67%) than in the whey fraction (20~26%). The high concentration of the enzymes in the whey fraction may be explained by the fact that these enzymes are released from the membranous fraction into the whey fraction by protease(s) which is abundant in human milk[23,24]. Ectoenzymes such as γ-glutamyl-

Table 5. Distribution of Ca²⁺-ATPase and other marker enzymes in human mature milk and the separated milk fractions .

	Whole milk		Cream		Whey		Skimmilk membrane		Casein	
	A	B	A	B	A	B	A	B	A	B
Ca²⁺-ATPase at pH 7	10.8± 7.1	100.0	118.7± 31.3	54.5± 15.0	2.9± 20.2	25.5± 11.5	35.8± 27.7	14.3± 5.7	17.6± 13.9	5.8± 3.8
Na⁺+K⁺-ATPace	1.2± 1.2	100.0	15.3± 7.2	49.4± 21.0	0.2± 0.4	7.9± 14.9	11.1± 8.3	25.4± 12.7	6.7± 7.3	17.3± 15.4
γ-Glutamly transpeptidase	89.3± 43.3	100.0	898.2± 348	53.3± 12.9	20.1± 8.9	34.7± 10	125.2± 80.7	7.9± 2.7	83.5± 43.4	4.2± 1.4
Alkaline phosphatase	2.0± 1.4	100.0	14.3± 4.9	28.7± 16.5	0.7± 0.5	48.5± 15.2	4.2± 4.7	7.0± 3.7	7.3± 6.4	16.4± 7.1
Protein		100.0		3.4± 1.8		83.9± 9.1		3.4± 1.7		3.2± 2.0

A, Specific activity(nmole/mg of protein/min) ; B, Relative activity was expressed as the ratio(%) over whole milk. Numerals show the mean±SD (n=8~14)

transpeptidase in the membrane are released from the matrix by the action of proteolytic enzymes, but retain their activity[25].

IV. DISCUSSION

In this study, we provide the first evidence that novel Ca^{2+}-ATPase is contained in the membrane fraction of human milk. Although, we have measured this enzyme activity with bovine milk, it was not founded even in colostrum membrane fraction. This Ca^{2+}-ATPase had optimal pH values 7.0, and basically required the Ca ion at different concentrations for full activation of the ATP hydrolysis reaction. Our Ca^{2+}-ATPase were distinguished from $(Na^{+}+K^{+})$-ATPase and mitochondrial ATPase by their lack of response to ouabain, oligomycin, and azide.

Although we have not yet measured a true K_m for Ca^{2+} of this ATPase, the requirement of the Ca ion for the maximal activation of Ca^{2+}-ATPase was high, when compared with that of rat liver plasma membrane[18] and sarcoplasmic reticulum of skeletal muscle[26] A low-affinity Ca^{2+}-ATPase has been found in various tissues, including placenta[27], kidney[28], liver[29], intestine[30], and corpus luteum[31]. However two apparent K_m parameters found for Ca^{2+}-ATPase have been reported from rabbits[32], human platelets[33], and sarcoplasmic reticulum[34].

Ca^{2+}-ATPase was not activated by added Mg^{2+}, but was inhibited more with CDTA than with EGTA, suggesting that this enzyme had been fully activated with endogenous Mg^{2+}. This has also been seen in the $(Ca^{2+}+Mg^{2+})$-ATPase in liver[35] fat cells[16] and corpus luteum[31] in which they were activated with Mg^{2+} at a low concentration, but not at a high concentration. CDTA had an equal affinity for Ca and Mg ions, while EDTA had a greater affinity for Ca than for Mg ions[31]. Ca^{2+}-ATPase was inhibited by lanthanide and vanadate, in agreement with the results for the erythrocyte calcium pump[15,16,20] Although their effect was small in this experiment, it may imply that Ca^{2+}-ATPase can be classified as a P type of ATPase[20].

The secretory cells of the mammary gland during lactation transport calcium accumulated from the blood (2~3 mM) into milk, in which its concentration ranges from 6~7 mM in human milk to greater than 100 mM in rat and rabbit milk[31]. Most or all of the secreted calcium forms a complex with casein and other smaller calcium-binding substances via the Golgi-derived vesicles of mammary secretion[31]. The total calcium content of the mammary gland was increased from about 2 to 12 mol /mg of tissue during the transition from pregnancy to lactation in mice, and at least two thirds of the total calcium was exchanged with external calcium in 6 hr in lactating mice[38]. Although the mechanism for this calcium transport process is unknown, Ca^{2+}-ATPase derived form the Golgi fraction has been reported to be responsible for transporting calcium from the cytoplasm into these membrane compartments[39,40]. This Ca^{2+}-ATPase, which may be localized on the plasma membrane, may mediate calcium transport between the cytoplasm and extracellular space in the human lactating mammary gland. The Ca^{2+}-ATPase activity in the

membrane fraction of human milk may represent an enzymatic basis for the calcium pump. The function and significance of Ca^{2+}-ATPase in human milk remains to be clarified. Purification and further characterization of Ca^{2+}-ATPase are shown in seminar presentation.

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