

Membrane $\text{Ca}^{2+} \text{Mg}^{2+}$ -ATPase activation by calcium-activated neutral protease (CANP) in conditions where there is elevated intracellular Ca^{2+} : An insight from erythrocytes of Duchenne muscular dystrophy (DMD)

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Abstract

CANP along with cytosolic calcium has been found to be elevated in erythrocytes from DMD patients. An enhanced association of this enzyme to the erythrocyte membrane is also observed in this disease condition. $\text{Ca}^{2+} \text{Mg}^{2+}$ -ATPase of DMD erythrocytes was also significantly elevated, indicative of the effort of the cells to pump out excess calcium. In normal erythrocytes incubated with calcium in the presence of Ionophore A 23187 there is a concomitant increase of the membrane-associated CANP and $\text{Ca}^{2+} \text{Mg}^{2+}$ -ATPase activity, with time. A model has been proposed to suggest that in conditions where there is an increase in intracellular calcium the membrane-bound active CANP increases the pumping efficiency of $\text{Ca}^{2+} \text{Mg}^{2+}$ -ATPase by limited proteolysis. This model has been supported by *in vitro* activation of $\text{Ca}^{2+} \text{Mg}^{2+}$ -ATPase by a homogeneous preparation of CANP.

Key words: DMD, RBC, CANP, activation, ATPase, proteolysis.

1. Introduction

Many of the alterations observed in the properties of DMD erythrocyte membrane are also observed in normal erythrocytes on treatment with Ionophore A23187 and calcium. These alterations include: 1) passive efflux of potassium¹⁻³; 2) changes in surface contour as observed by electron microscopy⁴⁻⁶ and cell deformability^{2,5,7}. These changes in the properties of DMD erythrocytes suggest a generalized defect of plasma membrane^{1,4,7-9} and it has also been suggested that there is an altered intracellular calcium status¹⁰.

Melloni *et al*¹¹ have shown that intracellular calcium regulates calcium-activated neutral protease activity. The pro-CANP (proenzyme form) is converted to active form (μ CANP) in the presence of elevated intracellular calcium¹².

CANP was found to increase in DMD muscle^{13,14}, which also registers an elevated intracellular calcium¹⁵. In normal erythrocytes the major form of CANP is μ CANP. The

physiological role of this enzyme is not clear, although it is known to cause limited proteolysis and in turn activate enzymes such as phosphorylase b kinase, tryptophan hydroxylase and glycogen synthetase¹⁶. CANP therefore could also activate Ca^{2+} -ATPase.

Since there are evidences to indicate increased intracellular calcium in DMD erythrocytes, the present investigation is to know the inter-relationship between cytosolic calcium and CANP (cytosolic and membrane bound) in DMD condition and relate the same to the activity of calcium ATPase in this condition.

2. Procedures

Diagnosis of DMD cases was based on clinical examination, electromyography, muscle biopsy analysis and estimation of serum creatine kinase (CK)¹⁴ and lactate dehydrogenase (LDH)²³. Blood was collected from confirmed cases of DMD and normals. Figure 1 gives the flow chart for the preparation of erythrocyte cytosol, membrane fractions and the purification of CANP from its endogenous inhibitor (ECI).

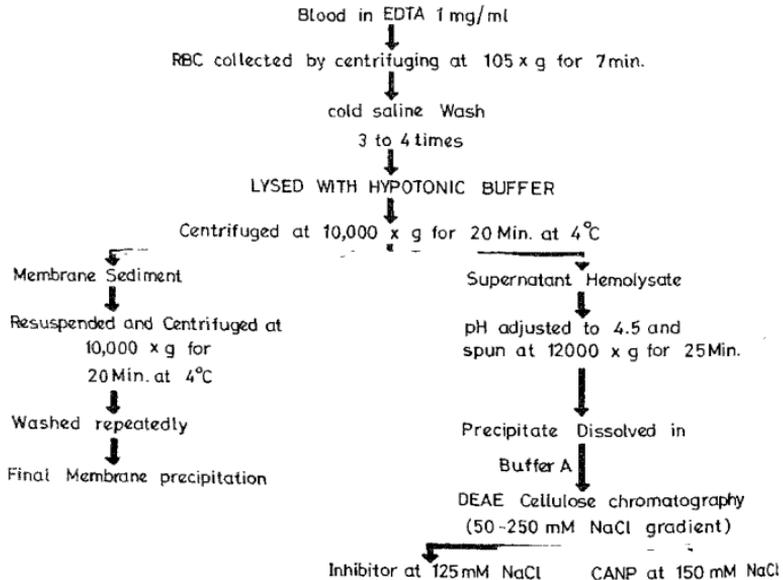


FIG. 1. Preparation of erythrocyte membrane and cytosolic fraction and purification of CANP from endogenous inhibitor.

2.1. Preparation of erythrocyte membrane

Blood was collected in EDTA (1 mg/ml) and the erythrocytes were pelleted by centrifuging in a table-top centrifuge at $108 \times g$ for five minutes. The cell pellet (2 ml) was washed thrice with ten volumes of cold isotonic saline. All subsequent operations were carried out at 4°C . The cells were lysed by suspending them in a 5 mM tris HCl buffer (pH 7.5) containing 5 mM disodium EDTA and 7 mM NaCl and was centrifuged at $10,000 \times g$ for 20 minutes to pellet the membrane. The membrane pellet thus obtained was resuspended in ten volumes of the above mentioned buffer. After sedimentation, the membrane pellet was re-suspended in a 20 mM tris HCl buffer (pH 7.5) containing 5 mM disodium EDTA, 150 mM NaCl and 10 mM 2-mercaptoethanol till a translucent membrane pellet was obtained (4-6 washes). For the purpose of CANP assay, membranes were cooled in ice, and sonicated using IMECO sonicator setting at maximum voltage for one minute (15 second bursts with intermittent cooling). 20-60 μg of protein was used for the assay.

2.2. Fractionation of CANP from ECI

Fractionation of CANP from its endogenous inhibitor (ECI) was carried out by a modified procedure of Murakami *et al*¹⁷. The pH of the membrane-free lysate was adjusted to 4.5 using 1N acetic acid and cooled in ice for 30 minutes. This suspension was centrifuged at $17,000 \times g$ for 20 minutes. The pellet thus obtained was dissolved in 20 mM tris HCl buffer (pH 7.5) containing 5 mM EDTA, 50 mM NaCl and 10 mM 2-mercaptoethanol (buffer A) and centrifuged at $17,000 \times g$ for 20 minutes. The clarified supernatant solution (10 mg of protein) was loaded on to a DEAE cellulose column (10 \times 1 cm) and was equilibrated with buffer A. The column was washed with the same buffer and eluted with 30 ml of linear gradient of NaCl (50 to 250 mM) in buffer A. Twenty fractions were collected and 30 μl aliquot from each fraction was assayed for CANP.

2.3. Assay of CANP

Assay of the proteolytic activity of CANP was carried out as described elsewhere¹⁴, except that only 100 μM CaCl_2 was present in the substrate. Protein was estimated according to the procedure of Lowry *et al*¹⁸.

2.4. Measurements of calcium

Saline-washed erythrocytes, suspended at a concentration of 2.5×10^9 cells/ml, were frozen away until the calcium measurements were carried out. The erythrocyte membranes were prepared as described earlier (2.1) and were washed 3-4 times and suspended in a buffer containing 5 mM tris HCl (pH 7.5) and 300 mM sucrose. The membrane samples were frozen till the calcium measurements were carried out. 50 μl of the erythrocytes (containing 10-15 mg of protein) and 100 μl membrane (containing to 2-4 mg protein) were used for digestion with 100 μl of concentrated HCl. The digested sample was made up to 1% lanthanum chloride in a total volume of 1 ml and was clarified by centrifugation at $800 \times g$ for 20 minutes. This was analysed for calcium using a Varian Techtron model 1000 atomic

absorption spectrophotometer. Calcium standards (1-4 $\mu\text{g/ml}$) were made in lanthanum chloride and were used for establishing a standard graph. The erythrocyte and membrane calcium were expressed as μg of calcium per mg of protein.

2.5. Ionophore A23187-dependent calcium uptake

Cold saline-washed erythrocytes were prepared as described in 2.1 and suspended in isotonic buffer containing 5 mM tris HCl (pH 7.5) and 300 mM sucrose. Ionophore A23187 was dissolved in 95% ethanol at a concentration of 1 mg/ml, and stored at -20°C . The stock solution of Ionophore A23187 was diluted ten times for the desired concentration in the suspension buffer. The concentration of ionophore was 1 $\mu\text{g/ml}$ of cell suspension and that of CaCl_2 , 1 mM. Ethanol contribution from the ionophore stock solution was less than 1 $\mu\text{l/ml}$. At this concentration of ethanol, the cells are not morphologically altered⁶. After incubation at 37°C for various time points, two aliquots of cells were withdrawn. One aliquot was cooled to 4°C , spun down ($150 \times \text{g}$ for 5 minutes), washed with saline 2-4 times and lysed. The lysate and the membrane were prepared as described earlier. The second aliquot of cells after 2-4 washes with isotonic sucrose was processed for the measurement of calcium. Cytosolic and membrane CANP and Ca^{2+} -ATPase were also quantified.

2.6. $\text{Ca}^{2+}\text{Mg}^{2+}$ -ATPase assay

$\text{Ca}^{2+}\text{Mg}^{2+}$ -ATPase activity was measured according to Zurini *et al*¹⁹. The ATPase activity was expressed as μ mole of the product formed per minute per mg of membrane protein.

2.7. Membrane incubation with purified CANP

Human placental μCANP (Sp.Ac.424.2 u/mg) and mCANP (442.0 u/mg) have been purified as described by Rabbani *et al*²⁰ and Radhika and Anandaraj²¹. Erythrocyte membranes from controls were prepared as mentioned earlier. 50 μg of membrane protein was incubated with 1 μg of the purified enzyme for 5 minutes at 30°C and assayed for Ca^{2+} -ATPase as described earlier.

3. Results and discussion

Table I shows the activities of μCANP in DMD erythrocytes. There was two-fold increase in CANP activity in the erythrocytes of DMD patients. This increased activity of cytosolic CANP could be due to the activation of proenzyme on the inner surface of erythrocyte membrane in the presence of increased cytosolic calcium^{11,12}.

To verify the hypothesis that intracellular calcium regulates CANP activity, membrane and cytosolic calcium were quantified (as given in 2.4) in DMD erythrocytes (Table II).

The total calcium in the erythrocyte from DMD patients is found to be elevated to about 35% compared to normal cells. Since total calcium is increased and membrane calcium is

Table I
Erythrocyte cytosolic CANP in DMD

Type of case	Sample size	CANP (u/mg)
1) DMD	6	mean \pm SD 35.89 \pm 4.87
2) Control	6	mean \pm SD 16.75 \pm 3.49
t > 0.05		

Table III
Membrane association of CANP in DMD

Type of case	Sample size	Membrane enzymes	
		LDS (u/mg)	CANP (u/mg)
1) DMD	6	mean \pm SD 8.64 \pm 2.43	11.5 \pm 2.38
1) Control	6	mean \pm SD 9.65 \pm 3.15	5.65 \pm 0.88
		t = insignificant	t > 0.05

Note: Cytosolic LDH in erythrocytes of DMD and control are 24.92 \pm 1.89, and 28.91 \pm 5.71 (n = 6), respectively.

decreased, cytosolic calcium must therefore be increased. This change is in keeping with the postulate that there is an increase in cytosolic calcium in non-muscle cells of DMD²². Hence increased cytosolic calcium must be responsible for an increase in proenzyme conversion to active form (μ CANP)¹³. This conversion step is mediated by increased proenzyme binding to the membrane. Hence, membrane-associated CANP was studied using membranes as enzyme source. The activities of CANP and LDH in membrane are given in Table III.

There was significant increase in the membrane-associated CANP in DMD erythrocytes. However this was not the case with LDH, another cytosolic enzyme. Thus the observed increase in the association of CANP to the membrane in DMD erythrocytes reflects an increase in the activation of pro-CANP. This association of CANP to the membrane could have pathological implications.

CANP is known to cause subtle changes in the membrane¹⁶. In fact, we find that CANP activates Ca²⁺-ATPase, implicating the role of the enzyme in conditions where there is an increased intracellular calcium. In order to verify the relationship between intracellular calcium and CANP activity, normal erythrocytes were incubated with calcium and ionophore A23187. Calcium and CANP (cytosolic and membrane) were monitored as described earlier. Concomitant increase in total erythrocyte calcium, cytosolic and membrane CANP follow a similar time course (Table IV) suggesting that the cytosolic calcium controls the level of active membrane and cytosolic CANP molecules.

Table II
Total calcium of erythrocyte and erythrocyte membrane in DMD

A)		Erythrocyte calcium (μ g/mg)
Type of case	Sample size	Mean \pm SD
1) DMD	9	0.276 \pm 0.035
2) Control	6	0.195 \pm 0.018
t > 0.05		
B)		Erythrocyte membrane calcium (μ g/mg)
Type of case	Sample size	Mean \pm SD
1) DMD	7	0.249 \pm 0.06
2) Control	6	0.506 \pm 0.0623
t > 0.05		

Table IV
Effect of Ionophore A23187 and calcium on normal erythrocytes

	0 min	15 min	30 min	45 min	1 h	3 h
Hemolysate* CANP u/mg	4.5	5.23	10.23	7.23	7.5	6.0
Membrane CANP u/mg	3.30	4.42	6.8	6.6	6.6	6.0
Erythrocyte calcium $\mu\text{g}/\text{mg}$	0.176	0.232	0.268	0.308	0.300	0.268
Membrane calcium $\mu\text{g}/\text{mg}$	0.633	0.655	0.597	0.568	0.438	0.429

* Hemolysate was directly loaded on to a DEAE-cellulose column and eluted with 0.125 and 0.150 M NaCl in buffer A. 0.150 M NaCl fractions were assayed for enzyme activity.

Table V
Erythrocyte membrane Ca^{2+} -ATPase

Type of case	Sample size	Ca^{2+} -ATPase u/mg
1) DMD	10	0.604 ± 0.122
2) Control	6	0.48 ± 0.09

$t > 0.05$

Table V shows Ca^{2+} -ATPase activity in DMD and control. There is an increase in Ca^{2+} -ATPase in DMD erythrocyte. Such an elevation in Ca^{2+} -ATPase has also been observed by several other workers²⁴⁻²⁷.

This increase in the specific activity of Ca^{2+} -ATPase could not be explained on the basis of either changes in membrane polypeptides, cytosolic activators (calmodulin) or changes in membrane phospholipids²⁷⁻³⁰.

As mentioned earlier erythrocyte Ca^{2+} , cytosolic CANP and membrane CANP were found to be elevated in DMD erythrocytes. It was therefore suggested that this increase in membrane CANP could cause subtle alterations in the DMD membrane. Thus the specific increase in Ca^{2+} -ATPase activity could be due to the action of membrane CANP (since CANP is known to activate enzymes by limited proteolysis¹⁶).

In order to verify if increase in intracellular Ca^{2+} increases Ca^{2+} -ATPase activity, erythrocytes from normal individuals were incubated in a medium containing Ionophore A23187 and calcium. Calcium, CANP and membrane ATPases were concomitantly monitored. Concomitant increase in total calcium, CANP and membrane CANP followed a similar time course. At these time points an increase in Ca^{2+} -ATPase was also observed (Table VI).

Moreover, in an *in vitro* experiment, where normal erythrocyte membranes were pre-treated with purified CANP (both m and μ CANP) from placental source (Table VII), increase in Ca^{2+} -ATPase was observed. Similar activation of Ca^{2+} -ATPase was observed *in vitro* when purified Ca^{2+} -ATPase was incubated with purified erythrocyte CANP³¹.

Table VI

Normal erythrocytes incubated with Ionophore A23187 and calcium: effect on ATPases

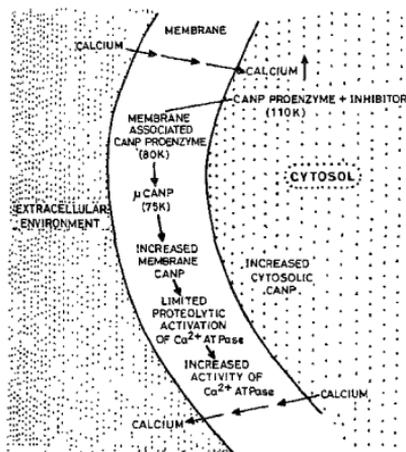
	0 min	15 min	30 min	45 min	1 h	3 h
Ca^{2+} -ATPase (u/mg)	0.549	0.556	0.592	0.668	0.752	1.040
Membrane CANP (u/mg)	3.3	4.42	6.8	6.6	6.6	6.0

Table VII

Membrane ATPase (control): effect of CANP

	Membrane	M + m CANP	M + μ CANP
Ca^{2+} -ATPase (u/mg)	0.464 ± 0.054	0.600 ± 0.038	0.653 ± 0.045

M = Membrane

FIG. 2 A model for activation of Ca^{2+} -ATPase by membrane CANP.

Thus these experimental evidences lend support to the fact that CANP activates Ca^{2+} -ATPase in conditions where there is increased intracellular calcium. Hence a model has been proposed, where the following sequence of events could occur in response to elevated intracellular calcium: 1) proenzyme binding to the membrane, 2) conversion to the active form, 3) increased membrane CANP activity, 4) limited proteolytic activation of CANP leading to activation of Ca^{2+} -ATPase, 5) increase in activity of Ca^{2+} -ATPase, and 6) increased efficiency in pumping out Ca^{2+} in order to maintain the low intracellular calcium level (fig. 2).

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