

## Copyright Undertaking

This thesis is protected by copyright, with all rights reserved.

**By reading and using the thesis, the reader understands and agrees to the following terms:**

1. The reader will abide by the rules and legal ordinances governing copyright regarding the use of the thesis.
2. The reader will use the thesis for the purpose of research or private study only and not for distribution or further reproduction or any other purpose.
3. The reader agrees to indemnify and hold the University harmless from and against any loss, damage, cost, liability or expenses arising from copyright infringement or unauthorized usage.

If you have reasons to believe that any materials in this thesis are deemed not suitable to be distributed in this form, or a copyright owner having difficulty with the material being included in our database, please contact [lbsys@polyu.edu.hk](mailto:lbsys@polyu.edu.hk) providing details. The Library will look into your claim and consider taking remedial action upon receipt of the written requests.

**The Hong Kong Polytechnic University**

**Department of Applied Biology and Chemistry Technology**

***A study of  $\text{Ca}^{2+}$ -ATPase in red cell aging***

**Thesis submitted to**

**Department of Applied Biology and Chemical Technology**

**In partial fulfillment of the requirement**

**For**

**The degree of Master of Philosophy of Science**

**by Lee Wai Yee**

**(July 1999)**



**Pao Yue-Kong Library  
PolyU • Hong Kong**

## **ACKNOWLEDGEMENT**

I would like to give gratitude to my supervisor, Dr. K. S. Lee for his guidance and advice throughout the study. I am grateful for support and encouragement from Dr. N. S. Wong, Mr. Y. L. Siu, B. L. Chan and C. Y. Chan. Lastly, I would be grateful for prayers from brothers and sisters of God's Family. Glory to God in the highest.

## LIST OF ABBREVIATIONS

$\text{Ca}^{2+}$ -ATPase	$\text{Ca}^{2+}$ -dependent adenosinetriphosphatase
PMCA	Plasma membrane $\text{Ca}^{2+}$ -ATPase
PMCAI	Plasma membrane $\text{Ca}^{2+}$ -ATPase protein inhibitor
<i>PMCA1</i>	Isoform 1 of plasma membrane $\text{Ca}^{2+}$ -ATPase
<i>hPMCA4</i>	Isoform 4 of human membrane $\text{Ca}^{2+}$ -ATPase
$V_{\text{max}}$	Maximum velocity
$K_m$	Michaelis constant
$K_a$	Activation constant
$K_i$	Inhibition constant
DTNB	5,5'-dithio-bis(2-nitrobenzoic acid)
EGTA	Ethyleneglycol-bis-( $\beta$ -amino-ethyl ether) $\text{N}'$ , $\text{N}'$ , $\text{N}'$ , $\text{N}'$ -tetraacetic acid
EDTA	Ethylenediamine tetraacetic acid
PK	Pyruvate kinase
LDH	Lactate dehydrogenase
PEP	Phosphoenolpyruvate
MCHC	Mean corpuscular hemoglobin concentration

## LIST OF TABLES

		Page
Table 1	ATPase activity of porcine erythrocyte membrane	49
Table 2	Age-markers of top 5% and bottom 5% erythrocytes	52
Table 3	Controlled tryptic proteolysis of membrane-bound $\text{Ca}^{2+}$ -ATPase in young and aged porcine erythrocytes	62
Table 4	Controlled calpain proteolysis of membrane-bound $\text{Ca}^{2+}$ -ATPase in young and aged porcine erythrocytes	64
Table 5	Controlled tryptic proteolysis of membrane-bound $\text{Ca}^{2+}$ -ATPase from fresh and outdated blood	81
Table 6	Controlled calpain proteolysis of membrane-bound $\text{Ca}^{2+}$ -ATPase from fresh and outdated blood	82
Table 7	Purification table of $\text{Ca}^{2+}$ -ATPase in young erythrocytes	88
Table 8	Purification table of $\text{Ca}^{2+}$ -ATPase in aged erythrocytes	90
Table 9	Purification table of $\text{Ca}^{2+}$ -ATPase in fresh blood	92
Table 10	Purification table of $\text{Ca}^{2+}$ -ATPase in outdated blood	95

## LIST OF FIGURES

	<b>Page</b>
Figure 1    Transport cycle of $\text{Ca}^{2+}$ -ATPase	8
Figure 2    Regulation of $\text{Ca}^{2+}$ -ATPase activity	17
Figure 3    Proteolysis model of $\text{Ca}^{2+}$ -ATPase	20
Figure 4    Proposed structural model of $\text{Ca}^{2+}$ -ATPase	23
Figure 5    Double-reciprocal plot: Response of $\text{Ca}^{2+}$ -ATPase in young and aged erythrocytes vs ATP	53
Figure 6    Double-reciprocal plot: Response of $\text{Ca}^{2+}$ -ATPase in young and aged erythrocytes towards calmodulin	55-56
Figure 7    Secondary plot: Response of $\text{Ca}^{2+}$ -ATPase in young and aged erythrocytes towards calmodulin	57
Figure 8    Double-reciprocal plot: Response of $\text{Ca}^{2+}$ -ATPase in young and aged erythrocytes towards oleic acid	59-60
Figure 9    Secondary plot: Response of $\text{Ca}^{2+}$ -ATPase in young and aged erythrocytes towards oleic acid	61
Figure 10   Elution profile of DEAE-Sephadex A-25 column chromatography for the purification of plasma membrane $\text{Ca}^{2+}$ -ATPase protein inhibitor (PMCAI)	66
Figure 11   Elution profile of DEAE-Superose column chromatography for the purification of plasma membrane $\text{Ca}^{2+}$ -ATPase protein inhibitor (PMCAI)	67
Figure 12   Double-reciprocal plot: Response of $\text{Ca}^{2+}$ -ATPase in young and aged erythrocytes towards PMCAI	69-70
Figure 13   Secondary plot: Response of $\text{Ca}^{2+}$ -ATPase in young and aged erythrocytes towards PMCAI	71
Figure 14   Double-reciprocal plot: Response of $\text{Ca}^{2+}$ -ATPase in fresh and outdated human blood vs ATP	73

	<b>Page</b>
Figure 15 Double-reciprocal plot: Response of $\text{Ca}^{2+}$ -ATPase in fresh and outdated human blood towards calmodulin	74-75
Figure 16 Secondary plot: Response of $\text{Ca}^{2+}$ -ATPase in fresh and outdated human blood towards calmodulin	76
Figure 17 Double-reciprocal plot: Response of $\text{Ca}^{2+}$ -ATPase in fresh and outdated human blood towards oleic acid	78-79
Figure 18 Secondary plot: Response of $\text{Ca}^{2+}$ -ATPase in fresh and outdated blood towards oleic acid	80
Figure 19 Double-reciprocal plot: Response of $\text{Ca}^{2+}$ -ATPase in fresh and outdated human blood towards PMCAI	84-85
Figure 20 Secondary plot: Response of $\text{Ca}^{2+}$ -ATPase in fresh and outdated human blood towards PMCAI	86
Figure 21 Affinity chromatography of Triton X-100 solubilized ghosts from young porcine erythrocytes on Sepharose 4B Calmodulin column	89
Figure 22 Affinity chromatography of Triton X-100 solubilized ghosts from aged porcine erythrocytes on Sepharose 4B Calmodulin column	91
Figure 23 Affinity chromatography of Triton X-100 solubilized ghosts from fresh human blood on Sepharose 4B Calmodulin column	93
Figure 24 Affinity chromatography of Triton X-100 solubilized ghosts from outdated human blood on Sepharose 4B Calmodulin column	96
Figure 25 SDS-PAGE electrophoresis gel (7.5%) of purified porcine $\text{Ca}^{2+}$ -ATPase	97
Figure 26 SDS-PAGE electrophoresis gel (7.5%) of purified human $\text{Ca}^{2+}$ -ATPase	98
Figure 27 $\text{Ca}^{2+}$ -ATPase in porcine erythrocyte ghost	100

	<b>Page</b>
Figure 28 $\text{Ca}^{2+}$ -ATPase in human erythrocyte ghost	101
Figure 29 Proposed mechanisms of increasing cytosolic calcium content during erythrocyte aging	111



**Abstract of thesis entitled 'A study of  $\text{Ca}^{2+}$ -ATPase in red cell aging' submitted by Lee Wai Yee for the degree of Master of Philosophy of Science at the Hong Kong Polytechnic University in July 1999**

Calcium homeostasis is important for cell functions. Elevation of intracellular calcium is commonly observed in aged erythrocytes. This change in calcium level may be due to alternation of  $\text{Ca}^{2+}$ -ATPase activity of erythrocyte. In this study, we looked into the changes of regulation of  $\text{Ca}^{2+}$ -ATPase during aging processes of erythrocyte.

Affinity of  $\text{Ca}^{2+}$ -ATPase towards ATP was found increased significantly with age while there was no significant difference between the maximal activities of the enzyme. In addition, response of the enzyme towards protein inhibitor, i.e. PMCAI was found largely increased whereas there was a decrease in affinity towards oleic acid in aged erythrocytes. The response and affinity of the enzyme towards calmodulin was found unchanged during aging. It was also found that activation effect of controlled proteolysis on  $\text{Ca}^{2+}$ -ATPase in aged erythrocyte was higher. In erythrocyte upon storage, similar trend of changes of regulation of  $\text{Ca}^{2+}$ -ATPase was observed.

The notion that  $\text{Ca}^{2+}$ -ATPase has undergone changes during aging in properties and structure was further illustrated in the immunoblotting study. More 40kDa fragment was found in aged erythrocyte. The pattern of proteolysis was observed to be different in out-dated bank blood too.

From our findings, we propose the mechanism of erythrocyte aging as follows. During erythrocyte aging, membrane barrier against calcium is gradually loosened. The response of  $\text{Ca}^{2+}$ -ATPase towards PMCAI is much increased while towards calmodulin does not change much. This leads to a decrease in capacity of the enzyme to restore intracellular calcium to original level. In addition, lipid content of cell also decreases during aging, thus availability of acid phospholipids to the enzyme may decrease. The elevated level of calcium also increases the activity of calpain and other proteases that act on various other proteins including  $\text{Ca}^{2+}$ -ATPase. The ATP-binding site of the enzyme becomes more exposed with gradual increase in susceptible sites for further proteolysis. Finally, extensive proteolysis causes the permanent inactivation of the  $\text{Ca}^{2+}$ -ATPase and the lost of calcium pump capacity. All factors of above may act together that account for the significant decrease in  $\text{Ca}^{2+}$ -ATPase activity and the increased in cell calcium observed in aged and outdated storage erythrocyte.

# TABLE OF CONTENT

	Page
Acknowledgements	
List of Abbreviations	
List of Tables	
List of Figures	
Abstract	
 Introduction	
1. Calcium homeostasis in the body	1
2. Plasma membrane $\text{Ca}^{2+}$ -ATPase	5
2.1 General properties of the pump	
2.2 Regulation of the enzyme activity	
2.2.1 <i>Calmodulin</i>	
2.2.2 <i>Lipids</i>	
2.2.3 <i>Proteolysis</i>	
2.2.4 <i>Endogenous protein inhibitor (PMCAI)</i>	
2.3 Isolation of the $\text{Ca}^{2+}$ -ATPase	
2.4 Proteolysis of the pump	
2.5 Functional domain of the pump	
2.6 Topology of the pump	
2.7 Isoform	
3. Study of aging process	26
3.1 Phenomena during erythrocyte aging	
3.2 Hypotheses of <i>in vivo</i> erythrocyte aging	
3.3 Aging and calcium	
 Methodology	36
Results	47
Discussion	102
Conclusion	115
References	116

# INTRODUCTION

## 1. Calcium homeostasis in the body

Calcium is important in mammalian body. It plays as an essential role in the function of cells and exerts a profound influence on many biological processes (Clapham, 1995; McCormack & Denton, 1988). It triggers activity of many enzyme systems in the cells, including kinases and phosphatases (Williamson & Cooper, 1980). Calcium also takes part in numerous processes in the body, e.g. muscle contraction, blood clotting, neurotransmitter release and microtubule formation etc (Farago & Nishizuca, 1990).

Since calcium is essential in cell, it is ubiquitous in the body. Majority of it is immobilized in bones and teeth in the form of hydroxyapatite  $[\text{Ca}_{10}(\text{PO}_4)_6(\text{OH})_2]$  while negligible amount of it can be found in extracellular and intracellular compartments (Carafoli, 1987, Borle, 1981). The influx and outflux of calcium ions from the bone controls calcium concentration in the extracellular fluid, including that in blood plasma. Thus, calcium content in the extracellular compartment is maintained at narrow range with approximately half of it in the form of ionized calcium ions. This ensures that a constant source of calcium can be available to cell.

Unlike many other secondary messenger molecules, calcium ions available to cell cannot be metabolized. Cell should tightly regulate the level of intracellular calcium ions through numerous systems (Clapham, 1995; Strynadka & James, 1988;

Carafoli, 1987; Blaustein & Nelson, 1982; Borle, 1981). In a cell, calcium ions can be complexed rapidly and reversibly by non-membranous anionic ligands and calcium-binding proteins, like troponin C and calbindin. The ions can also be temporarily sequestered by two intracellular calcium ion buffering organelles, i.e. mitochondrion and endoplasmic reticulum. In addition, there is also movement of calcium ions across the plasma membrane, so that calcium level can be maintained at a narrow range (Carafoli, 1987).

The plasma membrane has very limited and carefully controlled calcium permeability. In extracellular compartment of the body, free calcium ion concentration, of approximately 1.5mM, is very high when compared with that in the intracellular fluid, that is about  $0.1 \mu\text{M}$  (Carafoli & Zurini, 1982). This creates a very large inwardly directed calcium ion gradient across the plasma membrane. In the presence of such large electrochemical force, even very minor change of calcium permeability of the membrane would result in significant fluctuation in the cytosolic calcium concentration.

Though calcium is essential for cell growth and survival, uncontrolled influx of calcium ions would affect function of cell. Since many mammalian proteins, like troponin C, protein kinase C and parvalbumin, are triggered by calcium ions, fluctuation in cytosolic calcium ions may affect the modulation of calcium targets. Prolonged high calcium ion concentration would also lead to cleavage of DNA and chromatin by nucleases. This results in loss of structural integrity of chromatin and

affects cell cycle.

In addition, uncontrolled influx of intracellular calcium ions into erythrocyte would lead to cell death. There had been reports on the accumulation of cytosolic calcium ions during the early event in erythrocyte pathology, such as sickle cell anemia, thalassemia, hereditary spherocytosis and hemolytic anemia (Friederichs & Melselman, 1994; Damonte *et al*, 1992). Thus, much work has been focused on the disturbance of calcium homeostasis in erythrocyte (Schatzmann & Burgin, 1978). A slight elevation in intracellular calcium concentration would lead to a marked increase in potassium permeability of the plasma membrane. This would result in rapid potassium loss. If the loss would not be compensated by sodium influx, this would lead to osmotic water loss of the cell. The cell viscosity would then be increased. The continuous increase in calcium ions would lead to protrusion of teat-like evagination from the membrane and result in disc-shape transformation of cell. It is associated with cross-linking of nearest spectrin neighbors through disulfide couplings or formation of diacylglycerol in the inner half of cell membrane catalyzed by  $\text{Ca}^{2+}$ -activated cytoplasmic transglutaminase (Palek, 1978; Weed *et al*, 1969). The cell would lose its deformability and thus filter less rapidly through narrow blood vessel. Very high calcium concentration in the cytosol would block  $\text{Na}^+\text{-K}^+$  pump (Schatzmann & Vincenzi, 1969). In this case, the  $\text{Na}^+\text{/K}^+$  gradient across the membrane created by the pump could not be maintained. This would also affect the osmotic balance of the erythrocyte, leading to reduction in cell volume, an increase

in cell viscosity and ultimate hemolysis. Due to the physiological effects caused by disturbance of cell homeostasis, erythrocyte should be equipped with systems that can maintain calcium ion gradient across the plasma membrane.

Calcium ion gradient across plasma membrane can be maintained by the result of concerted operation of importing and exporting system of calcium on the plasma membrane, i.e.  $\text{Ca}^{2+}$  channel,  $\text{Ca}^{2+}$  pump and  $\text{Na}^+$ - $\text{Ca}^{2+}$  exchanger (Carafoli, 1991; Carafoli, 1987; Blaustein & Nelson, 1982).  $\text{Ca}^{2+}$  channel is gated by electrical potential across the plasma membrane. It is responsible for transporting calcium ions from extracellular fluid into the cell. In addition,  $\text{Na}^+$ /  $\text{Ca}^{2+}$  exchanger is a large capacity, low affinity carrier for calcium ions. Its maximal transport capacity is about 100 times greater than  $\text{Ca}^{2+}$  pump. It is particularly active in excitable plasma membranes and is responsible for the bulk ejection of calcium demanded by the functional cycle. It was found that erythrocyte may be the only cell where the exchanger is completely absent (Carafoli, 1987). On the other hand,  $\text{Ca}^{2+}$  pump, i.e.  $\text{Ca}^{2+}$ -dependent adenosinetriphosphatase (ATPase), is a low capacity, high affinity system for calcium ions. It is ubiquitous in eukaryotes. The high affinity of the enzyme for calcium enables the enzyme to satisfy the demand for fine-tuning of intracellular calcium concentration. Both  $\text{Na}^+$ - $\text{Ca}^{2+}$  exchanger and  $\text{Ca}^{2+}$  pump work in parallel with different relative efficiency in different cells.

As stated, even slight fluctuation of calcium would affect biological function of cells, study of fine-tuning of intracellular calcium by calcium pump would be

essential and valuable. Thus, much work has been focused on studying calcium pump on plasma membrane.

## **2. Plasma membrane $\text{Ca}^{2+}$ -ATPase**

The study of  $\text{Ca}^{2+}$ -ATPase has been started since 1960s. Dunham and Glynn first reported the existence of  $\text{Ca}^{2+}$ -ATPase in erythrocyte membrane in 1961 (Dunham & Glynn, 1961). Schatzmann then connected it to the pumping of calcium ion out of the cell in 1966 (Schatzmann, 1966). Since then, erythrocyte has been commonly used as a model for the study of  $\text{Ca}^{2+}$ -ATPase. Purification of the enzyme to homogenous forms and in a functionally active state from erythrocyte has been succeeded. Many researchers had concisely described the general properties of  $\text{Ca}^{2+}$ -ATPase (Carafoli, 1992, Carafoli, 1991). In recent years, particular attention has been put on the molecular properties of the enzyme (Carafoli *et al*, 1996).

### **2.1 General properties of the pump**

General properties of calcium pump have concisely described by many researchers (Carafoli, 1992; Carafoli, 1991).  $\text{Ca}^{2+}$ -ATPase is ubiquitous in plasma membrane of eukaryotic cells (Schatzmann, 1982). It can be found in erythrocyte, skeletal muscle, heart, kidney tubules, nervous cells etc. The properties of  $\text{Ca}^{2+}$ -ATPase are essentially similar in all plasma membranes. However, subtle differences may exist. Calcium pump has an apparent molecular weight of 134kDa (Carafoli,



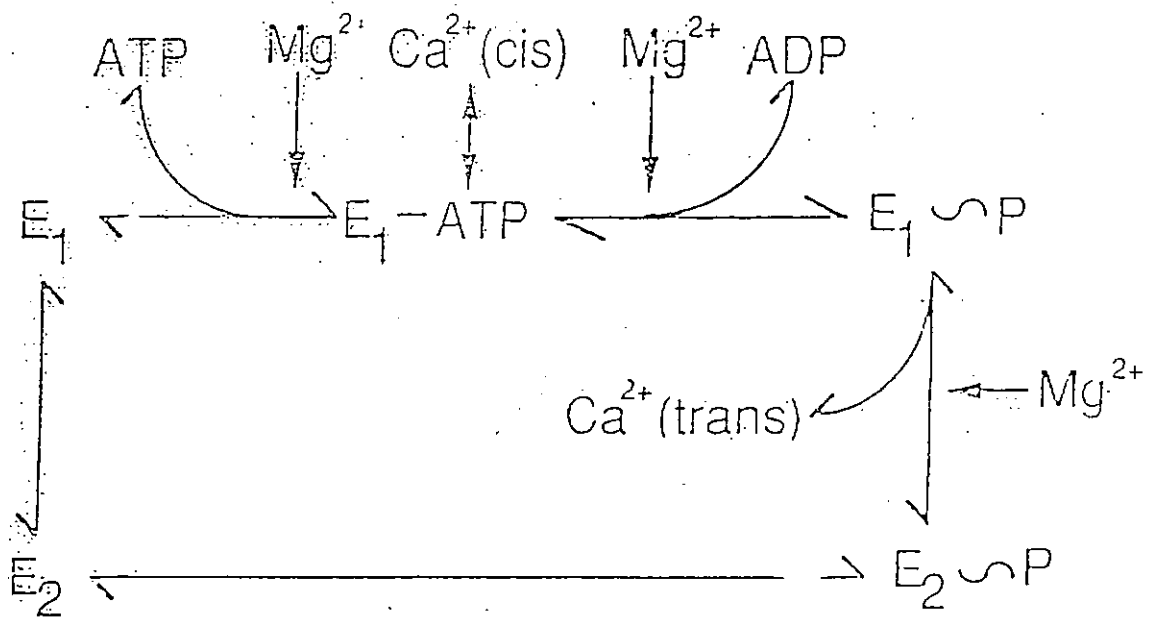
1992). It is of high affinity but low total capacity for calcium ions. It transports about 0.5nmol of calcium ions per mg of membrane protein per second (Caroni & Carafoli, 1981). Two ATP affinity sites are found in each calcium pump molecule. The high ATP affinity site is assumed to be the catalytic site of the enzyme with  $K_m$  for ATP between 1 and  $2.5 \mu\text{M}$ . Decomposition of phosphorylated intermediate is assumed to be related to the low affinity site which has  $K_m$  between 145 and  $180 \mu\text{M}$  (Richards, 1978).

$\text{Ca}^{2+}$ -ATPase is a P-class ATPase. It has typical properties that phosphorylates on an aspartate residue during the transport cycle and is inhibited by orthovanadate  $[\text{VO}_3(\text{OH})]^{2-}$  (Carafoli, 1992; Varecka & Carafoli, 1982; Rossi *et al*, 1981; Barrabin *et al*, 1980). The transport cycle of  $\text{Ca}^{2+}$ -ATPase begins with calcium-dependent transfer of terminal phosphate from ATP to the enzyme, with the formation of phosphorylated intermediate.

$\text{Ca}^{2+}$ -ATPase exists in two different states,  $E_1$  and  $E_2$ , corresponding to two different conformations of the enzyme (Carafoli, 1991; Carafoli & Zurni, 1982; Roufogalis, 1979). The formation of  $E_1\sim\text{P}$  is endergonic, however, the reaction is favorably towards the formation of  $E_2 + \text{P}$  as the reaction of  $E_2\sim\text{P}$  to  $E_2 + \text{P}_i$  is exergonic. The conformational transition from  $E_1\sim\text{P}$  to  $E_2\sim\text{P}$  is promoted by magnesium ion and its hydrolysis to  $E_2$  and  $\text{P}_i$  is assumed to be accelerated by the binding of ATP to the low affinity site of ATP of the enzyme (Garrahan & Rega, 1978). In the last step of the reaction cycle, the dephosphorylated  $E_2$  conformer

reverts back to the starting  $E_1$  conformer. The reaction scheme for plasma membrane  $\text{Ca}^{2+}$ -ATPase is shown in Figure 1 (Carafoli, 1991). Inhibition of the enzyme occurs when vanadate interacts with the  $E_2$  conformer, blocking the last step of the reaction cycle, i.e.  $E_2$  to  $E_1$  transition (Barrabin *et al*, 1980). Another inhibitor, lanthanum ( $\text{La}^{3+}$ ) blocks the transition between  $E_1\sim\text{P}$  and  $E_2\sim\text{P}$  (Luterbacher & Schatzmann, 1983). One point should be noticed that the translocation process of calcium ion is assumed to be related to the conformational change of the pump, i.e. the  $E_1\sim\text{P}$  to  $E_2\sim\text{P}$  transformation. Thus, the  $\text{Ca}^{2+}$ -binding site located on the interior side of the membrane will be converted to the external side of plasma membrane after the translocation step.

In calcium extrusion mechanism,  $\text{Ca}^{2+}$ -ATPase actually exists in three different states, i.e. A,  $B_1$  and  $B_2$  of which the activity of the enzyme is tuned up and down from one state to another (Al-Jobore & Roufogalis, 1981; Scharff & Foder, 1977). The A state may represent to the resting state of the enzyme while  $B_1$  and  $B_2$  states are the active enzyme. Actually, the shift between A state and  $B_1$  or  $B_2$  states of the pump is reversible. The A state is induced by exposing the enzyme to low calcium ion concentrations or by the presence of chelators in the medium used during the preparation of plasma membranes. It is characterized by its low calcium affinity and transport rate. In contrast, the  $B_1$  and  $B_2$  states are of high  $\text{Ca}^{2+}$  affinity and transport rate and are induced by the presence of micromolar calcium ions.  $V_{\text{max}}$  and calcium



**Figure 1** Transport cycle of  $\text{Ca}^{2+}$ -ATPase

$\text{Ca}^{2+}$ -translocating step is  $E_1P - E_2P$  transition (Carafoli, 1991)

affinity of B<sub>1</sub> and B<sub>2</sub> states can be regulated independently. The former is the same for both B<sub>1</sub> and B<sub>2</sub> states whereas the latter is higher for B<sub>2</sub> state.

Calcium pump is a regulatory enzyme. It is regulated by many factors in the body. The study of its regulation would be of great importance in order to help us to understand more of calcium extrusion mechanism.

## **2.2 Regulation of the enzyme activity**

Numerous factors are found to have stimulatory or inhibitory effects on Ca<sup>2+</sup>-ATPase (Sackett & Kosk-Kosicka, 1996; Carafoli, 1991; Kosk-Kosicka *et al*, 1989; Leclerc *et al*, 1988; Kosk-Kosicka & Bzdega, 1988; Iglesias & Rega, 1987; Niggli *et al*, 1982). Ca<sup>2+</sup>-ATPase is stimulated by calmodulin. Alternative treatments exhibit stimulation of the enzyme in the absence of calmodulin, including exposing the enzyme to acidic phospholipid and polyunsaturated fatty acid, proteolytic treatment, phosphorylation by protein kinase A or C and induction of an oligomeric state of the enzyme. This stimulation is predominately K<sub>m</sub> effect. Besides, there had been reports on inhibition by an endogenous protein inhibitor called PMCAI and other chemicals. These factors may interact with each other and control Ca<sup>2+</sup>-ATPase activity.

### **2.2.1 Calmodulin**

Calmodulin is one of the factors that have been studied for many years. Bond and Clough first observed stimulatory effect of calmodulin on Ca<sup>2+</sup>-ATPase in 1973 (Bond & Clough, 1973). They found that a soluble protein in the human erythrocyte activated human membrane Ca<sup>2+</sup>-ATPase while it exerted no effect on other ATPases. The soluble protein was purified and was confirmed as calmodulin because it shared many properties of calmodulin. Calmodulin is a single polypeptide with molecular weight of 16.7kDa and pI=4.2. It contains four homologous domains, each

containing helix-loop-helix structure that binds one calcium ion with dissociation constant ranging from 4 to 18  $\mu$  M. It is mostly found in brain, lung, kidney, liver, uterus and erythrocyte in micromolar concentration (Scharff, 1981).

Calmodulin stimulates  $\text{Ca}^{2+}$ -ATPase by direct interaction (Vorherr *et al*, 1992; O'Neil & Degrado, 1990; Agre *et al*, 1983; Vincenzi *et al*, 1980). Calmodulin itself is not active. Its active form is calmodulin- $\text{Ca}^{2+}$  complex (Cheung, 1980). The complex interacts with regulatory site of the enzyme, i.e. the calmodulin-binding domain, and changes the conformation of the pump. Actually, calmodulin requires calcium ion to increase its hydrophobicity. This makes it more accessible to active site of the enzyme. Once they interact, additional calcium ions may activate the enzyme directly by binding to the catalytic site of the enzyme as substrate. As a result of the interaction, the enzyme tends to switch from resting A state to the activated B state (Roufogalis & Mauldin, 1980; Scharff & Foder, 1978). The binding of calmodulin- $\text{Ca}^{2+}$  complex to A state of the enzyme may cause a redistribution of electric charges that leads to transition of B state (Scharff, 1981). This results in an increase affinity for calcium ion and maximal transport rate, with the former change being more prominent. The transition of state B to state A can be induced by removal

of calmodulin. In the presence of calmodulin,  $K_m$  ( $\text{Ca}^{2+}$ ) of the ATPase is decreased from  $20 \mu\text{M}$  to about  $0.5 \mu\text{M}$ . One point should be noticed that optimum saturation of the pump with calmodulin occurs only at calcium concentration (above  $10^{-6}\text{M}$ ) which is higher than that in cytosol in physiological state ( $\sim 10^{-7}\text{M}$ ) (Scharff, 1981). This decreases the efficiency of calmodulin interaction with the pump (Carafoli, 1991; Scharff, 1981; Roufogalis & Mauldin, 1980). Thus, other factors together with calmodulin may interact with calcium pump in order to attain optimum activation.

### 2.2.2 Lipids

Lipids may be one of these stimulatory factors.  $\text{Ca}^{2+}$ -ATPase is activated by acidic phospholipid or polyunsaturated fatty acid. The observation of sensitivity of  $\text{Ca}^{2+}$ -ATPase to lipid environments was shown by inactivation of the pump by phospholipase digestion. Activity of  $\text{Ca}^{2+}$ -ATPase was reactivated in the presence of acidic phospholipid or oleic acid (Ronner *et al*, 1977). Some researchers found that all acidic phospholipids and long chain polyunsaturated fatty acid could activate solubilized ATPase very efficiently while no stimulatory response was observed for neutral phospholipids (Wetzker *et al*, 1983; Carafoli & Zurini, 1982; Ronner *et al*, 1977). The stimulation of  $\text{Ca}^{2+}$ -ATPase by phospholipids and unsaturated fatty acid induce the transition of A state to  $B_2$  state or from  $B_1$  to  $B_2$  state and is predominantly a  $K_m$  effect (Enyedi *et al*, 1987; Carafoli & Zurini, 1982). Acidic phospholipid induces conformational change near the active site of the enzyme and increases its accessibility for calcium.

Some researchers found that acidic phospholipid was more effective than calmodulin for stimulating enzyme activity (Enyedi *et al*, 1987). It decreased  $K_m$  ( $Ca^{2+}$ ) to values lower than calmodulin did. Some of the reports mentioned that acidic phospholipid and polyunsaturated fatty acid may interact with calmodulin-binding domain of the enzyme (Brodin *et al*, 1992; Wetzker *et al*, 1983). This was supported by the observation that the enzyme was insensitive to calmodulin after purification in the presence of phosphatidylserine. It was not due to a state of permanent inhibition of the enzyme, but optimal activation had already been achieved in the absence of calmodulin (Carafoli & Zurini, 1982).

The regulation of the pump by acidic phospholipid is significant *in vivo*. In contrast to calmodulin, the amount of phosphatidylserine surrounding the membrane-bound ATPase is adequate to support about 50% of maximal activation. They are present in the inner leaflet of lipid bilayer where active si

### 2.2.3 Proteolysis :

$\text{Ca}^{2+}$ -ATPase can be activated by another treatment other than calmodulin and lipids, i.e. controlled treatment with trypsin. Activation was achieved by exposing the reconstituted enzyme to variable amounts of trypsin for a fixed time. Controlled trypsin proteolysis immediately produces a transient calmodulin-sensitive 90kDa fragment that is then rapidly degraded to two calmodulin-independent, highly active species of molecular size 81kDa and 76kDa. The proteolysis treatment results in loss of increasing C-terminal portion of the enzyme, which protrudes from the membrane into cytoplasm and contains the calmodulin-binding domain (Zurini, 1990). The pump progressively loses its response to calmodulin and it becomes permanently activated, independent of calmodulin. Thus, trypsin mimics the effect of calmodulin and the activated enzyme is no longer activated by further calmodulin treatment (Rossi & Schatzmann, 1982). This results in increase of calcium affinity and  $V_{\max}$ . Trypsin also removes N-terminal portion of the enzyme which is involved in acidic phospholipid response (Zurini, 1990). This portion has a strong basic character in its C-terminal half. The proteolytic treatment of the enzyme by trypsin is very useful for mapping of functionally domains of this enzyme molecule, however, it has no physiological meaning.

In contrast, effect of intracellular  $\text{Ca}^{2+}$ -dependent protease, i.e. calpain, is likely to be significant *in vivo* (Croall & Demartino, 1991; Wang *et al*, 1988). Calpain is a thio endoprotease, which is distributed in various locations in the body (Croall &



Demartino, 1991). It has a molecular weight of 110kDa, consisting of two non-identical subunits with 80kDa and 30kDa. Calcium ion concentration and an endogenous calpain inhibitor called calpastatin control the activity of calpain (Kawasaki *et al*, 1993; Kapprell & Goll, 1989; Melloni *et al*, 1982). In cell body, calpain is inactive at physiological intracellular calcium concentration and exists in a form of inactive proenzyme.

Conversion of inactive proenzyme to active calpain is a prerequisite step for the expression of proteolytic activity of the protease (Croall & Demartino, 1991; Suzuki *et al*, 1987; Pontremoli & Melloni, 1985). The regulatory subunit (30kDa) of the protease can be readily dissociated from the catalytic subunit (80kDa) in the presence of calcium ion. The 80kDa subunit exists in inactive proenzyme form containing catalytic and calcium binding sites. In the presence of calcium, the 80kDa proenzyme is transformed to a 75kDa species via autolysis, which is the active form of calpain. Actually, calpain is activated at calcium concentration higher than  $10\ \mu\text{M}$ . Intracellular calcium concentration must be increased to this level in the cytosol or near the plasma membrane for the function of calpain *in vivo*. In addition, there had been report that the autolysis of calpain may be regulated by a combination of calmodulin and phosphatidylinositol (Coolican & Hathaway, 1984). The active calpain is preferentially targeted to the carboxyl side of arginine residue in a protein, provided that the residue adjacent to arginine on the amino-terminal side is a hydrophobic amino acid like leucine. In  $\text{Ca}^{2+}$ -ATPase, this is possibly the

calmodulin-binding domain. Proteolytic effect of calpain increases the basal activity of calcium pump by reducing the enzyme to a fragment of about 124kDa, but the action is slower than that of extracellular proteases like trypsin. The reduction in size results from two-step removal of C-terminal portion of the pump that contains the calmodulin-binding domain (James *et al*, 1989). Consequently, this will eliminate the calmodulin reponsiveness.

#### **2.2.4 Endogenous protein inhibitor (PMCAI):**

Beside of the activator mentioned before, an endogenous protein inhibitor exerts inhibitory effect on calcium pump. An endogenous protein inhibitor of erythrocyte membrane  $\text{Ca}^{2+}$ -ATPase (PMCAI) was first isolated from pig membrane-free hemolysate by Au in 1978 (Au, 1978a). Several researchers have tried to purify it but they failed to obtain it in pure form (Au, 1996; Lee & Au, 1983; Wuthrich, 1982; Au & Lee, 1980; Au, 1978b). Au successfully achieved purifying PMCAI to homogeneity in 1996. The PMCAI was isolated by anion-exchange chromatography followed by  $\text{Ca}^{2+}$ -ATPase-Sepharose affinity column.

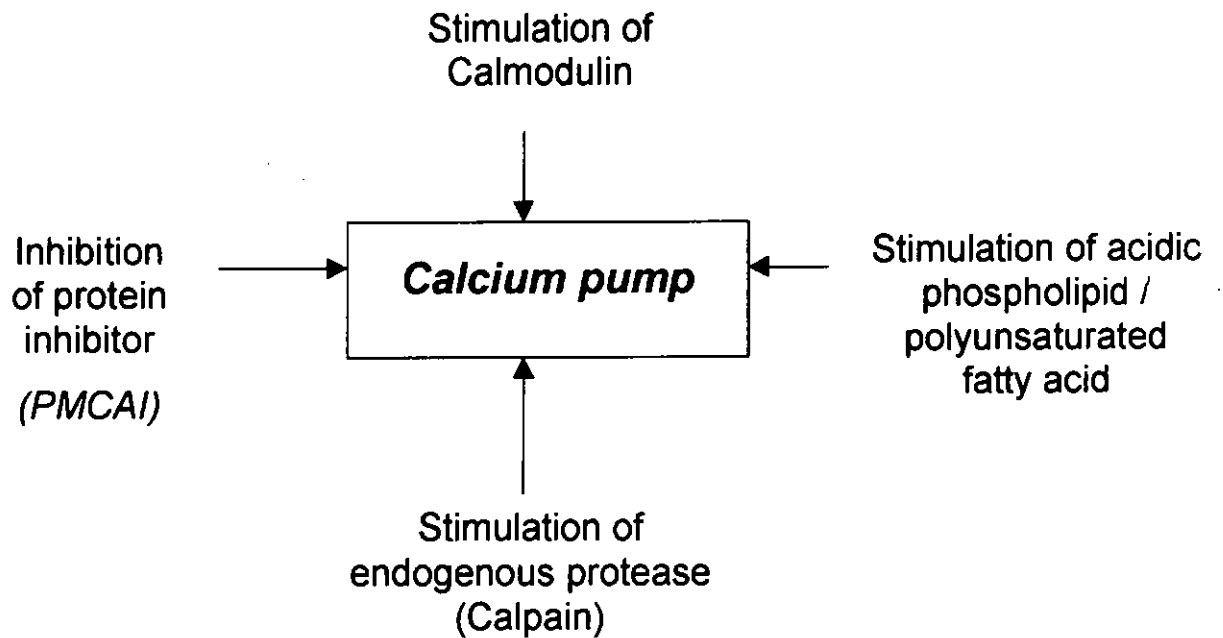
The PMCAI is protein in nature. It is susceptible to inactivation by trypsin, heat and lypophilization. It has a molecular weight of 7500Da with  $\text{pI}=4.8$  (Lee & Au, 1983). It inhibits  $\text{Ca}^{2+}$ -pump but exert no effect on  $\text{Mg}^{2+}$ -ATPase,  $(\text{Na}^{+}+\text{K}^{+})$ -ATPase and acetylcholinesterase. It is not a general protease. PMCAI is also not a calmodulin-binding protein as it exerts inhibition of the pump even with calmodulin-free membrane. The mode of inhibition of  $\text{Ca}^{2+}$ -ATPase by PMCAI is non-

competitive that it decreases  $V_{\max}$  of the pump while exerts no effect on  $K_m$  (ATP) (Au, 1996; Lee & Au, 1983). The interaction of PMCAI with the pump is still not clear. It was found that the inhibitor-binding site was different from the calmodulin-binding site. It has been postulated that in the presence of calcium, PMCAI exposes a hydrophobic patch, which allows it to interact with  $\text{Ca}^{2+}$ -ATPase at a site downstream of ion transport site (Au, unpublished data).

As stated, calcium pump is subjected to regulation by many factors. The interaction of these factors with calcium pump is summarized in Figure 2. These factors may interact with each other to exert rapid response to slight fluctuation of calcium concentration inside the cell.

### **2.3 Isolation of the $\text{Ca}^{2+}$ -ATPase**

Apart from studying the regulation of calcium pump, the study of the enzyme itself is also important. Thus, pure form of the enzyme is needed. Many researchers have attempted to purify  $\text{Ca}^{2+}$ -ATPase to homogeneity (Carafoli & Zurini, 1982; Gietzen & Kollandt, 1982; Niggli *et al*, 1979). However, difficulties were encountered in early attempts. The  $\text{Ca}^{2+}$ -ATPase is present in the plasma membrane in minute amounts and it tends to be co-purified with band III protein that is more abundant. The calcium pump is very labile in solubilized state and it is only active in appropriate phospholipid environment. The problem of lability can be solved by stabilizing the enzyme in the presence of detergent and it can be reactivated by a



**Figure 2 Regulation of  $\text{Ca}^{2+}$ -ATPase activity**

$\text{Ca}^{2+}$ -ATPase activity is regulated by several factors. These factors may interact with each other to exert rapid response to slight fluctuation of calcium concentration inside the cell

number of acidic phospholipid and long chain fatty acid (Ronner *et al*, 1977).

The observation that calmodulin-stimulated  $\text{Ca}^{2+}$ -ATPase provided the rationale for successful use of a calmodulin-affinity column for the purification of the enzyme (Niggli *et al*, 1979). The application of calmodulin-affinity column overcomes the difficulties during purification and yields a purified and functional enzyme at the end of a relatively rapid procedure. Detergent in the presence of phospholipid first solubilizes the calmodulin-free membrane-bound  $\text{Ca}^{2+}$ -ATPase. The solubilized enzyme is then loaded onto the column. Washing the column with calcium eluted majority of the protein applied and a large amount of ATPase activity. It is referred to as  $\text{Mg}^{2+}$ -ATPase, which does not require calcium and is calmodulin insensitive. The success of purification is based on different calmodulin affinities of various membrane protein, provided that the interaction of the enzyme with calmodulin is direct and not mediated by non-specific membrane phenomenon or by other protein components in the membrane. Thus, only  $\text{Ca}^{2+}$ -ATPase is eluted at the final EDTA elution step. Therefore, calmodulin-affinity column becomes a convenient means to purify  $\text{Ca}^{2+}$ -pump first from erythrocytes and then from other plasma membranes. This helps for the cloning of genes for calcium pump.

## **2.4 Proteolysis of the pump**

In order to obtain much information for the domain of calcium pump, controlled proteolysis of purified  $\text{Ca}^{2+}$ -ATPase can be used. Controlled proteolysis of the

purified erythrocyte  $\text{Ca}^{2+}$ -ATPase by trypsin produces the bands of 124kDa and following by fragments of 85 or 81kDa and 33.5kDa. Further tryptic proteolysis gives rise to 28kDa and 48kDa fragments. The model of proteolysis is summarized in Figure 3 (Zurini *et al*, 1984).

The model of proteolysis can be explained as follows. Early step of proteolysis results in changes in calmodulin response of the pump. In the transition from 90kDa to 81kDa fragment, the response of the fragment to calmodulin lose. This indicates that the fragment of ~9000 is calmodulin-interacting domain. The 85kDa-fragment, obtained during the transition from 90kDa to 81kDa fragments, binds to calmodulin but it is less responsive to calmodulin (Carafoli, 1991). In addition, the 81kDa-fragment has higher activity even in the absence of calmodulin. Therefore, calmodulin-binding domain was cut by two steps. The first cut is in the middle of the site. After prolonged digestion, a second cut is occurred at N-terminus of the domain, leading to a loss of calmodulin sensitivity and irreversible activation of the pump. The calmodulin-binding domain acts as an inhibitory polypeptide (Carafoli *et al*, 1992; Falchetto, 1991). It binds to a domain in the pump that is essential for full activation. The removal of it then increases the basal activity of the pump in the presence of acidic phospholipid.

Information of other functional domains is also obtained from the model of proteolysis of the calcium pump. The 48kDa-fragment derived from 76kDa-fragment is suggested to be the ATP-binding site as it can bind ATP analogue. On the

other hand, the 33.5kDa-fragment obtained by degradation of 35kDa- fragment interacts with hydrophobic probe and may contain hydrophobic intramembrane regions of the pump. The region may be at some distance from calmodulin-binding protrudes from TM domain 4 and it contains active site of the pump, i.e. the phosphorylation site and the ATP-binding domain. The last one protrudes from the last domain, which is the regulation site of the pump. It contains calmodulin-binding domain on its NH<sub>2</sub>-terminal side and is responsive to acidic phospholipid.

## **2.5 Functional domains of the pump**

Referring to the information of proteolysis of calcium pump, functional domains of the enzyme have been clearly put out. There are three important functional domains in Ca<sup>2+</sup>-ATPase, i.e. ATP- binding domain, the domain surrounding the apartylphosphate and the calmodulin-binding domain (Carafoli *et al*, 1992; Carafoli, 1991; Zurini, 1990). The calmodulin-binding domain has been identified using bifunctional, photoactivable and cleavable cross-linker coupled with calmodulin. The pump becomes labeled in a domain containing sequence predominance of basic amino acid. The domain has a tryptophan next to N-terminus and has high propensity to form amphiphilic helix. These are features common to putative calmodulin-binding domain of other calmodulin-modulated proteins. The propensity to form amphiphilic helix is particularly strong in the 5-6 COOH- and NH<sub>2</sub>-terminal residues of the domain. In the mid-portion, the domain tends to form  $\beta$ -sheet with a  $\beta$ -turn in the

middle separating the two halves of the domain. The minimal length compatible with adequate calmodulin affinity is about 20 residues. These residues are located C-terminally and interact with C-terminal calcium-binding lobe of calmodulin. Tryptophan in N-terminal portion together with phenylalanine next to it is required for optimal interaction with calmodulin (Vorherr *et al*, 1992). N-terminal portion of the calmodulin-binding domain of the pump interacts with the C-terminal portion of calmodulin while C-terminal portion of the domain interacts with the N-terminal portion of calmodulin.

The ATP binding domain and the phosphorylation domain share the similarity with that of other P-type ATPases (Filoteo, 1987; James, 1987). The three central residues of the domain surrounding the ATP binding domain is conserved in all P-type ion-motive domain. The sequence of phosphorylation domain also has canonical sequence in all P-type ATPases. In addition, there is a “hinge” domain connecting these two domains, permitting them to come close in space during the functional cycle. The information of functional domains of the enzyme can help for better understanding of the enzyme.

## **2.6 Topology of the pump**

After getting information of functional domains of the enzyme by controlled proteolysis, it helps us to look into the spatial arrangement of the domains. The calcium pump is organized in the membrane as the suggested topography of all other



P-type ATPases. The membrane topography of the pump is shown in Figure 4 (Carafoli *et al*, 1992). There are ten transmembrane TM domains and four main units protruding into cytoplasm. The first protruding unit is the N-terminal portion of the pump and the second is  $\beta$ -strand domain protruding from TM domain 2. The function of the third and the fourth units are known. The third unit protrudes from TM domain 4 and it contains active site of the pump, i.e. the phosphorylation site and the ATP-binding domain. The last one protrudes from the last domain, which is the regulation site of the pump. It contains calmodulin-binding domain, phosphorylation site for protein kinase A and protein kinase C, and calcium-binding site. Apart from the calmodulin-binding site, there is also a basic stretch located in the second cytosolic unit of which acidic phospholipid interacts. The calmodulin-binding domain also interacts with the second and third protruding units. The domains fold over the main body of the pump and the pump becomes inhibited in the absence of calmodulin (Falchetto, 1991). Thus, the interaction hinders free access of substrates to the active site of the pump. There is also a catalytic calcium-binding site apart from that in protruding unit 4 and it may be present in the TM portion of the molecule. The site may involve in channeling calcium ion through the membrane (Hofmann *et al*, 1993; Carafoli *et al*, 1992).

## 2.7 Isoform

Calcium pumps are essentially similar in all plasma membrane. They have

P-type ATPases. The membrane topography of the pump is shown in Figure 4 (Carafoli *et al*, 1992). There are ten transmembrane TM domains and four main units protruding into cytoplasm. The first protruding unit is the N-terminal portion of the pump and the second is  $\beta$ -strand domain protruding from TM domain 2. The function of the third and the fourth units are known. The third unit protrudes from TM domain 4 and it contains active site of the pump, i.e. the phosphorylation site and the ATP-binding domain. The last one protrudes from the last domain, which is the regulation site of the pump. It contains calmodulin-binding domain, phosphorylation site for protein kinase A and protein kinase C, and calcium-binding site. Apart from the calmodulin-binding site, there is also a basic stretch located in the second cytosolic unit of which acidic phospholipid interacts. The calmodulin-binding domain also interacts with the second and third protruding units. The domains fold over the main body of the pump and the pump becomes inhibited in the absence of calmodulin (Falchetto, 1991). Thus, the interaction hinders free access of substrates to the active site of the pump. There is also a catalytic calcium-binding site apart from that in protruding unit 4 and it may be present in the TM portion of the molecule. The site may involve in channeling calcium ion through the membrane (Hofmann *et al*, 1993; Carafoli *et al*, 1992).

## **2.7 Isoform**

Calcium pumps are essentially similar in all plasma membrane. They have

similar topography and functional domains, however, they may be of high isoform diversity.  $\text{Ca}^{2+}$ -ATPase has high isoform diversity generated from a multigene family comprising mammalian genes coding for plasma membrane  $\text{Ca}^{2+}$ -ATPase (PMCA) isoforms. Four different products of this multigene family are known for rat and human (Carafoli *et al*, 1996). The different gene products are classified as h (for human) and r (for rat) isoforms *PMCA1* to *PMCA4*. Each isoform corresponds to a family of alternatively spliced isoforms at three independent sites (Verma *et al*, 1996; Stauffer *et al*, 1993). The regions that are important to catalytic function of the P-type pump are highly conserved. In addition, the structure motifs responsible for membrane folding pattern of the pump are also conserved. The motifs include the TM domains and the protruding units. The variability among the isoforms concerns with the domains that are related to peculiar regulation properties of the pump, e.g. calmodulin-binding domain, phosphorylation site of protein kinase and putative domain responsive to acidic phospholipid. The main area of isoform diversity concerns the regulatory aspects of the pump, which is tissue-specific and differentiation-specific specialization (Hammes *et al*, 1994).

Relative abundance of the spliced mRNA variants may vary substantially with the tissue or cell type (Hammes *et al*, 1994). The first cloned human isoform is *hPMCA4*, which is still the most intensively studied one and the best known (Carafoli *et al*, 1996). Isoforms 1 and 4 are present in all tissues in large amount and are considered as products of housekeeping genes. Isoforms 2 and 3 are only found

in significant amount in brain. Isoform 2 is mainly in cerebellum while isoform 3 in choroid plexuses. In addition, the majority isoform in erythrocyte is isoform 4. Furthermore, the primary sequence of several isoforms had been established in human and rat tissues. Antibodies against the four isoforms of human PMCA were raised using an N-terminal sequence of the pump as epitope. The N-terminal region chosen for the production of isoform-specific antibodies had the lowest degree of homology among the four gene products (Stauffer *et al*, 1995).

### **3. Study of aging process**

The investigation of calcium homeostasis in the body may be one of the approaches to study the aging process, as some of the degenerative diseases are related to calcium accumulation in specific cells (Friederichs & Melselman, 1994; Damonte *et al*, 1992). Aging is an overall decline of physiological process that results in failure to adapt to environmental demands and leads to death. At the cellular level, the process leads to impaired cellular structure and diminished function and finally to cell death. The study of aging process is important and it helps in explaining the principles of aging and finding of means for delaying or treating the pathology of aging.

Age-related changes of membrane of the cell concern both major membrane constituents, i.e. lipids and proteins, and certain cytoskeletal elements that are membrane-bound (Timas, 1996). During aging, membrane lipids are susceptible to

peroxidation and membrane fluidity is markedly altered by free radical accumulation. Thus, capacity of membrane components to diffuse laterally along the plane of membrane decreases. In addition, alternation of membrane may affect architecture of cellular surface, e.g. receptor number and affinity, surface proteins that result in impairment of inter- and intracellular communication.

There are also age-related changes in cytoplasm and nucleus (Timas, 1996). Mitochondria are more exposed to damaging oxygen radicals during metabolism. Since mitochondrial DNA lacks protein coat for protection from damage of oxygen radicals, its damage and mutations increase with aging. In addition, numerous structural alternations in the nucleus have been associated with aging, e.g. irregularities of shape and invagination, chromatin condensation. This results in decrease in functional activity and metabolism of the cell.

The mammalian erythrocyte is a peculiar but useful model for studying of aging process at molecular and cellular levels (Bartos, 1996). Mature erythrocyte is devoid of nucleus and other organelles, and thus it is incapable of synthesizing enzymes and other proteins *de novo* (Danon, 1986). Although mature erythrocyte has all structure and functional components in the cell, it cannot replace the damaged one by re-synthesis. The energy necessary to maintain membrane structure, cation balance and hemoglobin function is solely derived from the anaerobic breakdown of glucose. Eventual destruction occurs after a remarkably long and constant time spent within the circulation. Normal aged erythrocytes are selectively removed from the

circulation via phagocytosis by reticuloendothelial system. Because of this, it can be a suitable model for investigating the mechanisms causing progressive deterioration of macromolecular constituents in aging cells (Danon, 1986). In addition, it can also help in studying of the recognition and removal of senescent or damaged cells.

### **3.1 Phenomena during erythrocyte aging**

Phenomena during erythrocyte aging are similar to that of other animal cells, however, information in this area is more comprehensive. Mature mammalian erythrocyte has a finite life span. During the life span of mammalian erythrocyte, it undergoes physical and chemical changes. These changes are observed in cell volume, in cell density, and in cytoplasmic and in membrane components. The mean corpuscular volume (MCV) of erythrocyte decreases during aging (Bartosz, 1996). The change may be due to the loss of potassium and membrane via microvesiculation. This results in cellular dehydration and an increase in cell density. It should be noticed that the change in cell density is the hallmark of erythrocyte aging in mammals and this serves as a basis for separating erythrocytes according to cell age (Vettore *et al*, 1980; Corash *et al*, 1974; Rahman *et al*, 1973; Murphy, 1973; Piomelli *et al*, 1967; Rigas & Koler, 1961). In addition, aged erythrocyte has higher mean corpuscular hemoglobin content (MCHC) (Ganzoni *et al*, 1976; Piomelli *et al*, 1967). Also, the rate of deformation of aged erythrocyte is lower while its minimum cylindrical diameter remains the same. Since deformability is of fundamental

importance for erythrocyte to pass through capillaries with smaller diameter than its own, the decreased deformability may be the most functional impairment occurring in aging erythrocyte and some reports suggested that impaired deformability might be a determinant of red cell life span (Marikovsky, 1996; Friederichs, 1992; Bartosz, 1991; Shiga *et al*, 1990; Nash *et al*, 1988).

Apart from changes in physical properties of erythrocyte, there are also biochemical changes during aging (Hrusova *et al*, 1993). The content of 2,3-diphosphoglycerate (2,3-DPG) in aged erythrocyte was found to reduce markedly (Haidas, 1973). Since 2,3-DPG is the main modulator for hemoglobin affinity for oxygen, the decrease leads to an increase in oxygen affinity of hemoglobin in aged erythrocyte. The aged cell is then less capable of releasing oxygen in tissues. This may be one of the most important impairments in the aging process (Kadlubowski & Agutter, 1977; Turner *et al*, 1974; Bertolini, 1962). In addition, one of the characteristics of erythrocyte aging is the decrease of creatine and inactivation of some of its enzymes, namely acetylcholinesterase, pyruvate kinase and glucose-6-phosphate dehydrogenase etc. Therefore, content of creatine and activities of enzymes can be employed as erythrocyte age index in assessing erythrocyte separations (Cohen *et al*, 1976; Corash *et al*, 1974).

The membrane of erythrocyte undergoes changes during the aging processes (Hensley *et al*, 1995; Schroeder *et al*, 1995; Kosower, 1993). Though there had been reports that there were no gross changes in any of the membrane components, subtle

changes in membrane components and organization can still be observed in aged erythrocyte (Cohen *et al*, 1976). Aged erythrocyte has lower total lipid content per cell and membrane lipid to protein ratio (Winterbourn & Batt, 1970). These changes agree with the hypothesis that aging involves a release of protein-poor vesicles from the cell (Cohen *et al*, 1976). There is also a decrease of ratio of unsaturated to saturated fatty acid residues. This may be due to lipid peroxidation during aging (Ando *et al*, 1995).

There had been reports on appearance of senescent neoantigen on the surface of aged erythrocyte (Chiarantini *et al*, 1995; Giger *et al*, 1995; Kay *et al*, 1991; Lornad, 1978; Tannert *et al*, 1977). This antigen is responsible for the binding of antibodies present in the blood plasma. These auto-antibodies are specific for band 3 polypeptide, band 3 protein carbohydrate sequence, desialylated glycophorin and  $\alpha$ -galactosyl residues. The binding of these antibodies results in recognition of erythrocyte and phagocytosis by macrophages, possibly with the participation of complement. This may accelerate the clearance of aged erythrocytes from the circulation (Kay *et al*, 1991; Tannert *et al*, 1977).

Many researchers also studied the changes of erythrocyte during blood storage (Godin & Caprani, 1997; Llanillo *et al*, 1995; Fernandez *et al*, 1993; Wolfe, 1989; Weed *et al*, 1969). They found that outdated bank blood has similar alteration as senescent erythrocytes, i.e. inactivation of most enzymes, appearance of senescent cell antigen on membrane surface, lipid peroxidation etc.



### **3.2 Hypotheses of *in vivo* erythrocyte aging**

Several factors may play a primary role in erythrocyte aging and trigger secondary aging phenomena (Tang, 1997; Aiken *et al*, 1995; Ando *et al*, 1995; King & Barnett, 1995; Seaman *et al*, 1980). Many hypotheses have been proposed and are summarized as follows:

#### **A. Mechanical fatigue:**

During the life span of erythrocyte, the cell undergoes numerous circulation. This may involve progressive denaturation of membrane proteins and the life span of the cell is then terminated.

#### **B. Metabolic depletion:**

Aged erythrocyte may lose its capacity of ATP synthesis by inactivating the key enzymes of glycolysis and pentose phosphate shunt. This may result in failure of ATP-dependent systems. In this case, the cell may be less able to respond to metabolic stress and is more vulnerable to events in the circulation that may require the ability to increase the basal rate. This may be the biochemical defect limiting the cell life span.

#### **C. Damaged by reactive oxygen species:**

Metabolic activation of oxygen leads to formation of reactive oxygen species (ROS), like superoxide radical anion  $O_2^{\cdot -}$  and hydrogen peroxide  $H_2O_2$ , and reactive hydroxyl radical  $OH^{\cdot}$ . These species are able to inactivate proteins and initiate lipid

peroxidation. Since erythrocyte is the cell that is most exposed to oxygen in the body, it is most susceptible to reactive oxygen species. It results in enzyme inactivation, lipid peroxidation, decreased lipid fluidity, impaired deformability, hemichrome binding to band 3 protein, protein oligomerization, increased K<sup>+</sup> leakage and increased IgG binding (Tang, 1997; Ando *et al*, 1995; Oliver *et al*, 1987). In addition, concentration of activities of enzymes decomposing reactive oxygen species, e.g. superoxide dismutase, catalase and glutathione reductase etc., also decrease during erythrocyte aging (Glass & Gershon, 1981). Thus, capability of defense against reactive oxygen species declines with age of erythrocyte.

It should be noticed that these proposed mechanisms are sometimes controversial. There is yet no established hypothesis that can best explain the aging process. Further studies are therefore required to unveil the full picture.

### **3.3 Aging and Calcium**

As stated, many degenerative diseases may be related to calcium accumulation in cell. It is of great interest to study the alternation of calcium homeostasis during the aging process. In recent years, the involvement of calcium in the aging process of erythrocytes was proposed (Aiken *et al*, 1992). There were reports on the elevation of calcium concentration in aged erythrocyte. Actually, increase in calcium content of erythrocyte affects rheologic behavior of the cell (Friederichs & Melselman, 1994). This leads to decrease of cell deformability through fixing spectrin network. Thus,

life span of erythrocyte is limited. In addition, calcium accumulation causes dose-dependent depression in erythrocyte glutathione regeneration. This results in increase in susceptibility to oxidative damage. Other researchers suggested that calcium potentiated the peroxidation of membrane lipids (Jain & Shohet, 1981). Since lipids are major components of erythrocyte membrane and are vital for maintaining its structural and functional integrity, this may be one of the determinants of red cell aging.

The elevation of calcium concentration during *in vivo* red cell aging is possible due to the enhancement of calcium permeability under high shear flow in the circulation. This may be primarily due to the declined ability of calcium extrusion during aging. Since calcium is extruded by calcium pump by the use of ATP, there had been reports on alternation of  $\text{Ca}^{2+}$ -ATPase during *in vivo* red cell aging. However, the results were controversial. Some researchers found that  $\text{Ca}^{2+}$ -ATPase was inactivated during aging and it was very sensitive to oxidative damage (Seidler & Swislocki, 1991; Vincenzi & Hinds, 1988). Others suggested that  $\text{Ca}^{2+}$ -ATPase activity remained unchanged during the process (Clark, 1988). In addition, some studies were concentrated on the regulators of the  $\text{Ca}^{2+}$ -ATPase system (Glaser *et al*, 1994; Samaja *et al*, 1989; Ekholm *et al*, 1981; Luthra & Kim, 1980). Luthra & Kim (1980) found that there was reduced affinity of aged  $\text{Ca}^{2+}$ -ATPase to calmodulin while Samaja and his co-workers (1989) did not. However, there is lack of study on the interaction of the enzyme with PMCAI during the aging process (Samaja *et al*,

1989).

Many researchers also studied calcium homeostasis during blood storage (Huang *et al*, 1995; Ferreira & Lew, 1975). There had been reports on alternation of  $\text{Ca}^{2+}$ -ATPase activity during blood preservation (Seidler & Swislocki, 1991). It was found that activity of  $\text{Ca}^{2+}$ -ATPase declined during storage.

The study of aging of erythrocyte and blood storage attracted the attention of many workers due to shortage supply of bank blood. It is because of many reasons. Shelf life of bank blood is limited, basically for 120 days. There may be a shortage problem when there is a dramatic increase of demand of blood transfusion or a limited supply of blood from donors. Thus, elongation of life span of erythrocyte during storage is highly desirable. Further study in this area is of practical importance. On the other hand, as a model of aging study, information of changes of erythrocyte in the circulation throughout its life span may help to explain the aging of cells and individuals.

In this study, we aim to study more about  $\text{Ca}^{2+}$ -ATPase and to see if its regulation is involved in the aging process of erythrocyte. Through the study of structural and functional changes of  $\text{Ca}^{2+}$ -ATPase of erythrocyte in aging and storage, we aim to correlate the changes with the aging process of other animal cells. Through the following objectives, we wish to achieve our goal.

**Objectives:**

1. To isolate different populations of erythrocyte according to their ages
2. To conduct comparative study of interaction of  $\text{Ca}^{2+}$ -ATPase in young and aged erythrocytes with regulators
3. To compare the interaction of  $\text{Ca}^{2+}$ -ATPase from fresh and outdated blood with regulators
4. To purify  $\text{Ca}^{2+}$ -ATPase from erythrocytes of different ages and from fresh and outdated blood
5. To observe structural changes of  $\text{Ca}^{2+}$ -ATPase during *in vivo* aging of erythrocyte and blood storage

## METHODOLOGY

### Materials :

Acetylthiocholine iodide, lactate dehydrogenase, pyruvate kinase, phosphoenolpyruvate, NADH-dipotassium salt, ATP-disodium salt, EGTA-disodium salt, EDTA-disodium salt, 5,5'-dithio-bis(2-nitrobenzoic acid) (DTNB), Stains-all, formamide, ouabain, sodium orthovanadate, calmodulin, Hepes, Tween 20, trypsin, soybean trypsin inhibitor, Drabkin's solution were products from Sigma Chemical Co. Calpain I and calpain inhibitor I were purchased from Calbiochem, asolectin from Fluka Chemie AG and Triton X-100 from Borehinger Mannheim. L-Histidine and disodium disulphite were obtained from E. Merck while 1-amino-2-naphthol-4-sulphonic acid, sodium sulphite were from BDH Laboratory Samples. Oleic acid was Peking's Chemical Work. DEAE-Sephadex A-25, DEAE-Superose, Calmodulin-Sepharose 4B were from Pharmacia Biotech Ltd and Dc Protein Assay Kit from Bio-Rad. PM-10 and YM-2 ultrafiltration membranes were purchased from Amicon. All other chemicals used were of analytical grades.

Human blood was freshly drawn from six volunteers while outdated human blood was obtained from Red Cross Blood Transfusion Service. Porcine blood was obtained from local Cheung Sha Wan Abattoir (Hong Kong).

## **Methods:**

### **1. Preparation of $(\text{Ca}^{2+}+\text{Mg}^{2+})$ -ATPase inhibitor and activator deficient membranes from porcine erythrocyte**

Fresh porcine blood was obtained utilizing heparin as an anticoagulant. The blood was centrifuged at 4,600 x g for 10 minutes to remove the supernatant, i.e. plasma and top layer of white blood cells. The erythrocytes obtained were washed three times with isotonic buffer (0.155M NaCl, 2mM histidine, 2mM EDTA) at pH 7.4. The cells were then lysed by hypotonic buffer (2mM histidine, 2mM EDTA, pH 7.4) in the ratio of 1 part cell to 9 parts buffer. The hemolysate was centrifuged at 39,000 x g for 30 minutes to sediment membranes. The membranes obtained were washed by the same buffer for three times and re-suspended in 10mM Tris-HCl, pH 7.4 buffer. Those were further washed 3 times to remove EDTA. The final re-suspended membranes were ready for activity and protein assay.

### **2. Partial purification of Plasma Membrane $\text{Ca}^{2+}$ -ATPase Protein Inhibitor (PMCAI) from porcine erythrocytes**

The purification of Plasma Membrane  $\text{Ca}^{2+}$ -ATPase Protein Inhibitor (PMCAI) was modified from that of Au (1996). Fresh porcine erythrocytes were obtained and lysed as described in the preparation of membranes. The hemolysate was centrifuged at 39,000 x g for 30 minutes to sediment membranes. The membrane-free hemolysate was then subjected to ultrafiltration through PM-

10 Amicon membrane. The ultra-filtrate obtained was then further concentrated 20-fold by YM-2 Amicon membrane. The concentrate was dialyzed (molecular weight cut off: 3500) against 50mM Imidazole buffer, pH 5.2.

The dialyzed protein sample was loaded onto DEAE-Sephadex A-25 anion exchange chromatography at 4°C. The column was first equilibrated by 50mM Imidazole, pH 5.2. The column had a bed volume of 120ml and a flow rate of 40ml/hr. The column was then washed by 50mM Imidazole, 0.1M NaCl, pH 5.2 after loading of protein sample until absorbance at 280nm returned to baseline level. The bound protein was eluted with a linear 0.1-0.25M NaCl gradient buffer lasting for 16 hours. The fractions collected were screened for inhibitor activity by inorganic phosphate assay and the active fractions were pooled for further study and PMCAI protein determination.

Besides, YM-2 concentrate was also loaded onto DEAE-Superose Fast Flow column (bed volume of 30ml) of AKTA Explorer 100. The purification step was the same as that for DEAE A-25 column chromatography except that the flow rate was changed to 5ml/min.

### **3. Isolation of membrane-bound $\text{Ca}^{2+}$ -ATPase from porcine erythrocytes according to different ages**

Porcine erythrocytes were separated into different age groups based on their density as described by Murphy (1973) with modifications. Fresh porcine blood



was first centrifuged at 4,600 x g for 10 minutes. Buffy coat was then removed while the plasma was saved. The packed cells obtained were suspended at a hematocrit of 90% with their own plasma. They were then put into centrifuge tubes (14 x 97mm) equipped with adapters and centrifuged at 30°C in a fixed-angle rotor at 34° at 39,000 x g for 1 hour. After centrifugation, the top 5% and bottom 5% cells were obtained. The procedures for preparation of membrane from top 5% and bottom 5% cells were the same as that of whole blood.

The mean corpuscular hemoglobin concentration (MCHC) of the cells and acetylcholinesterase activity of the membranes prepared were measured to assess the success of separation. Since MCHC increases while acetylcholinesterase activity decreases when erythrocyte ages (Piomelli *et al*, 1967, Ganzoni *et al* 1976, Kadlubowski & Agutter, 1977), success of separation of erythrocytes was confirmed by comparing the data obtained from top and bottom cells.

#### **4. Preparation of (Ca<sup>2+</sup>+Mg<sup>2+</sup>)-ATPase inhibitor and activator deficient membranes from fresh and outdated human blood**

Freshly drawn human blood was obtained utilizing citrate-phosphate-dextrose-adenine (CPD-A) solution as an anticoagulant. The procedures for preparation of membrane protein was the same as that for porcine erythrocytes.

Outdated bank blood was already removed from buffy coat. The procedure for preparation of membrane protein was the same as that for porcine erythrocyte.

## **5. Study of the interaction of membrane-bound $\text{Ca}^{2+}$ -ATPase from porcine erythrocytes and human blood with regulators**

Membrane-bound  $\text{Ca}^{2+}$ -ATPase activity was assayed by using coupled-enzyme assay. To determine the presence of  $\text{Ca}^{2+}$ -ATPase activity in the membrane prepared, the membrane was subjected to different treatments.  $\text{Ca}^{2+}$ -ATPase activity was determined in the presence of 10 units calmodulin, 3  $\mu\text{M}$  sodium orthovanadate or 0.025  $\mu\text{M}$  oleic acid.

To study the interaction of the membrane enzyme with its regulators, activities were determined in the presence of oleic acid (0.001-0.025  $\mu\text{M}$ ), calmodulin (0.05-1unit), PMCAI (0.02-0.2  $\mu\text{g}$ ) individually in the range of 0-1.25mM ATP in the assay medium.

To study the controlled proteolytic treatment of the membrane-bound enzyme, both trypsin and calpain were employed. For the tryptic effect, the membrane was incubated with 0.0025 x 10<sup>-3</sup> % trypsin at 37°C for 5 minutes. 10-fold soybean trypsin inhibitor then added to stop the reaction and  $\text{Ca}^{2+}$ -ATPase activity was then determined. The procedure for calpain digestion of the membrane was the same as that of trypsin. 1 unit of calpain I was added into the membrane and the mixture was incubated for 10 minutes. The reaction was stopped by 10-fold calpain inhibitor I.

## **6. Purification of $\text{Ca}^{2+}$ -ATPase from porcine and human erythrocytes**

The purification steps were based on the method described by Niggli *et al* (1979) with some modifications. Membrane-bound  $\text{Ca}^{2+}$ -ATPase was first solubilized in 50-fold volume 0.5% Triton X-100, 300mM KCl, 10mM Hepes, 1mM  $\text{MgCl}_2$ , 2mM DTT, pH 7.4 for each milligram membrane protein. The mixture was stirred gently at 4°C for 10 minutes. and it was subjected to centrifugation at 100,000 x g for 60 minutes. Clean supernatant was then added to equal volume of buffer (0.1% asolectin, 0.05% Triton X-100, 2mM DTT, pH 7.4). The solution was then loaded onto Calmodulin-Sepharose 4B column (bed volume of 5ml) with flow rate of 10 ml/hr.

The column was first equilibrated by 0.5% Triton X-100, 200mM KCl, 10mM Hepes, 1mM  $\text{MgCl}_2$ , 0.1mM  $\text{CaCl}_2$ , 2mM DTT, 0.1% Asolectin, pH 7.4. The column was washed by 0.05% Triton X-100, 300mM KCl, 10mM Hepes, 1mM  $\text{MgCl}_2$ , 0.5mM  $\text{CaCl}_2$ , 2mM DTT, 0.1% asolectin, pH 7.4 after sample loading until the absorbance at 280nm was returned to baseline. The column was then eluted by 0.05% Triton X-100, 300mM KCl, 10mM Hepes, 2mM DTT, 0.1% asolectin, 2mM EDTA, pH 7.4.  $\text{Ca}^{2+}$ -ATPase activity of each fraction was determined by coupled-enzyme assay and the active fractions were pooled.

## **7. SDS-polyacrylamide gel electrophoresis of $\text{Ca}^{2+}$ -ATPase**

$\text{Ca}^{2+}$ -ATPase was subjected to electrophoresis based on Laemmli (1970).

Aliquots of the enzyme samples were first precipitated with 10% Trichloroacetic acid. The precipitate was washed one time with 5% Trichloroacetic acid and then three times with deionized distilled water. The pellet was then resuspended in a buffer containing 60mM Tris-Cl, pH6.8, 2% SDS, 4M Urea, 0.001% bromophenol blue and 3% mercaptoethanol (v/v). The mixture was heated for 4 minutes in a boiling water bath and was then applied to polyacrylamide gel (Niggli *et al*, 1979). 7.5% polyacrylamide gel with a thickness of 1.5mm was used. The protein was visualized by staining with Coomassie brilliant blue R-250.

#### **8. Western-blot analysis of $\text{Ca}^{2+}$ -ATPase**

$\text{Ca}^{2+}$ -ATPase separated by SDS-polyacrylamide gel electrophoresis was transferred to PVDF membrane. The membrane was blocked with 3% bovine serum albumin (BSA) for 1 hour and washed with TBS-T (10mM Tris-HCl, 500mM NaCl, 0.05% Tween-20, pH 7.0). The membrane was then incubated with isoform specific antibody for *PMCA1* (1N) raised from rabbit (diluted 1/500 in TBS-T with 0.1% BSA, raised from rabbit) (Stauffer *et al*, 1995) overnight. After washing the membrane, it was then incubated with anti-rabbit IgG horseradish peroxidase conjugate (diluted 1/1000 in TBS-T with 0.1% BSA) for 1 hour. After the incubation, the transblotted proteins were detected by ECL method.

## **9. Protein determinations - Dc Protein Assay**

Dc Protein Assay Kit (Bio-Rad) was employed to measure protein content of the sample. The assay was a modified method of Lowry assay (Lowry *et al*, 1951). The protein content was calibrated against a bovine serum albumin (BSA) standard.

## **10. Ca<sup>2+</sup>-ATPase activity assay –Coupled-enzyme assay**

The Ca<sup>2+</sup>-ATPase activity of the enzyme sample was assayed by using coupled-enzyme assay (Au & Siu, 1992). This assay linked hydrolysis of ATP by Ca<sup>2+</sup>-ATPase in the presence of excess pyruvate kinase (PK), lactate dehydrogenase (LDH) and phosphoenolpyruvate (PEP). One unit of Ca<sup>2+</sup>-ATPase was defined as  $\mu$  mole NADH oxidized per minute.

The assay medium contained 80mM NaCl, 28mM KCl, 3.6mM MgCl<sub>2</sub>, 0.2mM CaCl<sub>2</sub>, 0.1mM EGTA, 2.5mM ATP, 0.1mM ouabain, 0.2mM NADH, 2.5mM PEP, 2 units PK, 5 units LDH, 80mM histidine, pH 7.4 in a final volume of 1ml. The reaction was monitored continuously by spectrophotometer at 37°C at 366nm by addition of sample (30  $\mu$ g protein). The mixture without addition of NADH was used as the reference. Each sample was assayed in triplicate and error within 5% was accepted.

## **11. PMCAI protein determinations-Stains-all absorption spectrum**

Stains-all absorption spectrum (Campbell *et al*, 1983) was employed to measure protein content of partially purified PMCAI. Standard solution contained 10mM Tris-base, 0.001% Stains-all, 0.1% formamide, pH 8.8 was prepared. Partially purified PMCAI (200  $\mu$ l) was added to 1ml standard solution. After incubation at room temperature in the dark for 30 minutes, absorbance at 600nm was measured against a control solution, i.e. containing no protein. The protein content was quantified by using calmodulin as standard.

## **12. Screening of inhibitory activity- Inorganic phosphate assay**

The assay was a modified method of Friske and Subbarow (1925) and was used for screening of inhibitory activity of post-DEAE-A25 and post-DEAE-Superose fractions. The assay medium contained 80mM NaCl, 28mM KCl, 3.6mM MgCl<sub>2</sub>, 0.2mM CaCl<sub>2</sub>, 0.1mM EGTA, 2.5mM ATP, 0.1mM ouabain, 80mM histidine, pH 8.0 in a final volume of 0.6ml. Porcine membrane-bound Ca<sup>2+</sup>-ATPase (60  $\mu$ g protein) was incubated for 0 and 1 hour at 44°C with or without addition of the column fraction.

The reaction was terminated by addition of 1ml ice-cold 10% trichloroacetic acid. Precipitates were removed by centrifuged at 1,500 x g for 10 minutes at 4°C. 1ml clean protein-free supernatant was then transferred to 3.3ml deionized distilled water to which 0.5ml 2.5% ammonium heptamolybdate in 5N H<sub>2</sub>SO<sub>4</sub>

and 0.2ml freshly prepared ANSA reagent (0.2% 1-amino-2-naphthol-4-sulphonic acid, 2% sodium sulphite, 6% disodium bisulphite) were added consecutively. Absorbance at 650nm was recorded 10 minutes after the addition of reagents. Amount of inorganic phosphate was calibrated against  $\text{KH}_2\text{PO}_4$  up to  $2 \mu$  mole. Percentage inhibition of the fraction was then determined by comparing with the basal activity of the membrane enzyme. Each sample was assayed in triplicate and error within 5% was accepted.

### **13. Assay of erythrocyte membrane acetylcholinesterase activity**

This enzyme assay, together with mean corpuscular hemoglobin concentration (MCHC) determination were assayed to assess the separation of young and old red cells. Acetylcholinesterase activity of erythrocyte membrane was measured (modified from Ellman and his co-workers, 1961). The assay medium contained 3ml 0.1M sodium phosphate buffer, pH 8,  $20 \mu$ l 75mM acetylthiocholine iodide and  $100 \mu$ l DTNB reagent (10mM DTNB, 18mM sodium hydrogen carbonate in 0.1M sodium phosphate buffer, pH 7). Membrane was added to the assay medium and the reaction was monitored spectrophotometrically at 412nm at 25°C against a reference without addition of membrane.

#### **14. Mean corpuscular hemoglobin concentration (MCHC) of blood sample determination**

Mean corpuscular hemoglobin concentration of blood sample was determined according to Bain (1994). 20  $\mu$ l blood sample was added into 5ml Drabkin's solution. After standing the mixture for 10 minutes, the absorbance at 540nm was measured by using Drabkin's solution as a blank. The amount of hemoglobin concentration (Hb) was calibrated against cyanmethemoglobin standard solution. Packed cell volume (PCV) of the blood sample was also measured. MCHC was then calculated by the following formula:

$$\frac{\text{Hb (g / 100ml)}}{\text{PCV (\%)}} \times 100 \%$$



## RESULT

### 1. Assay of erythrocyte membrane $\text{Ca}^{2+}$ -ATPase activity

In this study, we aim to investigate the alternation of  $\text{Ca}^{2+}$ -ATPase and its regulation during both *in vivo* aging of erythrocyte and blood storage.  $\text{Ca}^{2+}$ -ATPase is present in plasma membrane in minute amount and it is very labile in solubilized state. It is difficult to purify it in large quantities for the study of regulation of the enzyme. Whereas, membrane ghost is comparatively stable and it better represents the natural state of  $\text{Ca}^{2+}$ -ATPase. Large quantities of membrane ghosts can be easily prepared and it can be easily available for the enzyme study. Thus, the study was focused on the  $\text{Ca}^{2+}$ -ATPase of erythrocyte membrane. In the preliminary study, the protocol of preparation of  $\text{Ca}^{2+}$ -ATPase from erythrocyte membrane and the assay for  $\text{Ca}^{2+}$ -ATPase activity were optimized. The preparation of erythrocyte membrane from porcine blood was described in Methodology.

After separating porcine erythrocytes from plasma, they were lysed by hypotonic buffer containing EDTA. The membrane obtained was washed by same buffer and then by Tris.HCl buffer containing EDTA to remove membrane-bound calmodulin. Porcine erythrocyte membrane obtained was then subjected to coupled enzyme assay and inorganic phosphate assay to determine the presence of  $\text{Ca}^{2+}$ -ATPase activity. The assay medium contained 80mM NaCl, 28mM KCl, 3.6mM  $\text{MgCl}_2$ , 0.2mM  $\text{CaCl}_2$ , 0.1mM EGTA, 2.5mM ATP, 0.1mM ouabain, 80mM histidine, pH 7.4. The ATPase activity of the membrane was determined under

different treatments and the result is shown in Table 1.

Results in Table 1 show that the enzyme in the ghost is P-type ATPase as it was inhibited by vanadate (Rossi *et al*, 1981; Varecka & Carafoli, 1982; Carafoli, 1992). Majority of enzymes present in the porcine erythrocyte was ouabain-insensitive. In the presence of ouabain, only ouabain-insensitive ATPase activity was measured. Since Na<sup>+</sup>-K<sup>+</sup>-ATPase is sensitive to ouabain, majority of Na<sup>+</sup>-K<sup>+</sup>-ATPase activity of the membrane was suppressed in the assay medium. The amount of Na<sup>+</sup>-K<sup>+</sup>-ATPase present in the ghost is indeed minute. This is shown by the ATPase activity measured without the addition of ouabain as it was roughly the same as basal activity of the enzyme.

The findings obtained show that in the prepared membranes, there existed an enzyme with properties similar to that of Ca<sup>2+</sup>-ATPase. The enzyme was stimulated by calmodulin and oleic acid (Bond & Clough, 1973; Ronner *et al*, 1977) and inhibited by vanadate. In addition, activity of the enzyme was almost negligible in the absence of calcium. It shows that the membrane-bound enzyme was calcium-dependent ATPase and it is likely corresponding to calcium pump. The protocol of preparation of membrane ghost and the assay system were thus established in this study.

By comparing both coupled-enzyme and inorganic phosphate assay, similar results were obtained. Both methods were used for determining Ca<sup>2+</sup>-ATPase activity. In this study, it was found that sensitivity of coupled-enzyme assay was

**Table 1 ATPase activity of porcine erythrocyte membrane**

<i>Treatment</i>	<i>Specific activity of ATPase</i> (unit / mg membrane protein)	
	<i>Coupled-enzyme</i> (n=10)	<i>Inorganic phosphate</i> (n=10)
Basal	0.061±0.002	0.060±0.001
Assay medium without ouabain	0.065±0.001	0.067±0.003
Assay medium without Ca <sup>2+</sup>	0.001	0
Calmodulin (10 units)	0.170±0.01	0.160±0.02
Oleic acid (0.025µM)	0.214±0.01	0.200±0.02
Sodium orthovanadate (3µM)	0.011±0.001	0.010±0.002

ATPase activity of EDTA-washed membrane ghost from porcine erythrocyte was determined under different treatments by coupled-enzyme and inorganic phosphate assay. The assay medium contained 80mM NaCl, 28mM KCl, 3.6mM MgCl<sub>2</sub>, 0.2mM CaCl<sub>2</sub>, 0.1mM EGTA, 2.5mM ATP, 0.1mM ouabain, 80mM histidine, pH 7.4. For the coupled-enzyme assay, the reaction was monitored by spectrophotometer at 37°C at 366nm by addition of 30µg membrane protein. 1 unit of Ca<sup>2+</sup>-ATPase activity = 1 µmole NADH oxidized per min. For the inorganic phosphate assay, 60µg membrane protein was incubated with assay medium at 44°C and the reaction was terminated by addition of ice-cold trichloroacetic acid. The mixture was then subjected to Friske and Subbarow's method (1925) for determination of inorganic phosphate. Amount of inorganic phosphate liberated was measured at absorbance at 650nm. 1 unit of Ca<sup>2+</sup>-ATPase activity = 1 µmole Pi liberated per min. Each sample was assayed in triplicate and error within 5% was accepted. Data are mean ± SD from 10 experiments.

much higher. It could detect small changes of response even in minute amount of enzyme, e.g. 10  $\mu$ g membrane protein while the detection limit of inorganic phosphate assay was 50  $\mu$ g membrane protein. The coupled-enzyme assay was thus used for kinetic study of  $\text{Ca}^{2+}$ -ATPase. However, inorganic phosphate assay was convenient when  $\text{Ca}^{2+}$ -ATPase activity of batches of samples were measured. This method was employed for screening of inhibitory activity in the fractions collected during purification of protein inhibitor (PMCAI).

## **2. Preparation of membrane-bound $\text{Ca}^{2+}$ -ATPase from young and old porcine erythrocytes**

After establishing the assay system and the protocol of preparing of membrane-bound  $\text{Ca}^{2+}$ -ATPase, study of *in vivo* aging effect on the  $\text{Ca}^{2+}$ -ATPase was performed. Porcine erythrocytes were first separated into different populations of age group. The separation of erythrocytes in different age groups is well-established in rabbit and human (Vettore *et al*, 1980; Corash *et al*, 1974; Rahman *et al*, 1973), but not in porcine blood. Porcine erythrocytes were separated into different age populations on the basis of density differences by centrifugation (Murphy, 1973). After centrifugation, the top 5% and bottom 5% cells were collected. Since mean corpuscular hemoglobin concentration (MCHC) of erythrocyte increases (Piomelli *et al*. 1967; Ganzoni *et al* 1976) while its acetylcholinesterase activity decreases (Kadlubowski & Agutter, 1977), in the aging process, these two properties were used as markers to assess the success of

separation. The result is shown in Table 2.

From Table 2, it shows that the alternation of both acetylcholinesterase activity and MCHC of top 5% and bottom 5% cells followed the general trend of erythrocyte during aging. The ratio of acetylcholinesterase activity of top 5% to bottom 5% cells was 1.4: 1 while the MCHC ratio was 1: 1.1. The results obtained were reproducible for many trials (n=10). Thus, the bottom 5% cells separated by modified Murphy's method were indeed enriched with aged erythrocytes. It was the first attempt to separate porcine erythrocytes into different age groups according to their density differences. The protocol worked out in this study was successful and the isolated cells and their membranes were used for the following studies on regulation of  $\text{Ca}^{2+}$ -ATPase during aging.

### 3. Membrane-bound $\text{Ca}^{2+}$ -ATPase activity in young and aged erythrocytes

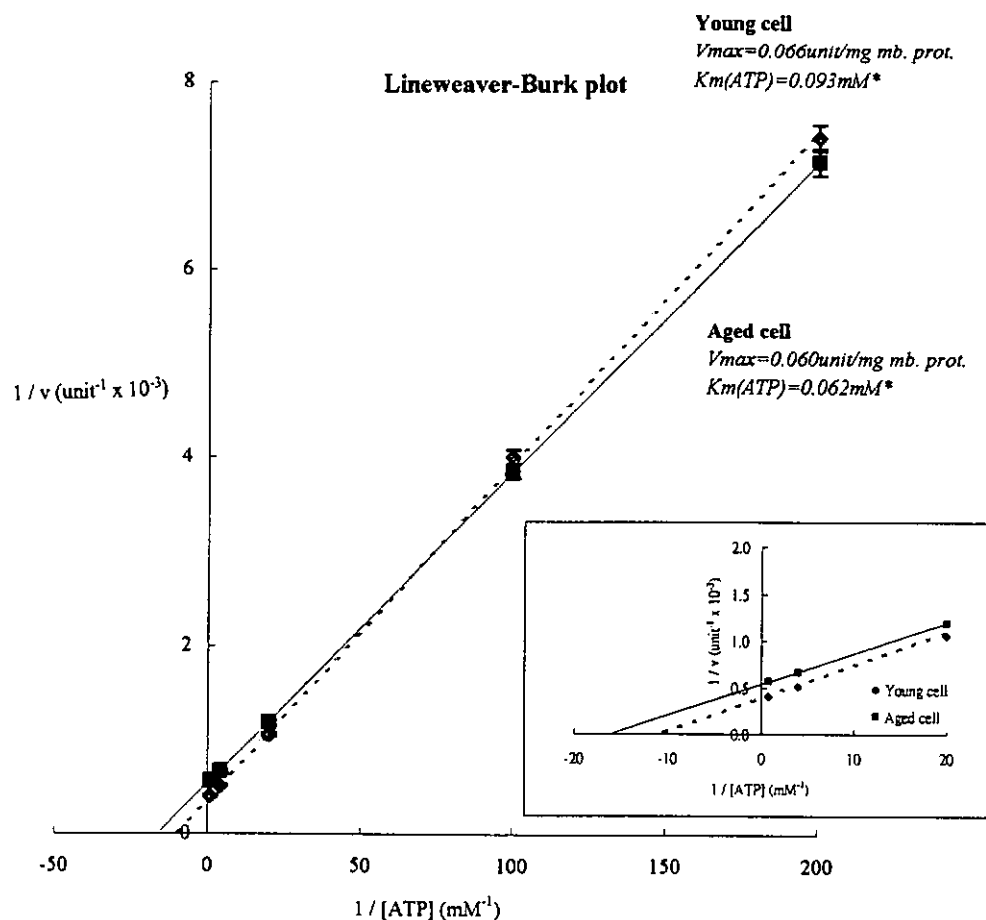
The membrane-bound  $\text{Ca}^{2+}$ -ATPase in young and aged erythrocytes were successfully prepared. They were then subjected to coupled-enzyme assay to find out if there were any alternations during aging of erythrocytes. Comparative study of membrane-bound  $\text{Ca}^{2+}$ -ATPase in young and aged erythrocytes with respect to their response towards substrate, i.e. ATP, was conducted. Double-reciprocal plot was obtained which was derived from a linear transformation of the Michaelis-

Menten equation , i.e. 
$$V_0 = \frac{V_{\max} [S]}{K_m + [S]}$$
 . Figure 5 shows the membrane

**Table 2 Age-markers of top 5% and bottom 5% erythrocytes**

<b><i>Marker</i></b>	<b><i>Top 5% cells (n=10)</i></b>	<b><i>Bottom 5% cells (n=10)</i></b>
Acetylcholinesterase activity (unit / g membrane protein)	0.031±0.002	0.022±0.001
MCHC (%)	28±0.5	31±0.3

Porcine erythrocytes (PCV=90%) were subjected to centrifugation at 30°C in a fixed-angle rotor at 34° at 39,000 x g for 1 hour. After centrifugation, top 5% and bottom 5% cells were obtained. Acetylcholinesterase activity and mean corpuscular hemoglobin concentration (MCHC) were then determined. Data are mean ± SD from 10 experiments. The differences between the age-markers in top cells and bottom cells significant at p<0.05 by student t-test.



**Figure 5 Double-reciprocal plot: Response of  $\text{Ca}^{2+}$ -ATPase in young and aged erythrocytes vs ATP**

$\text{Ca}^{2+}$ -ATPase activity of 30ug membrane protein was measured by coupled-enzyme assay in the presence of ATP (0 - 1.25mM). Each sample was assayed in triplicate and error within 5% was accepted. The plot represents means from 6 experiments. \* The differences are significant with  $p < 0.05$ .

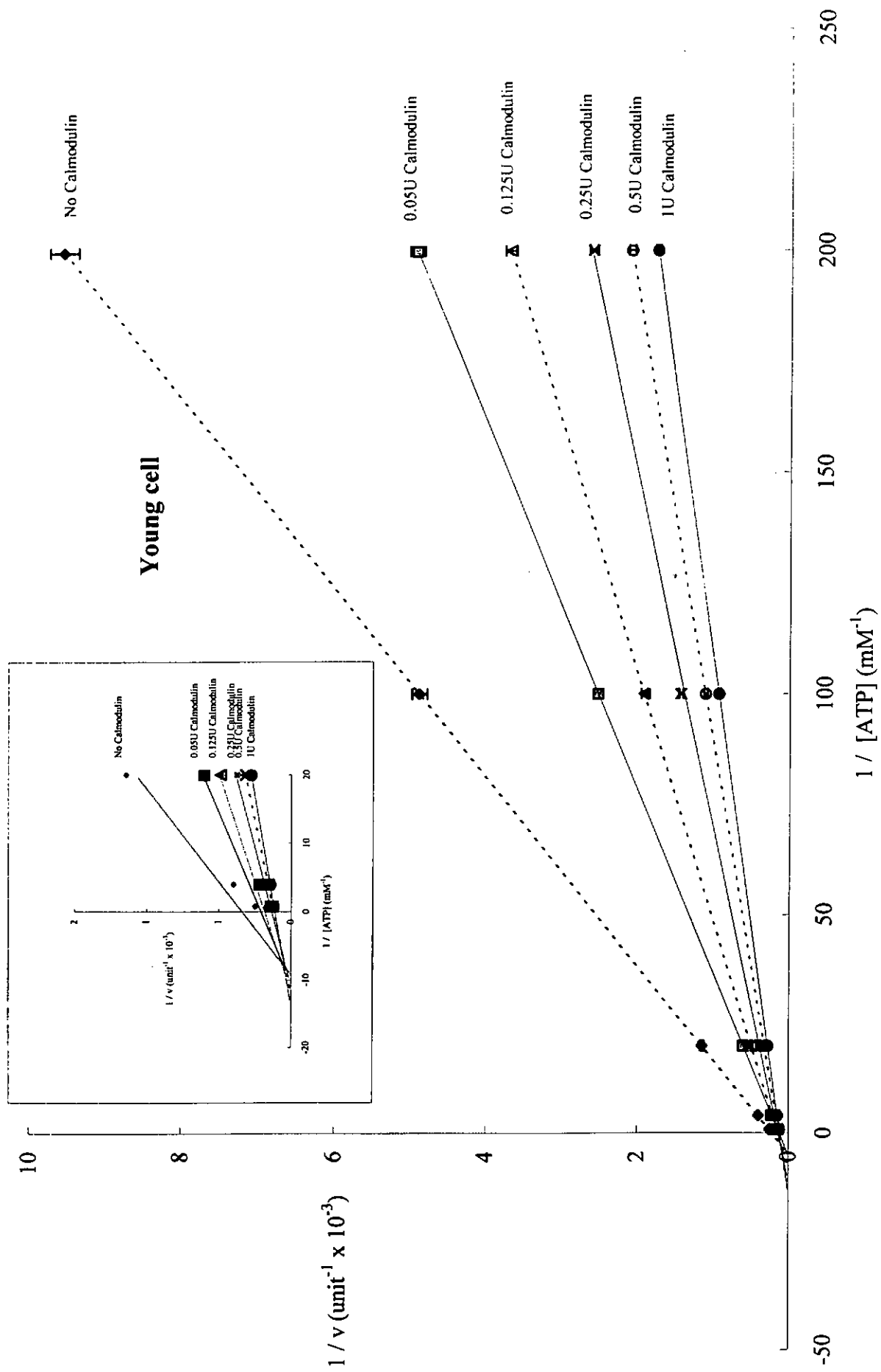
Ca<sup>2+</sup>-ATPase activity of the two different age groups with respect to ATP concentration. It was found that V<sub>max</sub> of Ca<sup>2+</sup>-ATPase of young and aged erythrocytes were 0.066 unit / mg membrane protein and 0.060 unit / mg membrane protein respectively. The difference was not significant. However, for K<sub>m</sub>(ATP) of Ca<sup>2+</sup>-ATPase, there was significant difference (n=6, p<0.05). The values for young and aged cells were 0.093mM and 0.062mM. The alternation may be due to alternation of regulation of the enzyme. Thus, interaction of membrane-bound Ca<sup>2+</sup>-ATPase from different age populations with regulators was further investigated.

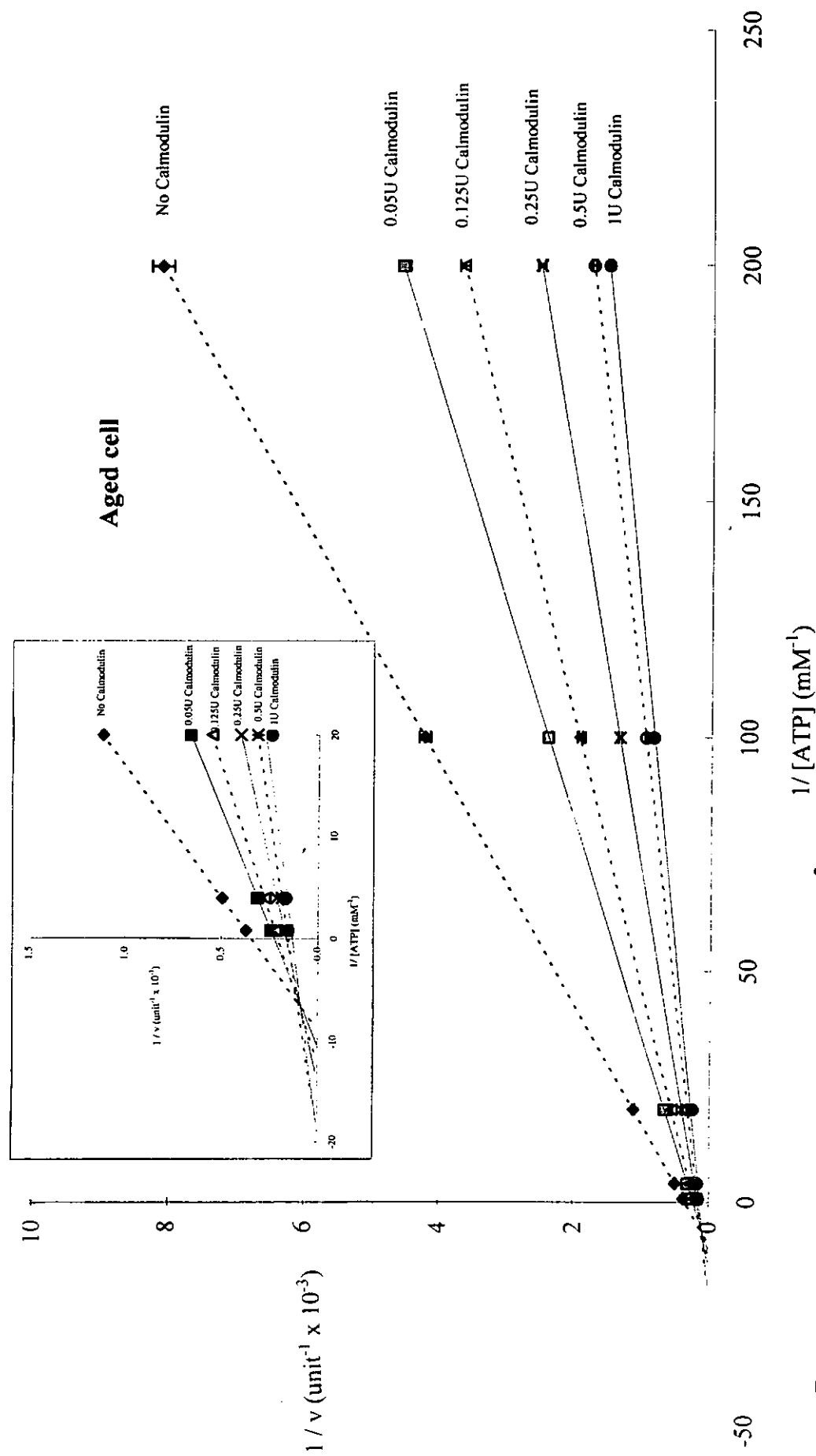
#### **4. Interaction of membrane-bound Ca<sup>2+</sup>-ATPase from different age populations with activators**

Ca<sup>2+</sup>-ATPase in natural state is subjected to regulation by endogenous regulators. In order to find out whether Ca<sup>2+</sup>-ATPase has changes in regulation during aging, its interactions with various regulators were studied. The membrane-bound Ca<sup>2+</sup>-ATPase was assayed in the presence of regulators individually by coupled-enzyme assay. The regulators being studied were calmodulin, oleic acid, trypsin and calpain.

The first regulator to be studied was calmodulin, which is a well-known activator of Ca<sup>2+</sup>-ATPase. It was found that the affinity and response of the enzyme towards calmodulin were about the same during the aging process (Figure 6 & 7).

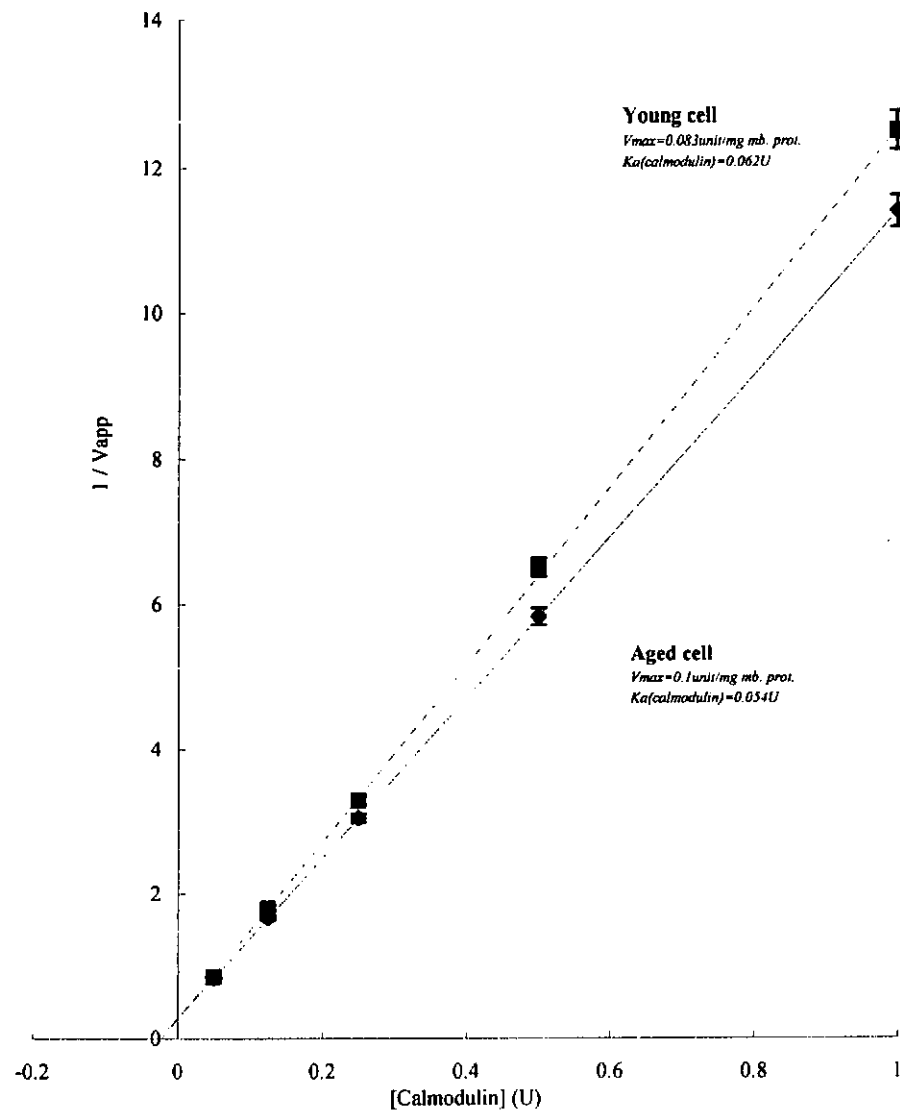






**Figure 6 Double-reciprocal plot: Response of  $\text{Ca}^{2+}$ -ATPase in young and aged erythrocytes towards calmodulin**

Double-reciprocal plot of  $\text{Ca}^{2+}$ -ATPase activity in young and aged erythrocytes vs ATP concentration.  $\text{Ca}^{2+}$ -ATPase activity of 30ug membrane protein was measured by coupled-enzyme assay in the presence of calmodulin (0 - 1 unit). Each test was assayed in triplicate and error within 5% was accepted. The plot above represents a typical membrane preparation.



**Figure 7 Secondary plot: Response of  $\text{Ca}^{2+}$ -ATPase in young and aged erythrocytes towards calmodulin**

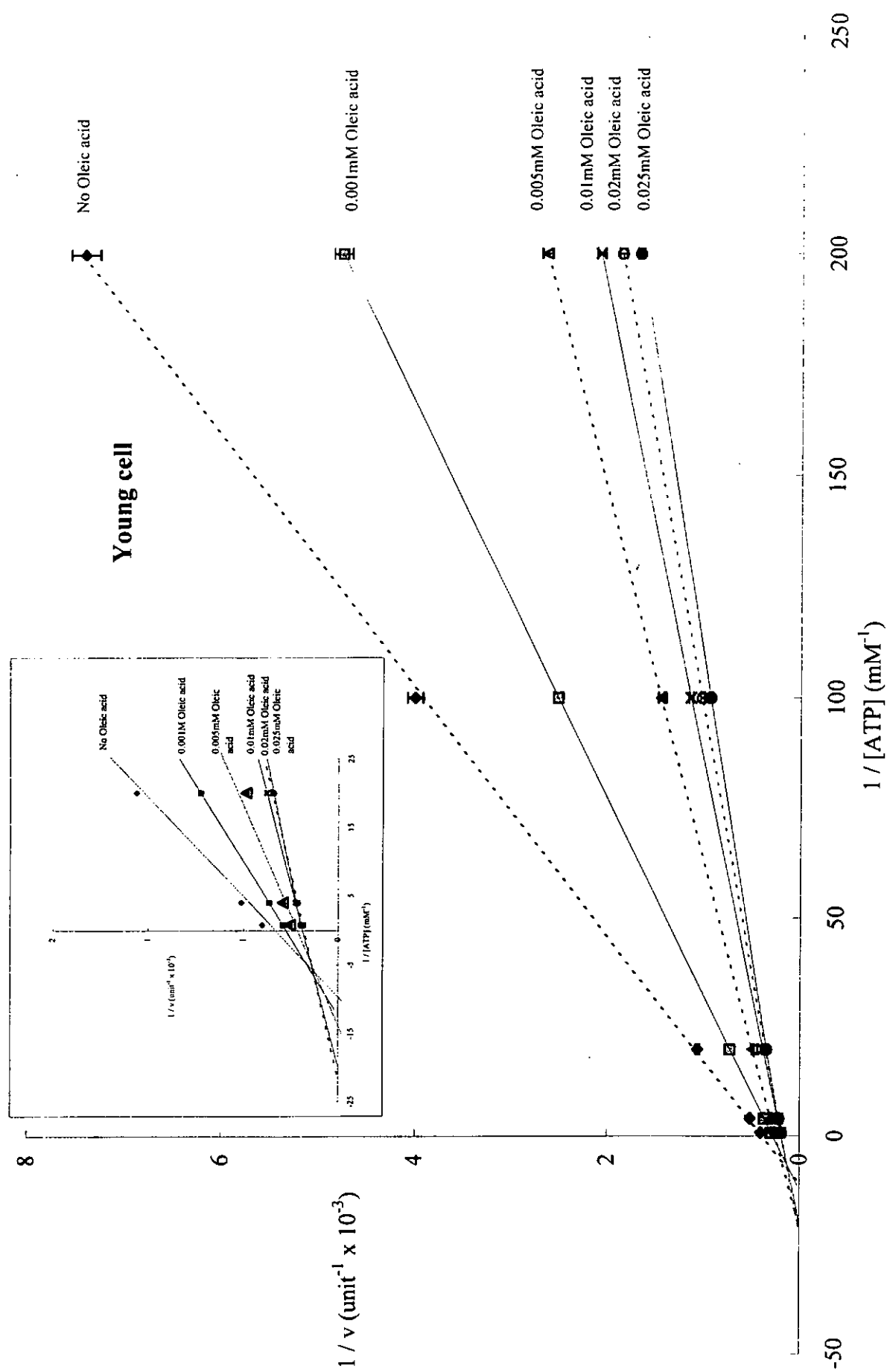
The plot represents means from 6 experiments.

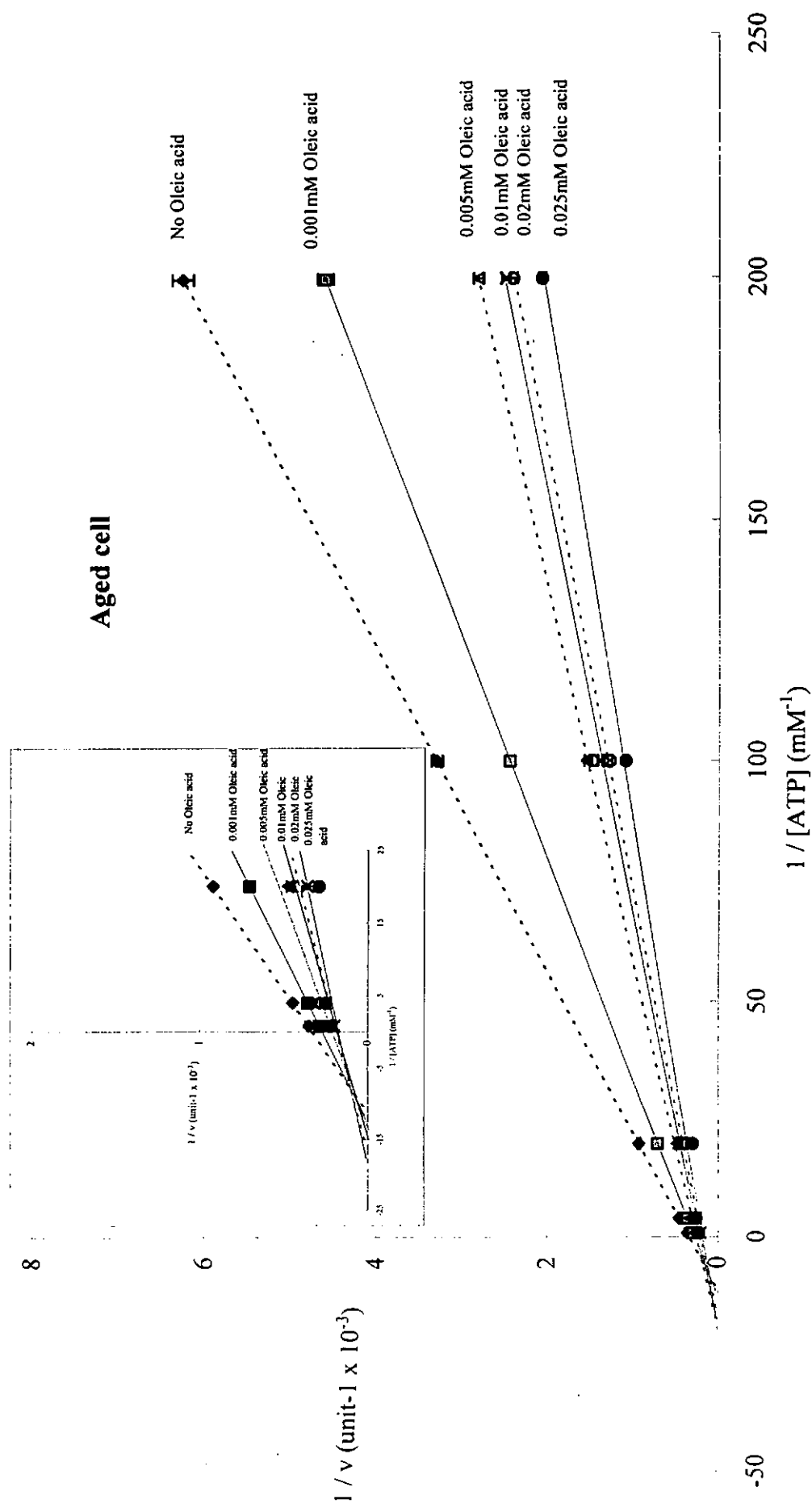
$K_a$  (calmodulin) and  $V_{max}$  were 0.062U and 0.083 unit / mg membrane protein for young cells while that for the aged was 0.054U and 0.1 unit / mg membrane protein respectively.

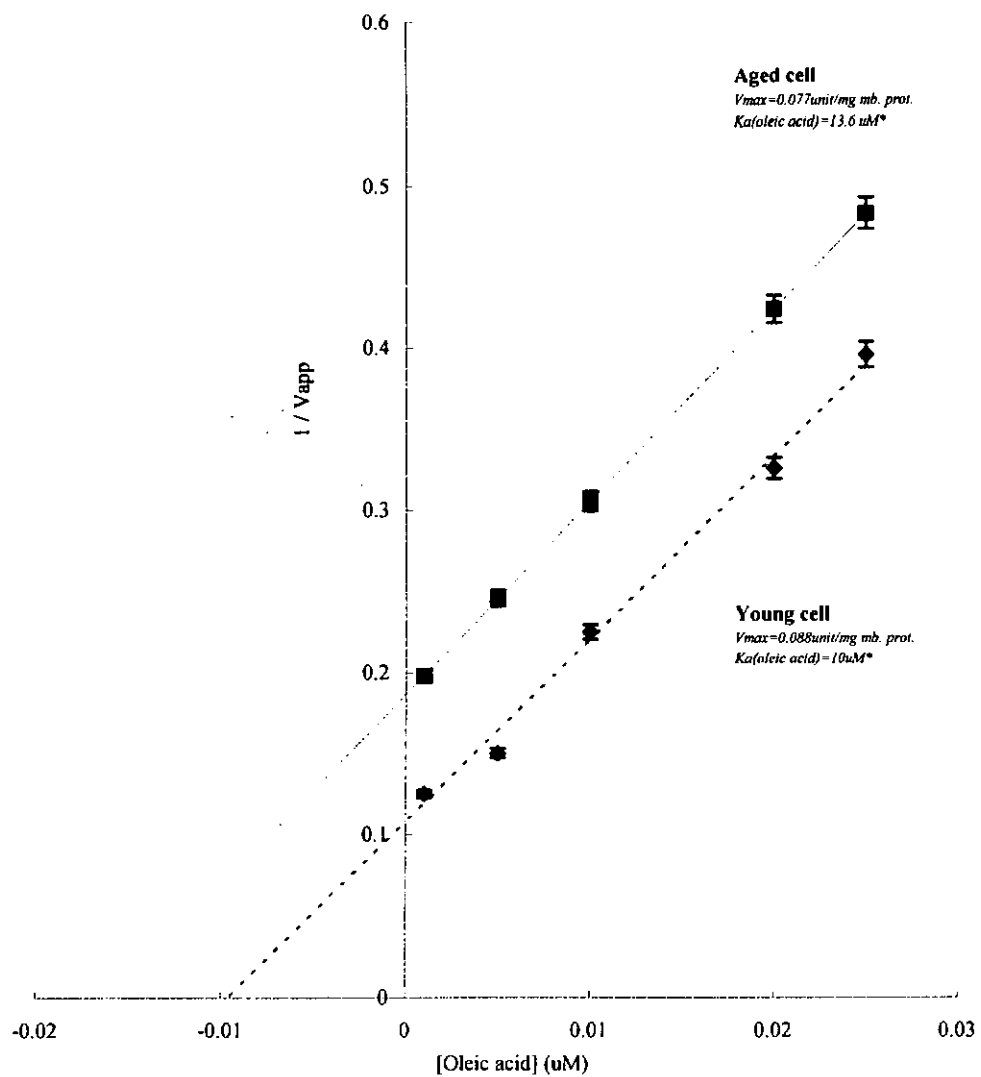
In addition, response of the enzyme towards oleic acid was studied. With respect to oleic acid (Figure 8 & 9),  $K_a$  (oleic acid) were found to be 10 and 13.6  $\mu$ M for young and aged cells ( $n=6$ ,  $p<0.05$ ). The maximal activities of the enzyme from young and aged cells were 0.088 and 0.077 unit / mg membrane protein. The difference was small and insignificant.

$Ca^{2+}$ -ATPase is also known to be stimulated by controlled proteolysis (Rossi & Schatzmann, 1982). Trypsin was first chosen for the study of effect of controlled proteolysis on the pump as it is easily available. In controlled tryptic proteolysis, the membrane was first incubated with  $0.0025 \times 10^{-3}\%$  trypsin for 5 minutes, then 10-fold soybean trypsin inhibitor was added to stop the reaction. The  $Ca^{2+}$ -ATPase activities of treated and untreated membranes were subsequently determined. From the result, it was found that membrane-bound  $Ca^{2+}$ -ATPase activity in aged erythrocyte was increased by around 80% while the activation effect was not significant in young cell (Table 3).

Trypsin was used in this study to mimic the effect of intracellular  $Ca^{2+}$ -dependent proteolysis effect. The finding indicates there is alternation in response of the enzyme towards trypsin during the aging process. It would be more preferable to study the effect of endogenous protease in the cell. Thus the effect







**Figure 9 Secondary plot: Response of  $\text{Ca}^{2+}$ -ATPase in young and aged erythrocytes towards oleic acid**

The plot represents means from 6 experiments. \* The differences are significant with  $p < 0.05$ .

**Table 3 Controlled tryptic proteolysis of membrane-bound  $\text{Ca}^{2+}$ -ATPase  
in young and aged porcine erythrocytes**

	<i>Specific activity of <math>\text{Ca}^{2+}</math>-ATPase</i> (unit / mg membrane protein)	
	<i>Young cell</i> (n=5)	<i>Aged cell</i> (n=5)
Basal	0.060 $\pm$ 0.001	0.062 $\pm$ 0.001
+0.0025 $\times 10^{-3}$ Trypsin	0.063 $\pm$ 0.001	0.113 $\pm$ 0.002
% Activation	5%	82%

40 $\mu\text{g}$  membrane protein was first incubated with 0.0025  $\times 10^{-3}$  trypsin at 37°C for 5 minutes and the proteolysis was stopped by addition of 10-fold of soybean trypsin inhibitor.  $\text{Ca}^{2+}$ -ATPase activity of the membrane was then determined by coupled-enzyme assay. Each sample was assayed in triplicate and error within 5% was accepted. Results are mean  $\pm$  1 SD obtained from 5 experiments.



of potent calcium-dependent protease, calpain, on  $\text{Ca}^{2+}$ -ATPase activity was further investigated. The membrane-bound enzyme was subjected to proteolysis by calpain instead of trypsin. The procedure for calpain digestion of the membrane was similar to that of trypsin proteolysis. Membrane-bound  $\text{Ca}^{2+}$ -ATPase was first incubated with 1 unit of calpain I and the reaction was stopped by 10-fold calpain inhibitor I. The enzyme activities of both treated and untreated membranes were then determined and the results are shown in Table 4. A similar trend of alteration of response as in previous tryptic treatment was observed. The activation effect of calpain on  $\text{Ca}^{2+}$ -ATPase in aged erythrocytes, however, was much higher, to be around 90%. The result shows that the response of  $\text{Ca}^{2+}$ -ATPase towards calpain increases during *in vivo* aging of erythrocyte.

#### **5. Partial purification of $\text{Ca}^{2+}$ -ATPase inhibitor protein (PMCAI) from porcine erythrocytes**

After studying the response of  $\text{Ca}^{2+}$ -ATPase towards activators during the aging process, study was then focused on the inhibitor of the enzyme.  $\text{Ca}^{2+}$ -ATPase inhibitor protein (PMCAI) is the least characterized candidate amongst the regulators. Before studying the interaction of  $\text{Ca}^{2+}$ -ATPase with its regulators,  $\text{Ca}^{2+}$ -ATPase inhibitor protein (PMCAI) was first purified from porcine erythrocytes. Plasma membrane  $\text{Ca}^{2+}$ -ATPase inhibitor protein (PMCAI) from porcine hemolysate was partially purified by anion-exchange chromatography. The YM-2 concentrate of PM-10 filtrate from porcine hemolysate was loaded onto DEAE-

**Table 4 Controlled calpain proteolysis of membrane-bound  $\text{Ca}^{2+}$ -ATPase in young and aged porcine erythrocytes**

	<i>Specific activity of <math>\text{Ca}^{2+}</math>-ATPase (unit/ mg membrane protein)</i>	
	<i>Young cell (n=5)</i>	<i>Aged cell (n=5)</i>
Basal	0.060 $\pm$ 0.001	0.062 $\pm$ 0.001
+ 1 unit Calpain I	0.065 $\pm$ 0.002	0.120 $\pm$ 0.003
% Activation	8%	94%

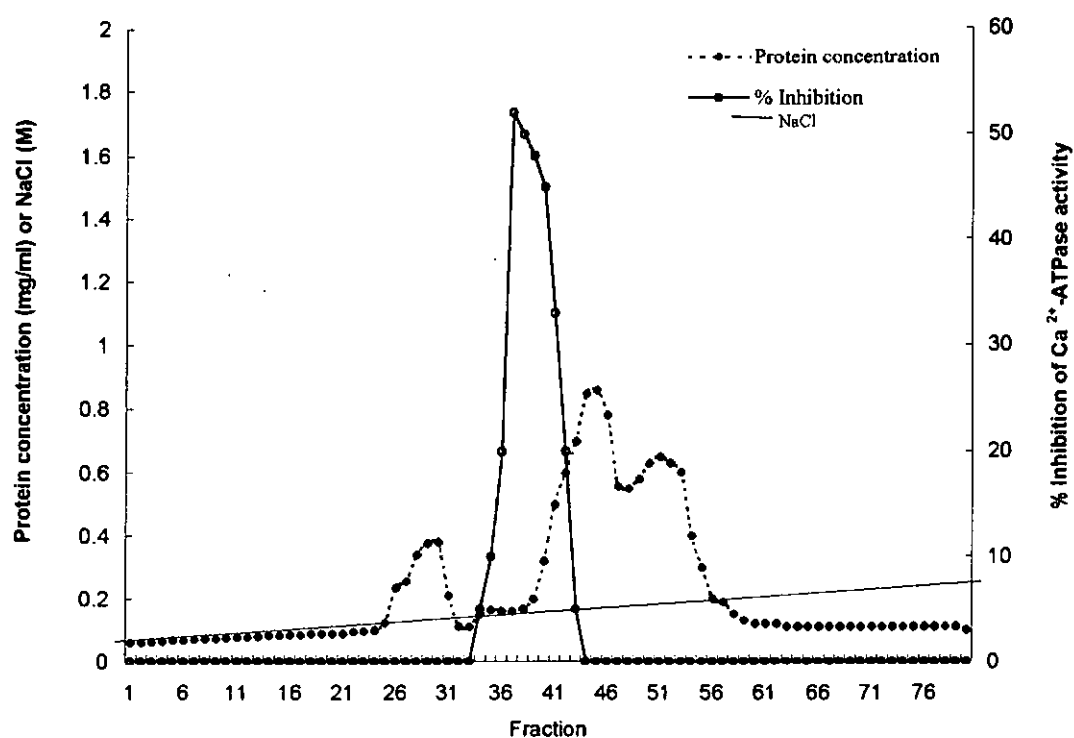
40 $\mu$ g membrane protein was first incubated with 1 unit calpain I at 37°C for 10 min. and the proteolysis was stopped by addition of 10-fold of calpain inhibitor I.  $\text{Ca}^{2+}$ -ATPase activity of the membrane was then determined by coupled-enzyme assay. Each sample was assayed in triplicate and error within 5% was accepted. Results are mean  $\pm$  1 SD obtained from 5 experiments.

Sephadex column (bed volume: 120ml) with flow rate of 40ml/hr. The column was first equilibrated with imidazole.HCl, pH 5.2 and the column was washed by 0.1M NaCl buffer after sample loading. The column was then eluted by linear 0.1-0.25M NaCl gradient. The fractions collected were screened for inhibitory activity by inorganic phosphate assay. The activity and elution profiles are shown as Figure 10.

From the profiles, it shows that fractions containing the PMCAI were eluted between 0.165 and 0.18M NaCl. The degree of inhibition of  $\text{Ca}^{2+}$ -ATPase was around 33 to 52%. The result obtained here agrees with that of Au (1996). The protein content of pooled active post-DEAE-Sephadex fractions was determined by Stains-all absorption spectrum and the estimated yield of partially purified PMCAI was 0.112 mg PMCAI/L packed cells.

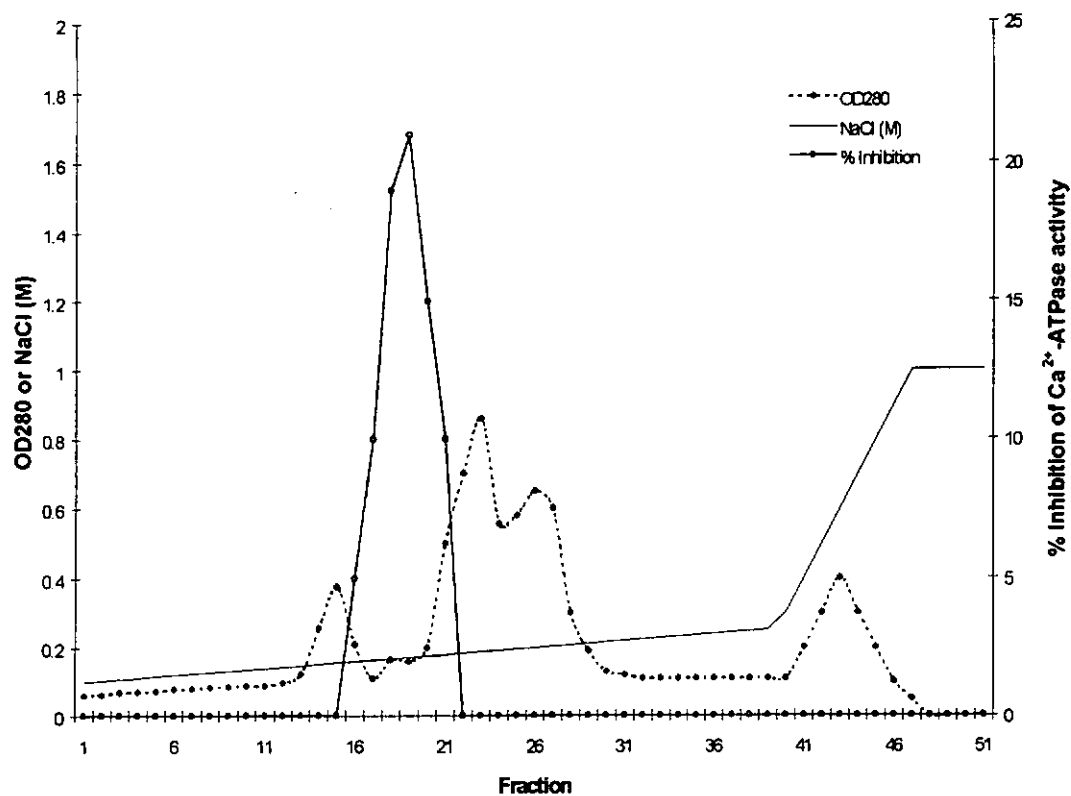
In addition, DEAE-Superose Fast Flow column of ATKA Explorer 100 was also used for the purification of PMCAI. The bed volume of the column was 30ml with the flow rate of 5ml/min instead. The elution profile of the column is shown in Figure 11. It was found that the elution profile of the column was similar to that of DEAE-Sephadex column (Refer to Fig. 10 ) except that the percentage of inhibition and the estimated yield of PMCAI were much lower.  $\text{Ca}^{2+}$ -ATPase activity was inhibited by 21% and the yield of partially purified PMCAI was only 0.03mg PMCAI/L packed cells.

It was the first time that DEAE-Superose column of AKTA system was employed for the preparation of PMCAI. The result obtained showed that this could



**Figure 10 Elution profile of DEAE-Sephadex A-25 column chromatography for the purification of plasma membrane  $\text{Ca}^{2+}$ -ATPase protein inhibitor (PMCAI)**

YM-2 concentrate from 200ml packed cells was loaded onto DEAE-Sephadex A-25 column (bed volume: 120ml) with flow rate of 40ml/min. The column was equilibrated by 50mM imidazole buffer, pH 5.2 and was eluted by 0.1-0.25M NaCl gradient. The fractions were screened for the presence of PMCAI by inorganic phosphate assay.



**Figure 11 Elution profile of DEAE-Superose column chromatography for the purification of plasma membrane  $\text{Ca}^{2+}$ -ATPase protein inhibitor (PMCAI)**

YM-2 concentrate from 200ml packed cells was loaded onto DEAE-Superose column (bed volume: 25ml) with flow rate of 5ml/min. The column was equilibrated with 50mM imidazole buffer, pH 5.2 and was eluted by 0.1-0.25M NaCl gradient. The fractions were screened for the presence of PMCAI by inorganic phosphate assay.

be an alternative choice. Though the running time for the DEAE-Sephadex column was much longer, it was more preferable for the purification of PMCAI for the following study on regulation of  $\text{Ca}^{2+}$ -ATPase.

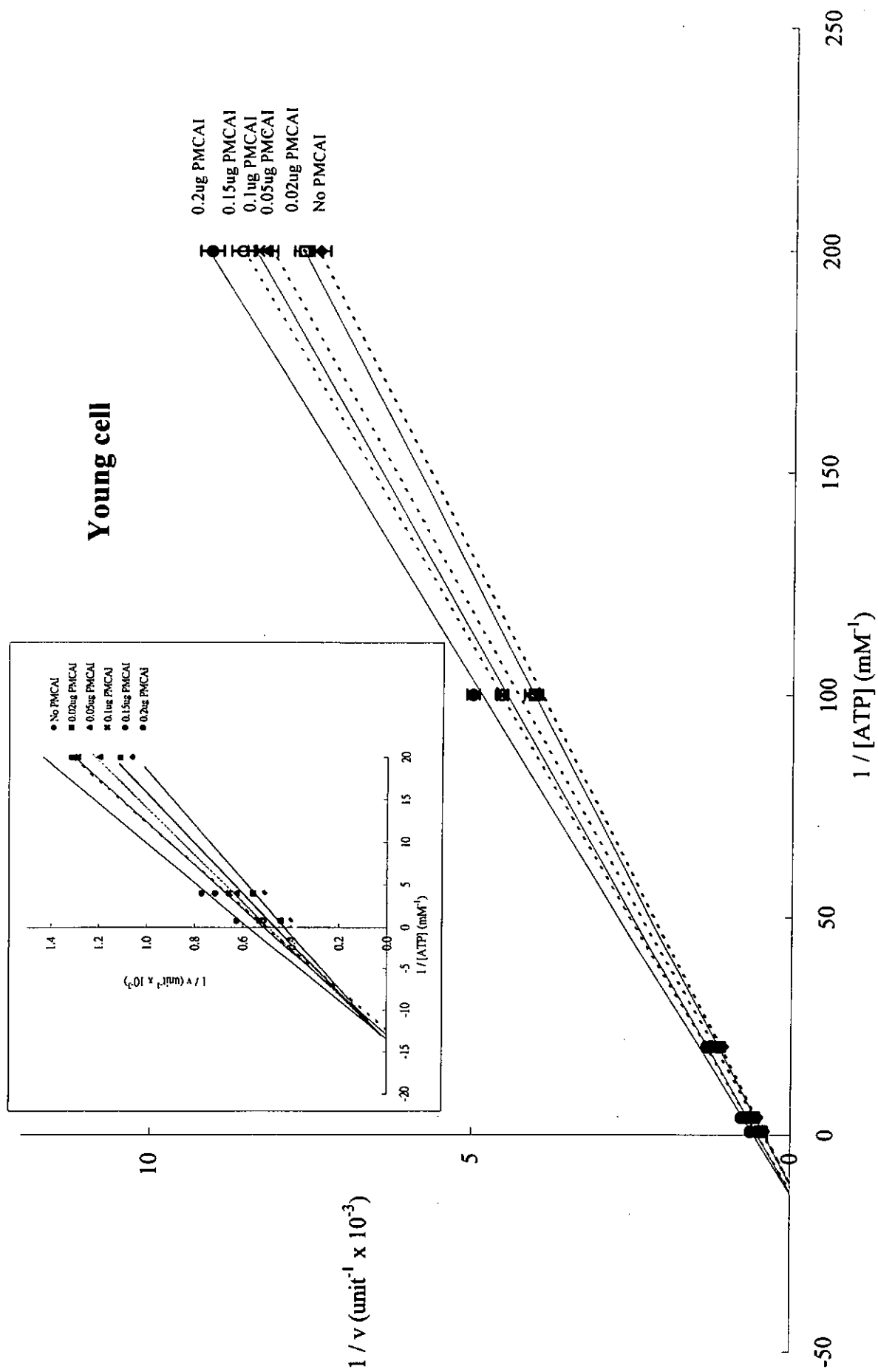
#### **6. Effect of endogenous PMCAI on membrane $\text{Ca}^{2+}$ -ATPase of young and old erythrocytes**

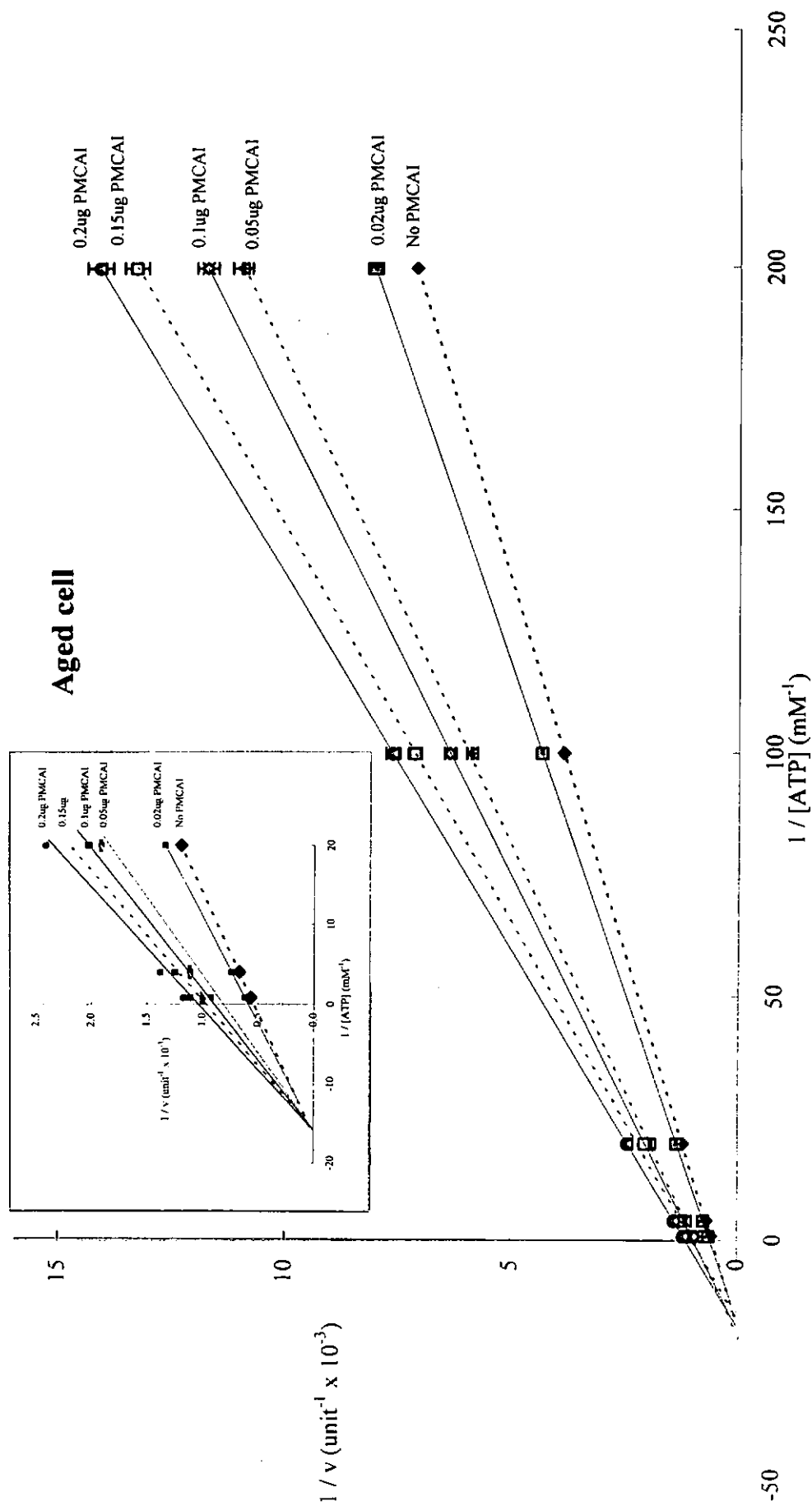
The response of  $\text{Ca}^{2+}$ -ATPase in the two age populations of erythrocytes towards its protein inhibitor (PMCAI) was then studied. As revealed from the double-reciprocal and secondary plot (Figure 12 & 13), the maximal response of membrane-bound enzyme in young and aged cells towards PMCAI were 0.022 and 0.011 unit / mg membrane protein. The difference is largely significant ( $n=6$ ,  $p<0.05$ ). However, the difference between the  $K_i$  (PMCAI) of the enzyme in young and aged cells was relatively small, i.e. 0.029 and 0.026  $\mu\text{g}$  respectively.

#### **7. Membrane $\text{Ca}^{2+}$ -ATPase activity in fresh and outdated human erythrocytes**

The findings of the changes in  $\text{Ca}^{2+}$ -ATPase and its regulation in young and old erythrocytes were informative. It provides us an area to look into the possible mechanism of aging. It would be beneficial if this animal model can apply to human bank blood too. The alternation of during blood storage and the preservation of bank blood have always been some of the areas of interest for blood transfusion. Thus, the work was then extended to study on the alternation of  $\text{Ca}^{2+}$ -ATPase during blood storage.

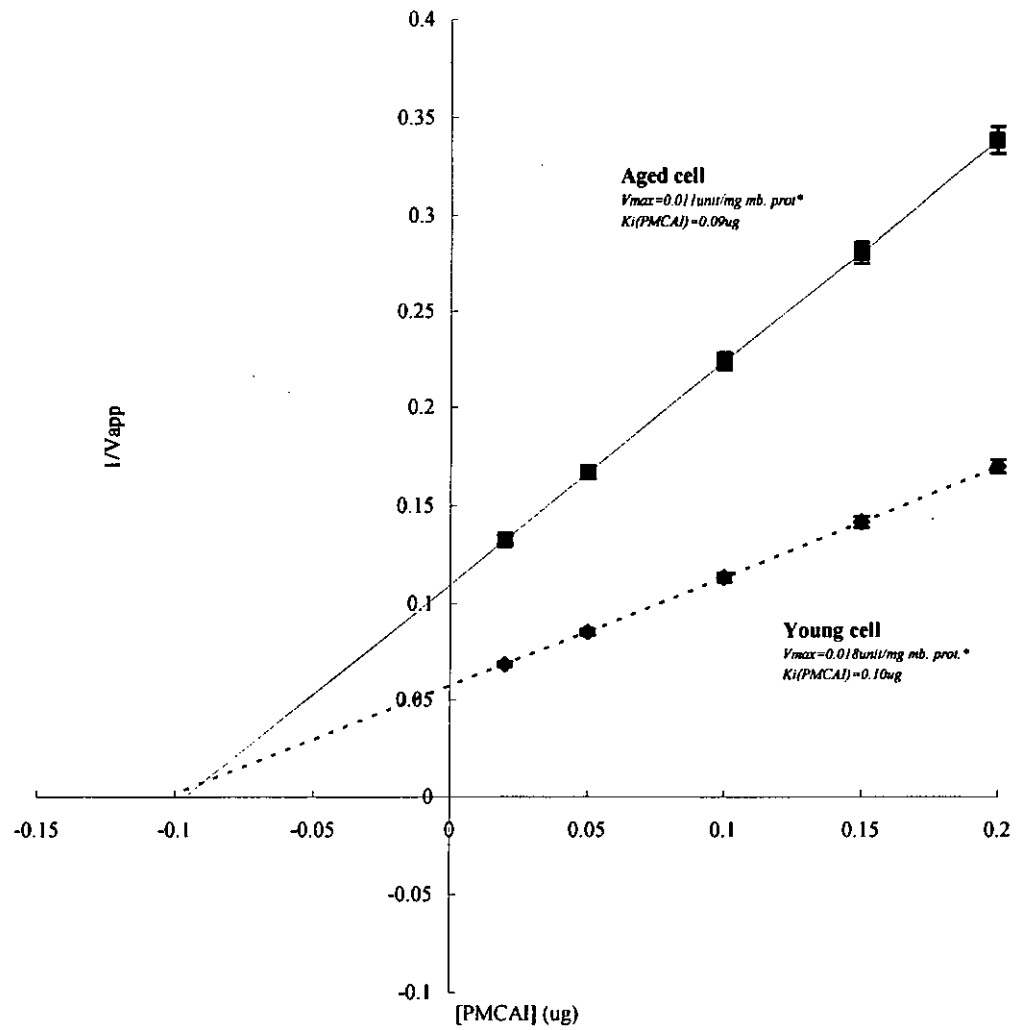
# Young cell





**Figure 12 Double-reciprocal plot: Response of  $\text{Ca}^{2+}$ -ATPase in young and aged erythrocytes towards PMCAI**  
 Double-reciprocal plot of  $\text{Ca}^{2+}$ -ATPase activity in young and aged erythrocytes vs ATP concentration.  $\text{Ca}^{2+}$ -ATPase activity of 30ug membrane protein was measured by coupled-enzyme assay in the presence of PMCAI (0 - 0.2ug). Each test was assayed in triplicate and error within 5% was accepted. The plot above represents a typical membrane preparation.





**Figure 13 Secondary plot: Response of  $\text{Ca}^{2+}$ -ATPase in young and aged erythrocytes towards PMCAI**

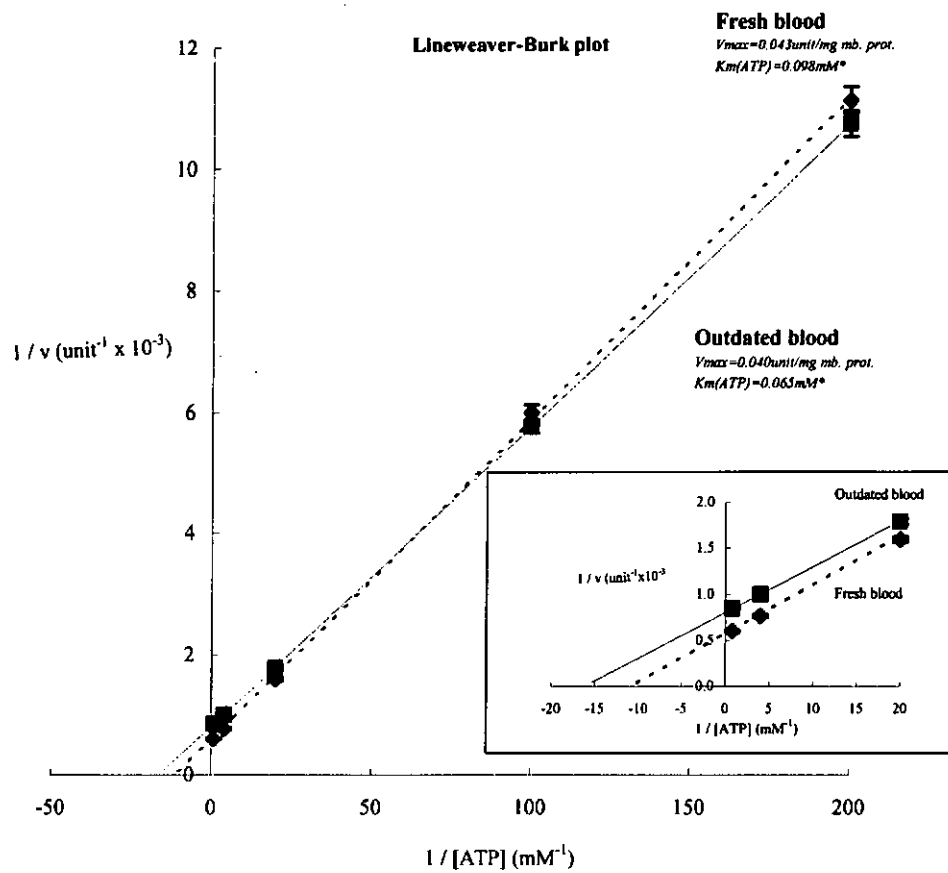
The plot represents means from 6 experiments. \* The differences are significant with  $p < 0.05$ .

For the comparative study in membrane  $\text{Ca}^{2+}$ -ATPase with respect to the response towards different concentrations of ATP (Figure 14), it shows that  $V_{\max}$  of  $\text{Ca}^{2+}$ -ATPase in fresh and outdated human erythrocytes were 0.043 unit/ mg membrane protein and 0.040 unit / mg membrane protein. The difference was found not significant. However, for  $K_m(\text{ATP})$  of  $\text{Ca}^{2+}$ -ATPase, there was significant difference ( $n=6$ ,  $p<0.05$ ). The values for fresh and outdated cells were 0.098mM and 0.065mM respectively.

#### **8. Study of interaction of membrane-bound $\text{Ca}^{2+}$ -ATPase with regulators during human blood storage with regulators**

Since affinity of  $\text{Ca}^{2+}$ -ATPase towards ATP changed during blood storage, there may be changes in the regulation of the enzyme by its regulators. The interactions of  $\text{Ca}^{2+}$ -ATPase with various regulators were studied in order to find out whether the enzyme has been altered in regulation during blood storage. The membrane-bound  $\text{Ca}^{2+}$ -ATPase was assayed in the presence of regulators individually.

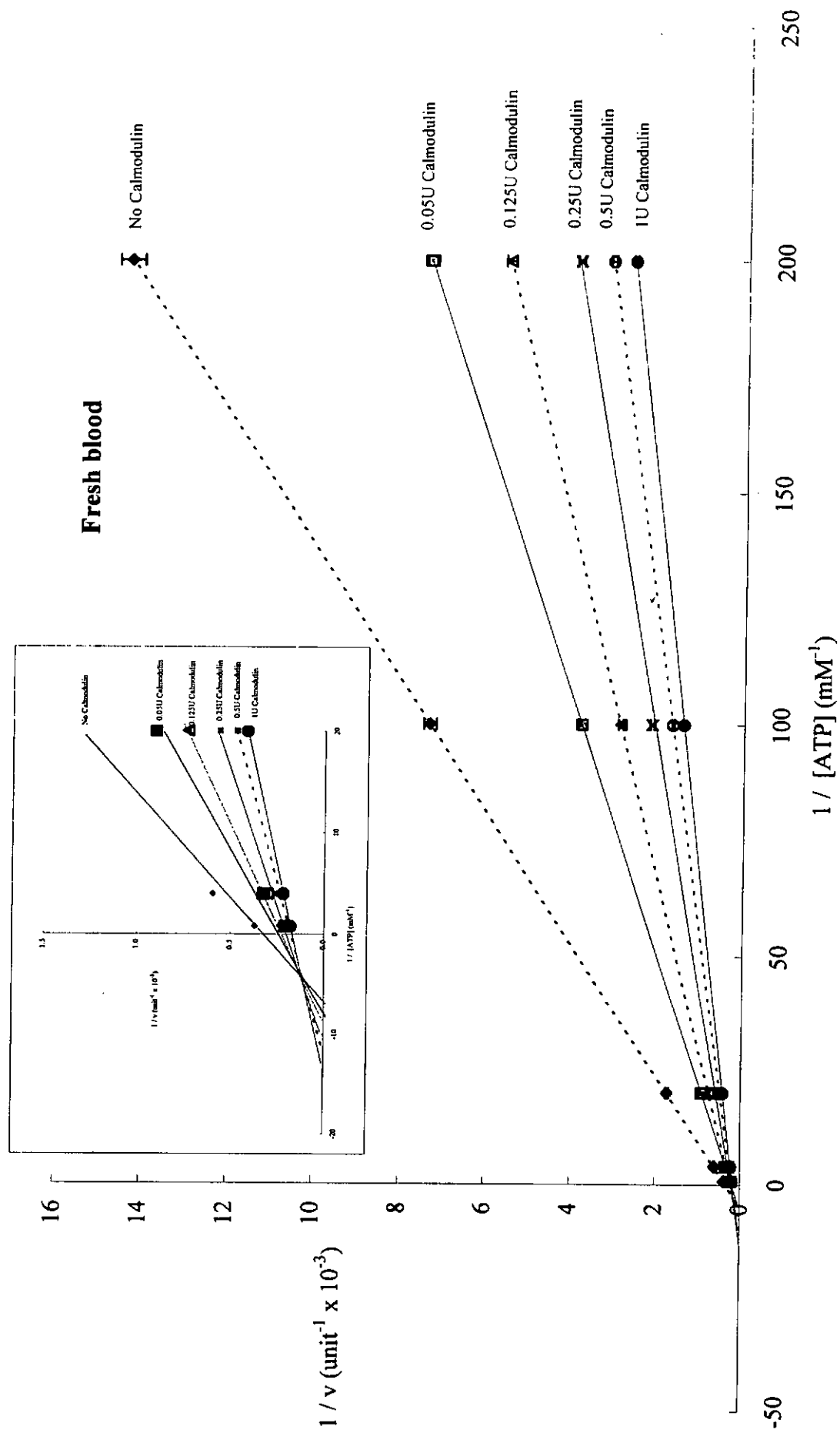
The affinity and response of the enzyme towards calmodulin was found to be nearly the same during the aging process (Figure 15 & 16).  $K_a$  (calmodulin) and  $V_{\max}$  were 0.060U and 0.058 unit / mg membrane protein for fresh blood while that for the outdated blood was 0.054U and 0.065 unit / mg membrane protein.

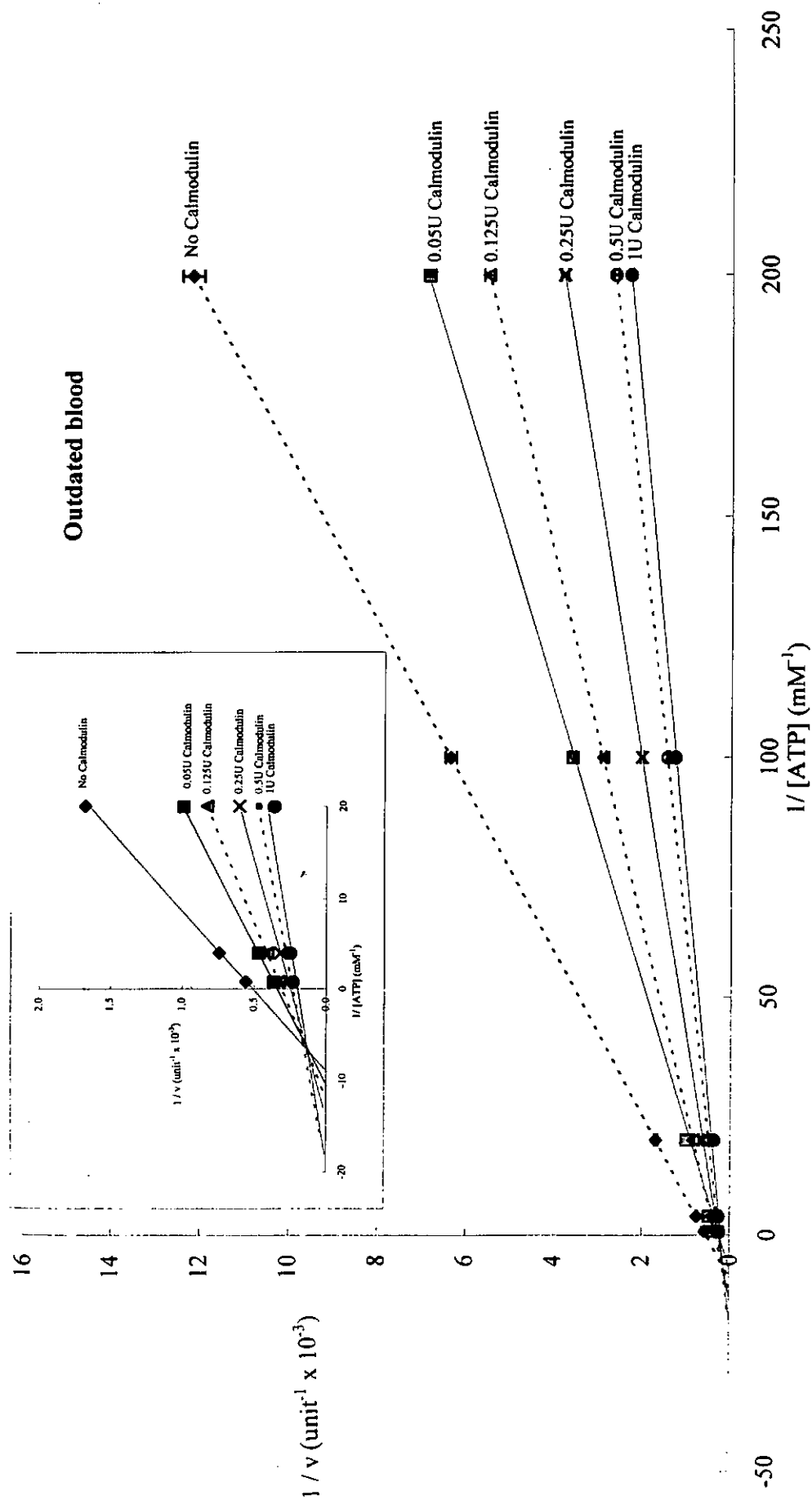


**Figure 14 Double-reciprocal plot: Response of  $\text{Ca}^{2+}$ -ATPase in fresh and outdated human blood vs ATP**

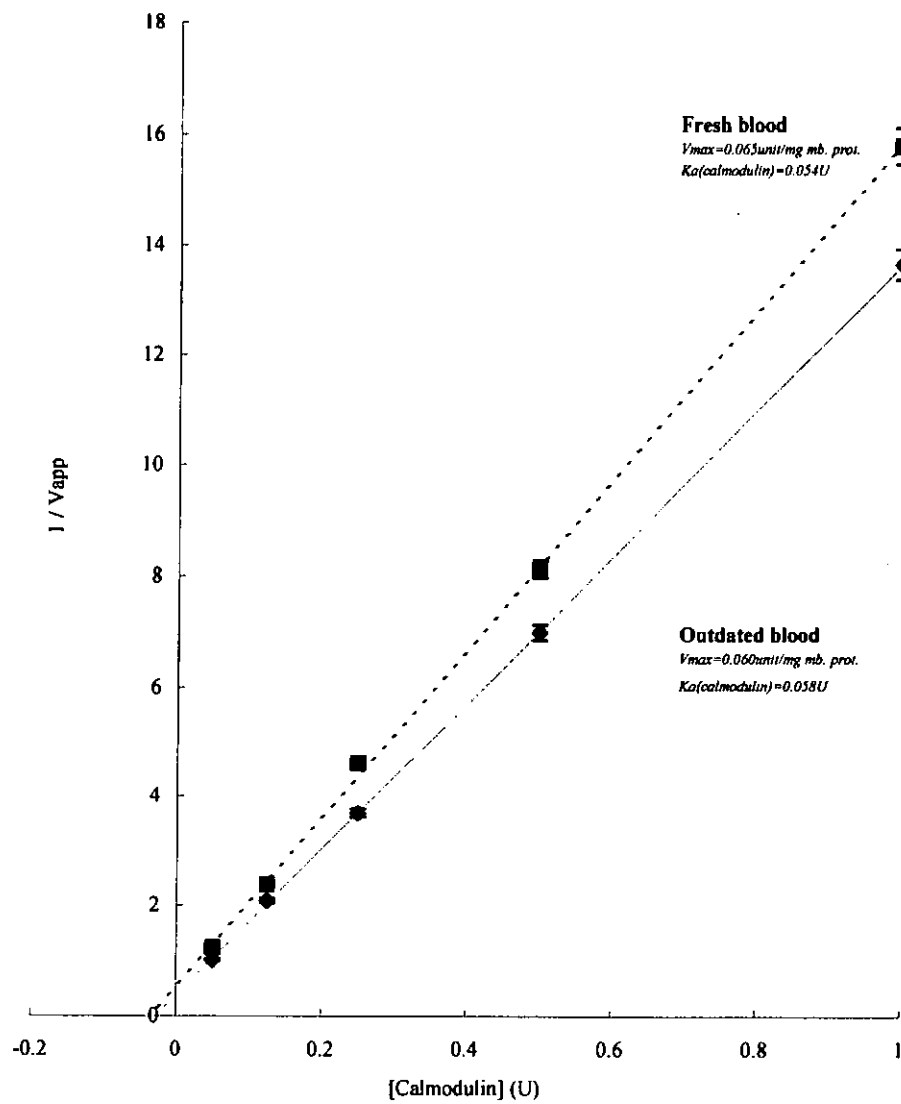
$\text{Ca}^{2+}$ -ATPase activity of 30ug membrane protein was measured by coupled-enzyme assay in the presence of ATP (0 - 1.25mM). Each sample was assayed in triplicate and error within 5% was accepted. The plot represents means from 6 experiments.

\* The differences are significant with  $p < 0.05$ .

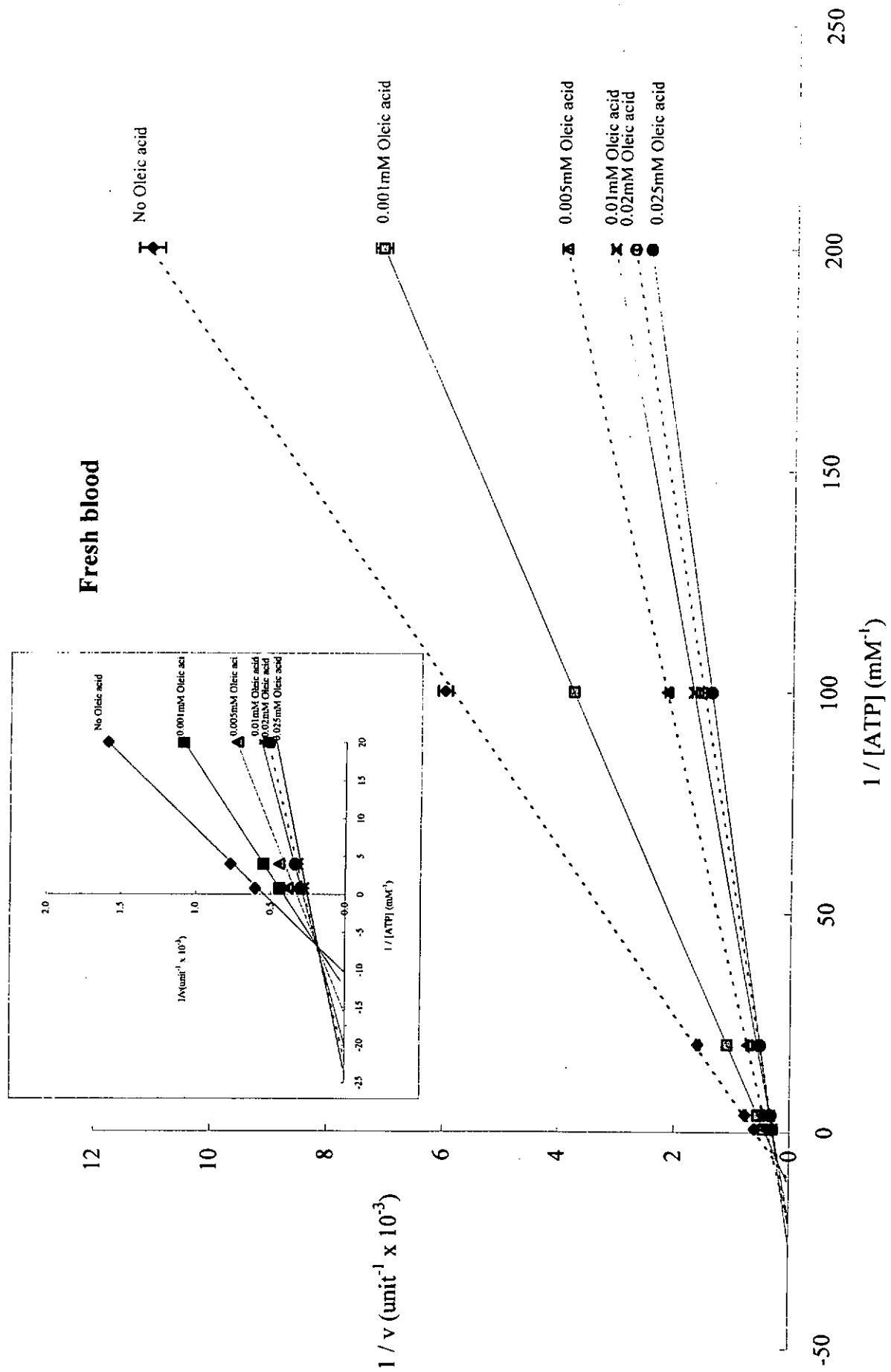




**Figure 15 Double-reciprocal plot: Response of  $\text{Ca}^{2+}$ -ATPase in fresh and outdated human blood towards calmodulin**  
 Double-reciprocal plot of  $\text{Ca}^{2+}$ -ATPase activity in fresh and outdated blood vs ATP concentration.  $\text{Ca}^{2+}$ -ATPase activity of 30ug membrane protein was measured by coupled-enzyme assay in the presence of calmodulin (0 - 1 unit). Each test was assayed in triplicate and error within 5% was accepted. The plot above represents a typical membrane preparation.



**Figure 16 Secondary plot: Response of  $\text{Ca}^{2+}$ -ATPase in fresh and outdated human blood towards calmodulin**  
 The plot represents means from 6 experiments.



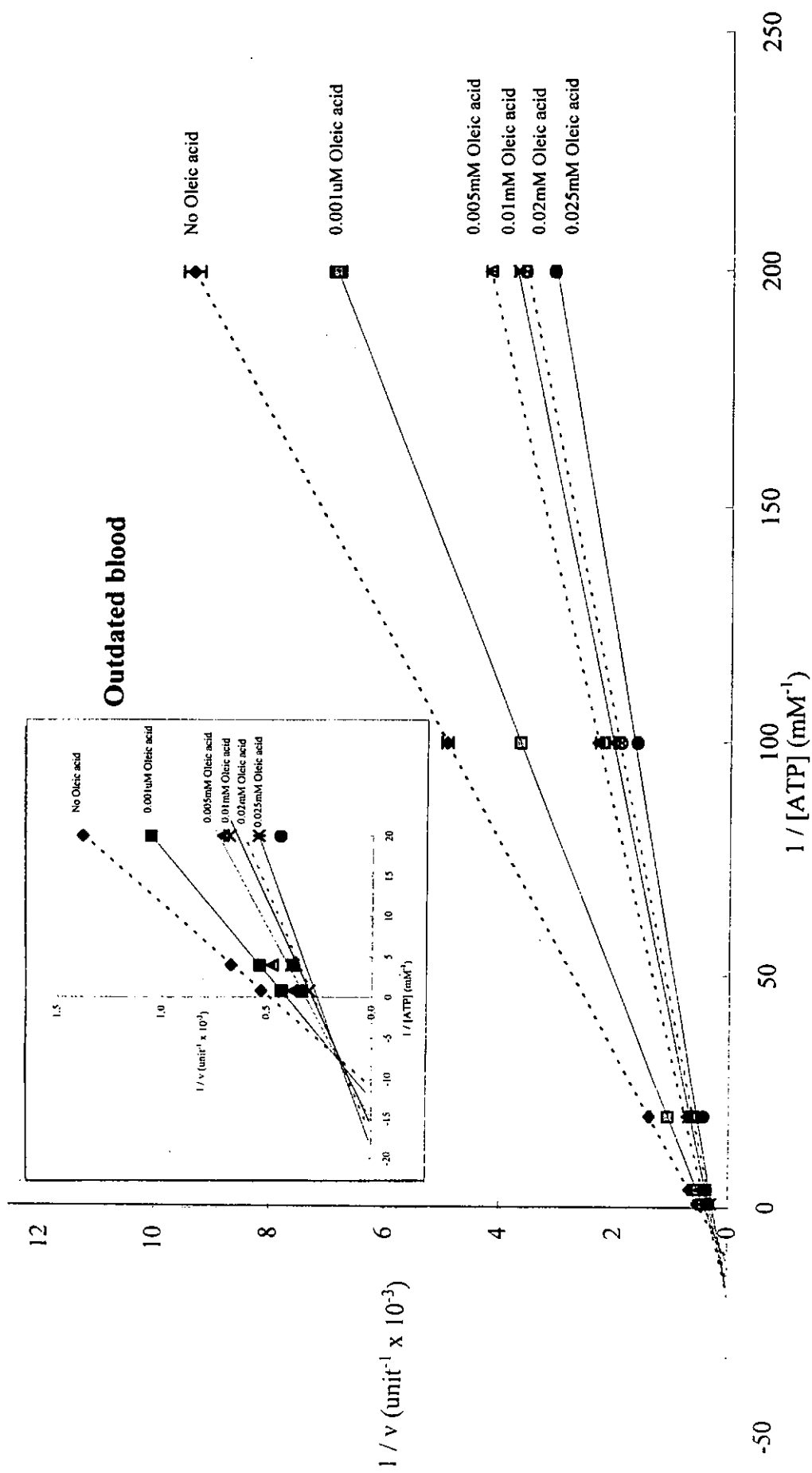
Response of the enzyme towards other activators such as oleic acid and proteolytic digestion were also studied. With respect to oleic acid (Figure 17 & 18),  $K_a$  (oleic acid) were found to be 11 and 14.5  $\mu$  M for fresh and outdated cells (n=6,  $p<0.05$ ). The maximal activity of the enzyme from fresh and outdated blood was 0.059 and 0.051 unit / mg membrane protein and the difference was small.

In controlled tryptic proteolysis, the membrane was first incubated with 0.0025 x 10<sup>-3</sup> % trypsin for 5 minutes, then 10-fold soybean trypsin inhibitor added to stop the reaction. The Ca<sup>2+</sup>-ATPase activities of treated and untreated membranes were determined. The membrane-bound Ca<sup>2+</sup>-ATPase in aged erythrocytes was found to increase by around 60% while the activation effect was not significant in fresh cells (Table 5).

The effect of calpain on Ca<sup>2+</sup>-ATPase activity was also studied. The result obtained was similar to that of controlled tryptic effect. Membrane-bound Ca<sup>2+</sup>-ATPase was first incubated with 1 unit of calpain I and the reaction was stopped by 10-fold calpain inhibitor I. The enzyme activities of both treated and untreated membranes were then determined and the result is shown in Table 6. There was similar activation effect of calpain on Ca<sup>2+</sup>-ATPase compared with tryptic treatment, however, the effect of calpain on the pump in outdated blood was much higher though not significant, to be 65%.

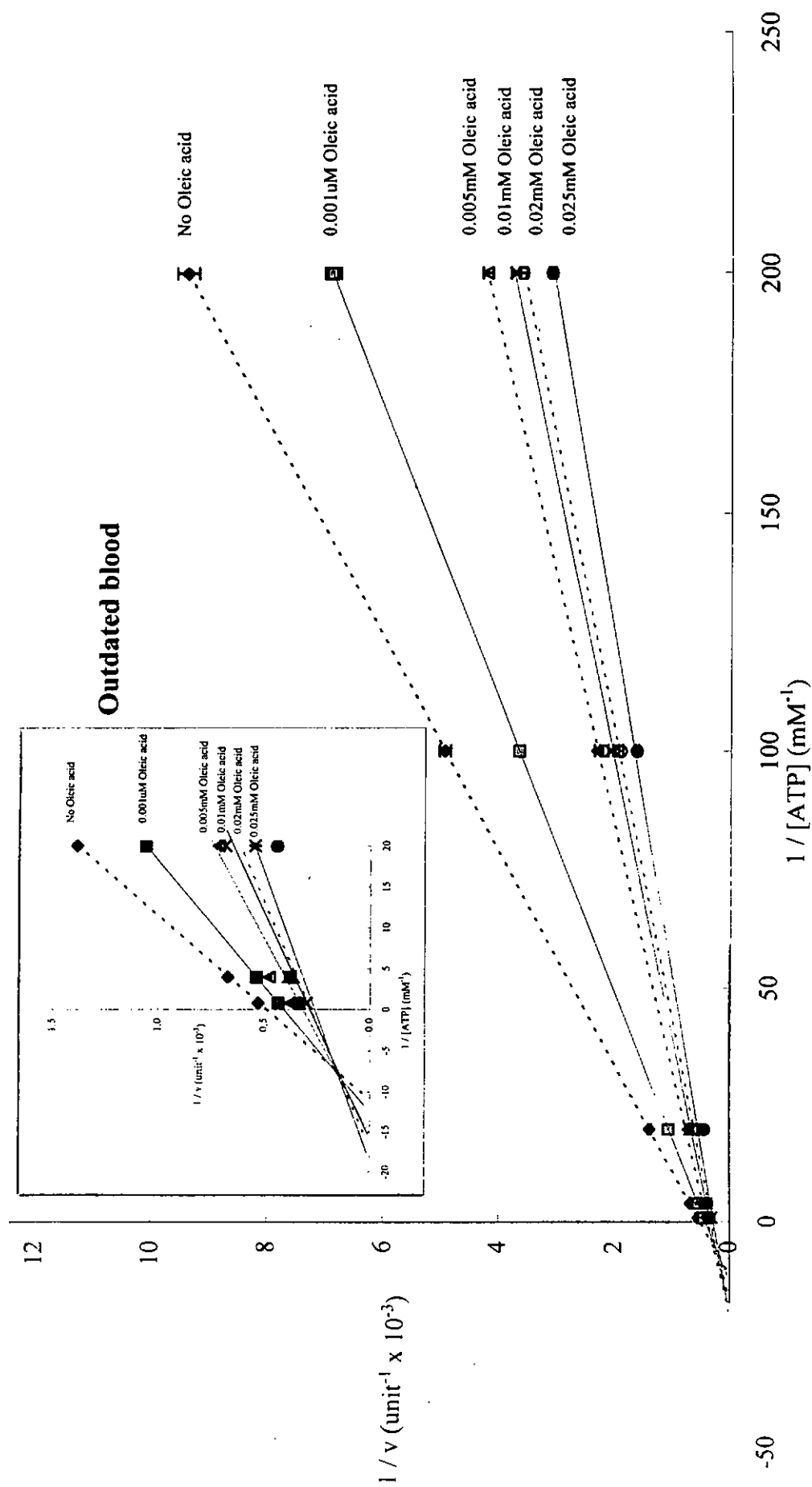
In addition, the response of Ca<sup>2+</sup>-ATPase towards its protein inhibitor





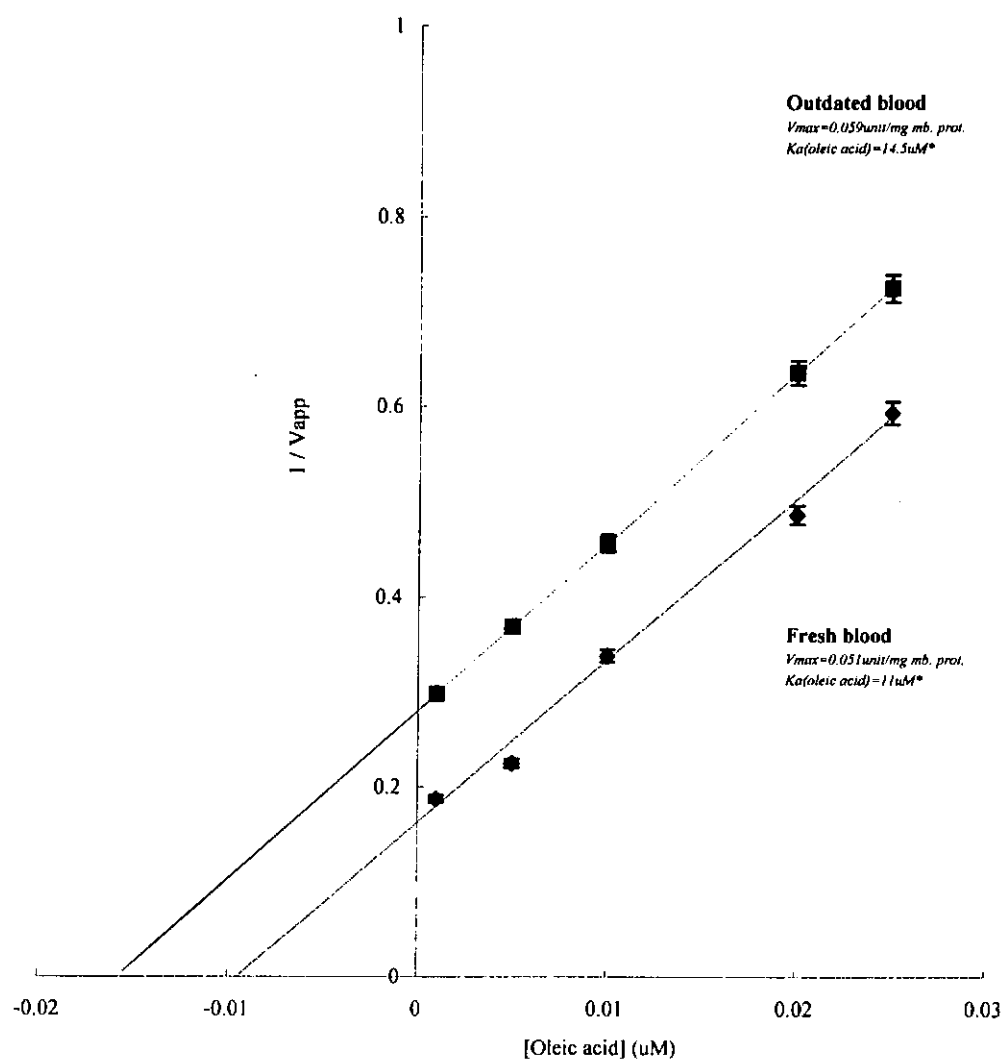
**Figure 17 Double-reciprocal plot: Response of  $\text{Ca}^{2+}$ -ATPase in fresh and outdated human blood towards oleic acid**

Double-reciprocal plot of  $\text{Ca}^{2+}$ -ATPase activity in fresh and outdated blood vs ATP concentration.  $\text{Ca}^{2+}$ -ATPase activity of 30  $\mu\text{g}$  membrane protein was measured by coupled-enzyme assay in the presence of oleic acid (0 - 0.025  $\text{mM}$ ). Each test was assayed in triplicate and error within 5% was accepted. The plot above represents a typical membrane preparation.



**Figure 17 Double-reciprocal plot: Response of  $\text{Ca}^{2+}$ -ATPase in fresh and outdated human blood towards oleic acid**

Double-reciprocal plot of  $\text{Ca}^{2+}$ -ATPase activity in fresh and outdated blood vs ATP concentration.  $\text{Ca}^{2+}$ -ATPase activity of 30  $\mu\text{g}$  membrane protein was measured by coupled-enzyme assay in the presence of oleic acid (0 - 0.025  $\text{mM}$ ). Each test was assayed in triplicate and error within 5% was accepted. The plot above represents a typical membrane preparation.



**Figure 18 Secondary plot: Response of  $\text{Ca}^{2+}$ -ATPase in fresh and outdated human blood towards oleic acid**

The plot represents means from 6 experiments. \* The differences are significant with  $p < 0.05$ .

**Table 5 Controlled tryptic proteolysis of membrane-bound  
Ca<sup>2+</sup>-ATPase from fresh and outdated blood**

	<i>Specific activity of Ca<sup>2+</sup>-ATPase (unit/ mg membrane protein)</i>	
	<i>Fresh blood (n=5)</i>	<i>Outdated blood (n=5)</i>
Basal	0.043±0.001	0.040±0.001
+0.0025×10 <sup>-3</sup> Trypsin	0.049±0.001	0.064±0.001
% Activation	14%	60%

40µg membrane protein was first incubated with 0.0025 x 10<sup>-3</sup> trypsin at 37°C for 5 minutes and the proteolysis was stopped by addition of 10-fold of soybean trypsin inhibitor. Ca<sup>2+</sup>-ATPase activity of the membrane was then determined by coupled-enzyme assay. Each sample was assayed in triplicate and error within 5% was accepted. Results are mean ± 1 SD obtained from 5 experiments.

**Table 6 Controlled calpain proteolysis of membrane-bound  $\text{Ca}^{2+}$ -ATPase from fresh and outdated blood**

	<i>Specific activity of <math>\text{Ca}^{2+}</math>-ATPase (unit/ mg membrane protein)</i>	
	<i>Fresh blood (n=5)</i>	<i>Aged blood (n=5)</i>
Basal	0.043±0.001	0.040±0.001
+ 1 unit Calpain I	0.050±0.001	0.066±0.002
% Activation	16%	65%

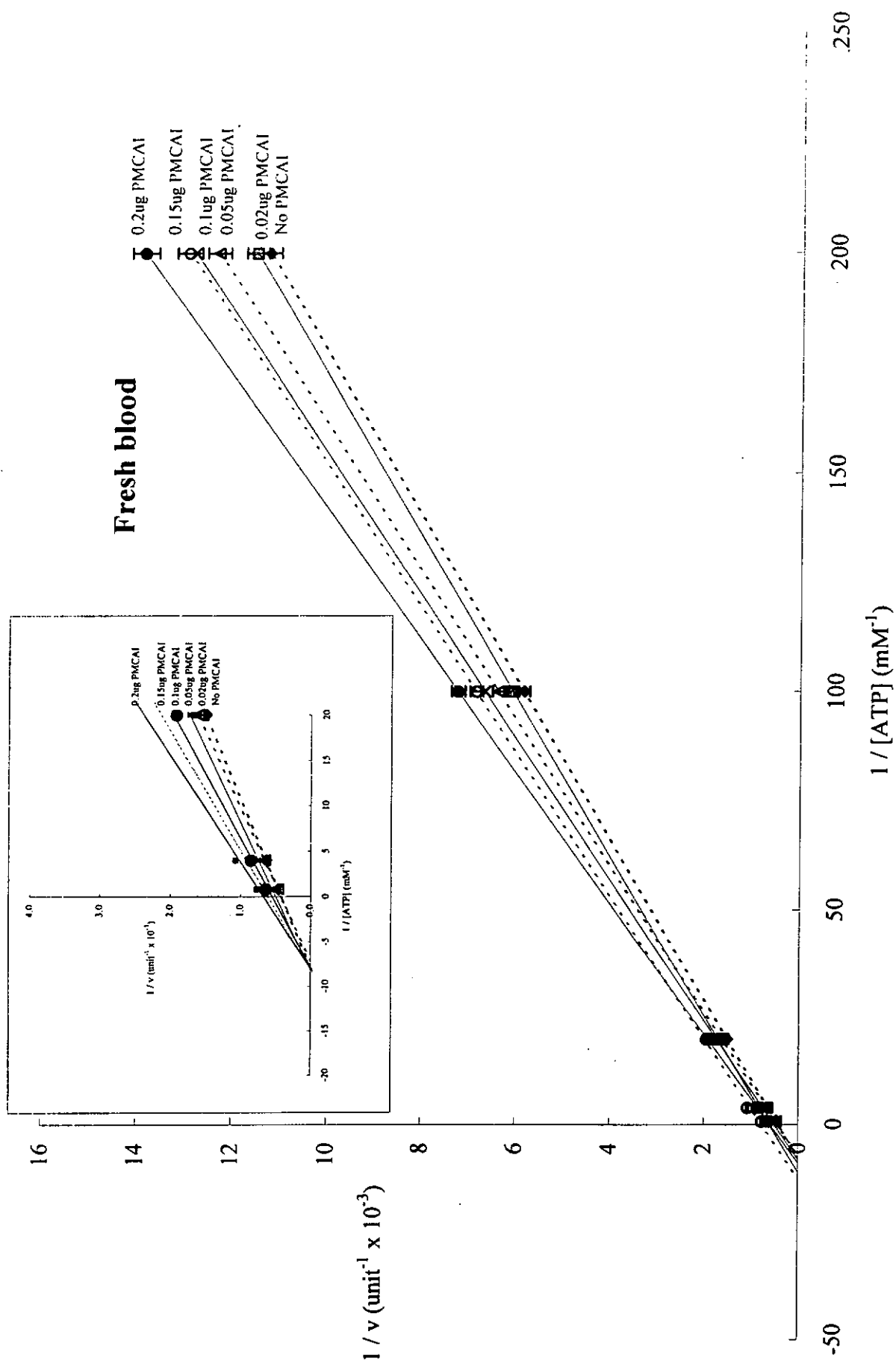
40µg membrane protein was first incubated with 1unit calpain I at 37°C for 10 min. and the proteolysis was stopped by addition of 10-fold of calpain inhibitor I.  $\text{Ca}^{2+}$ -ATPase activity of the membrane was then determined by coupled-enzyme assay. Each sample was assayed in triplicate and error within 5% was accepted. Results are mean ± 1 SD obtained from 5 experiments.

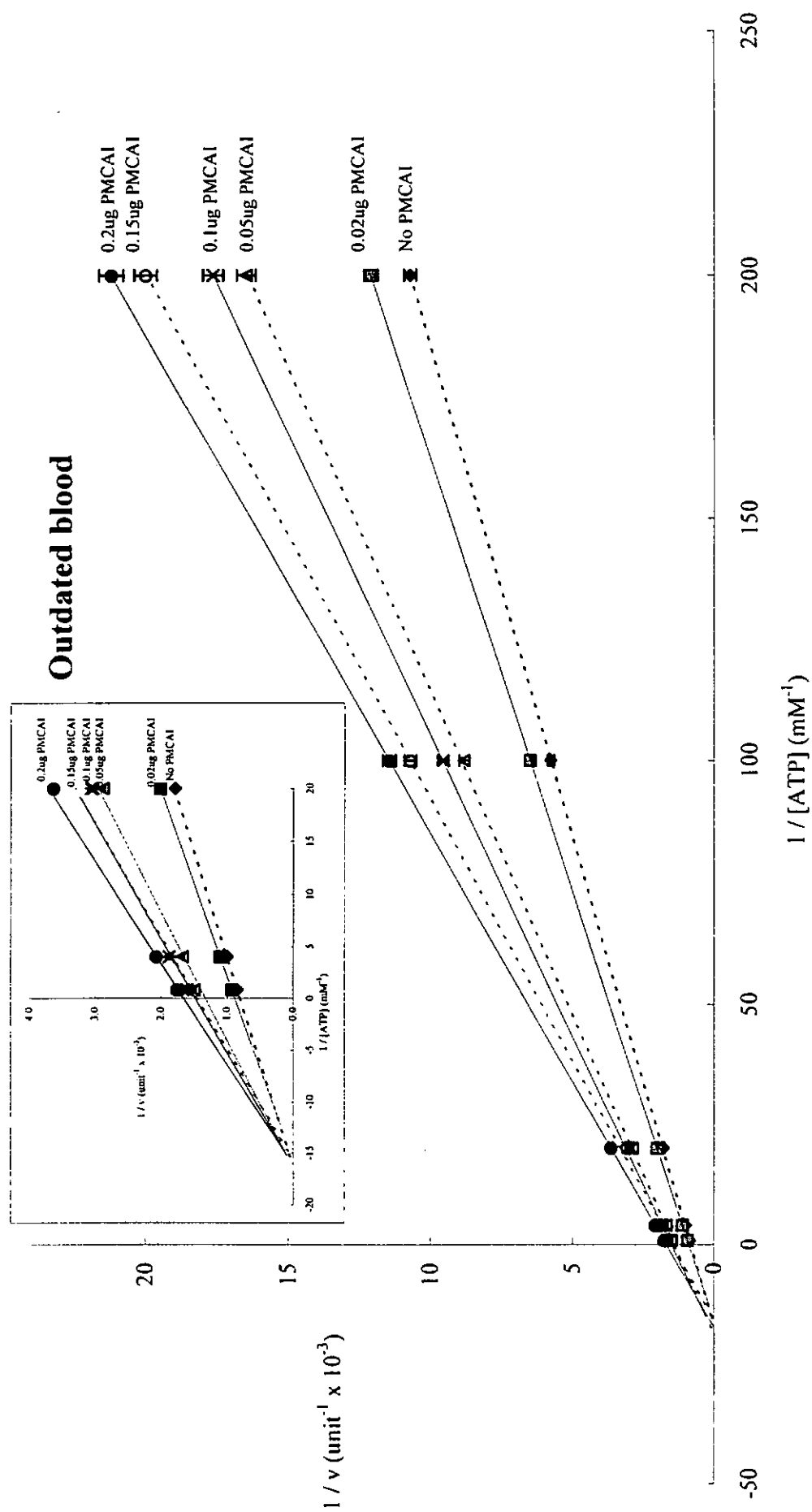
(PMCAI) during blood storage was studied. The double-reciprocal and secondary plot (Figure 19 & 20) shows that the response of membrane-bound enzyme from fresh and outdated blood towards PMCAI were 0.024 and 0.013 unit/mg membrane protein. The difference is largely significant ( $n=6$ ,  $p<0.05$ ). However, the difference between the  $K_i$ (PMCAI) of the enzyme in fresh and outdated blood found was small, i.e. 0.029 and 0.026  $\mu$ g respectively.

From the results on the regulation of  $\text{Ca}^{2+}$ -ATPase in fresh and outdated human blood, they show that there are changes in the regulation of the pump during blood storage. The alternation during both *in vivo* and *in vitro* aging of erythrocytes may be due to alternation of the calcium pump itself. Subsequently, the study was then focused on the calcium pump itself.

## **9. Purification of $\text{Ca}^{2+}$ -ATPase from porcine and human erythrocytes**

To study if there are any structural and conformational alteration of  $\text{Ca}^{2+}$ -ATPase itself during *in vivo* aging and blood storage, the enzymes should be purified first. Purification of membrane-bound  $\text{Ca}^{2+}$ -ATPase from young and aged erythrocytes by calmodulin-affinity column chromatography was performed in parallel. The membranes were first solubilized by Triton X-100 and were re-suspended in an environment with phosphatidylserine. The solubilized enzymes were then loaded onto the column. The column was eluted with calcium buffer and then followed by the EDTA buffer.

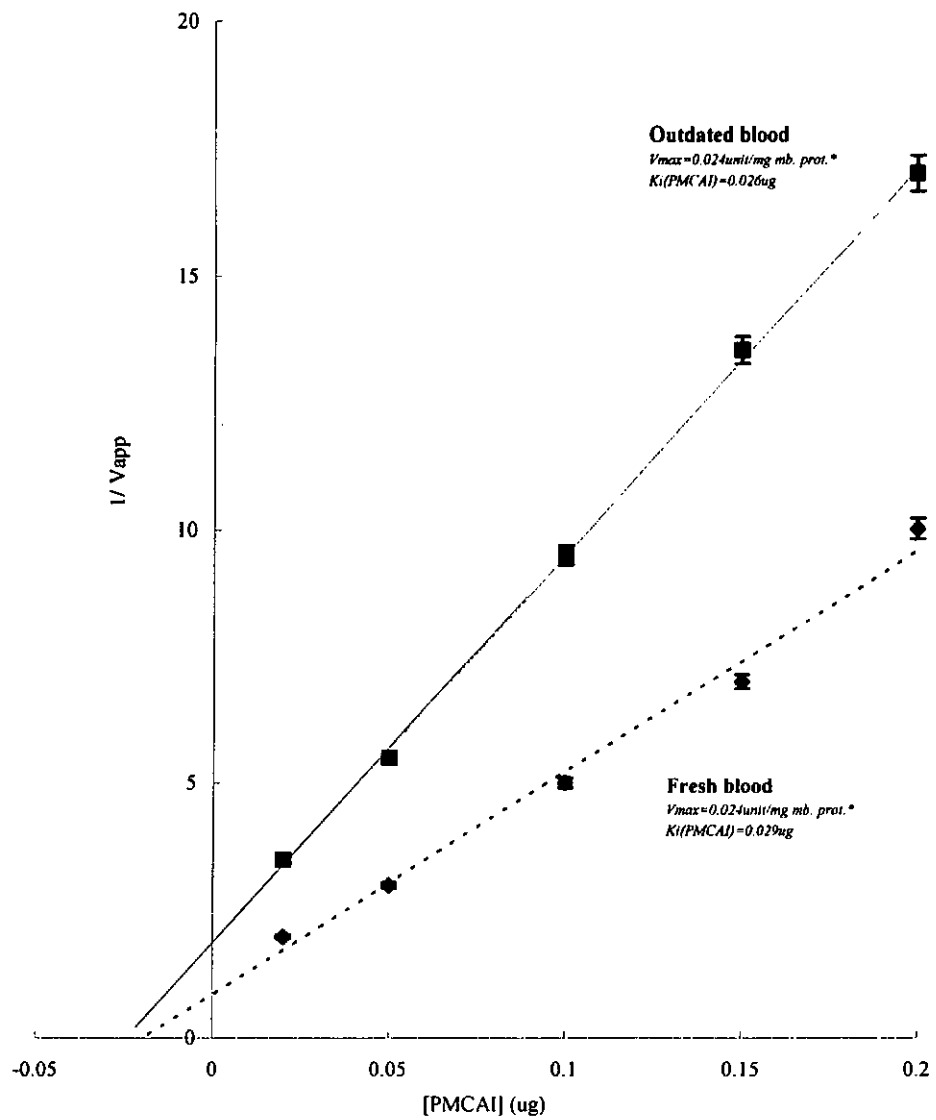




**Figure 19 Double-reciprocal plot: Response of  $\text{Ca}^{2+}$ -ATPase in fresh and outdated human blood towards PMCAI**

Double-reciprocal plot of  $\text{Ca}^{2+}$ -ATPase activity in fresh and outdated blood vs ATP concentration.  $\text{Ca}^{2+}$ -ATPase activity of 30ug membrane protein was measured by coupled-enzyme assay in the presence of PMCAI (0 - 0.2ug). Each test was assayed in triplicate and error within 5% was accepted. The plot above represents a typical membrane preparation.





**Figure 20 Secondary plot: Response of  $\text{Ca}^{2+}$ -ATPase in fresh and outdated human blood towards PMCAI**

The plot represents means from 6 experiments. \* The differences are significant with  $p < 0.05$ .

The purification table and elution profile of young porcine erythrocytes were obtained (Refer to Table 7 and Figure 21). It was found that washing of the column with calcium buffer eluted nearly 90% of the protein applied. The protein contained ATPase activity as determined by coupled-enzyme assay. Since this protein did not bind to calmodulin-affinity column, this was likely to be  $Mg^{2+}$ -ATPase or  $Ca^{2+}$ -ATPase with calmodulin-binding domain cleaved off. When column was eluted with EDTA buffer, a small peak of protein was obtained with nearly 50% recovery of total  $Ca^{2+}$ -ATPase activity. The fold of purification was 51-fold.

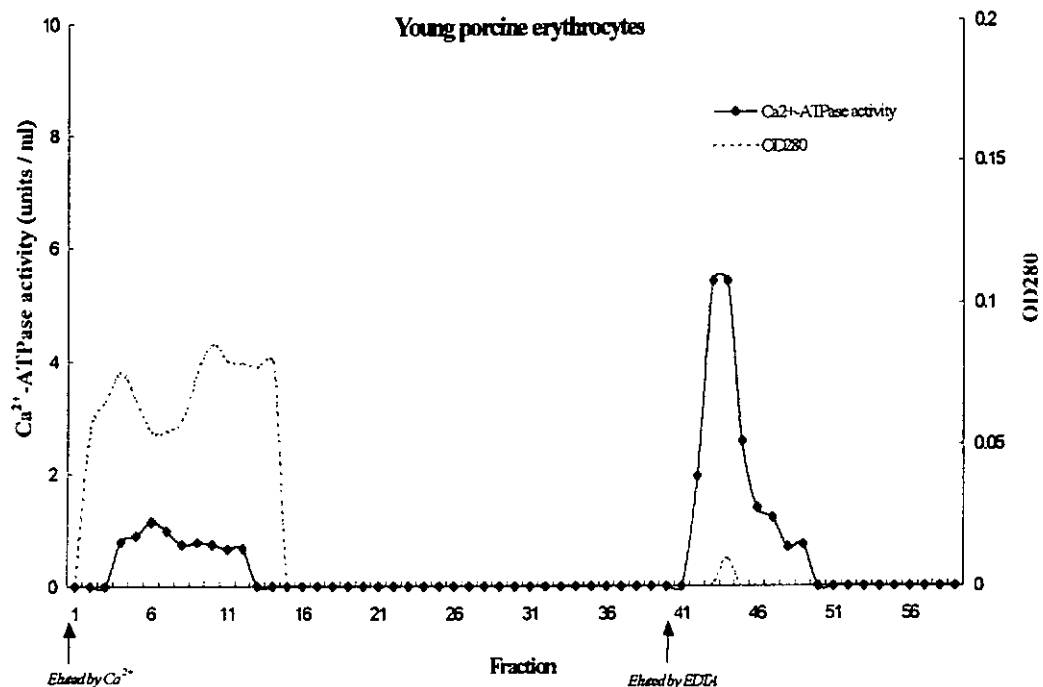
Similar elution profile was obtained for the purification of aged porcine erythrocytes (Figure 22 and Table 8). Nearly 70% of the protein applied was eluted by calcium buffer. This indicates that larger amount of calmodulin-sensitive protein was present in aged erythrocytes. In addition, it was found that only 18% of total  $Ca^{2+}$ -ATPase were obtained after EDTA elution. The fold of purification was 57-fold. The difference of folds of purification between that from young and aged cells was not significant.

In addition,  $Ca^{2+}$ -ATPase from fresh and outdated blood was also purified. From the purification table and elution profile of fresh blood (Refer to Table 9 and Figure 23), it was found washing of the column with calcium buffer eluted nearly 80% of the protein applied. When the column was eluted by EDTA buffer, a small peak of protein was obtained with nearly 37% of total  $Ca^{2+}$ -ATPase

**Table 7 Purification table of Ca<sup>2+</sup>-ATPase in young erythrocytes**

<i>Fraction</i>	<i>Total protein (mg)</i>	<i>Protein yield (%)</i>	<i>Total activity (unit)</i>	<i>Specific activity (unit/mg protein)</i>
Ghost	50	100	3.45	0.069
Solubilized ghost	12.5	25	2.44	0.195
Peak eluted with Ca <sup>2+</sup> buffer	11.2	22.4	0.77	0.069
Peak eluted with EDTA buffer	0.36	0.72	1.26	3.51

Total protein, protein yield, total activity and specific activity of the ghost, solubilized ghost and the fractions collected from calcium and EDTA eluate of Sepharose 4B Calmodulin column were determined. Ca<sup>2+</sup>-ATPase activity was determined by coupled-enzyme assay while Dc Protein Assay was employed for determining protein content of the enzyme. Table above represents the purification of a typical run. The experiment was repeated for three times and the results obtained were within a range of 5% deviations.



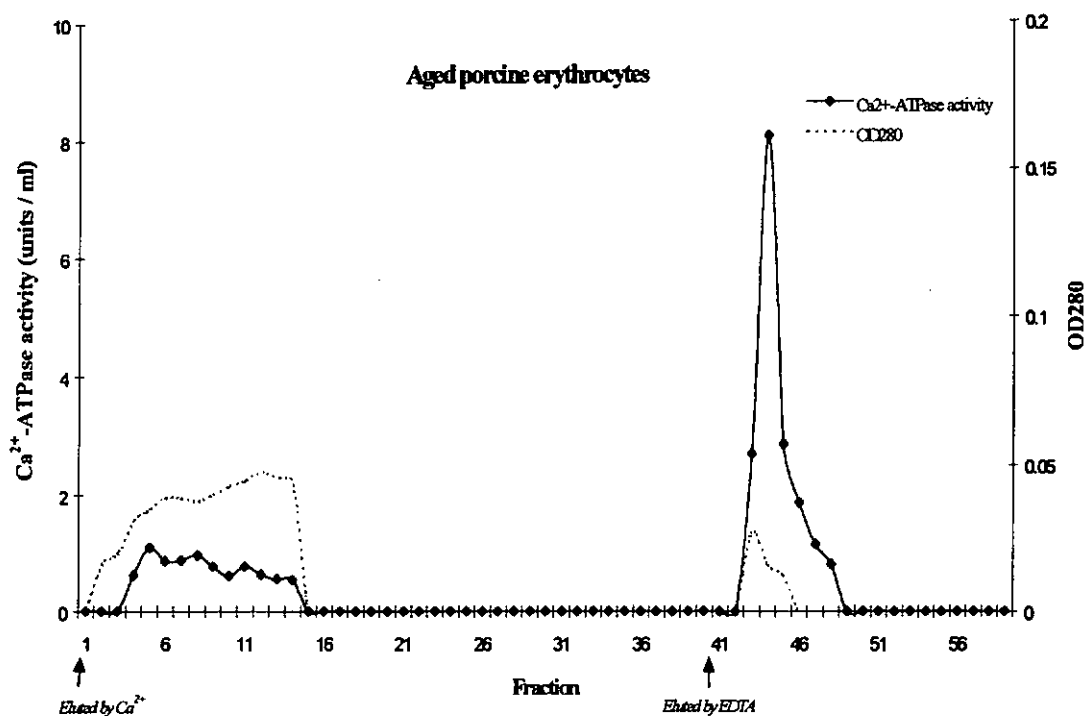
**Figure 21 Affinity chromatography of Triton X-100 solubilized ghosts from young porcine erythrocytes on Sepharose 4B Calmodulin column.**

50mg membrane protein from young erythrocytes was loaded onto the column (bed volume:5ml) with flow rate of 10ml/hr. Fractions of 2ml were collected.  $\text{Ca}^{2+}$ -ATPase activity was monitored by coupled-enzyme assay. The transmittance at 280nm was monitored. The column was first washed with 0.5mM  $\text{CaCl}_2$  and the buffer was then changed to 2mM EDTA.

**Table 8 Purification table of Ca<sup>2+</sup>-ATPase in aged erythrocytes**

<i><b>Fraction</b></i>	<i><b>Total protein (mg)</b></i>	<i><b>Protein yield (%)</b></i>	<i><b>Total activity (unit)</b></i>	<i><b>Specific activity (unit/mg protein)</b></i>
Ghost	50	100	3.1	0.062
Solubilized ghost	14	28	2.91	0.208
Peak eluted with Ca <sup>2+</sup> buffer	10	20	0.51	0.051
Peak eluted with EDTA buffer	0.15	0.3	0.53	3.51

Total protein, protein yield, total activity and specific activity of the ghost, solubilized ghost and the fractions collected from calcium and EDTA eluate of Sepharose 4B Calmodulin column were determined. Ca<sup>2+</sup>-ATPase activity was determined by coupled-enzyme assay while Dc Protein Assay was employed for determining protein content of the enzyme. Table above represents the purification of a typical run. The experiment was repeated for three times and the results obtained were within a range of 5% deviations.



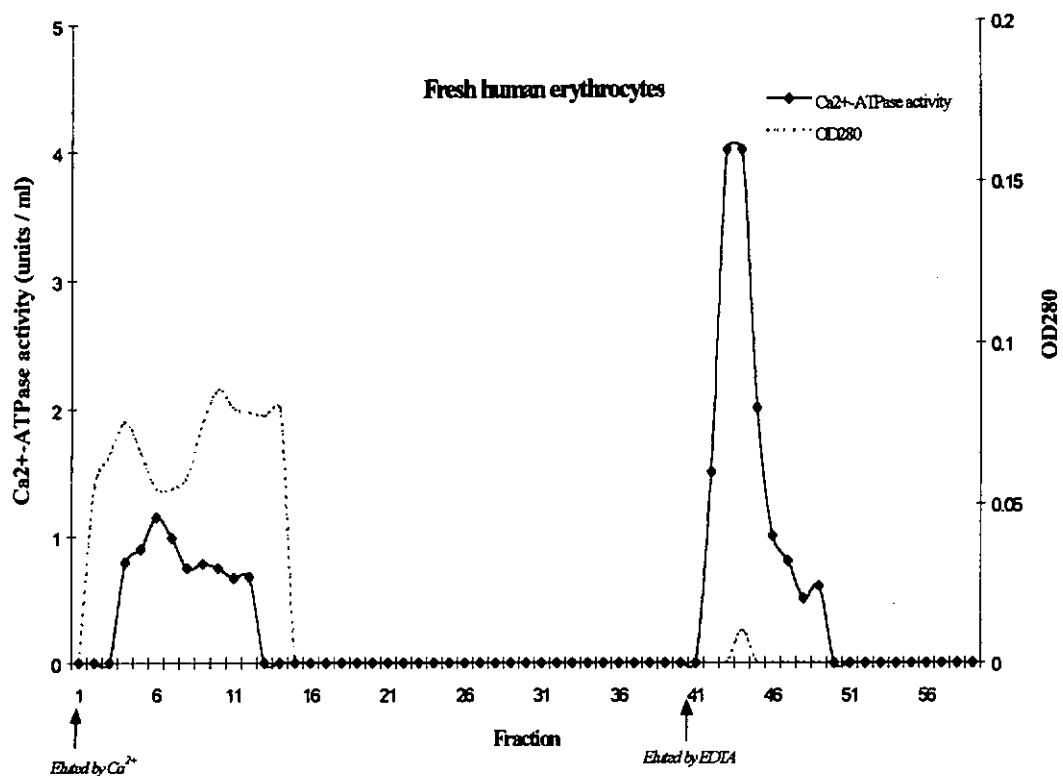
**Figure 22 Affinity chromatography of Triton X-100 solubilized ghosts from aged porcine erythrocytes on Sepharose 4B Calmodulin column.**

50mg membrane protein from aged erythrocytes was loaded onto the column (bed volume:5ml) with flow rate of 10ml/hr. Fractions of 2ml were collected. Ca<sup>2+</sup>-ATPase activity was monitored by coupled-enzyme assay. The transmittance at 280nm was monitored. The column was first washed with 0.5mM CaCl<sub>2</sub> and the buffer was then changed to 2mM EDTA.

**Table 9 Purification table of Ca<sup>2+</sup>-ATPase in fresh blood**

<i><b>Fraction</b></i>	<i><b>Total protein (mg)</b></i>	<i><b>Protein yield (%)</b></i>	<i><b>Total activity (unit)</b></i>	<i><b>Specific activity (unit/mg protein)</b></i>
Ghost	50	100	2.3	0.046
Solubilized ghost	11	22	1.43	0.13
Peak eluted with Ca <sup>2+</sup> buffer	9	18	0.42	0.047
Peak eluted with EDTA buffer	0.36	0.72	0.86	2.4

Total protein, protein yield, total activity and specific activity of the ghost, solubilized ghost and the fractions collected from calcium and EDTA eluate of Sepharose 4B Calmodulin column were determined. Ca<sup>2+</sup>-ATPase activity was determined by coupled-enzyme assay while Dc Protein Assay was employed for determining protein content of the enzyme. Table above represents the purification of a typical run. The experiment was repeated for three times and the results obtained were within a range of 5% deviations.



**Figure 23 Affinity chromatography of Triton X-100 solubilized ghosts from fresh human blood on Sepharose 4B Calmodulin column.**

50mg membrane protein from fresh blood was loaded onto the column (bed volume:5ml) with flow rate of 10ml/hr. Fractions of 2ml were collected. Ca<sup>2+</sup>-ATPase activity was monitored by coupled-enzyme assay. The transmittance at 280nm was monitored. The column was first washed with 0.5mM CaCl<sub>2</sub> and the buffer was then changed to 2mM EDTA.



activity loaded onto the column. The fold of purification was 52-fold.

Elution profile obtained for the purification of outdated blood was similar to that for fresh blood (Figure 24 and Table 10). Nearly 50% of the protein applied was eluted by calcium buffer. Half amount of protein was remained bound to calmodulin in the column. Furthermore, only 17% of total  $\text{Ca}^{2+}$ -ATPase were obtained after EDTA elution. The fold of purification was 57-fold.

The results from both porcine and human erythrocytes indicate that there might be alteration of the enzyme itself during both *in vivo* aging and blood storage. Thus, the purified enzymes were subjected to further analysis by SDS-polyacrylamide gel electrophoresis and western-blot.

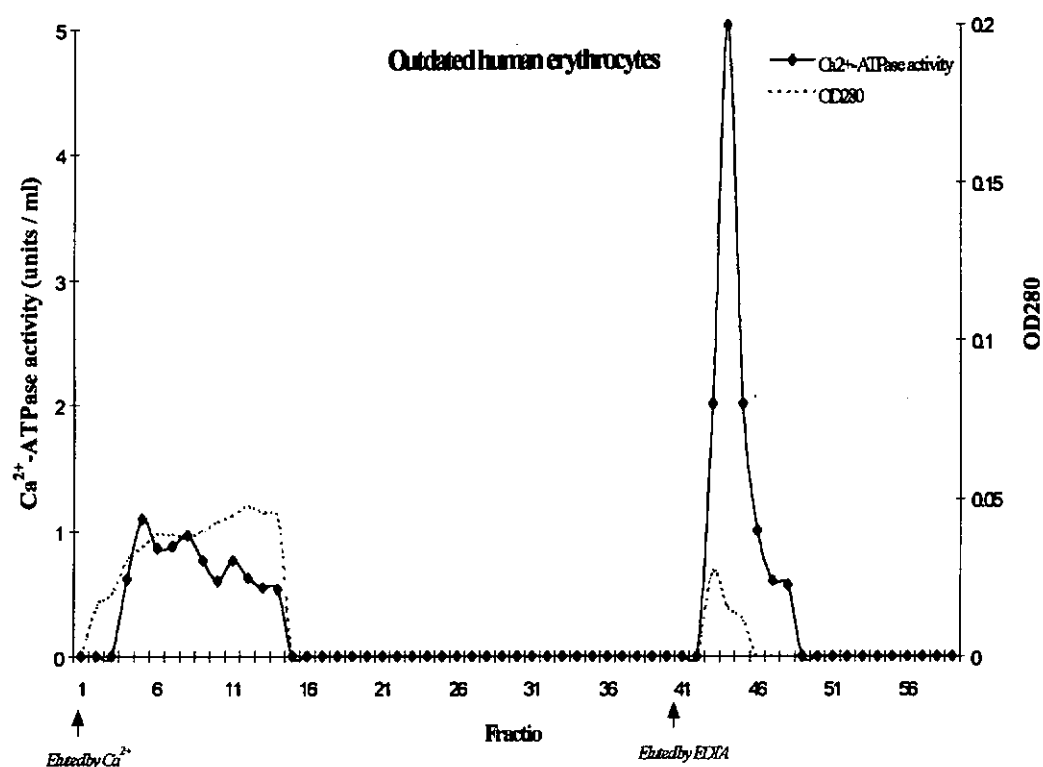
#### **10. SDS-polyacrylamide gel electrophoresis of $\text{Ca}^{2+}$ -ATPase**

10  $\mu\text{g}$   $\text{Ca}^{2+}$ -ATPase was loaded onto 7.5% SDS-PAGE gel with thickness of 1.5mm and the gel was stained with Coomassie blue. For porcine erythrocytes (Figure 25), one major band with apparent molecular weight of 130kDa was observed in the post-calmodulin affinity chromatography purified fractions. There were also minor bands with apparent mass of 110k, 89k and 40kDa in both young and aged cells. However, the gel patterns for human blood were slightly different (Figure 26). A major band with 130kDa with four minor bands, i.e. 110k, 89k, 45k and 42kDa were observed for fresh samples. On the contrary, 40k and 28kDa bands were obtained instead of 45k and 42kDa bands in outdated samples.

**Table10 Purification table of Ca<sup>2+</sup>-ATPase in outdated blood**

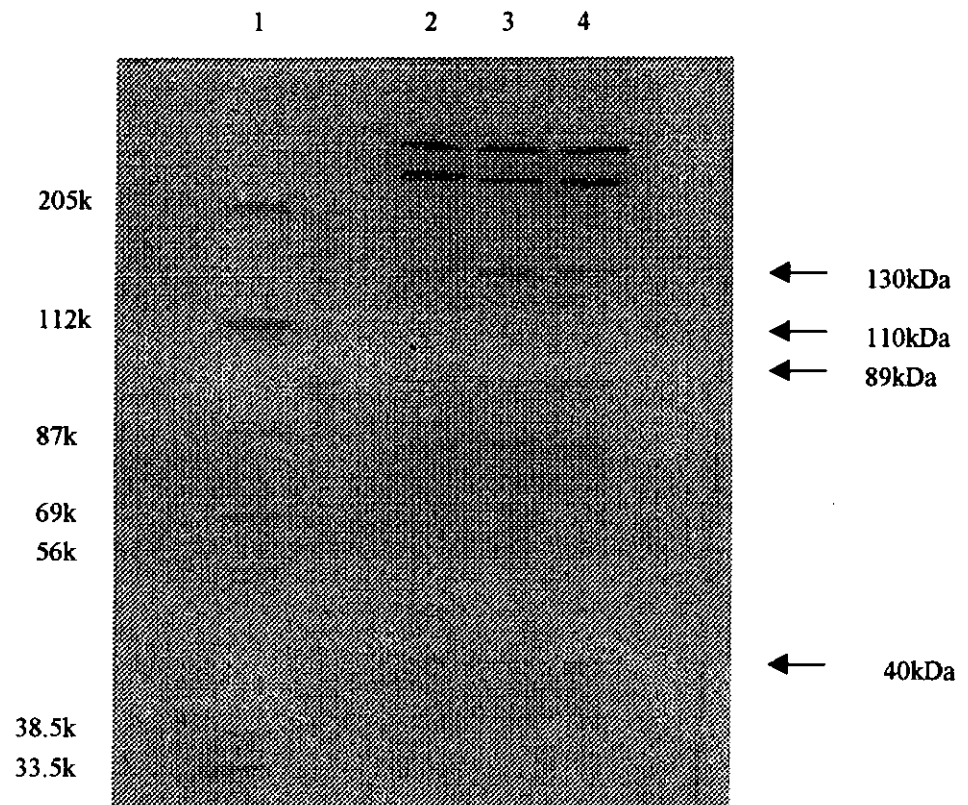
<i><b>Fraction</b></i>	<i><b>Total protein (mg)</b></i>	<i><b>Protein yield (%)</b></i>	<i><b>Total activity (unit)</b></i>	<i><b>Specific activity (unit/mg protein)</b></i>
Ghost	50	100	2.01	0.041
Solubilized ghost	14	28	1.96	0.14
Peak eluted with Ca <sup>2+</sup> buffer	7	14	0.24	0.034
Peak eluted with EDTA buffer	0.15	0.3	0.35	2.34

Total protein, protein yield, total activity and specific activity of the ghost, solubilized ghost and the fractions collected from calcium and EDTA eluate of Sepharose 4B Calmodulin column were determined. Ca<sup>2+</sup>-ATPase activity was determined by coupled-enzyme assay while Dc Protein Assay was employed for determining protein content of the enzyme. Table above represents the purification of a typical run. The experiment was repeated for three times and the results obtained were within a range of 5% deviations.



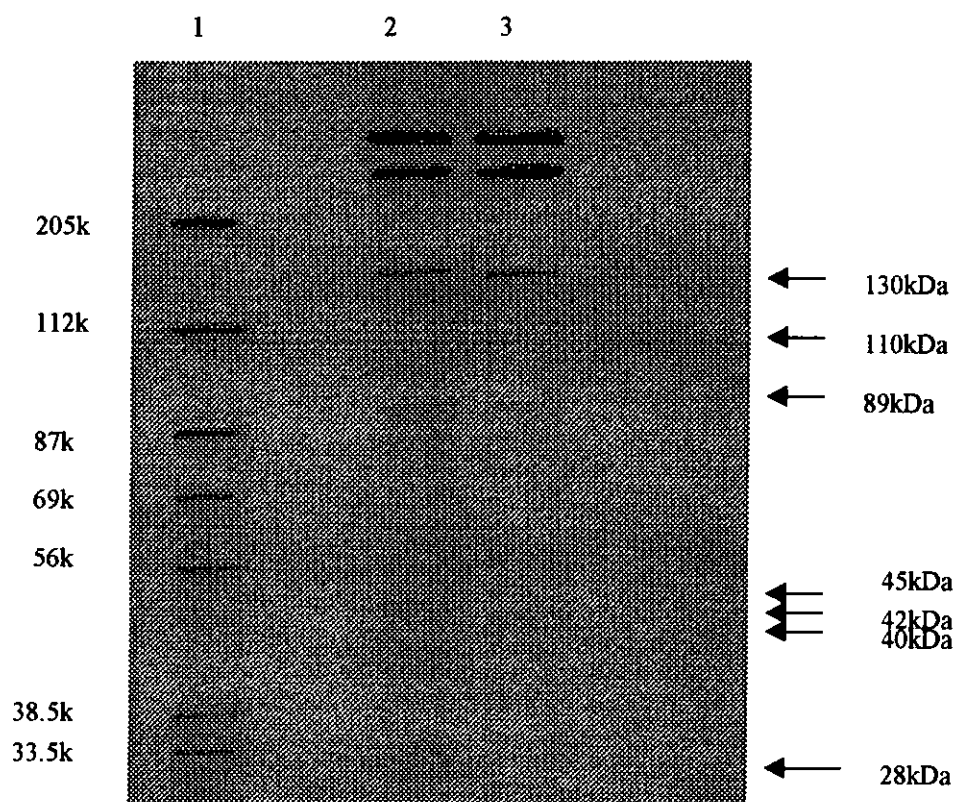
**Figure 24 Affinity chromatography of Triton X-100 solubilized ghosts from outdated human blood on Sepharose 4B Calmodulin column.**

50mg membrane protein from outdated blood was loaded onto the column (bed volume:5ml) with flow rate of 10ml/hr. Fractions of 2ml were collected.  $\text{Ca}^{2+}$ -ATPase activity was monitored by coupled-enzyme assay. The transmittance at 280nm was monitored. The column was first washed with 0.5mM  $\text{CaCl}_2$  and the buffer was then changed to 2mM EDTA.



**Figure 25 SDS-PAGE electrophoresis gel (7.5%) of purified porcine  $\text{Ca}^{2+}$ -ATPase**

30 $\mu\text{g}$  protein of pooled post-Sepharose 4B Calmodulin column fractions was loaded onto each lane of the gel. Lane 1: marker; lane 2: aged erythrocyte; lane 3: young erythrocyte; lane 4: whole blood.



**Figure 26 SDS-PAGE electrophoresis gel (7.5%) of purified human  $\text{Ca}^{2+}$ -ATPase**

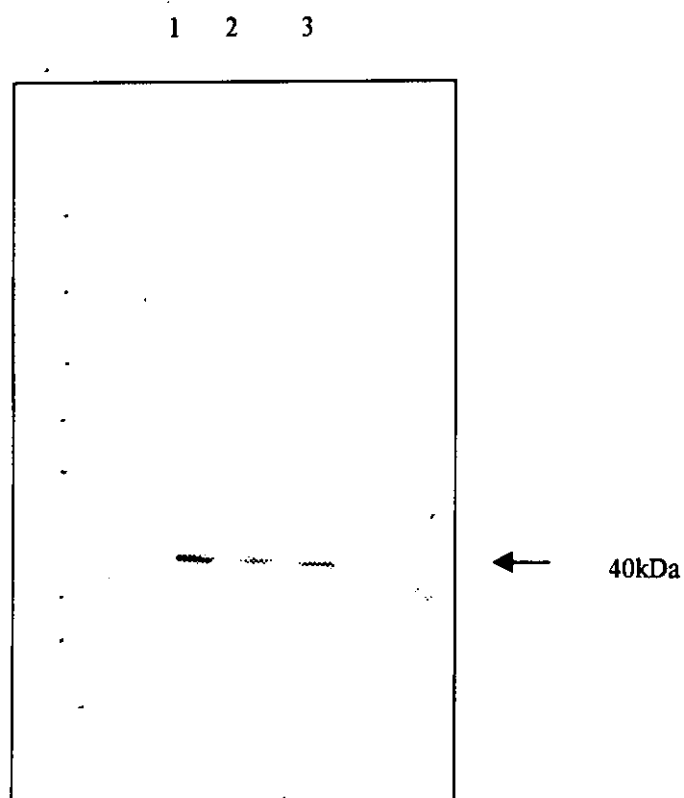
30 $\mu\text{g}$  protein of pooled post-Sepharose 4B Calmodulin column fractions was loaded onto each lane of the gel. Lane 1: marker; lane 2: fresh blood; lane 3: outdated blood.

There were size differences observed in the SDS-PAGE gel analysis. The results suggest that there are structural changes during blood storage.

#### 11. Western-blot analysis of $\text{Ca}^{2+}$ -ATPase

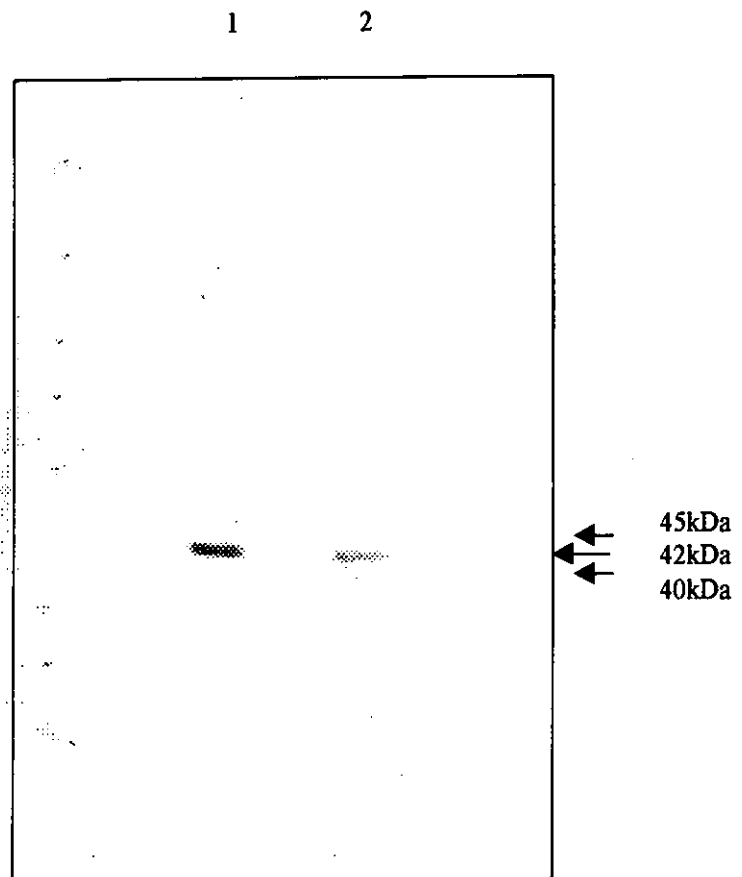
To further confirm the changes of molecular size of  $\text{Ca}^{2+}$ -ATPase during *in vivo* and *in vitro* aging process of erythrocyte, western-blot analysis was performed. The purified  $\text{Ca}^{2+}$ -ATPase separated by SDS-polyacrylamide gel electrophoresis was transferred to PVDF membrane and incubated with specific antibody for plasma membrane  $\text{Ca}^{2+}$ -ATPase (1N). The transblotted protein was detected by ECL method and the result is showed in Figure 27 & 28. For porcine erythrocytes (Figure 27), one band with apparent mass of 40kDa was observed for both young and aged cells. However, they were of different intensities, i.e. the one from young cells was 60% of that from aged cells. There seemed to be alternation of size of  $\text{Ca}^{2+}$ -ATPase during *in vivo* aging process of erythrocyte that resulted in different abundance of the transblot protein.

However, the transblot for human sample was slightly different (Figure 28). Only the band with apparent molecular weight of 40kDa was observed in samples from outdated blood. In contrast, 45k and 42kDa bands were observed instead in fresh blood. The result further suggests that there was changes in size of the  $\text{Ca}^{2+}$ -ATPase during blood storage.



**Figure 27  $\text{Ca}^{2+}$ -ATPase in porcine erythrocyte ghost**

30 $\mu\text{g}$  membrane protein of erythrocyte ghost were prepared and tested for the presence of PMCA1 isoform of  $\text{Ca}^{2+}$ -ATPase using specific antibodies 1N. Lane 1: aged erythrocyte; lane 2: young erythrocyte; lane 3: whole blood.



**Figure 28  $\text{Ca}^{2+}$ -ATPase in human erythrocyte ghost**

30 $\mu\text{g}$  membrane protein of erythrocyte ghost were prepared and tested for the presence of *PMCA1* isoform of  $\text{Ca}^{2+}$ -ATPase using specific antibodies 1N. Lane 1: fresh blood; lane 2: outdated blood.



## DISCUSSION

Calcium homeostasis is important in mammalian cells. Disturbance of calcium level in the cell would lead to adverse physiological effects of the body. Prolonged high calcium ion level in the cell would lead to cleavage of DNA and chromatin by nucleases. That results in loss of structural integrity of chromatin and affects cell cycle. Fluctuation in cytosolic calcium ion level may affect modulation of calcium targets. These phenomena may be an early event in pathology and cell death (Friederichs & Melselman, 1994; Damonte *et al*, 1992). Elevation of intracellular calcium concentration would also lead to adverse effects in erythrocyte, leading red cell aging and cell death. In this project, erythrocyte was chosen as a model for the study of aging process.

There had been reports on elevation of calcium concentration in senescent erythrocyte (Aiken *et al*, 1992). One of the possible reasons may be due to an increase in membrane permeability to calcium ion. Thus, increased calcium influx was resulted. There were also suggestions that erythrocyte aging may be due to a decrease in  $\text{Ca}^{2+}$ -ATPase activity during the aging process (Seidler & Swislocki, 1991; Vincenzi & Hinds, 1988). However, no established agreement came up.  $\text{Ca}^{2+}$ -ATPase in natural state is subjected to regulation by endogenous regulators. In order to find out whether  $\text{Ca}^{2+}$ -ATPase undergone changes in regulation during aging, its interactions with various regulators may be of great interest. In this study, we propose to look into the change of regulation of  $\text{Ca}^{2+}$ -ATPase during aging process

and in blood storage.

### **Regulation of $\text{Ca}^{2+}$ -ATPase during aging:**

In the study of aging process in erythrocyte, comparative study in membrane-bound  $\text{Ca}^{2+}$ -ATPase from young and aged porcine erythrocytes with respect to their response towards the substrate, ATP, was conducted. The result indicates that affinity of  $\text{Ca}^{2+}$ -ATPase towards ATP increases significantly with age (Figure 5). The change in affinity to the substrate may be due to alternation of molecular configuration of the enzyme molecule during aging. Thus, the ATP binding site of the enzyme molecule may be more exposed and easily accessible to the substrate. The findings obtained here suggest that there is an alternation of  $\text{Ca}^{2+}$ -ATPase of erythrocyte during the aging process. ATP content in the cell decreased during the aging process of erythrocyte (Cohen *et al*, 1976). For young erythrocyte, the ATP content is 3.74 – 5.22  $\mu\text{mole/g}$  hemoglobin. The ATP content in the aged erythrocyte is much lower, i.e. 3.52 – 3.53  $\mu\text{mole/g}$  hemoglobin. Since ATP content in the cell during the aging process of erythrocyte decreased, less ATP would be available for the membrane  $\text{Ca}^{2+}$ -ATPase in aged cell (Clark, 1988). The increase in affinity of  $\text{Ca}^{2+}$ -ATPase towards ATP may be one of the adaptations of erythrocyte during aging. This results in an increase in availability of substrate for the phosphorylation process of  $\text{Ca}^{2+}$ -ATPase.

In contrast, when comparing the maximal activities of  $\text{Ca}^{2+}$ -ATPase in young

and aged erythrocytes, there was no significant difference between them. The capacity of the enzyme to pump calcium ions without the presence of regulators did not change much.  $\text{Ca}^{2+}$ -ATPase is a regulatory enzyme in erythrocyte. It seldom works at maximal capacity in physiological conditions. It is more likely that there may be alternation of regulation of the enzyme during the aging process. Thus, study was concentrated on the interaction between the enzyme and its regulators.

In order to find out whether regulation of  $\text{Ca}^{2+}$ -ATPase has been altered during aging, interactions of the membrane-bound enzyme in young and aged erythrocytes with various regulators were studied. For the first time, the interaction of  $\text{Ca}^{2+}$ -ATPase of different age populations with its protein inhibitor, PMCAI was studied. It was found that  $V_{\max}$  of the enzyme in the presence of PMCAI was largely decreased during aging (Figure 12 & 13). The finding indicates that the PMCAI may play a role in the process. This agrees with the hypothesis raised by Samaja and his co-workers in 1989. He suggested that aging process in erythrocyte involve inhibitor protein rather than  $\text{Ca}^{2+}$ -ATPase itself or calmodulin. However, he did not carry out comparative study of the interaction of  $\text{Ca}^{2+}$ -ATPase with PMCAI. Thus, regulation of the enzyme with PMCAI was investigated in this study to confirm such notion. Though affinity of the enzyme towards PMCAI remained unchanged during aging, the change in  $V_{\max}$  with the inhibitor may be one of the possible reasons for the depression of  $\text{Ca}^{2+}$ -ATPase activity during *in vivo* aging process of erythrocyte.

Apart from the decrease in  $V_{\max}$  of  $\text{Ca}^{2+}$ -ATPase in the aged erythrocyte

towards inhibitor, there may also be change in regulation of the enzyme by activators during the aging process. The finding obtained shows that there was a decrease in affinity of membrane-bound  $\text{Ca}^{2+}$ -ATPase towards oleic acid for aged erythrocyte (Figures 8 & 9). The phenomena may be a consequence of change of hydrophobicity of the whole enzyme molecule or of the area near the binding site of oleic acid. The change in affinity of the enzyme towards oleic acid may result in depression of  $\text{Ca}^{2+}$ -ATPase activity during the aging process.

Many researchers had studied the response of  $\text{Ca}^{2+}$ -ATPase towards calmodulin. Luthra and Kim (1980) found that there was a reduced affinity of aged membrane to calmodulin while Samaja and his co-workers (1989) found that there was no significant difference during *in vivo* aging of erythrocyte. The results obtained by different groups were controversial and this might be due to different preparations and condition of membrane enzyme, e.g. some researchers studied inside-out vesicles while other employed solubilized enzyme (Samaja *et al*, 1989). In this study, interaction of membrane-bound  $\text{Ca}^{2+}$ -ATPase with calmodulin was investigated. It was found that response and affinity of membrane-bound  $\text{Ca}^{2+}$ -ATPase towards calmodulin remained unchanged during aging (Figure 6 & 7).

The interaction between  $\text{Ca}^{2+}$ -ATPase with protease is also important in the aging process of erythrocyte. From the result, it was found that activation effect of controlled trypsin proteolysis on  $\text{Ca}^{2+}$ -ATPase in aged erythrocyte was higher (Table 3). In this study, trypsin was used to mimic the effect of intracellular  $\text{Ca}^{2+}$ -dependent

protease, calpain, on  $\text{Ca}^{2+}$ -ATPase. It could be more direct to employ calpain for the study of its activation effect on membrane-bound  $\text{Ca}^{2+}$ -ATPase. Though here in this study, we found that the use of trypsin was satisfactory, similar trend was obtained for treating the membranes with calpain as that for controlled trypsin proteolysis (Table 4). The result showed that both trypsin and calpain exerted similar effect on  $\text{Ca}^{2+}$ -ATPase during the aging process of erythrocyte, though the effect of calpain was more remarkable. Proteolysis results in removal of inhibitory polypeptide around active site of the  $\text{Ca}^{2+}$ -ATPase molecule (Carafoli *et al*, 1992; Falchetto, 1991). The finding here indicates that the inhibitory polypeptide may be more susceptible to protease during aging of erythrocyte and thus the activation effect may be larger.

The result obtained in this study indicates that there was an alternation of regulation of  $\text{Ca}^{2+}$ -ATPase in aging. Actually, erythrocyte membrane proteins are subjected to proteolysis by cytosolic protease throughout its life span. The extent of proteolysis of membrane  $\text{Ca}^{2+}$ -ATPase in young and aged erythrocytes may be different. Intact  $\text{Ca}^{2+}$ -ATPase (apparent molecular weight of ~130kDa) in young and aged porcine erythrocytes were obtained (Figure 25). Fragments with apparent molecular weight of 110kDa, 89kDa and 40kDa were also obtained. These may be the proteolytic products of  $\text{Ca}^{2+}$ -ATPase resulted from the action of endogenous protease. From the immunoblotting study of both enzymes (Figure 27), fragment with 40kDa was recognized by isoform specific antibody for *PMCA1* (1N). The 40kDa-fragment may be the proteolytic product from intact  $\text{Ca}^{2+}$ -ATPase. The

abundance of fragment with 40kDa in young cell was 60% of that in the aged. As erythrocyte ages, its membrane is subjected to proteolysis by endogenous protease and the intact  $\text{Ca}^{2+}$ -ATPase may be cleaved into 40kDa-fragment in subsequent proteolysis steps. The extent of proteolysis in aged erythrocyte is thus anticipated to be higher, in accordance to our finding, abundance of 40kDa-fragment in aged erythrocyte is much more.

The susceptibility of erythrocyte membrane to endogenous protease can help to explain the alternation of regulation of  $\text{Ca}^{2+}$ -ATPase during *in vivo* aging of erythrocyte. The tertiary structure of  $\text{Ca}^{2+}$ -ATPase composes of many regulatory domains (Figure 4) (Carafoli *et al*, 1992; Carafoli, 1991). Some of them may be very close together. There may be hindrance between them. The proteolysis effect of  $\text{Ca}^{2+}$ -ATPase during the aging of erythrocyte may be specific to several regions of the enzyme molecule. This may help to expose some of the regulatory domains of the enzyme and the affinity towards specific substrate or regulator be increased. This could be the reason for the increase of affinity of  $\text{Ca}^{2+}$ -ATPase towards ATP in the aged erythrocytes.

In this study, it was found that the proteolysis of membranous  $\text{Ca}^{2+}$ -ATPase might be limited. The proteolysis is thus moderate and incomplete. It can be explained by the observation of activation effect of membrane-bound  $\text{Ca}^{2+}$ -ATPase by the treatment of the enzyme with exogenous calpain and trypsin. As stated before, the extent of proteolysis of  $\text{Ca}^{2+}$ -ATPase in the aged erythrocyte was much higher,

then the susceptible site for exogenous protease may be more exposed. This results in an increase of activation effect of membranous  $\text{Ca}^{2+}$ -ATPase by exogenous protease.

### **Regulation of $\text{Ca}^{2+}$ -ATPase during blood storage:**

The alternation of interaction of membrane-bound  $\text{Ca}^{2+}$ -ATPase with ATP and regulators during human blood storage was also studied. It was found that similar trend of changes were observed as that during *in vivo* aging of erythrocytes (Figure 14). Increased affinity of  $\text{Ca}^{2+}$ -ATPase towards ATP was found. In addition,  $V_{\max}$  of the enzyme in the presence of PMCAI decreased while its affinity towards oleic acid decreased during blood storage (Figure 17-20). Alternation of regulation of  $\text{Ca}^{2+}$ -ATPase is first described in this study occur during blood storage. This might be one of the cause for limited shelf life of bank blood.

In this study, it was found that the extent of proteolysis of membranous  $\text{Ca}^{2+}$ -ATPase in fresh and outdated human blood was different. From the SDS-polyacrylamide gels electrophoresis (Figure 26), apart from the intact  $\text{Ca}^{2+}$ -ATPase, fragments with apparent molecular weight of 110kDa, 89kDa, 45kDa and 42kDa were observed in samples obtained from fresh blood. However, in the outdated sample, we only found the 110kDa, 89kDa, 40kDa and 28kDa-fragments. This demonstrates that there are structural changes of  $\text{Ca}^{2+}$ -ATPase during blood storage. This may be due to proteolysis of the action of endogenous protease of erythrocytes during the process. In addition, 45kDa and 42kDa fragments were recognized by

antibody of *PMCA1*, i.e. *IN*, while only the 40kDa fragment was recognized by the antibody (Figure 28). During blood storage,  $\text{Ca}^{2+}$ -ATPase of the stored blood was subjected to proteolysis by endogenous protease. This would be aggravated with the shelf life of bank blood. Thus, different fragments of  $\text{Ca}^{2+}$ -ATPase were recognized by *PMCA1* for both fresh and outdated blood. However, further study should be carried out to confirm if the 40kDa fragment from outdated blood was the proteolytic product of 45kDa and 42kDa fragments of fresh blood.

#### **Decrease in total $\text{Ca}^{2+}$ -ATPase activity in both *in vivo* aging of erythrocyte and blood storage:**

Apart from the alternation of regulation of  $\text{Ca}^{2+}$ -ATPase during both *in vivo* and *in vitro* aging process, the decrease of  $\text{Ca}^{2+}$ -ATPase activity during the processes may be due to a decrease in the amount of active  $\text{Ca}^{2+}$ -ATPase in the plasma membranes. When comparing the purification table of  $\text{Ca}^{2+}$ -ATPase in young and aged erythrocytes (Tables 7-8), it shows that for the same amount of  $\text{Ca}^{2+}$ -ATPase loaded onto the column, only 18% of total  $\text{Ca}^{2+}$ -ATPase activity was recovered from the aged cell. In contrast, nearly 50% of total enzyme activity was obtained for the young one. Similar result obtained when we repeated the experiment by changing the two columns. This may be due to many possible reasons. During the purification step of the enzyme by using calmodulin-affinity chromatography, we were trying to bind enzyme to the immobilized ligand at the calmodulin-binding domain. The diminished

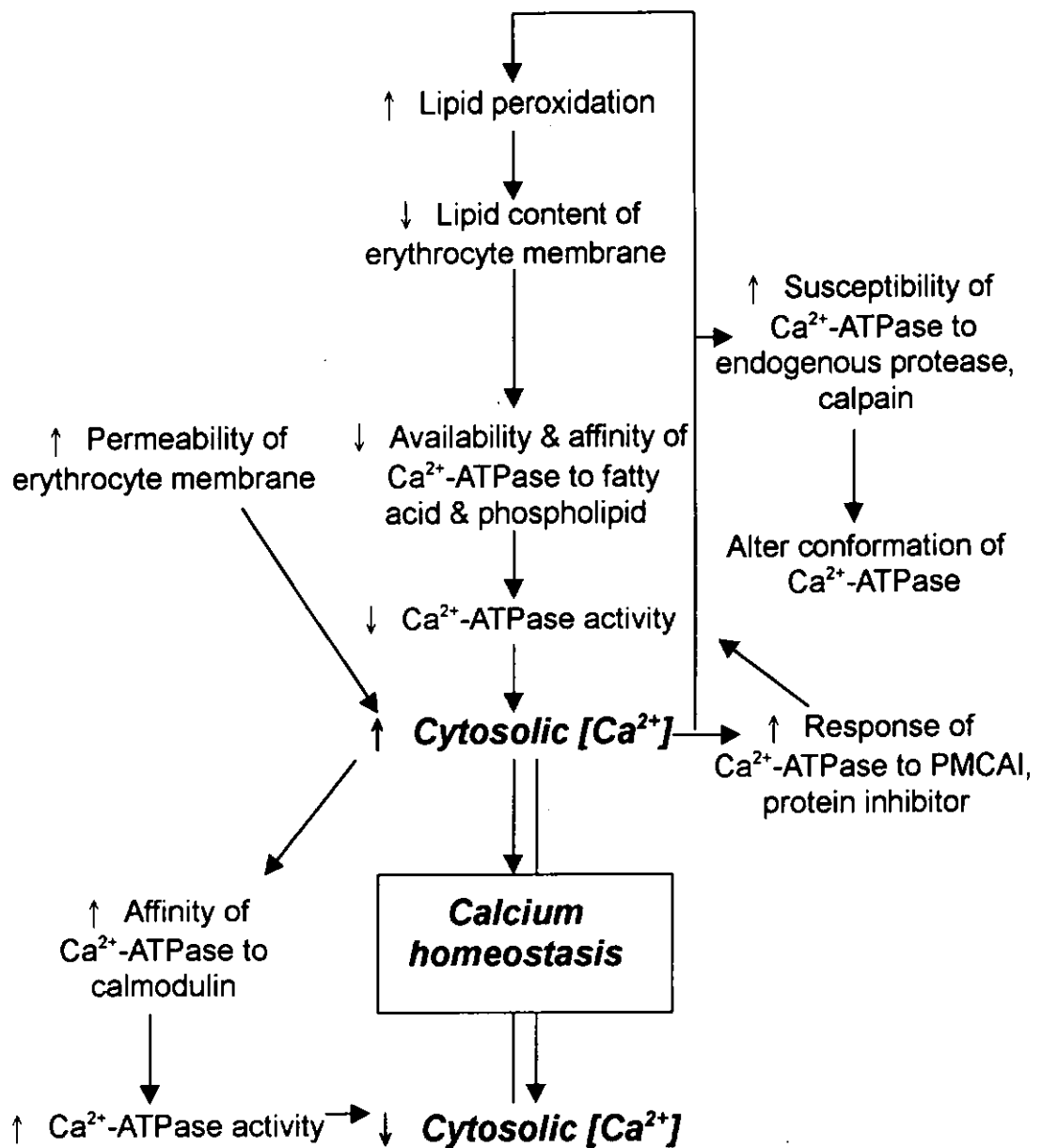


total enzyme activity during aging process may be due to decrease of abundance of calmodulin-sensitive, active  $\text{Ca}^{2+}$ -ATPase in aged erythrocyte. The calmodulin-binding domain of the enzyme from aged erythrocyte may be altered, leading to diminished affinity to the “hook” (Au *et al*, 1989). This can be confirmed by further study on the interaction of calmodulin with the enzyme by using fluorescence spectrophotometer. In addition, similar trend of decrease in total  $\text{Ca}^{2+}$ -ATPase was observed for fresh and outdated blood. It shows that there was also a decrease of active  $\text{Ca}^{2+}$ -ATPase during human blood storage.

### **Proposed mechanisms of aging:**

Many possible mechanisms of increasing cytosolic calcium content in aging process of erythrocyte have been raised. As stated before, the primary cause for the increase in calcium ion content may be due to calcium leakage of the plasma membrane, i.e. increasing permeability of the erythrocyte cell membrane. Another possible cause may be a decrease in  $\text{Ca}^{2+}$ -ATPase activity of erythrocyte. The findings obtained in this study substantiate the hypothesis that there is an alternation of  $\text{Ca}^{2+}$ -ATPase itself and its regulation during both processes, namely *in vivo* aging and blood storage.

The possible mechanism of aging in erythrocyte is put forward as follows (Figure 29). During the life span of erythrocyte, the permeability of the membrane to calcium ions through leakage is not significant. Even if so, the increase of calcium



**Figure 29 Proposed mechanisms of increasing cytosolic calcium content during aging of erythrocyte**

ion influx can be balanced by the pumping action of  $\text{Ca}^{2+}$ -ATPase. As erythrocyte ages, the permeability of the plasma membrane to calcium ion is largely increased. The pumping action of  $\text{Ca}^{2+}$ -ATPase should be largely increased to cope with the fluctuation of calcium level. However, during the aging process, the response of  $\text{Ca}^{2+}$ -ATPase to protein inhibitor, i.e. PMCAI, is much increased while response to other activators does not change much. This leads to a decrease in capacity of the enzyme. In addition to the decrease in lipid content of the cell, availability and affinity of the  $\text{Ca}^{2+}$ -ATPase to fatty acid or phospholipid may be decreased. This further aggravates the depression of  $\text{Ca}^{2+}$ -ATPase activity. On the other hand, membrane proteins of erythrocyte are susceptible to endogenous protease action throughout its life span. Since the increase in calcium ion content in the cell leads to an increase in activation of calpain (Schwarz-Benmeir *et al*, 1994), aged and outdated erythrocytes proteins are more susceptible to proteolysis. The proteolysis may alter the conformation of the ATPase enzyme molecule. This leads to an increase in exposure of ATP-binding site to ATP and susceptible sites on the enzyme molecule for further proteolysis. Further proteolysis by endogenous proteases may result in permanent inactivation of membranous  $\text{Ca}^{2+}$ -ATPase. That results in a decrease in total active  $\text{Ca}^{2+}$ -ATPase of the cell. Actually, the factors mentioned above may act together and a decrease in  $\text{Ca}^{2+}$ -ATPase activity of aged and outdated erythrocytes would be resulted.

### **Significance and value:**

The findings obtained in this study can help us to depict a clearer picture of *in vivo* aging of erythrocyte. We propose that erythrocyte in the circulation is subjected to proteolysis by endogenous proteases throughout its life span. This results in alternation of conformational change of the enzyme molecule. Together with the alternation of regulation of the enzymes with its regulators, total active  $\text{Ca}^{2+}$ -ATPase of the cell decreases and this leads to decrease of  $\text{Ca}^{2+}$ -ATPase activity in aged cell. Similar alternation also occurs during blood storage. Thus, further study in this area can help to provide more information of both *in vivo* aging process and blood storage.

It is noted that there are alternation of regulation of  $\text{Ca}^{2+}$ -ATPase with regulators during erythrocyte aging and blood storage, namely PMCAI, oleic acid, calmodulin, calpain and trypsin. It is still unknown if there are changes in the regulators themselves in level and activity during both processes (Ekholm *et al*, 1981). Further study can be followed on the isolation of regulators from different populations of erythrocytes. The regulators isolated are then subjected to comparative study of interaction with the enzyme from the same population of erythrocyte. If there is different response of the regulators from different population of erythrocytes with the same enzyme, it is possibly that the regulator may be altered during the process. Then, further study can then be concentrated on the regulators by investigating its conformational and structural change during the aging process.

Furthermore, it is proposed that there are changes of the enzyme molecule during both processes. This may be due to decrease in number of copy of  $\text{Ca}^{2+}$ -ATPase molecule on the plasma membrane or affinity of ATP-binding site towards the substrate. This can be further studied by applying radiolabeled *PMCA1* or ATP to the enzyme. The abundance of the enzyme molecule can be observed on radiography while interaction of the enzyme with substrate can be assayed by using fluorescence spectrophotometer.

In this study, we are beginning to unveil the alteration of regulation of  $\text{Ca}^{2+}$ -ATPase in both erythrocyte aging and blood storage. To obtain more information in this area, more populations of erythrocytes from different stages in the circulation and bank blood can be employed. Thus, a more complete profile of both processes can be obtained. This would finally lead us to a better understanding of the red cell aging.

## CONCLUSION

The present work focused on the study of both *in vivo* erythrocyte aging and bank blood storage. In the past, many possible mechanisms of increasing cytosolic calcium content during both processes have been raised. Our findings substantiate the hypothesis that there is an alternation of  $\text{Ca}^{2+}$ -ATPase itself and its regulation during the processes that would lead to elevation of calcium concentration in the cells. It was found that affinity and response of the enzyme to its regulators changed. They may act together and result in decrease in  $\text{Ca}^{2+}$ -ATPase activity of aged and outdated erythrocytes. Thus, study of red cell aging can be focused on the regulation of  $\text{Ca}^{2+}$ -ATPase and this would finally lead us to a better understanding of the process.

## REFERENCES

1. Agre, P., Gardner, K. and Bennett, V. "Association between human erythrocyte calmodulin and the cytoplasmic surface of human erythrocyte membranes". *Journal of Biological Chemistry*, Vol. 258, pp.6258-6265 (1983)
2. Aiken, N. R., Galey, W. R. and Satterlee, J. D. "A peroxidative model of human erythrocyte intracellular calcium changes with *in vivo* cell aging: measurement by  $^{19}\text{F}$ -NMR spectroscopy". *Biochimica et Biophysica Acta*, Vol. 1270, pp.52-57 (1995)
3. Aiken, N. R., Satterlee, J. D. and Galey, W. R. "Measurement of intracellular  $\text{Ca}^{2+}$  in young and old human erythrocytes using  $^{19}\text{F}$ -NMR spectroscopy". *Biochimica et Biophysica Acta*, Vol. 1136, pp.155-160 (1992)
4. Al-Jobore, A. and Roufogalis, B. D. "Influence of EGTA on the apparent calcium affinity of  $\text{Mg}^{2+}$ -dependent,  $\text{Ca}^{2+}$ -stimulated ATPase in the human erythrocyte membrane". *Biochimica et Biophysica Acta*, Vol. 645, pp.1-9 (1981)
5. Ando, K., Beppu, M. and Kikugawa, K. "Evidence for accumulation of lipid hydroperoxides during the aging of human red blood cells in the circulation". *Biology and Pharmacology Bulletin*, Vol. 18, pp.659-663 (1995)
6. Au, K. S. "Purification of a protein inhibitor of erythrocyte plasma membrane  $\text{Ca}^{2+}$ -ATPase-Sepharose affinity chromatography". *Biochemistry and Molecular Biology Investigation*, Vol. 39, pp.197-203 (1996)
7. Au, K. S. "An endogenous inhibitor of erythrocyte membrane ( $\text{Ca}^{2+}+\text{Mg}^{2+}$ )-ATPase". *International Journal of Biochemistry*, Vol. 9, pp.477-480 (1978a)
8. Au, K. S. "Studies on an endogenous protein activities of pig erythrocyte membrane ( $\text{Ca}^{2+}+\text{Mg}^{2+}$ )-ATPase". *International Journal of Biochemistry*, Vol. 9, pp.735-743 (1978b)
9. Au, K. S. and Lee, K. S. "An endogenous inhibitor of erythrocyte membrane ( $\text{Ca}^{2+}+\text{Mg}^{2+}$ )-ATPase involved in calcium transport". *International Journal of Biochemistry*, Vol. 11, pp.177-181 (1980)

10. Au, K. S., Lee, M. F. and Siu, Y. L. "Ca<sup>2+</sup>-mediated activation of human erythrocyte membrane Ca<sup>2+</sup>-ATPase". *Biochimica et Biophysica Acta*, Vol. 978, pp.197-202 (1989)
11. Au, K. S. and Siu, Y. L. "Variation in Ca<sup>2+</sup>-mediated activation of Ca<sup>2+</sup>-ATPase and its associated inhibition in erythrocyte". *International Journal of Biochemistry*, Vol. 24, pp.1169-1173 (1992)
12. Bain, B. F. "Basic hematological techniques". In Dacie, J. V. and Lewis, S. M. eds., *Practical Haematology*, Churchill Livingstone, Edinburgh, pp.49-82 (1994)
13. Barrabin, H., Garrahan, P. J. and Rega, A. F. "Vanadate inhibitor of the Ca<sup>2+</sup>-ATPase from human red cell membranes". *Biochimica et Biophysica Acta*, Vol. 600, pp.796-804 (1980)
14. Bartosz, G. "Erythrocyte aging". In Timiras, P. S. and Bittar, E. E., eds., *Advances in Cell Aging and Gerontology. Some Aspects of the Aging Process*, JAI Press Inc., London, pp.63-88 (1996)
15. Bartosz, G. "Erythrocyte aging: physical and chemical membrane changes". *Gerontology*, Vol. 37, pp.33-67 (1991)
16. Bertolini, A. M. "Modifications of cellular enzymatic systems during aging". *Gerontologia*, Vol. 6, pp.175-187 (1962)
17. Blaustein, M. P. & Nelson, M. T. "Sodium-calcium exchange: Its role in the regulation of cell calcium". In Carafoli, E., ed., *Membrane transport of calcium*, Academic Press Inc., London, pp.217-236 (1982)
18. Bond, G. H. and Clough, D. L. "A soluble protein activator of (Mg<sup>2+</sup>+Ca<sup>2+</sup>)-ATPase in human red cell membranes". *Biochimica et Biophysica Acta*, Vol. 323, pp.592-599 (1973)
19. Borle, A. B. "Control, modulation, and regulation of cell calcium". *Reviews of Physiology, Biochemistry and Pharmacology*, Vol. 90, pp.15-153 (1981)
20. Brodin, P., Falchetto, R., Vorherr, T. and Carafoli, E. "Identification of 2 domains which mediate the binding of activating phospholipids to the plasma-



membrane  $\text{Ca}^{2+}$  pump". *European Journal of Biochemistry*, Vol. 204, pp.939-946 (1992)

21. Campbell, K. P., MacLennan, D. H. and Jorgensen, A. O. "Staining of the  $\text{Ca}^{2+}$ -binding proteins, calsequestrin, calmodulin, troponin C, and S-100, with cationic carbocyanine dye, Stains-all". *Journal of Biological Chemistry*, Vol. 258, pp.11267-11273 (1983)
22. Carafoli, E. "The  $\text{Ca}^{2+}$ -pump of the plasma membrane". *Journal of Biological Chemistry*, Vol. 267, pp.2115-2118 (1992)
23. Carafoli, E. "Calcium pump of the plasma membrane". *Physiological Reviews*, Vol. 71, pp.129-158 (1991)
24. Carafoli, E. "Intracellular calcium homeostasis". *Annals of Reviews of Biochemistry*, Vol. 56, pp.395-433 (1987)
25. Carafoli, E. and Zurini, M. "The  $\text{Ca}^{2+}$ -pumping ATPase of plasma membranes. Purification, reconstitution and properties". *Biochimica et Biophysica Acta*, Vol. 683, pp.279-301 (1982)
26. Carafoli, E., Kessler, F., Falchetto, R., Heim, R., Quadroni, M., Krebs, J., Strehler, E. E. and Vorherr, T. "The molecular basis of the modulation of the plasma membrane calcium pump by calmodulin". *Annals New York Academy of Sciences*, Vol. 671, pp.58-68 (1992)
27. Carafoli, E., Martin, E. G. and Guerini, D. "The plasma membrane calcium pump: recent developments and future perspectives". *Experientia*, Vol. 52, pp.1091-1100 (1996)
28. Caroni, P. and Carafoli, E. "The  $\text{Ca}^{2+}$ -pumping ATPase of heart sarcolemma. Characterization, calmodulin dependence and partial purification". *Journal of Biological Chemistry*, Vol. 256, pp.3263-3270 (1981)
29. Cheung, W. Y. "Calmodulin plays a pivotal role in cellular regulation". *Science*, Vol. 207, pp.19-27 (1980)
30. Chiarantini, L., Rossi, L., Fraternale, A. and Magnani, M. "Modulated red blood

- cell survival by membrane protein clustering". *Molecular and Cellular Biochemistry*, Vol. 144, pp.53-59 (1995)
31. Clapham, D. E. "Calcium signaling". *Cell*, Vol. 80, pp.259-268 (1995)
  32. Clark, M. R. "Calcium extrusion by high-density human red blood cells". *Blood Cells*, Vol. 14, pp.119-131 (1988)
  33. Cohen, N. S., Ekholm, J. E., Luthre, M. G. and Hanahan, D. J. "Biochemical characterization of density-separated human erythrocytes". *Biochimica et Biophysica Acta*, Vol. 419, pp.229-242 (1976)
  34. Coolican, S. A. and Hathaway, D. R. "Effect of L- $\alpha$ -phosphatidylinositol on a vascular smooth muscle  $\text{Ca}^{2+}$ -dependent protease". *Journal of Biological Chemistry*, Vol. 259, pp.11627-11630 (1984)
  35. Corash, L. M., Piomelli, S., Chen, H. C., Seaman, C. and Gross, E. "Separation of erythrocytes according to age on a simplified density gradient". *Journal of Laboratory and Clinical Medicine*, Vol. 84, pp.147-151 (1974)
  36. Croall, D. E. and Demartino, G. N. "Calcium activated neutral protease (calpain) system: structure, function and regulation". *Physiological Reviews*, Vol. 71, pp.813-847 (1991)
  37. Damonte, G., Guida, L., Sdraffa, A., Benatti, U., Melloni, E., Forteleoni, G., Meloni, T., Carafoli, E. and De Flora, A. "Mechanisms of perturbation of erythrocyte calcium homeostasis in favism". *Cell Calcium*, Vol. 13, pp.649-658 (1992)
  38. Danon, D. "Aging of the erythrocyte". In Cristofalo, V. J., eds., *CRC Handbook of Cell Biology of Aging*, CRC Press Inc., USA, pp.317-340 (1986)
  39. Dunham, E. T. and Glynn, I. M. "Adenosine-triphosphatase activity and the active movements of alkali metal ions". *Journal of Physiology*, Vol. 156, pp.274-293 (1961)
  40. Ekholm, J. E., Shukla, S. D. and Hanahan, D. J. "Change in cytosolic calmodulin activity of density (age) separated human erythrocytes towards membrane

- Ca<sup>2+</sup>/Mg<sup>2+</sup> ATPase". *Biochemical and Biophysical Research Communications*, Vol. 103, pp.407-413 (1981)
41. Ellman, G. L., Courtney, K. D., Andres, V. and Featherstone, R. M. "A new and rapid colorimetric determination of acetylcholinesterase activity". *Biochemical Pharmacology*, Vol. 7, pp.88-95 (1961)
  42. Enyedi, A., Flura, M., Sarkadi, B., Gardos, G. and Carafoli, E. "The maximal velocity and the calcium affinity of the red cell calcium pump may be regulated independently". *Journal of Biology Chemistry*, Vol. 262, pp.6425-6430 (1987)
  43. Falchetto, R. "The plasma membrane Ca<sup>2+</sup> pump contains a site that interacts with its calmodulin-binding domain". *Journal of Biological Chemistry*, Vol. 266, pp.2930-2936 (1991)
  44. Farago, A. and Nishizuca, Y. "Protein kinase C in transmembrane signaling". *FEBS Letters*, Vol. 268, pp.350-354 (1990)
  45. Fernandez, A., Sanchez-Yague, J., Martin-Valmaseda, M., Felipe, A., Cabezas, J. A. and Llanillo, M. "Changes in the fatty acid composition of stored erythrocytes from sheep of different ages". *Mechanisms of Aging and Development*, Vol. 71, pp.189-198 (1993)
  46. Ferreira, H. G. and Lew, V. L. "Calcium transport and calcium pump reversal in human red blood cells". *Proceedings of the Physiological Society*, 86P-87P (1975)
  47. Filoteo, A. G. "The ATP-binding site of the erythrocyte membrane calcium pump". *Journal of Biological Chemistry*, Vol. 262, pp.6526-6530 (1987)
  48. Friederichs, E. "Influence of calcium permeabilization and membrane-attached hemoglobin on erythrocyte deformability", *American Journal of Hematology*, Vol. 41, pp.170-177 (1992)
  49. Friederichs, E. and Melselman, H. J. "Effects of calcium permeabilization on red blood cell rheologic behavior". *Biorheology*, Vol. 31, pp.207-215 (1994)
  50. Friske, C. H. and Subbarow, Y. "The colorimetric determination of

- phosphorous". *Journal of Biological Chemistry*, Vol. 66, pp.375-400 (1925)
51. Ganzoni, A. M., Barras, J. P. and Marti, H. R. "Red cell aging and death". *Vox Sang*, Vol. 30, pp.161-174 (1976)
  52. Garrahan, P. J. and Rega, A. F. "Activation of partial reactions of the  $\text{Ca}^{2+}$ -ATPase from human red cells by  $\text{Mg}^{2+}$  and ATP". *Biochimica et Biophysica Acta*, Vol. 513, pp.59-65 (1978)
  53. Gietzen, K. and Kolandt, J. "Large-scale isolation of human erythrocyte  $\text{Ca}^{2+}$ -transport ATPase". *Biochemical Journal*, Vol. 207, pp.155-159 (1982)
  54. Giger, U., Sticher, B., Naef, R., Burger, R. and Lutz, H. U. "Naturally occurring human anti-band 3 autoantibodies accelerate clearance of erythrocytes in guinea pigs". *Blood*, Vol. 85, pp.1920-1928 (1995)
  55. Glaser, T., Schwarz-Bermeir, N. S., Barnoy, S., Barak, S., Eshhar, Z. and Kosower, N. S. "Calpain ( $\text{Ca}^{2+}$ -dependent thiol protease) in erythrocytes of young and old individuals". *Proceedings of National Academy of Science*, Vol. 91, pp.7879-7883 (1994)
  56. Glass, G. A. and Gershon, D. "Enzymatic changes in rat erythrocytes with increasing cell and donor age: loss of superoxide dismutase activity associated with increases in catalytically defective forms". *Biochemical and Biophysical Research Communications*, Vol. 103, pp.1245-1253 (1981)
  57. Godin, C. and Caprani, A. "Effect of blood storage on erythrocyte/wall interactions: implications for surface charge and rigidity". *European Biophysics Journal*, Vol. 26, pp.175-182 (1977)
  58. Haidas, S. "2, 3-diphosphoglycerate content and oxygen affinity as a function of red cell age in normal individuals". *Blood*, Vol. 38, pp.463-467 (1973)
  59. Hammes, A., Oberdorf, S., Strehler, E. E., Stauffer, T., Carafoli, E., Vetter, H. and Neyses, L. "Differentiation-specific isoform mRNA expression of the calmodulin-dependent plasma membrane  $\text{Ca}^{2+}$ -ATPase". *FASEB Journal*, Vol. 8, pp.428-435 (1994)

60. Hensley, K., Howard, B. J., Carney, J. M. and Butterfield, D. A. "Membrane protein alternations in rodent erythrocytes and synaptosomes due to aging and hyperoxia". *Biochimica et Biophysica Acta*, Vol. 1270, pp.203-206 (1995)
61. Hofmann, F., James, P., Vorherr, T. and Carafoli, E. "The C-terminal domain of the plasma membrane calcium pump contains 3 high affinity calcium binding sites". *Journal of Biological Chemistry*, Vol. 268, pp.10252-10259 (1993)
62. Hrusova, H., Strunecka, A., Piacentini, M. P., Accorsi, A. and Magnani, M. "Red blood cell age dependent modifications of inositol 1,4,5-trisphosphate". *Mechanism of Aging and Development*, Vol. 67, pp.13-19 (1993)
63. Huang, Y. H., Li, X. D., Yang, J. L., Liu, J. F. and Xu, J. Y. "Observation on the membrane  $\text{Ca}^{2+}$ -ATPase activity and oxygen consumption rate of preserved red blood cells". *Bulletin of Human Medical University*, Vol. 20, pp.341-343 (1995)
64. Iglesias, R. O. and Rega, A. F. "Gramicidin S inhibition of the  $\text{Ca}^{2+}$ -ATPase of human red blood cells". *Biochimica et Biophysica Acta*, Vol. 905, pp.383-389 (1987)
65. Jain, S. K. and Shohet, S. B. "Calcium potentates the peroxidation of erythrocyte membrane lipids". *Biochimica et Biophysica Acta*, Vol. 642, pp.46-54 (1981)
66. James, P. E. "The amino acid sequence of the phosphorylation domain of the erythrocyte  $\text{Ca}^{2+}$ -ATPase". *Biochemical, Biophysical Research Communication*, Vol. 149, pp. 7-12 (1987)
67. James, P., Vorherr, T., Krebs, J., Morelli, A., Castello, G., McCormick, D., Penniston, J. T., De Flora, A. D. and Carafoli, E. "Modulation of erythrocyte  $\text{Ca}^{2+}$ -ATPase by selective calpain cleavage of the calmodulin-binding domain". *Journal of Biological Chemistry*, Vol. 264, pp.8289-8296 (1989)
68. Kadlubowski, M. and Agutter, P. S. "Changes in the activities of some membrane-associated enzymes during in vivo aging of the normal human erythrocyte". *British Journal of Haematology*, Vol. 37, pp.111-125 (1977)
69. Kapprell, H. and Goll, D. E. "Effect of  $\text{Ca}^{2+}$  on binding of the calpains to

- calpastatin". *Journal of Biological Chemistry*, Vol. 264, pp.17888-17896 (1989)
70. Kawasaki, H., Emori, Y. and Suzuki, K. "Calpastatin has 2 distinct sites for interaction with calpain. Effect of calpastatin fragments on the binding of calpain to membranes". *Archives of Biochemistry and Biophysics*, Vol. 305, pp.467-472 (1993)
  71. Kay, M. M. B., Marchalonis, J. J., Schluter, S. F. and Bosman, G. "Human erythrocyte aging: cellular and molecular biology". *Transfusion Medicine Reviews*, Vol. 5, pp.173-195 (1991)
  72. King, C. M. and Barnett, Y. A. "Oxidative stress and human aging". *Biochemical Society Transactions*, Vol. 23, 375S (1995)
  73. Kosk-Kosicka, D. and Bzdega, T. "Activation of the erythrocyte  $\text{Ca}^{2+}$ -ATPase by either self-association or interaction with calmodulin". *Journal of Biological Chemistry*, Vol. 263, pp.19184-18189 (1988)
  74. Kosk-Kosicka, D., Bzdega, T. and Wawrzynow, A. "Fluorescence energy transfer studies of purified erythrocyte  $\text{Ca}^{2+}$ -ATPase". *Journal of Biological Chemistry*, Vol. 264, pp.19495-19499 (1989)
  75. Kosower, N. S. "Altered properties of erythrocytes in the aged". *American Journal of Hematology*, Vol. 42, pp.241-247 (1993)
  76. Laemmli, U. K. "Cleavage of structural proteins during the assembly of the head of bacteriophage T4". *Nature*, Vol. 227, pp.680-685 (1970)
  77. Leclerc, L., Vasseur, C., Bursaux, E., Marden, M. and Poyart, C. "Inhibition of membrane erythrocyte  $(\text{Ca}^{2+}+\text{Mg}^{2+})$ -ATPase by hemin". *Biochimica et Biophysica Acta*, Vol. 946, pp.49-56 (1988)
  78. Lee, K. S. and Au, K. S. "A protein inhibitor of erythrocyte membrane  $(\text{Ca}^{2+}+\text{Mg}^{2+})$ -ATPase". *Biochimica et Biophysica Acta*, Vol. 742, pp.54-62 (1983)
  79. Llanillo, M., Sanchez-Yague, J., Checa, A., Martin-Valmaseda, E. M. and Felipe, A. *et al* "Phospholipid and fatty acid composition in stored sheep erythrocytes of different densities". *Experimental Hematology*, Vol. 23, pp.258-264 (1995)

80. Lorand, L. "Formation of  $\gamma$ -glutamyl- $\epsilon$ -lysine bridges between membrane protein by a  $\text{Ca}^{2+}$ -regulated enzyme in intact erythrocyte". *Journal of Supramolecular Structure*, Vol. 9, pp.427-440 (1978)
81. Lowry, O. H., Rosebrough, N. J., Farr, A. L. and Randall, R. J. "Protein measurement with folin phenol reagent". *Journal of Biological Chemistry*, Vol. 193, pp.265-275 (1951)
82. Luterbacher, S. and Schatzmann, H. J. "The site of action of  $\text{La}^{3+}$  in the reaction cycle of the human red cell membrane  $\text{Ca}^{2+}$ -pump ATPase". *Experientia*, Vol. 39, pp.311-312 (1983)
83. Luthra, M. G. and Kim, H. D. " $(\text{Ca}^{2+}+\text{Mg}^{2+})$ -ATPase of density-separated human red cells. Effects of calcium and a soluble cytoplasmic activator (calmodulin)". *Biochimica et Biophysica Acta*, Vol. 600, pp.480-488 (1980)
84. Marikovsky, Y. "The cytoskeleton in ATP-depleted erythrocytes: the effect of shape transformation". *Mechanisms of Aging and Development*, Vol. 86, pp.137-144 (1996)
85. McCormack, J. G. and Denton, R. M. "The regulation of mitochondrial function in mammalian cells by calcium ions". *Biochemistry Society Transactions*, Vol. 109, pp.523-527 (1988)
86. Melloni, E., Sparatore, B., Salamino, F., Michetti, M. and Pontremoli, S. "Cytosolic calcium dependent proteinase of human erythrocytes: formation of an enzyme-neutral inhibitor complex induced by calcium ions". *Biochemical, Biophysical Research Communication*, Vol. 106, pp.731-740 (1982)
87. Murphy, J. R. "Influence of temperature and method of centrifugation on the separation of erythrocytes". *Journal of Laboratory and Clinical Medicine*, Vol. 82, pp. 334-341 (1973)
88. Nash, G. B., Linderkamp, O., Pfafferoth, C. and Melselman, H. J. "Changes in human red cell mechanisms during *in vivo* aging: possible influence on removal of senescent cells". In Platt, D., eds., *Blood cells, rheology and aging*, Springer-

Verlag, Berlin, pp.99-112 (1988)

89. Niggli, V., Adunyan, E. S., Penniston, J. T. and Carafoli, E. "Purified ( $\text{Ca}^{2+}+\text{Mg}^{2+}$ )-ATPase of the erythrocyte membrane". *Journal of Biological Chemistry*, Vol. 256, pp.395-401 (1981)
90. Niggli, V., Penniston, J. T. and Carafoli, E. "Purification of the ( $\text{Ca}^{2+}+\text{Mg}^{2+}$ )-ATPase from human erythrocyte membranes using a calmodulin affinity column". *Journal of Biological Chemistry*, Vol. 254, pp.9955-9958 (1979)
91. Niggli, V., Sigel, E. and Carafoli, E. "Inhibition of the purified and reconstituted calcium pump of erythrocytes by  $\mu\text{M}$  levels of DIDS and NAP-taurine". *FEBS Letters*, Vol. 138, pp.164-166 (1982)
92. O'Neil, K. T. and Degrado, W. F. "How calmodulin binds its targets: sequence independent recognition of amphiphilic  $\alpha$ -helices". *TIBS*, Vol. 15, pp.59-64 (1990)
93. Oliver, C. N., Ahu, B., Moerman, E. J., Goldstein, S. and Stadtman, E. R. "Age-related changes in oxidized proteins". *Journal of Biological Chemistry*, Vol. 262, pp.5488-5491 (1987)
94. Palek, J. *et al* "Polymerization of red cell membrane protein contributes to spherocytocyte shape irreversibly". *Nature*, Vol. 274, pp.505-507 (1978)
95. Piomelli, S., Lurinsky, G. and Wasserman, L. R. "The mechanism of red cell aging. I Relationship between cell age and specific gravity evaluated by ultracentrifugation in a discontinuous density gradient". *Journal of Laboratory and Clinical Medicine*, Vol. 69, pp.659-674 (1967)
96. Pontremoli, S. and Melloni, E. "Human erythrocyte  $\text{Ca}^{2+}$ -dependent neutral proteinase. New approaches to the understanding of the physiological function". *Progress in Clinical and Biological Research*, Vol. 180, pp.127-140 (1985)
97. Rahman, V. E., Elson, D. L. and Cerny, E. A. "Studies on the mechanism of erythrocyte aging and destruction. I Separation of rat erythrocytes according to age by ficoll gradient centrifugation". *Mechanisms of Aging and Development*,



Vol. 2, pp.141-150 (1973)

98. Richards, D. E. "Two classes of site for ATP in the  $\text{Ca}^{2+}$ -ATPase from human red cell membranes". *Biochimica et Biophysica Acta*, Vol. 511, pp.194-201 (1978)
99. Rigas, D. A. and Koler, R. D. "Ultracentrifugal fractionation of human erythrocytes on the basis of cell age". *Journal of Laboratory and Clinical Medicine*, Vol. 58, pp.242-246 (1961)
100. Ronner, P., Gazzotti, P. and Carafoli, E. "A lipid requirement for the  $(\text{Mg}^{2+}+\text{Ca}^{2+})$ -activated ATPase of erythrocyte membrane". *Archives of Biochemistry and Biophysics*, Vol. 179, pp.578-583 (1977)
101. Rossi, J. P. and Schatzmann, H. J. "Trypsin activation of the red cell  $\text{Ca}^{2+}$ -pump ATPase is calcium-sensitive". *Cell Calcium*, Vol. 3, pp.583-590 (1982)
102. Rossi, J. P., Garrahan, P. J. and Rega, A. F. "Vanadate inhibition of active calcium transport across human red cell membranes". *Biochimica et Biophysica Acta*, Vol. 648, pp.145-150 (1981)
103. Roufogalis, B. D. "Regulation of calcium translocation across the red cell membrane". *Canadian Journal of Physiology and Pharmacology*, Vol. 57, pp.1331-1349 (1979)
104. Roufogalis, B. D. and Mauldin, D. "Regulation by calmodulin of the calcium affinity of the calcium-transport ATPase in human erythrocytes". *Canadian Journal of Biochemistry*, Vol. 58, pp.922-927 (1980)
105. Sackett, D. L. and Kosk-Kosicka, D. "The active species of plasma membrane  $\text{Ca}^{2+}$ -ATPase are a dimer and a monomer-calmodulin complex". *Journal of Biological Chemistry*, Vol. 27, pp.9987-9991 (1996)
106. Samaja, M., Rubinacci, A., Ponti, A. D. and Portinaro, N. "The effect of *in vitro* and *in vivo* cellular aging on the active calcium transport in human inside-out red cell membrane vesicles". *Biochemical and Biophysical Research Communication*, Vol. 159, pp.432-438 (1989)
107. Scharff, O. "Calmodulin and its role in cellular activation". *Cell Calcium*, Vol. 2,

pp.1-27 (1981)

108. Scharff, O. and Foder B. "Reversible shift between two states of  $\text{Ca}^{2+}$ -ATPase in human erythrocyte mediated by  $\text{Ca}^{2+}$  and a membrane-bound activator". *Biochimica et Biophysica Acta*, Vol. 509, pp.67-77 (1978)
109. Scharff, O. and Foder, B. "Low calcium concentration controlling two kinetic states of  $\text{Ca}^{2+}$ -ATPase from human erythrocytes". *Biochimica et Biophysica Acta*, Vol. 483, pp.416-424 (1977)
110. Schatzmann, H. J. "The plasma membrane calcium pump of erythrocyte and other animal cells". In Carafoli, E. eds., *Membrane Transport of Calcium*, Academic Press inc., London, pp.41-108 (1982)
111. Schatzmann, H. J. "ATP-dependent  $\text{Ca}^{2+}$ -extrusion from human red cells". *Experientia*, Vol. 22, pp.364-365 (1966)
112. Schatzmann, H. J. and Burgin, H. "Calcium in human red blood cells". *Annals New York Academy of Sciences*, Vol. 307, pp.125-147 (1978)
113. Schatzmann, H. J. and Vincenzi, F. F. "Calcium movements across the membrane of human red cells". *Journal of Physiology*, Vol. 201, pp.369-395 (1969)
114. Schroeder, F., Woodford, J. K., Kavecansky, J., Wood, W. G. and Joiner, C. "Cholesterol domains in biological membranes". *Molecular Membrane Biology*, Vol. 12, pp.113-119 (1995)
115. Schwarz-Benmeir, N., Tova, G., Barnoy, S. and Kosower, N. S. "Calpastatin in erythrocytes of young and old individuals". *Biochemical Journal*, Vol. 304, pp.365-370 (1994)
116. Seaman, C., Wyss, S. and Piomelli, S. "The decline in energetic metabolism with aging of the erythrocyte and its relationship to cell death". *American Journal of Hematology*, Vol. 8, pp.31-42 (1980)
117. Seidler, N. W. and Swislocki, N. I. " $\text{Ca}^{2+}$  transport activities of inside-out vesicles prepared from density-separated erythrocytes from rat and human",

- Molecular and Cellular Biochemistry*, Vol. 105, pp.159-169 (1991)
118. Shiga, T., Maeda, N., Kon, K. "Erythrocyte rheology". *Critical Reviews in Oncology/Hematology*, Vol. 10, pp.9-48 (1990)
  119. Stauffer, T. P., Guerini, D. and Carafoli, E. "Tissue distribution of the 4 genes products of the plasma membrane calcium pump". *Journal of Biological Chemistry*, Vol. 270, pp.12184-12190 (1995)
  120. Stauffer, T. P., Hilfiker, H., Carafoli, E. and Strehler, E. E. "Quantitative analysis of alternative splicing options of human plasma membrane calcium pump genes". *Journal of Biological Chemistry*, Vol. 268, pp.25993-26003 (1993)
  121. Strynadka, N. C. J. and James, M. N. G. "Crystal structures of the helix-loop-helix calcium-binding protein". *Annuals of review of biochemistry*, Vol. 58, pp.951-998 (1988)
  122. Suzuki, K., Imajoh, S., Emori, Y., Kawasaki, H., Minami, Y. and Ohno, S. "Calcium-activated neutral protease and its endogenous inhibitor". *FEBS Letters*, Vol. 220, pp.271-277 (1987)
  123. Tang, T. K. "Free radical theory of erythrocyte aging". *Journal of Formos Medical Association*, Vol. 96, pp.779-783 (1997)
  124. Tannert, C., Schmidt, G., Klatt, D. and Rapoport, S. M. "Mechanism of senescence of red blood cells". *Acta Biol. Med. Germ.*, Vol. 36, pp.831-836 (1977)
  125. Timiras, P. S. "The cell aging process". In Timiras, P. S. and Bittar, E. E. eds., *Advances in Cell Aging and Gerontology*, JAI Press Inc., London, pp.1-29 (1996)
  126. Turner, B. M., Fisher, R. A. and Harris, H. "The age-related loss of activity of four enzymes in the human erythrocyte". *Clinica Chimica Acta*, Vol. 50, pp. 85-95 (1974)
  127. Varecka, L. and Carafoli, E. "Vanadate induced movements of  $\text{Ca}^{2+}$  and  $\text{K}^{+}$  in human red blood cells". *Journal of Biological Chemistry*, Vol. 257, pp.7414-

7421 (1982)

128. Verma, A. K., Enyedi, A., Filoteo, A. G., Strehler, E. E. and Penniston, J. T. "Plasma membrane calcium pump isoform 4a has a longer calmodulin-binding domain than 4b". *Journal of Biological Chemistry*, Vol. 271, pp.3714-3718 (1996)
129. Vettore, L., De Matteis, M. C. and Zampini, P. "A new density gradient system for the separation of human red blood cells". *American Journal of Hematology*, Vol. 8, pp.291-297 (1980)
130. Vincenzi, F. F. and Hinds, T. R. "Decreased calcium pump ATPase activity associated with increased density in human red blood cells". *Blood Cells*, Vol. 14, pp.139-148 (1988)
131. Vincenzi, F. F., Hinds, T. R. and Raess, B. U. "Calmodulin and the plasma membrane calcium pump". *Annals New York Academy of Sciences*, Vol. 80, pp.232-244 (1980)
132. Vorherr, T., Quadroni, M., Krebs, J. and Carafoli, E. "Photoaffinity labeling study of the interaction of calmodulin with the plasma membrane  $\text{Ca}^{2+}$  pump". *Biochemistry*, Vol. 31, pp.8245-8251 (1992)
133. Wang, K. K., Villalobo, A. and Roufogalis, B. D. "Activation of the  $\text{Ca}^{2+}$ -ATPase of human erythrocyte membrane by an endogenous  $\text{Ca}^{2+}$ -dependent neutral protease". *Archives of Biochemistry and Biophysics*, Vol. 260, pp.696-704 (1988)
134. Weed, R. I., LaCelle, P. L. and Merrill, E. W. "Metabolic dependence of red cell deformability". *Journal of Clinical Investigation*, Vol. 48, pp.765-809 (1969)
135. Wetzker, R., Klinger, R. and Frunder, H. "Effects of fatty acids on activity and calmodulin binding of  $\text{Ca}^{2+}$ -ATPase of human erythrocyte membranes". *Biochimica et Biophysica Acta*, Vol. 730, pp.196-200 (1983)
136. Williamson, J. R. and Cooper, R. H. "Regulation of the citric acid cycle in mammalian systems". *FEBS Letters*, Vol. 117, K73-K85 (1980)

137. Winterbourn, C. C. and Batt, R. D. "Lipid composition of human red cells of different ages". *Biochimica et Biophysica Acta*, Vol. 202, pp.1-8 (1970)
138. Wolfe, L. C. "Oxidative injuries to the red cell membrane during conventional blood preservation". *Seminars in Hematology*, Vol. 26, pp.307-312 (1989)
139. Wuthrich, A. "Isolation from haemolysate of a proteinaceous inhibitor of the red cell  $\text{Ca}^{2+}$ -pump ATPase. Its action on the kinetics of the enzyme". *Cell Calcium*, Vol. 3, pp.201-214 (1982)
140. Zurini, E. "Mapping of functional domains in the plasma membrane  $\text{Ca}^{2+}$  pump using trypsin proteolysis". *Biochemistry*, Vol. 29, pp.8070-8076 (1990)
141. Zurini, M., Krebs, J., Penniston, J. T. and Carafoli, E. "Controlled proteolysis of the purified  $\text{Ca}^{2+}$ -ATPase of the erythrocyte membrane". *Journal of Biological Chemistry*, Vol. 259, pp.618-627 (1984)