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CHANGE IN CYTOSOLIC CALMODULIN ACTIVITY OF DENSITY (AGE) SEPARATED HUMAN ERYTHROCYTES TOWARDS MEMBRANE Ca^{2+}/Mg^{2+} ATPase

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SUMMARY: The membrane Ca^{2+}/Mg^{2+} ATPase of density (age) separated human erythrocytes was examined for its stimulation by the cytosols of these cell groups. On the assumption that the stimulatory activity in the cytosol is only calmodulin, it was consistently observed that the young cytosol had a significantly higher activity towards the membrane Ca^{2+}/Mg^{2+} ATPase activity (from any age group) than did the old cell cytosol. The data clearly demonstrates decided differences in the expression of calmodulin activity in cytosols from young and old erythrocytes and would support the conclusion that calmodulin activity is altered during in vivo aging of these cells. Possible mechanisms for these alterations are discussed.

INTRODUCTION

Current theories regarding mammalian membrane structure and function have derived in the main from an enormous number of studies on the human erythrocyte (1) which have provided an exciting insight into the arrangement of lipids and proteins in these cells. However, a factor largely overlooked in most of these investigations has been that the cell samples were in point of fact a mixed age population with a finite life span (for example, 120 days for humans). That there are biochemical differences in various age groups of human erythrocytes has been shown in several reports. For example, as human erythrocytes age there is a decrease in size (2), an increase in hemoglobin concentration (3), a decrease in cholesterol and phospholipid (3), and a change in membrane ATPase (4,5) as well as other membrane associated enzymes (6) and alterations in cytosolic enzymes (7,8). Our interest in the Ca^{2+}/Mg^{2+} ATPase of human erythrocytes (9-11) has led to the speculation that changes in this enzyme system, including its activator, calmodulin, may be related closely to the increased calcium level in old erythrocytes and possibly be directly related to the ultimate demise of this cell. The availability of the excellent technique of Murphy (12) for the separation of human erythrocytes into age groups which has been confirmed by this lab (3) as well as that

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Abbreviations used: NaCl-His buffer, 0.155 M NaCl, 5 mM histidine, pH 7.6

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of Sears and Luthra (13) provided a reproducible method for obtaining sufficient amounts of erythrocytes of different age groups. Although it had been shown earlier (4,5) that membrane Ca^{2+}/Mg^{2+} ATPase activity does decrease with age of the cell, there is no report as to any possible differences in calmodulin activity of cytosol with cell aging. The data presented in this communication would support the conclusion that calmodulin activity is altered on an age related basis.

MATERIALS AND METHODS

Disodium ATP, EGTA, L-histidine and ouabain octahydrate were from Sigma Chemical Co. Saponin from Calbiochem was passed through BioRad AG50W-X8, 200-400 mesh resin for removal of any contaminating Ca^{2+}.

Separation of Erythrocytes into Age Groups

Erythrocytes were separated into different age groups based on their density as described by Murphy (12) and further examined by Cohen et al. (3). Blood was collected from the anticubital vein of healthy adult human volunteers (ages 20-35 years) into heparinized Becton Dickinson vacutainer tubes. The blood was centrifuged for 8 to 10 minutes at 1500 x g at 4°C, the plasma removed and saved, and the buffy coat aspirated carefully with minimal loss of erythrocytes. The remaining packed cells were resuspended in original plasma and the above washing procedure repeated to remove any additional buffy coat. Erythrocytes resuspended at 90% hematocrit in the plasma recovered from the second wash were centrifuged in a Sorvall Model RC-2B equipped with an SS-34 rotor for one hour at 30,000 x g at 25-30°C. The plasma and any remaining buffy layer were removed carefully by suction. Measured fractions of the packed cells, based on volume, were withdrawn from the top of the erythrocyte pack by means of a 1 ml disposable syringe. Only the top 10% (young) and bottom 10% (old) cells were used in this study.

Cell fractions were washed three times with at least two volumes of NaCl-His buffer and adjusted to a 10% hematocrit suspension. Hemoglobin was determined by the method of Kachmar (14). Hematocrits were measured in tubes spun for five min at room temperature in an Adams micro-hematocrit centrifuge (Clay Adams, Inc., New York).

Preparation of Saponin EGTA Membranes

Erythrocytes were lysed by adding saponin to a 10% cell suspension in NaCl-His buffer containing 2 mM EGTA (15). The final concentration of saponin was 0.1 mg/ml. After standing for 10 to 15 min at room temperature, the membranes were washed four times with six volumes of cold NaCl-His buffer by centrifugation at 30,000 x g for 35 min at 4°C. The first two washes included 2 mM EGTA. The resulting white membranes were resuspended in a volume of NaCl-His buffer equivalent to the starting volume of the original 10% erythrocyte suspension. It should be noted that after the first centrifugation the membranes were carefully separated from a small dense button. Protein was assayed by the Lowry procedure (16).

Membranes prepared by saponin lysis were found to be superior to other membrane preparations in expression of maximal Ca^{2+}/Mg^{2+} ATPase activity (4). The Ca^{2+}/Mg^{2+} ATPase of saponin EGTA membranes is stimulated approximately 2.5 fold by the addition of cytosol derived from a 10% (mixed age) erythrocyte suspension. In addition, these membranes may be stored frozen at −80°C for many months with no observable loss in activity and thus provided a very reproducible and convenient source of enzyme for the calmodulin activity.

Preparation of Saponin Cytosol

Ten percent hematocrit suspensions of young and old erythrocytes (see above) were lysed with saponin (but no EGTA) as mentioned above. The hemolysates were centrifuged at 30,000 x g for 35 min at 4°C to obtain a membrane-free supernatant or cytosol. Cytosols from young and old erythrocyte hemolysates (31.8 ± 0.36 mg Hgb/ml and 37.1 ± 0.65 mg Hgb/ml, respectively, expressed as mean ± S.E.M.) were then serially diluted and assayed for their effect on (saponin EGTA) membrane Ca^{2+}/Mg^{2+} ATPase.

Ca^{2+}/Mg^{2+} ATPase Assay

The Ca^{2+}/Mg^{2+} ATPase activity was measured as previously described (4). Membrane protein, ranging from 0.025 to 0.028 mg per 0.05 ml aliquot was combined with 0.05 ml cytosol followed by 0.5 ml addition of ATPase cocktail and incubated for two hours at 44°C. Final concentrations of reagents in the incubation mixture were 3.6 mM $MgCl_2$, 80 mM NaCl, 33 mM KCl, 2.5 mM Na_2ATP, 0.9 mM ouabain and 0.05 mM $CaCl_2$, buffered with 80 mM histidine, pH 7.6. The reaction was terminated by the addition of 1.0 ml ice-cold TCA. The protein precipitate was removed by centrifugation at 1000 x g for three min at room temperature, and 1.0 ml of the supernatant was assayed for inorganic phosphate (17).

Assay for Ca^{2+}/Mg^{2+} Stimulating Activity (Calmodulin)

Cytosol samples were serially diluted 1:1. To each 0.05 ml aliquot of the diluted samples, an equal volume of a 10% membrane preparation and 0.5 ml of ATPase cocktail were added. Details of the assay conditions were described above. Basal membrane activity was determined by substituting NaCl-His buffer for the cytosol. The largest observed activity, where increased amounts of cytosol had no further stimulating effect was considered as the maximum activity level (100% stimulation).

The amount of inorganic phosphate released by Ca^{2+}/Mg^{2+} ATPase activity was plotted against n (the number of times a cytosol preparation was diluted on a 1:1 basis). For an undiluted sample, n would be zero. One unit of calmodulin activity has been arbitrarily defined as the volume of the undiluted cytosol which provided a 50% stimulation of Ca^{2+}/Mg^{2+} ATPase per ml of assay medium.

RESULTS AND DISCUSSION

The results presented in this report establish that there is a significant decrease in the Ca^{2+}/Mg^{2+} ATPase stimulating (calmodulin) activity in the cytosols of density separated old erythrocytes as compared to young erythrocytes. An overall interpretation of the data would suggest a general conclusion that the Ca^{2+}/Mg^{2+} ATPase itself has not changed with aging of the cell; rather, that the expression of calmodulin activity is altered.

Addition of mixed membranes to serially diluted cytosol from young and old erythrocytes revealed that young cell cytosol contained a (greater) Ca^{2+}/Mg^{2+} ATPase stimulating activity than old cell cytosol (Fig. 1). Undiluted cytosol from young and old cells, where n = 0 (see Materials and Methods), stimulated membrane Ca^{2+}/Mg^{2+} ATPase to an equal extent when assayed with a mixed population of red cell membranes. However, at a 32-fold dilution (n = 5) differences were apparent. Addition of these two cytosols separately on mixed age membranes clearly showed that the differences in Ca^{2+}/Mg^{2+} ATPase stimulating activity are the properties inherent in cytosols.

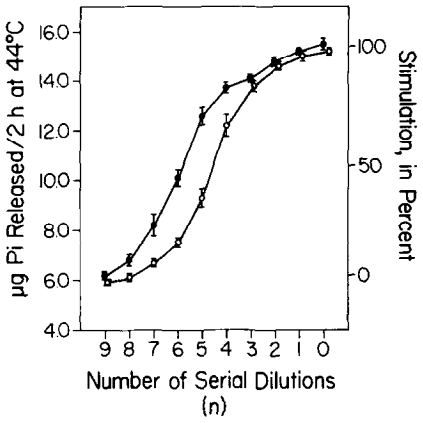

Figure 1. Stimulation of Membrane Ca^{2+}/Mg^{2+} ATPase of Non-Density Separated Erythrocytes by Young and Old Erythrocyte Cytosol

Cytosols obtained from saponin lysis of 10% suspension of young (•—•) and old (o—o) erythrocytes, were serially diluted and assayed for Ca^{2+}/Mg^{2+} ATPase stimulating activity as described in Materials and Methods. Each point is the mean ± S.E.M. of duplicate analyses on three separate preparations.

In order to investigate this effect more thoroughly, cytosol from young and old erythrocytes were added to the membranes obtained from young and old erythrocytes in various combinations and have been shown in Figure 2 and Table 1. In Figure 2a, young and old erythrocyte cytosols were assayed with young erythrocyte membranes. The stimulating ability of each cytosol was comparable to that observed when mixed membranes were used (i.e., young cytosol stimulates to a greater extent than old cytosol). Old erythrocyte membrane Ca^{2+}/Mg^{2+} ATPase also showed greater activity in the presence of young erythrocyte cytosol as compared to the old erythrocyte cytosol (Fig. 2b). The basal activity of the two (age group) saponin-EGTA membrane preparations was the same, but maximal activity was slightly more in young membranes regardless of the age of erythrocyte cytosol added (Fig. 2a,b).

In Table I, units of calmodulin activity in the cytosols have been expressed per ml of the original 10% hematocrit. However, since the old erythrocytes contain more hemoglobin per ml packed cells than the young erythrocytes, units were also calculated on the basis of hemoglobin as hemoglobin remains at a constant level in cells throughout aging (3). Some membrane protein is lost as the cell ages (3) which may affect the expression of ATPase activity and its ability to be stimulated by the cytosol. Therefore, units of Ca^{2+}/Mg^{2+} ATPase stimulating activity in the cytosol were also compared with the level of membrane protein used in the assay. It was apparent that incubation of young erythrocyte cytosols with young and old erythrocyte membranes showed a higher Ca^{2+}/Mg^{2+}

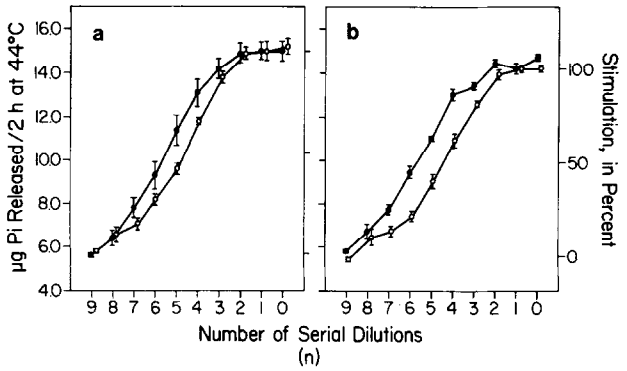

Figure 2. Stimulation of Young and Old Erythrocyte Membrane Ca^{2+}/Mg^{2+} ATPase by Serially Diluted Cytosols

Experimental conditions are the same as those described in Figure 1.

a. Young erythrocyte membranes were added to young (•–•) and old (o–o) ervthrocyte cytosols.

b. Old erythrocyte membranes were added to young (•–•) and old (o–o) erythrocyte cytosols.

ATPase stimulating activity whereas the incubation of old erythrocyte cytosol either with young or with old erythrocyte membranes always gave a lower stimulating activity (Table I). This further establishes that the differences in stimulating properties are due to cytosols rather than the membranes.

The data obtained in this paper clearly demonstrates that there are significant differences in the expression of Ca^{2+}/Mg^{2+} ATPase stimulating (calmodulin) activity of cytosols obtained from young and old erythrocytes. There are at least five possibilities which could account for the higher Ca^{2+}/Mg^{2+} ATPase stimulating activity (calmodulin activity) in young erythrocyte cytosol as compared to that observed in old erythrocyte cytosol and are formulated as follows: 1) an inhibitory component may be present in the cytosol of old erythrocytes. However, this is rather unlikely since the mixtures of young and old erythrocyte cytosols in ratios of 1:3, 1:1 and 3:1 provided intermediary levels of stimulating activity to that shown in Figure 1 (data not shown). In the 1:3 ratio, any free inhibitor type molecule present in the old erythrocyte cytosol would have been expected to abolish the activating property of young erythrocyte cytosol, but this did not occur. 2) The concentration of activator (calmodulin) protein may be lower in the old erythrocyte cytosol compared to young erythrocyte cytosol. 3) A Ca^{2+}/Mg^{2+} ATPase stimulating protein (other than calmodulin) may be present in young erythrocyte cytosol which is depleted during aging. In this regard there is a report of such a protein present in human erythrocyte membranes, which has Ca^{2+}/Mg^{2+} ATPase stimulating activity (18).

TABLE I

Calmodulin Activity in Young and Old Erythrocyte Cytosols as Measured on Membrane Ca^{2+}/Mg^{2+} ATPase of Young and Old Erythrocyte Membranes

	Calmodulin Activities* in					
	Young Erythrocyte Cytosol			Old Erythrocyte Cytosol		
	Units/ml Cytosol	Units/mg Cytosol Hgb	Units/mg Membrane Protein	Units/ml Cytosol	Units/mg Cytosol Hgb	Units/mg Membrane Protein
A.	553±29 (3)	17.3±0.9 (3)	1103±67 (2)	240±20 (3)	6.4±0.6 (3)	444±61 (2)
B.	544±37 (3)	17.0±1.2 (3)	1061±22 (2)	232±22 (3)	6.2±0.7 (3)	400±60 (2)

A. Young erythrocyte membranes

B. Old erythrocyte membranes

*For non-density separated erythrocyte, cytosol values of calmodulin activity assayed with a mixed population of erythrocyte membranes (three separate donors) were as follows: 346±18.8 units/ml cytosol, 10.2±0.5 units/mg Hgb, 596±33 units/mg protein.

Units of calmodulin activity were calculated as described in Materials and Methods. Specific activity, based on mg hemoglobin, provided direct comparison between young and old erythrocyte cytosol activities since the amount of hemoglobin per cell remains constant throughout cell aging. In addition, specific activity was also calculated on the basis of membrane protein used in the assay. Results are given for duplicate assays and are expressed as the mean ± S.E.M. (no. of subjects).

4) The molecular nature of the stimulatory protein (calmodulin) in young and old erythrocyte cytosols may be different. It is relevant to mention that modification of calmodulin has been shown to affect its activity. Recently methylation of calmodulin has been found to reduce its activating properties (19). It has also been reported that at the same concentration, calmodulin obtained from different sources, exhibit a varying degree of brain phosphodiesterase stimulation. It was suggested that this was due to molecular changes in calmodulin during evolution. In addition, the present communication is another argument for molecular alteration of calmodulin activity in erythrocytes during their aging process in circulation. 5) A difference in the affinity of calmodulin in the young and old erythrocyte cytosols for Ca^{2+} may be another important factor.

A molecular explanation as to which of the above possibilities could account for the differences in calmodulin activity of young and old erythrocyte cytosols, together with the

physiological significance of these subtle differences in Ca^{2+} regulation in this cell must await further investigation.

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