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$(Ca^{2+} + Mg^{2+})$-ATPase OF DENSITY-SEPARATED HUMAN RED CELLS

EFFECTS OF CALCIUM AND A SOLUBLE CYTOPLASMIC ACTIVATOR (CALMODULIN) *

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Summary

The effect of calcium and a soluble cytoplasmic activator on $(Ca^{2+} + Mg^{2+})$-ATPase of density-separated human red cells was investigated. At all calcium concentrations tested, dense (old) lysed cells and their isolated membranes displayed lower activities as compared to the light (young) cells and their membranes. Isolated membranes from all density red cell fractions showed two distinct $(Ca^{2+} + Mg^{2+})$-ATPase activities; one at low calcium and another at moderate calcium concentrations. At high calcium concentration, $(Ca^{2+} + Mg^{2+})$-ATPase activity of isolated membranes was low in all cell fractions. In contrast to the isolated membranes, lysed cells from all density fractions had a maximum $(Ca^{2+} + Mg^{2+})$-ATPase activity only at a low concentration of calcium, while moderate and high calcium concentrations produced low activity. Upon isolation of membranes, a substantial loss of $(Ca^{2+} + Mg^{2+})$-ATPase activity took place from all density cell fractions. Upon membrane isolation, the relative loss of $(Ca^{2+} + Mg^{2+})$-ATPase activity at low Ca^{2+} concentration was greater in older cells. The extent of stimulation of $(Ca^{2+} + Mg^{2+})$-ATPase by the activator at low calcium concentration was 3—4-fold greater in older cell membranes than in the young ones.

These data suggest that the lower $(Ca^{2+} + Mg^{2+})$-ATPase activity in old cells could be accounted for by a selective loss of $(Ca^{2+} + Mg^{2+})$-ATPase activity at low Ca^{2+} concentration presumably due to reduced affinity of old cell membranes to activator protein.

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Introduction

In the companion paper [1], evidence has been presented in support of a view that the stimulation of human red cell membrane (Ca^{2+} + Mg^{2+})-ATPase activity by a cytoplasmic activator protein is highly dependent upon the Ca^{2+} concentration. It was found that the Ca^{2+} concentration at which the cytoplasmic activator protein elicits a maximal stimulation of the membrane enzyme permitted an expression of only a small fraction of the optimal (Ca^{2+} + Mg^{2+})-ATPase activity of the membrane alone. These findings imply that Ca^{2+} concentration itself together with the activator protein play a decisive role in governing various state of (Ca^{2+} + Mg^{2+})-ATPase. This paper is concerned with the question of how various enzyme states affected by Ca^{2+} and cytoplasmic activator protein might undergo changes in the course of cell aging.

Materials and Methods

Materials used were the same as in the preceding paper [1].

Density separation of human red cells. Blood was collected from healthy adult humans into Becton-Dickinson vacutainer tubes containing 143 USP * units of heparin. Cells were separated by using the method of Murphy [2] as outlined previously [3—5]. Seven equal fractions from top to bottom of the centrifuge tube were collected and numbered 1 to 7 from top to bottom, respectively. Only fractions 1, 4 and 7 were used in this study and designated as the top, middle and bottom fractions, respectively.

Preparation of red cells and membranes. Density-separated red cells were washed three times in 0.172 M Tris-HCl buffer, pH 7.6, and resuspended in the same buffer to 50% hematocrit. One aliquot was stored on ice and another aliquot was used for isolation of membranes in 20 imosM Tris-HCl, pH 7.6, as described previously [6]. Membranes were resuspended and treated with saponin as described in the preceding paper [1]. Hemoglobin was measured in washed cells by using the method of Kachmar [7].

Preparation of membrane-free hemolysate. During the first step of hypotonic lysis, the supernatant after centrifugation was saved [6] and used as a source of (Ca^{2+} + Mg^{2+})-ATPase activator without further concentration. Unless otherwise stated, each membrane derived from density-separated cells was tested with its own corresponding hemolysate. Measurement of (Ca^{2+} + Mg^{2+})-ATPase activity was carried out by using the method described in the preceding paper [1]. Likewise, concentration of calcium in the assay varied from no calcium, low calcium, moderate calcium and high calcium as described in the preceding paper [1]. (Na^+ + K^+)- and Mg^{2+}-dependent ATPase activities were determined and subtracted as detailed in the preceding paper [1].

Results

Effect of calcium on (Ca^{2+} + Mg^{2+})-ATPase activity of density-separated red cells and their isolated membranes

Fig. 1 shows the (Ca^{2+} + Mg^{2+})-ATPase activities of various density-separated

* USP, United States Pharmaecopeia.

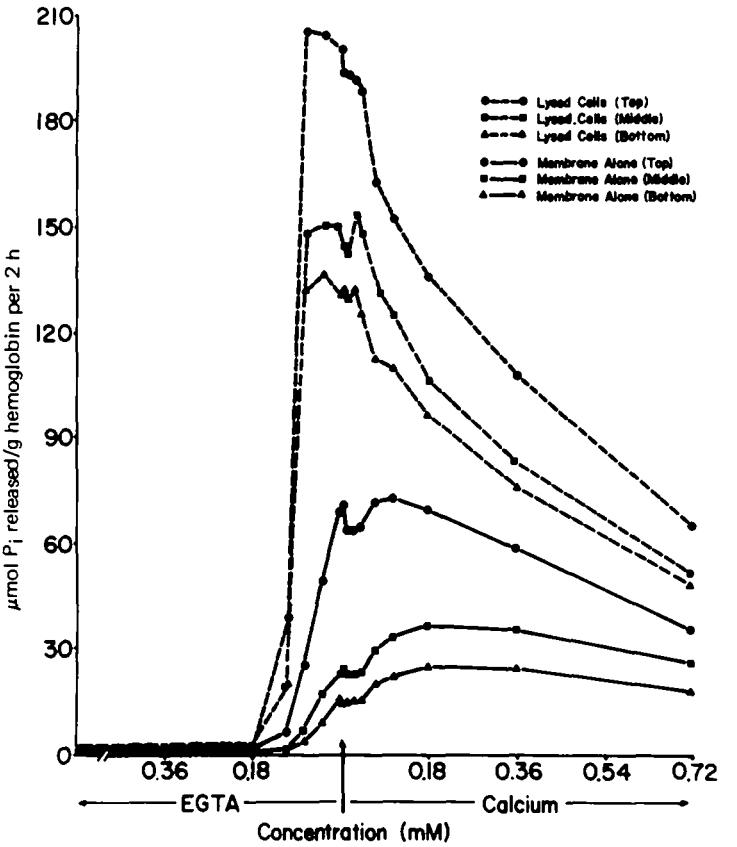

Fig. 1. Effect of calcium on (Ca^{2+} + Mg^{2+})-ATPase activity of various density-fractionated cells and their isolated membranes. Cells were separated into various density (age) cohorts. The membranes were isolated and (Ca^{2+} + Mg^{2+})-ATPase activities were measured in saponin-treated cells and membranes at various concentrations of either EGTA or calcium as described in Materials and Methods.

red cell lysates and membranes as a function of calcium concentration in the assay medium. The optimum activity was obtained in all cases at low calcium concentrations (i.e., 0.071 mM EGTA to 0.08 mM Ca^{2+}). Further increase in calcium concentration in the assay medium inhibited (Ca^{2+} + Mg^{2+})-ATPase activity and at 0.72 mM added calcium, approx. 65% of the (Ca^{2+} + Mg^{2+})-ATPase activity was lost from all the cells. At all calcium concentrations, the levels of (Ca^{2+} + Mg^{2+})-ATPase activities were highest in the lighter (young) cells followed by the middle and bottom fraction cells. However, the magnitude of differences in (Ca^{2+} + Mg^{2+})-ATPase activities was greater in low calcium concentration than in higher Ca^{2+} concentrations as shown in Fig. 2 in which (Ca^{2+} + Mg^{2+})-ATPase activity of the middle and bottom fraction cells were normalized relative to that of the top fraction cells. Thus, these data suggest that not all the calcium-dependent ATPase activities were lost in a uniform manner, but rather preferential loss of more of the low calcium-stimulated ATPases took place during human red cell aging.

As in red cells, the ATPase activities obtained at all calcium concentrations

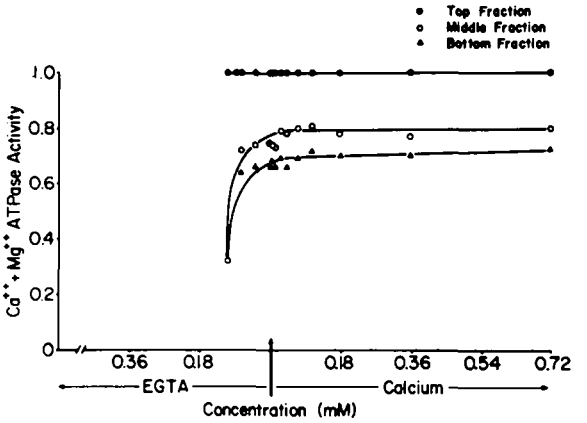

Fig. 2. Relative (Ca^{2+} + Mg^{2+})-ATPase activities in different density-separated cells as a function of calcium concentration. The relative activities of the middle and bottom fraction cells were normalized relative to the low-density (young cell) fraction at various concentration of EGTA and calcium from Fig. 1.

were highest in lighter (young) cell membranes and lowest in denser (old) cell membranes (Fig. 1). On the other hand, in contrast to red cells, all membranes displayed two distinct calcium-stimulated ATPase activities; one in the region of low calcium concentrations and the other at moderate calcium concentrations (Fig. 1). It was also found that in contrast to red cells, inhibition of (Ca^{2+} + Mg^{2+})-ATPase activities in isolated membranes was detectable only at moderate calcium concentrations (Fig. 1), in keeping with the result reported in the companion paper [1]. At all calcium concentrations, the recovery of (Ca^{2+} + Mg^{2+})-ATPase activities in isolated membrane from the enzyme activity of the cells was lower in dense (old) cells when compared to light (young) cells (Fig. 3). The results imply that in the course of membrane preparation, the relative recovery of low calcium-stimulated ATPase

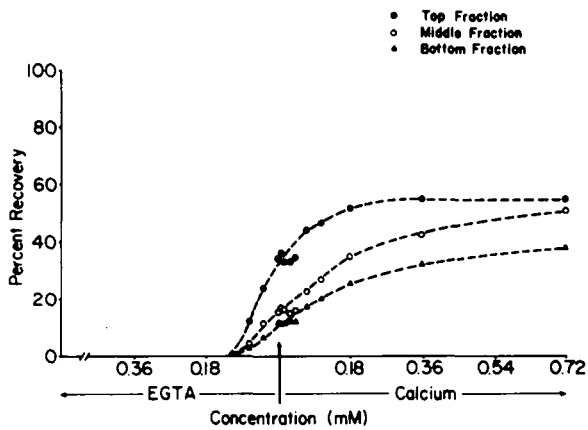

Fig. 3. Recovery of (Ca^{2+} + Mg^{2+})-ATPase activities in isolated membranes of different density (age) red cells at different concentrations of calcium or EGTA. Percent recovery ATPase activity was calculated from their respective lysed red cell activities from Fig. 1.

activity was smaller in dense (old) cells than in light (young) cells, possibly due to more retention of a soluble cytoplasmic activator on membranes of low-density (young) cells.

Effect of cytoplasmic activator on various calcium-stimulated ATPase activities of isolated membranes from density-separated red cells

Upon addition of hemolysate containing a soluble cytoplasmic activator to isolated membranes from various density-separated red cells, the (Ca^{2+} + Mg^{2+})-ATPase activities were found to increase at various calcium concentrations in a manner shown in Fig. 4. The extent of stimulation by the activator of (Ca^{2+} + Mg^{2+})-ATPase activities calculated from Fig. 4 at various calcium concentrations is shown in Fig. 5. It is clear that optimum activation was achieved in the regions of low calcium concentration in all membranes (Fig. 5). The percent activation was 3-fold higher in dense (old) cell membranes (1300%) than that found in light (young) cell membranes (400%).

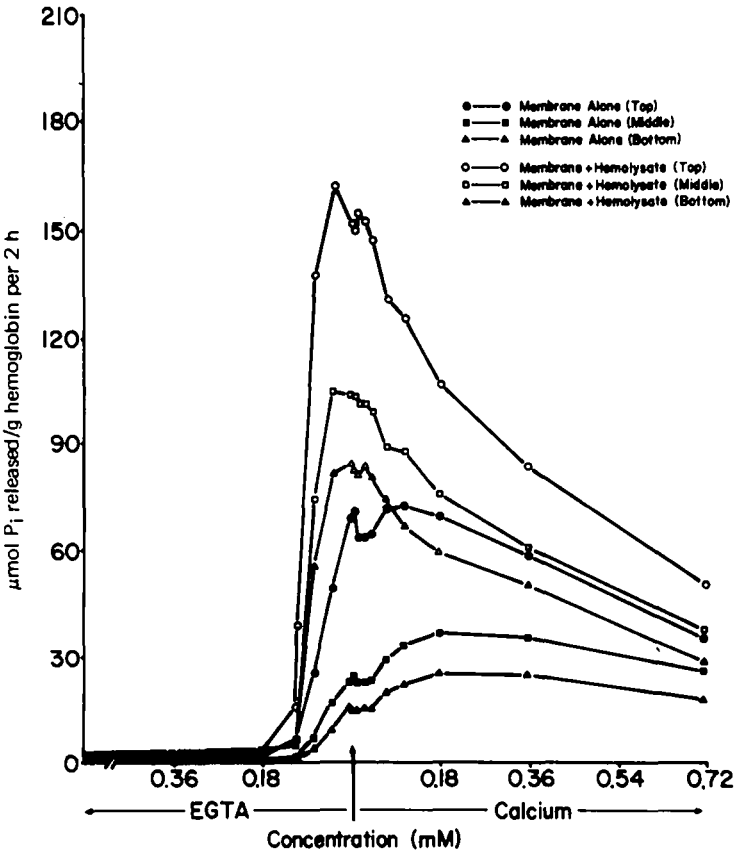

Fig. 4. (Ca^{2+} + Mg^{2+})-ATPase activities of isolated membranes from different density red cells at various concentrations of calcium in the presence or absence of hemolysate. Cells were separated into various density (age) cohorts. The membranes were isolated and (Ca^{2+} + Mg^{2+})-ATPase activities were measured in the absence (●——●, ▲——▲, ■——■) or presence (○——○, △——△, □——□) of their respective hemolysates.

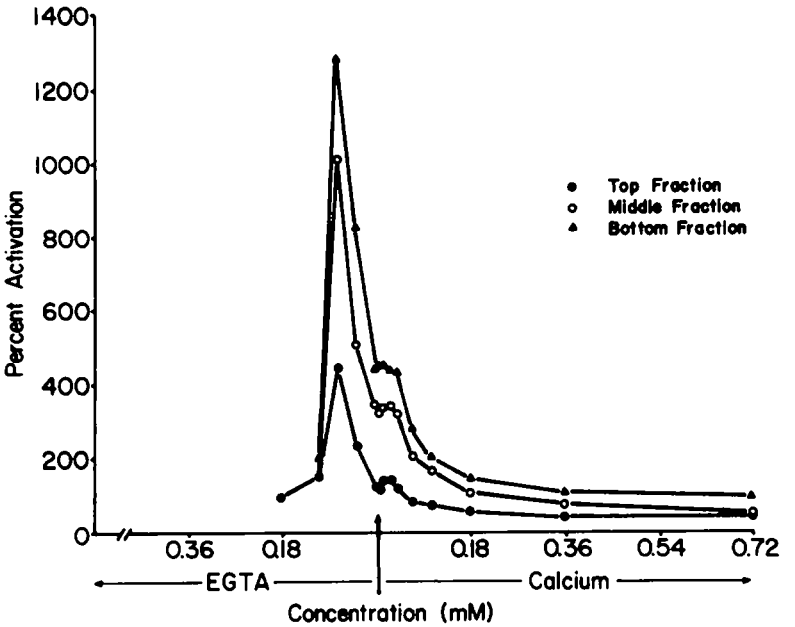

Fig. 5. Percent stimulation of $(Ca^{2+} + Mg^{2+})$-ATPase activities of isolated membranes by the activator protein calculated from Fig. 4.

However, exact quantitative assessment of stimulation is difficult to make since the extent to which the cytoplasmic activator is bound to various hypotonically prepared membranes is unknown. In any case, the data presented in Fig. 6 depict that a maximal recovery of $(Ca^{2+} + Mg^{2+})$-ATPase in reconstituted system containing the membrane plus the activator from all density fractions failed to reach the levels of enzyme activity seen in saponin-lysed

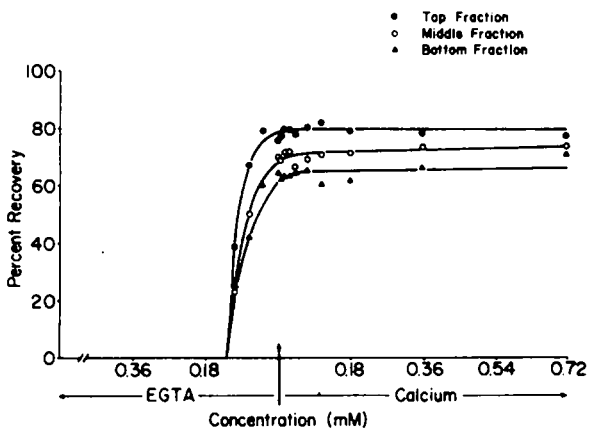

Fig. 6. Recovery of $(Ca^{2+} + Mg^{2+})$-ATPase activities in reconstituted systems containing membrane and hemolysate from various density-separated red cells. $(Ca^{2+} + Mg^{2+})$-ATPase activities in various density red cell membranes and their respective hemolysates shown in Fig. 4 were taken in computing percent recoveries of ATPase activities from their respective lysed red cell activities shown in Fig. 1.

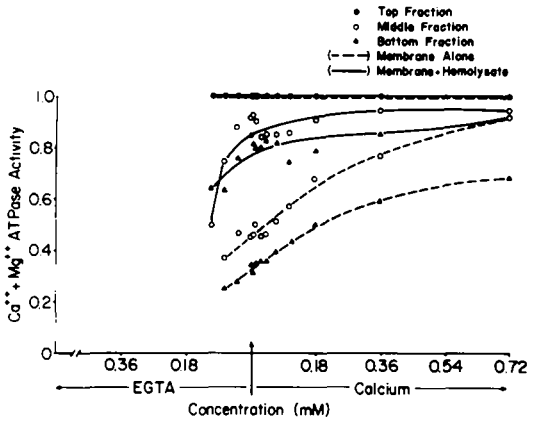

Fig. 7. Comparison of $(Ca^{2+} + Mg^{2+})$-ATPase activities in membranes and membranes plus hemolysates from cells of various densities. Activities in membranes alone (— — —) and membranes plus the hemolysate of the middle and bottom fraction (——) were normalized relative to the corresponding activities in the young cell fraction at various concentrations of calcium shown in Fig. 4.

cells, a result similar to that presented in the preceding paper [1]. Furthermore, the recovery of $(Ca^{2+} + Mg^{2+})$-ATPase activity was substantially small in low calcium regions even in the presence of hemolysate. However, the relative recovery of $(Ca^{2+} + Mg^{2+})$-ATPase activity either in reconstituted systems or in membranes alone was still higher in young cell membranes as compared to membranes isolated from old cells (Fig. 7). Figs. 4—7, show that even though the percent activation of $(Ca^{2+} + Mg^{2+})$-ATPase was smaller in light cell (young) membranes, the absolute magnitudes of the enzyme activities were still higher than the dense (old) cell membranes. Moreover, in addition to a higher ATPase activity at low Ca^{2+} concentrations in young cells, it seems likely that a greater retention of a soluble cytoplasmic activator on membranes from young red cells than from old red cells takes place when isolated under similar conditions.

Discussion

It has been found that old red cells have a higher calcium content than young cells. Elevated cell calcium is implicated in causing a number of deleterious effects on red cells [9]. Studies conducted on the total population of red cells indicated that the low levels of calcium [9] in human red cells is maintained through an active extrusion of calcium from cells mediated by a membrane-bound $(Ca^{2+} + Mg^{2+})$-ATPase [10—12]. To delineate, the underlying mechanism by which older cells accumulate more $(Ca^{2+} + Mg^{2+})$-ATPase of red cells was investigated [3,13]. It was noted that the low-density (young) cells and their membranes unexpectedly showed lower $(Ca^{2+} + Mg^{2+})$-ATPase activity than the higher-density (older) cells in earlier studies [12,13]. Furthermore, $(Ca^{2+} + Mg^{2+})$-ATPase activity of isolated membranes from young cells was not stimulated by the cytoplasmic activator protein [13]. In all these studies, the freeze-thaw technique was used to expose $(Ca^{2+} + Mg^{2+})$-ATPase activity. Recently, Hanahan and Ekholm [14] explored other methods of exposing ATPase activities of human red cells and they found that saponin

lysis was the preferred technique for this purpose [14]. Using this technique, these authors found much higher levels of $(Ca^{2+} + Mg^{2+})$-ATPase activity in low-density (young) cells than the other density classes and that there was a progressive decline in the level of this activity with age. Our findings confirm and corroborate their observations [14]. However, in our previous studies [3,13] including studies of Hanahan and Ekholm [14], only one set of calcium concentrations was used to measure the levels of $(Ca^{2+} + Mg^{2+})$-ATPase activities.

As shown in the companion paper [1], Ca^{2+} concentrations influence greatly the different states of $(Ca^{2+} + Mg^{2+})$-ATPase and their activation by the cytoplasmic activator. Accordingly, we carried out studies to investigate the effect of calcium and cytoplasmic activator on membrane $(Ca^{2+} + Mg^{2+})$-ATPase in aging red cells.

We found, in general, that the absolute calcium-dependence and various forms of calcium-dependent ATPase activities seem to exist in the membranes isolated from all density-separated red cells. Similarly, a low calcium concentration requirement for both the optimum activation of membrane $(Ca^{2+} + Mg^{2+})$-ATPase by hemolysate and the optimum expression of $(Ca^{2+} + Mg^{2+})$-ATPase activities in red cells is a common feature of all density-separated red cells. However, the levels of $(Ca^{2+} + Mg^{2+})$-ATPase activities obtained at various concentrations of calcium decreased as the density of the cell was increased. Of particular interest is the finding that low calcium concentration stimulated ATPase activities and a stimulation of isolated membrane $(Ca^{2+} + Mg^{2+})$-ATPase activity produced by the soluble cytoplasmic activator undergo opposite changes during cell aging. The loss of $(Ca^{2+} + Mg^{2+})$-ATPase stimulation in low concentrations of calcium could possibly occur due to the loss of a form of $(Ca^{2+} + Mg^{2+})$-ATPase activity activated by the activator or to the change in its affinity towards the activator or both. The data obtained on the activation of $(Ca^{2+} + Mg^{2+})$-ATPase by the activator indicate that the percentage activation in old cell membranes is greater than the young cell membranes, while the absolute activity of young cell membranes in the presence of hemolysate is higher than the old cell membranes. Similarly, red cells also show higher activity in young cells than in old cells.

In conclusion, the data presented in this communication suggest that: (1) there is an overall decrease in calcium-dependent ATPase activities during cell aging; (2) the isolated membranes from cells of all age cohorts have a similar requirement of a low calcium concentration in order to produce a maximum stimulation by a soluble cytoplasmic activator; and (3) there is more selective loss of low calcium-stimulated ATPase activity during red cell aging. These changes can conceivably be brought about both by the loss of low calcium-stimulated ATPase activity and by the change in its affinity toward the soluble cytoplasmic activator or possibly towards the inhibitory protein of $(Ca^{2+} + Mg^{2+})$-ATPase [15].

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