Biochimica et Biophysica Acta, 419 (1976) 180–186

BBA 77159

OBSERVATIONS ON THE $(Ca^{2+}+Mg^{2+})$-ATPase ACTIVATOR FOUND IN VARIOUS MAMMALIAN ERYTHROCYTES

MADAN G. LUTHRA[a], GEORGE R. HILDENBRANDT[a], H. D. KIM[b] and D. J. HANAHAN[a]

[a]*Department of Biochemistry and* [b]*Department of Physiology, College of Medicine, University of Arizona, Tucson, Ariz. 85724 (U.S.A.)*

(Received June 24th, 1975)

SUMMARY

1. A soluble activator of membrane $(Ca^{2+}+Mg^{2+})$-ATPase is present in hemolysates of the newborn calf and cow, the newborn and adult pig as well as human erythrocytes.

2. The activator is also found in reticulocytes of the adult pig.

3. The activator obtained from any of the above species is capable of stimulating the membrane $(Ca^{2+}+Mg^{2+})$-ATPases of the other species, regardless of the age of the animals.

4. The results obtained from density fractionation of human erythrocytes revealed that the soluble factor has little stimulatory effect on membranes of young erythrocytes from which it is derived but caused a marked stimulation on $(Ca^{2+}+Mg^{2+})$-ATPase activity of the intermediate aged and old erythrocyte membranes.

5. The above observations support the following conclusions: (a) the extremely low levels of $(Ca^{2+}+Mg^{2+})$-ATPase in cow erythrocytes is not due to the lack of a $(Ca^{2+}+Mg^{2+})$-ATPase activator; (b) the distribution of $(Ca^{2+}+Mg^{2+})$-ATPase activator is not species specific and the differences in the level of membrane $(Ca^{2+}+Mg^{2+})$-ATPase activity in various species of cells is an inherent property of that particular membrane; (c) the $(Ca^{2+}+Mg^{2+})$-ATPase activator is present at least from the time of reticulocyte formation and remain during the life span of the erythrocyte.

INTRODUCTION

In the preceding paper, it was shown that a soluble activator of the $(Ca^{2+}+Mg^{2+})$-stimulated ATPase can be recovered from hemolysate of human erythrocytes, a result in agreement with the finding of Bond and Clough [1]

However, the physiological functions of this activator are poorly understood and its potential presence in other species is not established. The elucidation of the functional role of this activator could conceivably explain, for example, the well established observation on the postnatal loss of $(Ca^{2+}+Mg^{2+})$-ATPase activity in

calf erythrocytes as this animal ages [2] and the different levels of $(Ca^{2+}+Mg^{2+})$-ATPase found among various species.

The results obtained in the present communication show that the activator is present in several different mammalian species but is not species specific. Furthermore, the differences in membrane $(Ca^{2+}+Mg^{2+})$-ATPase observed in various mammalian erythrocytes could not be attributed to the activator alone but rather due to the inherent property of the particular membrane.

EXPERIMENTAL

Methods

Erythrocyte isolation. Blood samples from various mammalian species were collected in heparinized tubes and the plasma and buffy coat were removed after centrifugation at $1900 \times g$ for 15 min at 4 °C. In experiments where further density separation was required, packed cells were resuspended in plasma and the various density (age distribution) erythrocytes were separated by centrifugation using the method described by Murphy [3]. The cells were then washed three times with 0.172 M Tris · HCl buffer pH 7.6 and frozen-thawed ($\times 3$) prior to ATPase assay.

Reticulocyte isolation. Reticulocytosis in adult pig was induced by phenylhydrazine administration [4] and blood was collected at the highest level of reticulocyte count and further fractionation on basis of reticulocyte density (age distribution) was achieved by a method similar to that recently developed by Murphy [3] for human erythrocytes. Packed reticulocytes were then washed with 0.172 M Tris · HCl buffer pH 7.6.

Preparation of hemolysate and membranes. Washed erythrocytes and reticulocytes were hemolyzed at pH 7.6 as described in the preceding paper. In the case of cow erythrocyte hemolysis, 5 mM $MgCl_2$ was added to stop the fragmentation of membranes [5]. Supernatants and pellets were isolated from various hemolysates by centrifugation at $27\,000 \times g$ for 30 min at 4 °C. The supernatants (membrane free hemolysate) were used as source of $(Ca^{2+}+Mg^{2+})$-ATPase activator. The pellets were further washed ($\times 3$) in their respective medium at the same osmolarity to recover the membranes.

$(Ca^{2+}+Mg^{2+})$-ATPase assay. ATPase assays were carried out by methods described in the preceding paper [6]. Unless otherwise stated, activity was expressed as μmol of P_i released by one ml of packed cell equivalent membrane. The value of the latter was obtained by recording the hematocrit of starting erythrocyte suspension.

RESULTS AND DISCUSSION

In confirmation of previous observations [2] the present data show that membranes prepared by hypotonic lysis of cow erythrocytes express very low levels of $(Ca^{2+}+Mg^{2+})$-ATPase activity as compared to calf erythrocyte membranes. It was also found that the membrane-free hemolysate obtained during hypotonic lysis of cow erythrocytes was capable of stimulating the $(Ca^{2+}+Mg^{2+})$-ATPase of the membranes obtained from cow, calf, pig and human erythrocytes (Table I). Conversely, the membrane free hemolysate obtained from calf, pig and human erythrocytes was capable of stimulating cow erythrocyte membrane $(Ca^{2+}+Mg^{2+})$-ATPase (Table I). However,

TABLE I

EFFECT OF MEMBRANE-FREE HEMOLYSATE FROM VARIOUS MAMMALIAN ERYTHROCYTES ON MEMBRANE (Ca^{2+} + Mg^{2+}) ATPase

Membranes and hemolysates were prepared as described in Methods. Membrane (Ca^{2+} + Mg^{2+})-ATPase was assayed in the presence of increasing volume of membrane-free hemolysate in a total assay volume of 0.75 ml at 37° C. 1 ml of membrane free hemolysate from each species represents 33 µl of their erythrocytes.

Reconstitution conditions		(Ca^{2+} + Mg^{2+}) ATPase activity (µmol P_i/ml packed cells per h) Hemolysate added (µl)		
Erythrocyte preparation	Hemolysate (source added)	0	25	50
A. Cow				
1. Intact cells	None	2.82	—	—
2. Membranes	None	0.74	—	—
3. Membranes	Pig	—	1.5	1.4
4. Membranes	Cow	—	1.1	1.1
5. Membranes	Calf	—	1.8	1.4
6. Membranes	Human	—	1.0	1.4
B. Calf				
1. Intact	None	31.3	—	—
2. Membranes	None	5.5	—	—
3. Membranes	Pig	—	13.3	14.9
4. Membranes	Cow	—	13.2	14.9
5. Membranes	Calf	—	12.5	14.1
6. Membranes	Human		12.9	13.9
C. Human				
1. Intact cells	None	13.4	—	—
2. Membranes	None	5.1	—	—
3. Membranes	Pig	—	8.6	9.9
4. Membranes	Cow	—	8.9	9.8
5. Membranes	Calf	—	9.0	9.9
6. Membranes	Human	—	9.1	9.9
D. Pig				
1. Intact	None	32.2	—	—
2. Membranes	None	7.7	—	—
3. Membranes	Pig	—	18.1	21.3
4. Membranes	Cow	—	17.3	20.6
5. Membranes	Calf	—	17.4	20.9
6. Membranes	Human	—	17.6	20.1

the activity of (Ca^{2+}+Mg^{2+})-ATPase of cow erythrocyte membranes never reached the levels of calf erythrocyte membranes, even under conditions in which an excess amount of activator was used for reconstitution (Table I). These results suggest that extremely low levels of (Ca^{2+}+Mg^{2+})-ATPase activity in cow erythrocytes is attributable neither to a change in specificity nor to a lack of (Ca^{2+}+Mg^{2+})-ATPase activator. In view of the fact that the membrane-free hemolysate from any given erythrocyte could stimulate membrane (Ca^{2+}+Mg^{2+})-ATPase obtained from various mammalian erythrocytes (Table I), it seems reasonable to conclude that the distribution of (Ca^{2+}+Mg^{2+})-ATPase activator is not species specific. Data presented in this table also show that human erythrocytes express low levels of (Ca^{2+}+Mg^{2+})-ATPase

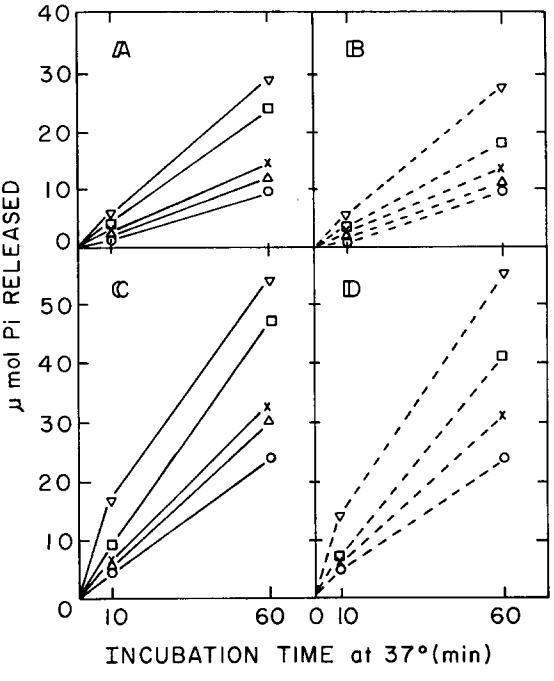

Fig. 1. Effect of membrane-free hemolysate from adult pig erythrocytes on newborn pig erythrocyte membrane ($Ca^{2+}+Mg^{2+}$)-ATPase and vice-versa. Erythrocyte membranes from adult (A, B) and new born (C, D) pigs were incubated in a total volume of 1.5 ml ATPase assay medium with increasing volume of membrane-free hemolysate (○–○, 0.1 ml; △–△, .03 ml; ×–×, .06 ml; □–□, 0.15 ml; ▽–▽, 0.4 ml) from new born (– – –) and adult (—) pig erythroytes.

activity as compared to calf and pig erythrocytes. However, the percentage recovered of ($Ca^{2+}+Mg^{2+}$)-ATPase in human erythrocyte membranes upon hypotonic lysis was higher than calf and pig erythrocyte membranes (Table I). These observations can perhaps be interpreted as an inherent difference in ($Ca^{2+}+Mg^{2+}$)-ATPase activity in erythrocyte membranes obtained from various mammalian species (e.g. pig, human and calf).

Fig. 1 shows that newborn pig erythrocyte membranes exhibited higher ($Ca^{2+}+Mg^{2+}$)-ATPase activity as compared to the adult pig erythrocyte membranes. The decrease in ($Ca^{2+}+Mg^{2+}$)-ATPase activity from newborn to adult pig is similar to that obtained in the case of bovine erythrocyte membranes but the magnitude of difference in activities of newborn and adult pig is smaller than in the case of calf and cow (Table I and Fig. 1). Furthermore, the variations seen in ($Ca^{2+}+Mg^{2+}$)-ATPase activity of newborn and adult pig were not caused by the lack or change in the specificity of ($Ca^{2+}+Mg^{2+}$)-ATPase activator, since the membrane-free hemolysate from adult pig erythrocyte was capable of stimulating the newborn pig erythrocyte membrane ($Ca^{2+}+Mg^{2+}$)-ATPase and vice versa (Fig. 1). In view of the comparable increase in ($Ca^{2+}+Mg^{2+}$)-ATPase activity by activation between the newborn and adult pig erythrocyte membrane (Fig. 1), it seemed unlikely that an excessive retention of ($Ca^{2+}+Mg^{2+}$)-ATPase activator on membranes had occurred during membrane preparation. These results once again supported the conclusion that the

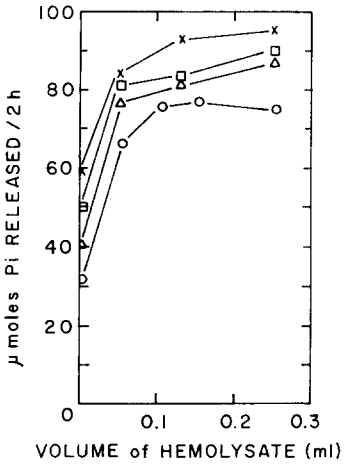

Fig. 2. Effect of membrane-free reticulocyte hemolysate on membrane $(Ca^{2+}+Mg^{2+})$-ATPase. After centrifugation of an 85 % suspension of reticulocytes or erythrocytes at 15 000 rev./min for 30 min at 30 °C in a Sorvall centrifuge using SW34 rotor, 10 % of top; low density (×–×), 10 % bottom; high density (△–△) and unfractionated (□–□) reticulocytes and erythrocytes (○–○) were washed and hemoglobin-free membranes were prepared as described in Methods. Membranes were reacted with ATP for 2 h at 37 °C in a total volume of 0.75 ml ATPase assay medium with increasing volumes of their own membrane-free hemolysate.

difference in membrane $(Ca^{2+}+Mg^{2+})$-ATPase activator of newborn and adult pig erythrocyte is an inherent variation in $(Ca^{2+}+Mg^{2+})$-ATPase of adult and newborn pig erythrocyte membranes.

In addition to the postnatal decrease in $(Ca^{2+}+Mg^{2+})$-stimulated ATPase level, the enzymatic activity also undergoes a significant reduction in the course of reticulocyte maturation in the pig [4]. When the crude membrane-free reticulocyte hemolysates were tested for activation of reticulocyte membrane $(Ca^{2+}+Mg^{2+})$-ATPase, a considerable stimulation was displayed irrespective of reticulocyte age (Fig. 2). Thus, it would appear that the $(Ca^{2+}+Mg^{2+})$-activator is synthesized prior to reticulocyte formation and persists throughout the life span of the erythrocyte.

TABLE II

$(Ca^{2+}+Mg^{2+})$ ATPase ACTIVITIES OF AGE-SEPARATED ERYTHROCYTES AND THEIR HEMOGLOBIN-FREE MEMBRANES

See text for details of separation

Erythrocyte fraction	μmol P_i released/g hemoglobin at 37 °C for 60 min	
	Erythrocytes	Membranes*
Top 10 %	54	9.0
Middle 80 %	90	20.0
Bottom 10 %	72	18.0

* This activity was related to the hemoglobin (g) present in intact cells from which the membranes were prepared.

Using the facile method of Murphy [3] for density fractionation of human erythrocytes, it was found that low density (young) erythrocytes express low $(Ca^{2+}+Mg^{2+})$-ATPase activity as compared to middle and high density ("old") cells. A typical result is shown in Table II. It should be noted that a similar pattern of activities was observed in hypotonically lyzed membranes but with an overall reduction in $(Ca^{2+}+Mg^{2+})$-ATPase activity due to the removal of $(Ca^{2+}+Mg^{2+})$-ATPase activator (Table II). Further it was noted that the membrane free hemolysate from low density (young) erythrocytes was not capable of stimulating its own erythrocyte membrane but activated the membrane $(Ca^{2+}+Mg^{2+})$-ATPase from middle and high density (old) erythrocytes (Fig. 3). Fig. 3 also shows that membrane free hemolysate from middle and high density (old) erythrocytes can stimulate their own mem-

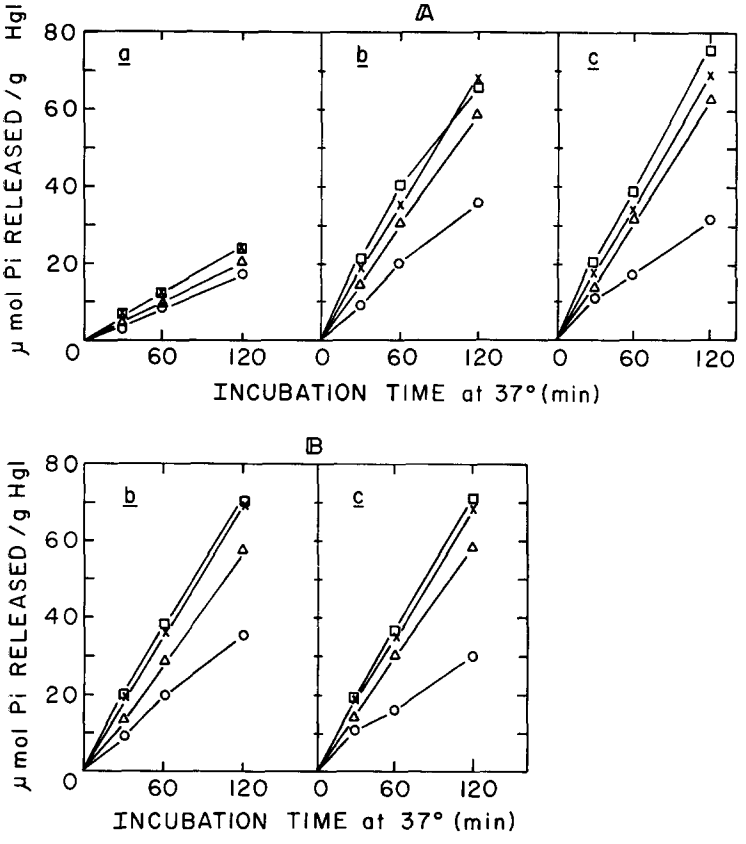

Fig. 3. (A). Effect of membrane-free hemolysate from low density (young) human erythrocytes on membrane $(Ca^{2+}+Mg^{2+})$-ATPase from middle and high density (old) erythrocytes. Hemoglobin-free membranes from (a) top 10% ;(b) bottom 10%; and (c) middle 80% fractions of human erythrocytes were prepared as described in Methods. Membranes were reacted with ATP for various time intervals in a total assay volume of 0.75 ml with increasing volumes of membrane-free hemolysate (○–○, 0 ml; △–△, .05 ml, ×–×, 0.1 ml and □–□, 0.2 ml) from top 10% low density erythrocytes. (B) Effect of membrane-free hemolysate from middle and high density erythrocytes on their own membrane $(Ca^{2+}+Mg^{2+})$-ATPase. Membrane-free hemolysates were obtained from middle and high density erythrocytes were reacted with their own membranes under conditions given in Fig. 3(A).

brane $(Ca^{2+}+Mg^{2+})$-ATPase activities. These results suggest that the $(Ca^{2+}+Mg^{2+})$-ATPase activator remains functional throughout the life span of the erythrocyte. On the other hand it is puzzling that the $(Ca^{2+}+Mg^{2+})$-ATPase activity of young erythrocyte membranes is not stimulated by the activator from the same cells.

The results on the stimulation of $(Ca^{2+}+Mg^{2+})$-ATPase reported here are reminiscent of similar observations made on the muscle contractile protein, myosin. In the latter case the dissociation of the so-called light and heavy chains caused a drastic reduction of Ca^{2+}-ATPase activity and subsequent recombination of these two chains under certain conditions resulted in a partial recovery of the Ca^{2+}-ATPase activity [7, 8]. Of some considerable significance, claims have been made on the identification and isolation of (muscle-like) contractile proteins in erythrocyte membranes [9, 10]. Thus, it is provocative to speculate that the contractility of the erythrocyte may bear a close relationship to the $(Ca^{2+}+Mg^{2+})$-ATPase activity and its "activator".

ACKNOWLEDGEMENTS

This investigation was supported by grant No. AM-17723, United States Public Health Service, a General Research Support grant to the University of Arizona College of Medicine, and a grant from the Arizona Heart Association, all to H. D. K. and a grant No. HL 14521-04, United States Public Health Service, to D. J. H.

REFERENCES

1 Bond, G. H. and Clough, D. L. (1973) Biochim. Biophys. Acta 323, 592–599
2 Hanahan, D. J. (1973) Biochim. Biophys. Acta 300, 319–340
3 Murphy, J. R. (1973) J. Lab. Clin. Med. 82, 334–341
4 Luthra, M. G., Hildenbrandt, G. R. and Kim, H. D. (1975) Fed. Proc. 34 (3) 498
5 Burger, S. P., Fujii, T. and Hanahan, D. J. (1968) Biochemistry 7, 3682–3700
6 Luthra, M. G., Hildenbrandt, G. R. and Hanahan, D. J. (1976) Biochim. Biophys. Acta 419, 164–179
7 Kim, H. D. and Mommaerts W. F. H. M. (1971) Biochim. Biophys. Acta 245, 230–234
8 Dreizen, P. and Gersham, L. C. (1970) Biochemistry 9, 1688–1693
9 Rosenthal, A. S., Kregenow, F. M. and Moses, H. L. (1970) Biochim. Biophys. Acta 196, 254–262
10 Stewart, G. J., Schrager, S. M. and Galvin, P. D. (1973) in Erythrocytes, Thrombocytes and Leukocytes (Gerlach, E., Moser, K., Deutsch, E., Wilmanns, W. eds), pp. 53–57, Thieme, Stuttgart