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ENHANCED ACTIVATION OF HUMAN ERYTHROCYTE Ca^{2+}-ATPase BY CALMODULIN AFTER STORAGE OR BRIEF EXPOSURE TO DISULFITE

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Summary

Ca^{2+}-ATPase of human erythrocyte membranes which are prepared from freshly drawn human blood can be activated by the calmodulin present in the hemolysate to 1.5-times the basal level. However, when the membranes are prepared from blood stored for 5—14 days the activation by calmodulin reaches 2.5-times the basal level. An enhanced reactivity to calmodulin of similar magnitude was produced by brief exposure of fresh erythrocytes to 25 mM $Na_2S_2O_5$ prior to isolation of the membranes. Reincubation of the activated cells in a disulfite-free medium restored the membrane-bound Ca^{2+}-ATPase to a state of normal reactivity to calmodulin. It is hypothesized that these results are related to the level of cytoplasmic Ca^{2+} which is partly controlled by complex formation with 2,3-diphosphoglycerate, the concentration of which is diminished when its specific phosphatase is activated by $Na_2S_2O_5$.

Introduction

The Ca^{2+}-ATPase of human erythrocyte membranes has been quantitated in several disease states, including psychiatric depression (Hesketh et al. [1], Choi et al. [2]). In all studies reported to date the enzyme activity was determined on the nearly white membranes which are prepared, with minor modifications of the method of Dodge et al. [3], by hypotonic lysis and, in some instances, extensive washing with EDTA. Recently, Farrance and Vincenzi

Abbreviation: EGTA, ethylene glycol bis-(β-aminoethyl ether)-*N,N'*-tetraacetic acid.

[4] described the preparation of membranes employing isosmotic lysis as a first step, and showed that such preparations contained a more active Ca^{2+}-ATPase than membranes prepared by hypotonic lysis. They and others also demonstrated that the increased activity due to a cytoplasmic protein which could also augment the activity of membranes prepared by hypotonic lysis, and several groups (Farrance and Vincenzi [5], Jarrett and Penniston [6], Luthra et al. [7], Scharff and Foder [8], Hinds et al. [9], Sarkadi et al. [10]) described further properties of this substance, termed calmodulin, which is now known to be ubiquitous in animal tissues.

The existence of calmodulin within the erythrocyte offers an opportunity to reassess some previously published data on human erythrocyte Ca^{2+}-ATPase. In the process of defining the exact conditions under which we would proceed we noted that Farrance and Vincenzi [4] had isolated membranes from outdated blood samples obtained from a blood bank. We therefore sought to determine whether the age of the blood sample had any effect on the analytical results. After observing that the calmodulin-activated Ca^{2+}-ATPase increased with the length of time that the intact cells were stored prior to membrane preparation, we began to explore the possibility of accelerating this process by brief pre-incubation of erythrocytes under varying conditions. This paper compares the results obtained by brief preincubation with sodium disulfite to those resulting from storage of whole erythrocytes.

Materials and Methods

Heparinized blood was collected in acid/citrate/dextrose solution from volunteers and patients attending the Lithium Clinic at the New York State Psychiatric Institute; the additional tube of blood needed for this study was obtained with the informed consent of the patient.

Membranes were prepared in the hypotonic I-20 medium of Farrance and Vincenzi [4] and stored at 4°C until analysis the following day. Ca^{2+}-ATPase activity was assayed in 1 ml final volume of a medium containing 3 mM ATP, 80 mM NaCl, 15 mM KCl, 3 mM $MgCl_2$, 18 mM histidine/18 mM imidazole buffer at pH 7.1, 0.1 mM ouabain, 0.1 mM EGTA, and 0.3—0.8 mg membrane protein. Hemolysate (0.1 ml) and $CaCl_2$ (0.2 mM final concentration) were added to selected tubes. All assays were done in duplicate. Except where noted, each membrane preparation was incubated with its own hemolysate.

Inorganic phosphate was measured by the method of Fiske and SubbaRow [11] and protein by the procedure of Lowry et al. [12] with bovine serum albumin as standard. The results were calculated as μmol inorganic phosphate released/mg protein per h. Basal Ca^{2+}-ATPase was calculated as the increment due to Ca alone and activated Ca^{2+}-ATPase as the increment due to Ca plus hemolysate. The quantity of hemolysate used gave maximal activity in all cases.

Preincubation was carried out as follows. Plasma and buffy layer was removed from centrifuged blood and the cells were then washed 2-times in a modified Ringer-glucose medium of the following composition in mequiv/l: Na^+, 145; K^+, 3.9; Mg^{2+}, 2.5; Ca^{2+}, 2.8; PO_4^{2-}, 2.3; HCO_3^-, 18.2; Cl^-, 130. This solution was saturated with 95% O_2/5% CO_2 to obtain a final pH of 7.4.

Glucose was added to a final concentration of 200 mg/100 ml. The washed cells were then suspended in the same medium at a hematocrit of about 35% and a solution of $Na_2S_2O_5$ was added to a final concentration of 25 mM. After 30 min incubation at 37°C in a shaker-incubator the suspension was centrifuged at 4°C and the cells were washed twice with 0.9% NaCl. Membranes were then prepared from the packed cells.

Results

Before investigating the parameters of interest it was necessary to clarify a technical point in the ATPase assay. Many workers have employed the technique of freeze-thawing to 'expose' the enzyme to the reagents; Scharff [13] has apparently accomplished the same result by subjecting the membrane to a shear stress in a continuous flow centrifuge. However, Farrance and Vincenzi [4] reported that when membranes which had been prepared by hemolysis in isosmotic buffer were subjected to freeze-thawing a marked decrease in the Ca^{2+}-ATPase activity occurred. Subsequently, Gopinath and Vincenzi [14] reported that calmodulin also activated the Ca^{2+}-ATPase of membranes prepared by hypotonic hemolysis; they did not state whether or not this preparation had been subjected to freeze-thawing prior to assay. To examine this question we prepared membranes as described above and either analyzed for Ca^{2+}-ATPase directly or subjected them either to 30 s mixing with a vortex mixer (Labline Instruments, 'fast' setting) or to freeze-thawing twice. The data of Table I show that vortexing did not significantly increase the observed activity, while freeze-thawing approximately doubled the values obtained for activated Ca^{2+}-ATPase. Consequently, all determinations described below were done after freeze-thawing.

When membranes were prepared according to the hypotonic hemolysis procedure we found that the extent of activation of membrane-bound Ca^{2+}-

TABLE I

EFFECT OF FREEZE-THAWING ON Ca^{2+}-ATPase

Membranes from individual blood samples were prepared as described in the text and divided into the three portions shown in columns 3—5. The aged samples were stored 4—6 days.

Sample	*N*	Ca^{2+}-ATPase (μM P_i/mg protein per h at 37°C)		
		Assayed directly	Vortexed	Freeze-thawed
Fresh blood	10			
Basal		0.543 ± 0.191	0.568 ± 0.220	0.888 ± 0.189 (a)
Activated		0.541 ± 0.187	0.568 ± 0.225 (b)	1.123 ± 0.274 (c)
Aged blood	5			
Basal		0.751 ± 0.068	0.686 ± 0.102	0.815 ± 0.145
Activated		1.09 ± 0.344	1.11 ± 0.381	1.71 ± 0.59 (d)

Paired *t*-test values between column 3 and column 4 or 5 were: (a) $t = 3.45$, $P = 0.006$; (b) $t = 2.85$, $P = 0.016$; (c) $t = 5.31$, $P = 0.003$; (d) $t = 3.66$, $P = 0.0096$. The values shown are ± S.D. The *t* and *P* values were calculated with the aid of the Applied Statistics Module of the Texas Instruments 59 Programmable Calculator, and refer to a two-tailed test.

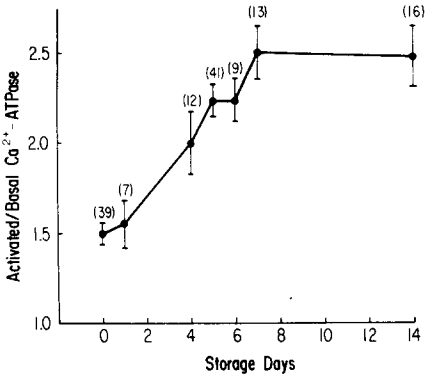

Fig. 1. Time-dependent increase in the ratio of activated to basal Ca^{2+}-ATPase. Membranes were prepared by hypotonic hemolysis after storage in the cold with added acid-citrate/dextrose for 0—14 days. Means ± S.E. are shown for the number of samples indicated above each point.

ATPase depended upon the length of time the cells were stored prior to membrane preparation. Maximum activity, with a mean value of 2.5 μmol P_i/mg protein per h, was obtained at about 7 days. The data of Fig. 1 show that membranes prepared from cells drawn the same day could only be activated to a mean of 150% of the basal level. In contrast, when blood stored in acid/citrate/dextrose at 4°C, was used to prepare membranes, the activated Ca^{2+}-ATPase ranged from 224 to 250% of the basal level for storage periods ranging from 5 to 14 days. With storage time there was also an increase in the basal Ca^{2+}-ATPase activity from 0.807 to 1.00 μmol P_i/mg protein per h. This change, which was significant at the $P = 10^{-5}$ level, was observed in 46 samples stored 4 or more days compared with membranes isolated without storage from 43 blood samples. These data for the entire subject population are reinforced by the data of Table II, which shows the increase in activated/basal Ca^{2+}-ATPase when individual blood samples were divided into two portions and

TABLE II

EFFECT OF ERYTHROCYTE STORAGE TIME ON THE RATIO OF ACTIVATED TO BASAL Ca^{2+}-ATPase

The differences in the means of the ratio of activated to basal Ca^{2+}-ATPase are shown in column 3 for the time interval shown in column 2, where the larger mean was always obtained at the second time point. The ratios were calculated from paired samples which were obtained by dividing the blood sample of each subject into two portions and storing in cold acid/citrate/dextrose solution. Membranes isolated from the 33 samples of fresh blood shown in the first three lines had a basal Ca^{2+}-ATPase of 0.795 ± 0.198 and an activated Ca^{2+}-ATPase of 1.17 ± 0.412 μmol P_i/mg protein per h at 37°C; the ratio of activated to basal was thus 1.47. Statistics were obtained as stated in the legend to Table I.

N	Comparison interval (days)	Mean difference	Paired *t*-test	*P*
5	0 to 1	0.18 ± 0.19	2.14	0.056
5	0 to 4	0.50 ± 0.49	2.30	0.046
23	0 to 5	0.76 ± 0.61	5.98	0.00001
5	5 to 7	0.24 ± 0.50	1.00	0.20
8	5 to 12	0.13 ± 0.38	0.92	0.24

stored for varying periods of time. A statistically significant paired t value was obtained when blood stored for 5 days was compared with fresh blood.

To determine whether these changes were dependent upon interaction of plasma with the stored erythrocytes, cells from individual subjects were divided into two portions. One portion was stored as above in acid-citrate/dextrose; the other was stored in Ringer solution plus acid-citrate/dextrose after removing the plasma. The mean ratio of activated/basal Ca ATPase from nine

TABLE III

EFFECT OF HEMOLYSATE SOURCE ON ACTIVATION OF Ca^{2+}-ATPase

The data were obtained from ten individual samples which were divided into two portions. Membranes were obtained on the same day from one portion and after 5 days storage in cold acid-citrate/dextrose solution from the other portion. The ratio of activated to basal Ca^{2+}-ATPase is shown in the first line. The t-tests are for equality between the adjacent means.

Membranes from:	Fresh blood		Aged blood	
Hemolysate from:	Fresh blood	Aged blood *	Aged blood **	Fresh blood ***
Mean ratio	1.697	1.644	2.45	2.329
S.D.	0.313	0.292	0.322	0.324
Paired t-test	1.838		3.803	
Mean difference	0.035		0.121	
S.D. of difference	0.060		0.101	
P	0.079		0.003	

* This hemolysate was obtained from one previous preparation of membranes obtained from stored blood of a different subject.

** From the hemolysates of the 5-day-old blood of each subject.

*** The hemolysate from the fresh blood of each subject was stored cold until the assay was done.

TABLE III A

ORIGINAL DATA

These are the original data from which Table III was constructed. The data are representative of all of the data in the other tables and Fig. 1. The values tabulated are enzyme activity, expressed as the increase in μmol P_i/mg protein per h. In the case of the basal activity, the actual value is the value obtained in the presence of added Ca^{2+}, minus the value obtained in the presence of EGTA. The activated value is similarly corrected for the blank. Each line is for a single subject.

Membranes from fresh blood			Membranes from aged blood		
Activated Ca^{2+}-ATPase: Hemolysate from		Basal	Activated Ca^{2+}-ATPase: Hemolysate from		Basal
Fresh blood	Aged blood		Fresh blood	Aged blood	
1.68	1.64	0.96	2.01	1.97	0.975
1.52	1.46	0.722	1.60	1.55	0.833
1.68	1.69	0.811	2.41	2.30	1.07
1.69	1.62	0.869	2.23	2.09	0.978
0.895	0.958	0.703	2.76	2.76	0.992
0.747	0.747	0.453	2.16	1.94	0.799
0.671	0.627	0.445	1.66	1.52	0.697
0.759	0.759	0.656	2.29	2.29	0.836
1.44	1.36	0.887	2.30	2.06	0.907
1.10	1.07	0.645	2.88	2.75	1.01

subjects was 2.28 ± 0.385 for samples stored in their own plasma and 2.29 ± 0.390 for the paired samples stored in Ringer solution. It is clear that activation by hemolysate occurred equally well in either medium.

To determine whether the storage-induced changes were due primarily either to alterations in the cytosol or the membrane, the experiment outlined in Table III was performed. It was found that membranes prepared from fresh blood gave the same ratio of activated to basal Ca^{2+}-ATPase whether they were incubated with their own (fresh) hemolysate or with hemolysate obtained from the stored blood of another subject. Thus the hemolysate from aged blood does not contain some special factor or, conversely, the hemolysate from fresh blood does not lose some special factor, which is responsible for the lesser activation of membranes from fresh blood. Similarly, membranes from aged blood showed the same degree of activation whether they were incubated with their own hemolysate or with hemolysate which had been obtained from fresh blood. Thus the storage-induced changes can be attributed to some time-dependent change in membrane-bound Ca^{2+}-ATPase.

The possibility that the time required for increasing the enzyme response to a saturating dose of calmodulin could be shortened was investigated by preincubation of membranes prepared from fresh blood with $Na_2S_2O_5$. As shown in Table IV, preincubation in Ringer-glucose medium with 25 mM $Na_2S_2O_5$ for 30 min produced a significant increase in Ca^{2+}-ATPase activity in the presence of calmodulin. This effect of $Na_2S_2O_5$ could be reversed by reincubating for 15—60 min in a $Na_2S_2O_5$-free medium. $Na_2S_2O_5$ was also effective in incrementing the calmodulin-activated Ca^{2+}-ATPase of membranes prepared from aged cells, although the proportionate increase was lower than that with fresh cells (Table IV). The basal Ca^{2+}-ATPase of membranes prepared from fresh cells was also increased by preincubation with $Na_2S_2O_5$; in 14 samples the mean increased from 0.734 to 0.966 μmol P_i/mg protein per h (t = 2.55 and P = 0.022).

TABLE IV

EFFECT OF DISULFITE ON ACTIVATED Ca^{2+}-ATPase

I. Each of 14 individual samples of fresh blood, collected in acid/citrate/dextrose solution, were divided into four portions and membranes were prepared either (a) directly: (b) after 30 min incubation with 25 mM $Na_2S_2O_5$: (c) after a subsequent reincubation in $Na_2S_2O_5$-free medium for 15—60 min; or (d) after a control incubation in $Na_2S_2O_5$-free medium for 30—120 min. The means and statistical evaluation are shown on line 1 for the sample pair (a) vs. (b); on line 2 for (b) vs. (c); on line 3 for (a) vs. (d); and on line 4 for (a) vs. (c). II. Same as for I, for membranes prepared from six individual samples aged for varying periods of time in cold acid/citrate/dextrose solution.

Paired sample groups	$\bar{X}_1$	$\bar{X}_2$	Mean difference ± S.D.	t	P
I. Fresh blood					
Control vs. $Na_2S_2O_5$ incubation	1.03	1.73	0.699 ± 0.663	3.95	0.0016
$Na_2S_2O_5$ incubation vs. reincubation	1.73	1.18	0.539 ± 0.650	3.10	0.0081
Control vs. control incubation	1.03	1.21	0.172 ± 0.594	1.09	0.21
Control vs. $Na_2S_2O_5$ reincubation	1.03	1.18	0.250 ± 0.816	0.78	0.28
II. Blood aged in acid/citrate/dextrose for 7.3 ± 2.9 days					
Control vs. $Na_2S_2O_5$ incubation	2.15	2.68	0.53	2.48	0.034

Discussion

The foregoing results demonstrate that 'basal' membranes prepared after storage or preincubation of whole cells with $Na_2S_2O_5$ have an enhanced reactivity to calmodulin *, compared with 'basal' membranes prepared from fresh cells. This response must be due to an organizational change in the membrane which is preserved under conditions of hypotonic hemolysis; the organizational change is directed more toward the activated than the basal state of Ca^{2+}-ATPase, since the increment in calmodulin-activated enzyme activity was proportionately greater than the increment in basal Ca^{2+}-ATPase.

Although most of these data were obtained from the blood of 60 patients undergoing treatment with Li_2CO_3 or other psychotropic medications, similar changes were observed with blood from nine medication-free normal controls. The ratio of activated-to-basal Ca^{2+}-ATPase from this control group was 1.48 ± 0.22 with membranes from fresh blood, and 2.41 ± 0.85 with membranes from aged blood; the differences were significant at $P = 0.006$. However, these limited data do not rule out the possibility that quantitative differences exist between the two groups.

The increased activity was shown to be due to changes occurring in the membrane of the intact erythrocyte. Two explanations are possible: (1) the aging process, as a result of structural rearrangements, may make the membrane more receptive to calmodulin; (2) freshly prepared membranes may contain variable amounts of a Ca^{2+}-ATPase inhibitor which slowly breaks down on storage. The occurrence of an inhibitor is consistent with the following: Au [15] has reported on the existence of an inhibitor in pig erythrocytes and Kobayashi et al. [16] have shown that some drugs can prevent calmodulin activation of human erythrocyte Ca^{2+}-ATPase. Larsen et al. [17] had demonstrated previously that a modulator-binding protein isolated from bovine brain [18] inhibited the activation of Ca^{2+}-ATPase by calmodulin.

If structural rearrangements, rather than an inhibitor, are responsible for the increased activation brought about both by aging and by preincubation with disulfite, it is useful to consider whether a common mechanism could be involved. The increased reactivity to calmodulin brought about by $Na_2S_2O_5$ could have resulted from the following sequence of events: (a) $Na_2S_2O_5$ activated diphosphoglycerate phosphatase, causing a decrease in its intracellular substrate, 2,3-diphosphoglycerate; (b) Ca^{2+} normally complexed to 2,3-diphosphoglycerate was released as Ca^{2+}; (c) the increased intracellular Ca^{2+} promoted the binding of calmodulin to Ca^{2+}-ATPase; (d) the increase in membrane-bound calmodulin either displaced an inhibitor or produced a primary rearrangment which then facilitated the post-hemolysis binding of increased amounts of calmodulin (cooperative binding).

Supporting data for the foregoing postulate is as follows: $Na_2S_2O_5$ has been reported to activate diphosphoglycerate phosphatase [19]; under conditions

* It is implied that the observed activation of Ca^{2+}-ATPase is due to the calmodulin in the hemolysate. To investigate this further, calmodulin was isolated from bovine brain by the procedure of Watterson et al. [23]. Activation of membranes was accomplished with 2.5 μg calmodulin added to 1.0 ml assay medium. The ratio of activated-to-basal Ca^{2+}-ATPase from membranes obtained from aged cells was 2.23 ± 0.633 ($N = 10$), and for membranes from fresh cells, 1.08 ± 0.364 ($N = 6$). Thus the increased activation of old cells can be observed with purified calmodulin, confirming that the results do not depend upon changes occurring in the activating factors of the hemolysate.

similar to those detailed above we have observed that erythrocyte 2,3-diphosphoglycerate is decreased to less than 50% of its initial value after 30 min incubation with 25 mM $Na_2S_2O_5$. Complexes of calcium with 2,3-diphosphoglycerate have been described and 2,3-diphosphoglycerate, along with ATP, is regarded as a major cytoplasmic buffer of ionic calcium [20]. The rapid loss of intracellular K^+ which occurs in the presence of $Na_2S_2O_5$ [21] is consistent with the operation of the well-described Ca^{2+}-gated K^+ channel in erythrocyte membranes [22], and thus implies an increase in intracellular Ca^{2+}; we have also observed that intra-erythrocyte K^+ is decreased to about 85% of its initial level after only 15 min incubation with 7.5 mM $Na_2S_2O_5$.

The foregoing speculation appears to us to be reasonable. If, as suggested, increased intracellular Ca^{2+} is the event which leads to a structural rearrangement of the membrane that persists even after hemolysis, then the effect of storage could be due to a slow leak of Ca^{2+} into the cells, and the similar selective reponse of the membrane to calmodulin activation after either storage or preincubation with $Na_2S_2O_5$ could have elevated cytoplasmic Ca^{2+} as a common factor. However, other mechanisms, including the possibility that disulfite acts directly on Ca^{2+}-ATPase or on its lipid environment, cannot be ruled out at this stage.

Finally, since the disulfite effect is reversible, it is not unlikely that in addition to the basal Ca^{2+}-ATPase state, the Ca^{2+} pump in erythrocytes in vivo can rapidly switch on and off between a normal calmodulin-activated state and an enhanced calmodulin-activated state in response to a challenge by elevated intracellular Ca^{2+}.

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