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CALCIUM EFFECTS ON HUMAN ERYTHROCYTE MEMBRANE PROTEINS

LLOYD E. KING, Jr.* and MARTIN MORRISON

Department of Biochemistry, St. Jude Children's Research Hospital, 332 North Lauderdale, Memphis, Tenn. 38101 and Veterans Administration Hospital, Departments of Medicine (Dermatology and Anatomy), The University of Tennessee Center for the Health Sciences, Memphis, Tenn. 38103 (U.S.A.)

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Summary

The effects of Ca^{2+} on human erythrocyte membrane proteins were examined by sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis. Ca^{2+} had several effects on normal human erythrocyte membrane proteins. It affected the binding of cytoplasmic proteins to the membrane, produced a non-reversible aggregation of several membrane proteins and activated apparent proteolysis of membrane proteins. The Ca^{2+} effect could be obtained with isolated, washed membranes when the erythrocyte cytoplasm was added. These studies indicate that the Ca^{2+}-induced membrane proteolysis and aggregation effects are not due simply to its presence at the time of hemolysis as previously suggested (Carraway, K.L., Triplett, R.B. and Anderson, D.R. (1975) Biochim. Biophys. Acta 379, 571—581), but are the result of more complex interactions between the erythrocyte membrane and cytoplasmic factors.

In the mammalian erythrocyte membrane, calcium has been noted to play an important role in the maintenance of red cell conformation [1, 2, 3], in the synthesis of phosphoinositides [4], in the aggregation of red cell membrane proteins and in the activation of protease activity [5, 6]. Carraway et al. have suggested that the latter phenomena, aggregation and proteolysis of membrane proteins, occur only if calcium is present during hemolysis. They suggest that reorganization of the membrane takes place during hemolysis and may be a major factor involved in these calcium-related phenomena. We have re-investigated the Ca^{2+} effect on erythrocyte membrane proteins and report that they can be produced using lysed cells or isolated, washed membranes. The

*Present address: Veterans Administration Hospital, Department of Medicine (Dermatology), Vanderbilt University, Nashville, Tennessee, U.S.A.

calcium-induced events are the result of interactions between the erythrocyte membranes and cytoplasmic factors.

Human erythrocyte membranes were prepared by the procedures of Dodge et al. [7] as modified below. Cells from normal human subjects were hemolyzed using one of the following solutions with or without added $CaCl_2$ (0.01—10 mM): distilled water; 5 mM phosphate buffer, pH 7.4; 10 mM Tris·HCl, pH 7.4, or GIMS buffer (10 mM glucose, 10 mM imidazole, 2 mM $MgCl_2$, 50 mM NaCl, pH 7.4). The supernatant, free of membranes and cells, was used as indicated in the different experiments.

To determine the conditions under which Ca^{2+} effects on the membrane can be observed, the Ca^{2+} concentration was varied from 0.01 to 10 mM before, during, or after hemolysis. The relationship of Ca^{2+}-mediated changes to membrane and cytoplasmic components were also studied. Membranes were isolated and washed. They were then incubated with various dilutions of the initial lysate with and without Ca^{2+}.

Polyacrylamide gel electrophoresis of erythrocyte membranes was per-

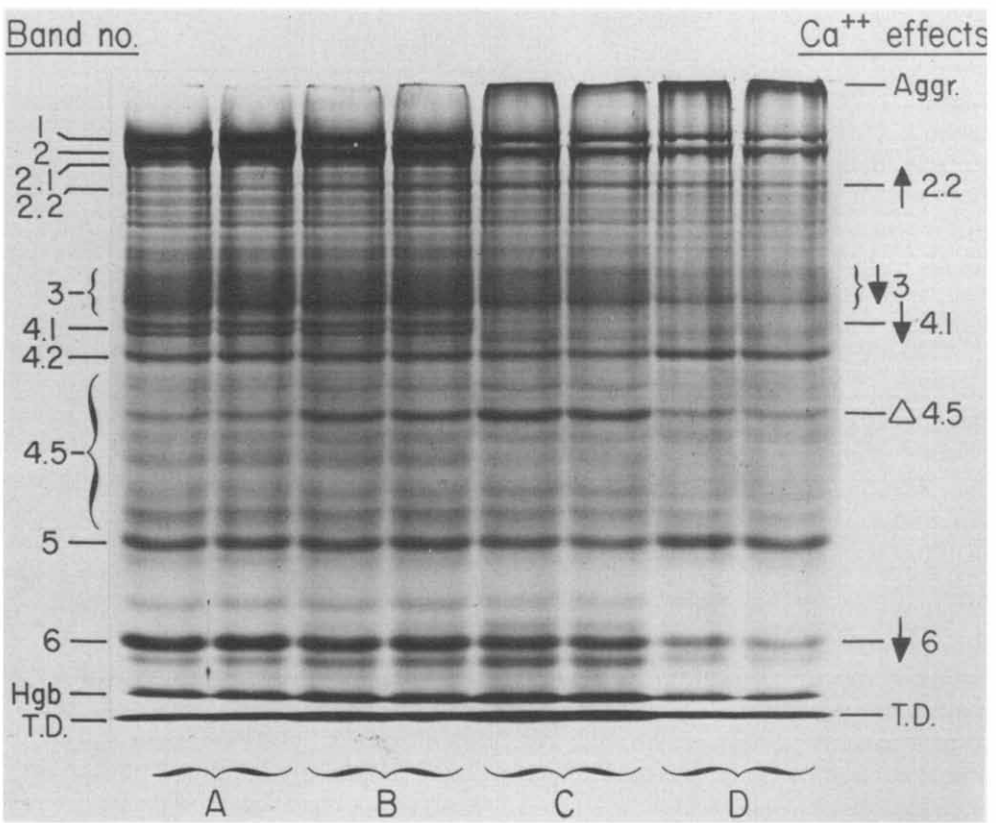

Fig. 1. The effect of varying calcium concentrations on erythrocyte membrane proteins. 1 ml of washed, packed erythrocytes were hemolyzed at 22°C by the addition of 10 ml of 11 mM Tris·HCl buffer, pH 7.4. A solution of 0.11 M calcium chloride dissolved in the 11 mM Tris buffer was added to this hemolysate in varying quantities to make the final concentration of the added calcium 0.1 mM (B), 1 mM (C), 10 mM (D) and 0 mM (A). The solutions were incubated at 37°C with shaking for 4.5 h. At the end of this time, the solutions were centrifuged. The membranes were isolated, washed and the membrane proteins separated by SDS-acrylamide gel electrophoresis as outlined in the text.

formed on a slab gel electrophoresis apparatus by the method of Laemmli [8]. Each well contained 80—100 μg of membrane protein. Gels were stained with Coomassie Blue [9], periodic-acid Schiff's reagent (PAS) [10, 11], or by a previously reported Stains-All (SA) method [12]. The separated polypeptides were numbered by a decimal notation according to Fairbanks et al. [9] although there were some differences due to the enhanced resolution obtained with the methods used in the present study. The most obvious differences are an increased number of peptides in the region between spectrin bands 1, 2, 2.1 and band 3 (2.2 region, Fig. 1); increased resolution between bands 4.2 and 5 (4.5 region, Fig. 1); and the splitting of band 4.1 into a doublet in the absence of excess Ca^{2+} (4.1, Fig. 1). In the presence of 0.1—1 mM Ca^{2+} under appropriate incubation conditions, the 4.1 doublet became a triplet (Fig. 1c, 3c).

When Ca^{2+} was removed from the incubation medium before lysis, no membrane alterations were observed. However, when Ca^{2+} was present at the time of lysis, the effects noted by Carraway et al. [6] were reliably reproduced.

Fig. 1 shows that Ca^{2+} (0.1 mM Ca^{2+}, B) added to cells after lysis resulted in increased staining of polypeptides in the 2.2 and 4.5 regions and hemoglobin. At 1 mM Ca^{2+} (C) a high molecular weight aggregate appeared at the top of the gel; bands 1 and 2, which are the spectrin peptides, as well as band 3 and the 4.1 doublet decreased. In addition, there is an increase in bands in the 2.2 and 4.5 regions and several of the lower molecular weight components below band 5. At 10 mM Ca^{2+} (D), these changes are even more marked. The high molecular weight aggregate and one peptide in the 2.2 region are more pronounced and the loss of spectrin, band 3 and 4.1 doublet are also obvious. However, at this concentration of Ca^{2+}, bands 4.5 and 6.0, as well as low molecular weight components including hemoglobin, are now decreased compared to that noted in 1 mM Ca^{2+}.

When 1—10 mM iodoacetate is added to the calcium-containing lysed cell suspension, no high molecular weight aggregate appears at the top of the gel and spectrin and protein 3 do not decrease in concentration. However, the calcium effects on bands 2.2, 4.1 and 4.5 can still be observed.

The major membrane sialoglycoproteins (PAS I—III) are not appreciably involved in these effects. PAS and SA staining of comparable gels shows no alterations in sialoglycoproteins.

Fig. 2 shows the effects are proportioned to time. The temperature of the reaction mixtures also influences the rate of the reaction. At 4°C, the same changes are observable but at much longer time intervals.

Fig. 3 shows that Ca^{2+} induced the same effects on well-washed white membranes when cytoplasmic factors are present. These effects are attributable to cytoplasmic proteins since dialyzed preparations of lysate retain activity and these activities are destroyed by heating at 100°C for 10 min. The rate of aggregation and the effects on 4.1 and 2.2 are proportional to the lysate concentration.

The present study indicates that increased concentrations of calcium have a number of effects on erythrocyte membrane proteins. There is (1) an increased binding of cytoplasmic components to the membrane, (2) an increase in the aggregation of membrane proteins, and (3) activation of an apparent

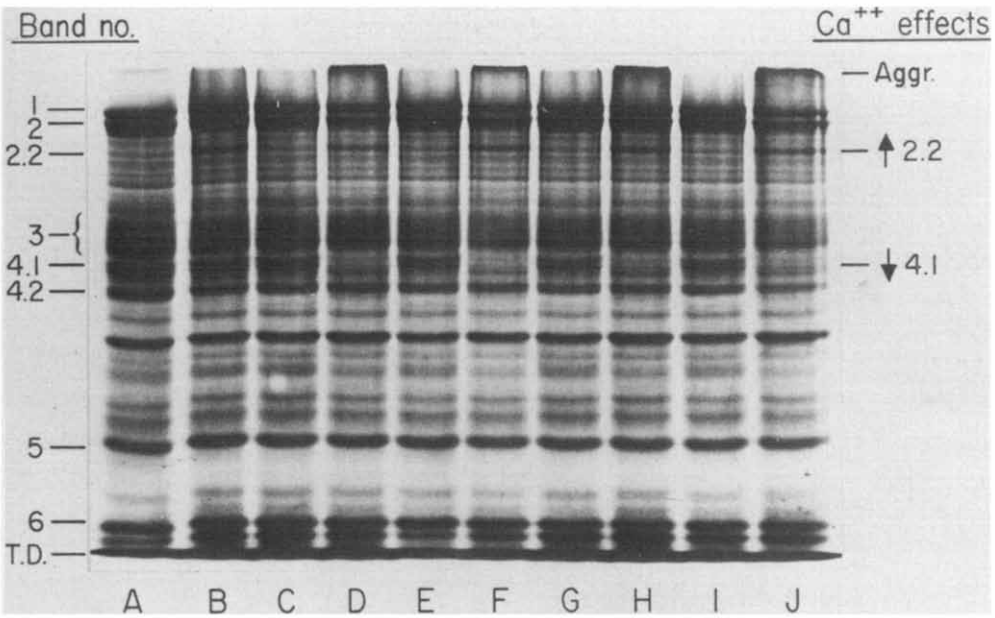

Fig. 2. Time course of calcium-induced changes in erythrocyte membranes. An erythrocyte hemolysate was prepared as described in Fig. 1 and brought to a final concentration of 1 mM calcium. The suspension was incubated at 37°C with shaking. At varying time intervals after the calcium addition, aliquots were centrifuged and the membranes were washed at least 5 times prior to separation of the membrane proteins. Samples A, C, E, G and I contain no added calcium, while B, D, F, H and J contain calcium. A and B were incubated for 5 min, C and D for 30 min, E and F for 1 h, G and H for 2 h, and I and J for 4 h.

protease activity resulting in the hydrolysis of some of the membrane proteins.

Binding of the cytoplasmic components is not a simple phenomenon. At relatively low concentrations of calcium, 0.1—1 mM, there is a progressive increase in the binding of proteins to the membrane such as hemoglobin. At higher concentrations of calcium, there does not appear to be any great enhancement of binding. As a matter of fact, with some membrane components, less protein is bound at the higher concentrations of calcium as indicated in Fig. 1. Hemoglobin is most obviously decreased in its binding and other low molecular weight components are clearly affected. Hanahan [13—15] has observed changes in enzyme activities bound to the membrane with calcium.

The sialoglycoproteins do not seem to be involved in aggregation of membrane proteins while band 3 is contained in the aggregate. When the SDS-polyacrylamide gels are stained with the Stains-All procedure, band 3 takes on the unique purple color, and this makes it easier to detect the gradual decrease in protein 3 intensity with a corresponding increase in the higher molecular weight aggregate in the gel. Lactoperoxidase-catalyzed iodination of intact membrane proteins labels primarily the sialoglycoproteins and protein 3 [16]. Table I shows that in cells labeled in this way, about 80% of protein 3 is incorporated into the aggregate. The sialoglycoproteins are unaffected. Probably the most sensitive changes in the erythrocyte membrane in response to the added calcium are an increase in the 2.2 region and a loss of 4.1 doublet. The loss of spectrin

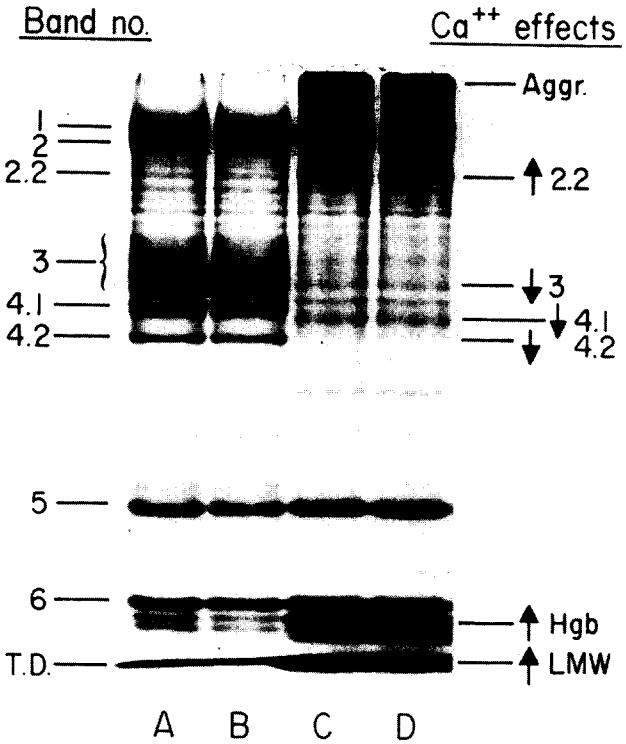

Fig. 3. The effects of cytoplasmic proteins, Ca^{2+} and phenylmethylsulfonyl fluoride on washed human erythrocyte membranes. Erythrocyte membranes were washed free of hemoglobin as described in the text. 10 ml of the lysate (3.4 g hemoglobin %) were added to 1 ml of the washed white stroma (8 mg membrane protein/ml). Ca^{2+} (C, D) and phenylmethylsulfonyl fluoride (B, D) were added to a final concentration of 10 mM and 2 mM, respectively. All samples were then incubated at 37°C for 30 min. Membranes were isolated and polypeptides separated by SDS electrophoresis as described in the text. Aggr., high molecular weight aggregate; Hgb, hemoglobin; LMW, low molecular weight region; T.D., tracking dye front.

and band 3 is not as apparent, and this may be due to their higher concentration in the membrane.

Iodoacetate inhibits transferase activity and with iodoacetate present, the high molecular weight aggregate is not formed in the presence of calcium. However, the changes in bands 2.2, 4.1 and 4.5 are still apparent. Since these changes take place in the absence of transferase activity, they may be attributable to a calcium-activated protease.

The three effects observed may be sequential. The first observable effect is the binding of cytoplasmic proteins to the membrane. This may be followed by the production of low molecular weight polypeptides, presumably produced by a protease. However, the apparent protease activity was not blocked by the serine protease inhibitor, phenylmethylsulfonyl fluoride. This is shown in Fig. 3 where even in the presence of this inhibitor there is an increase in low molecular weight components after Ca^{2+} additions. The loss of 4.1 and increase in low molecular weight components is detectable before any noticeable change in aggregation of spectrin and band 3 is detectable. This calcium-activated protease may, in fact, be directly involved in the aggregation phenomenon or

TABLE I

AGGREGATION OF PROTEIN 3 BY Ca^{2+}

Washed intact erythrocytes were labeled with ^{125}I employing the lactoperoxidase procedure. The cells were then washed twice and lysed by addition of 10 volumes of 10 mM Tris buffer, pH 7.4. This mixture was made 10 mM with respect to Ca^{2+} and was incubated at 37°C for 30 min. The membranes were then isolated and membrane proteins separated by the SDS polyacrylamide gel electrophoresis system. The gels were dried and a radioautograph obtained. The percentage of radioactivity in each band was determined.

	% Total radioactivity	
	Control	Ca^{2+}
High molecular weight aggregate	0	37
Band 3	50	10
Sialoglycoproteins	50	53

be involved in an activation of an enzyme which catalyzes the aggregation. The aggregate of membrane protein as Carraway et al. [6] have pointed out, appears to involve covalent linkage of membrane polypeptides. This type of aggregate can be produced by an enzyme such as the transferase involved in fibrinogen clotting. Lorand et al. have shown that such an enzyme will act on erythrocyte membrane proteins [17]. The calcium-activated phenomenon appears to be irreversible. If this system is important in vivo and is nonreversible, it may well be involved in erythrocyte uptake and the signal for aging. If the aggregation is reversible in vivo, then these Ca^{2+}-activated events may play an important control point for altering cell membrane rigidity via its effects on spectrin and related membrane proteins.

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Addendum

After this work was completed, the interesting paper by Lorand et al. [18] appeared showing the effect of Ca^{2+} on human erythrocytes involving glutamyl transferase activity.

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