CALCIUM EFFECTS ON ERYTHROCYTE MEMBRANE PROTEINS

Richard B. Triplett, James M. Wingate and Kermit L. Carraway

Department of Biochemistry, Oklahoma State University
Stillwater, Oklahoma 74074

Received September 19,1972

Summary: The addition of calcium (>1 mM) during hemolysis of human erythrocytes results in three significant changes in the membrane proteins, as determined by electrophoresis of the ghosts solubilized in sodium dodecyl sulfate. There is an appearance of an aggregate of material which is not dissociated by dodecyl sulfate, the appearance of a polypeptide of molecular weight 174,000 and the disappearance of a polypeptide of molecular weight 91,000. The latter two changes appear to result from proteolysis of the membrane proteins. Treatment of isolated erythrocyte membranes with calcium by the same procedure has no effect on the membrane proteins as determined by electrophoresis. This suggests a possible difference in the protein organization of the ghost when compared to the membrane of the intact cell.

Calcium ion plays an important role in a number of cellular membrane processes (1). In the human erythrocyte Ca^{++} accumulates as a result of ATP depletion. Changes in cellular deformability (2), shape and critical hemolytic volume (3) are also noted. Weed (4) has suggested that alterations in red cell deformability may be directly related to shortened red cell life in vivo. Changes in erythrocyte membrane deformability and ghost size are noted if Ca^{++} is introduced into resealed ghosts during the hemolysis stages (5,6). The mechanism of Ca^{++} action is still rather obscure, although it has been suggested that a Ca^{++}-activated ATPase may play a role in these phenomena (7). An additional possibility is that ATP controls the concentration of membrane-bound Ca^{++} by acting as a chelating agent (8). In the present communication we wish to describe effects of Ca^{++} on the proteins of the erythrocyte membrane, as studied by sodium dodecyl sulfate acrylamide electrophoresis. Two effects were observed, an aggregation of the membrane proteins and a degradation of specific proteins which apparently results from protease action.

Methods. Blood was obtained from the Dallas Community Blood Bank and used within one week of the withdrawal date. Red cells were washed with 10 mM Tris buffer in 0.15 M sodium chloride (pH 7.4). Particular care was taken to remove white cells, which are a source of protease contamination (9). For hemolysis and preparation of ghosts in the presence or absence of effector substances the following 4 step procedure was used:

Step 1: Packed red cells were hemolyzed in 10 volumes of 10 mM Tris buffer (pH 7.4) at 4° for 10 minutes. Step 2: Resealing was achieved by addition of 3.0 M NaCl or a mixture of 1.42 M KCl and 0.28 M NaCl to give a final salt concentration of 0.17 M and incubation at 37° for 30 minutes (10). Step 3: Resealed ghosts were hemolyzed in 10 mM Tris (pH 7.4) at 4° for 1 hour. Step 4: Membranes were washed with 10 mM Tris buffer at 4° until essentially free of hemoglobin.

Effectors (cations or chelating agents) could be added in appropriate concentrations at Steps 1-3. Hemoglobin-free ghosts were also prepared directly by hemolysis and washing in 10 mM Tris (pH 7.4) without the intermediate steps. Ghosts prepared by either of the two procedures were solubilized in 1.3% dodecyl sulfate in 0.1 M phosphate (pH 7.8) and 1% mercaptoethanol at room temperature (9). They were subjected to electrophoresis on 5% polyacrylamide gels in 0.1% SDS as described previously (9,11). The gels were stained with coomassie blue by the procedure of Fairbanks, et al. (12), with the elimination of the third staining step.

Results and Discussion. The addition of calcium during the hemolysis of erythrocytes (first step) causes significant alterations in the proteins of the membranes as isolated by the 4 step procedure. Figure 1 shows the changes in membrane protein electrophoretic patterns as a function of Ca^{++} or Mg^{++} concentrations. Gels A-I were from samples incubated with increasing concentrations of Ca^{++} (0-5.0 mM). Gels J-L are from samples incubated with Mg^{++} (5-20 mM) during hemolysis. Because of the experimental procedure used, there are some variations in the total amount of protein

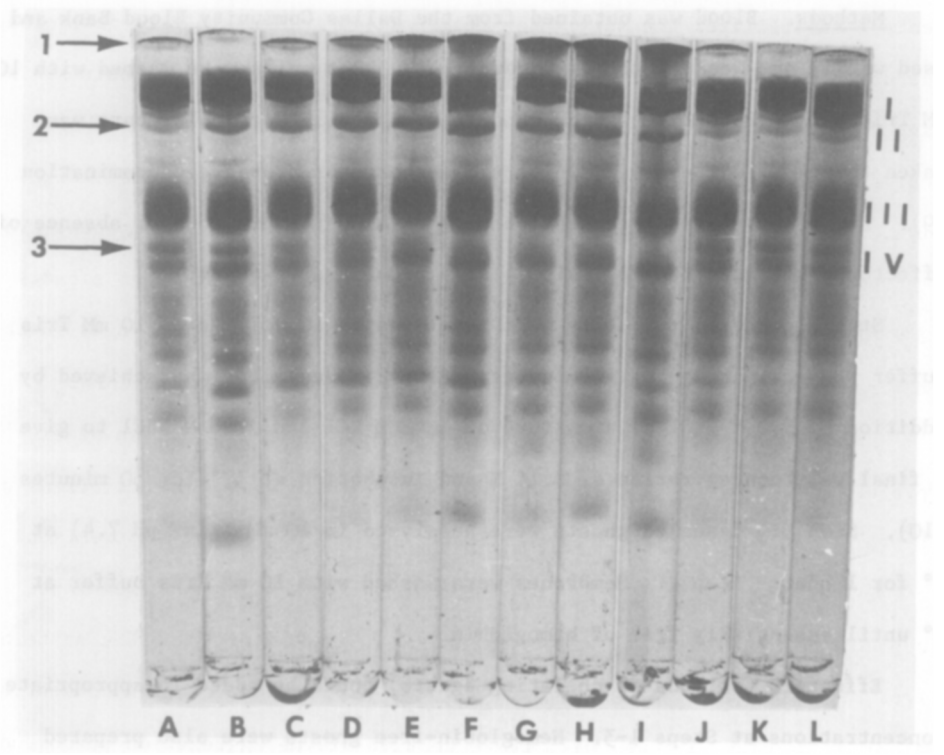

Fig. 1 - Effects of adding calcium or magnesium during hemolysis on proteins of the human erythrocyte membrane. Human erythrocytes washed in 0.15 M NaCl in 10 mM Tris, pH 7.4, were hemolyzed for ten minutes at 4° in 10 mM Tris, pH 7.4, containing 0-5 mM Ca^{++} or 5-20 mM Mg^{++}. The hemolysate was taken to isotonic conditions by the addition of 3 M NaCl followed by incubation at 37° for thirty minutes. The membranes were washed two times with NaCl-Tris buffer and membranes were isolated with 10 mM Tris. Electrophoresis and staining of gels were performed as described in Methods. Gel A, untreated control; Gel B, 0.1 mM Ca^{++}; Gel C, 0.3 mM Ca^{++}; Gel D, 0.5 mM Ca^{++}; Gel E, 0.7 mM Ca^{++}; Gel F, 1.0 mM Ca^{++}; Gel G, 2.0 mM Ca^{++}; Gel H, 3.0 mM Ca^{++}; Gel I, 5.0 mM Ca^{++}; Gel J, 5.0 mM Mg^{++}; Gel K, 10.0 mM Mg^{++}; Gel L, 20.0 mM Mg^{++}. Bands are labeled as previously described (9,13).

---

applied to each gel. The areas of significant changes in protein patterns are denoted by the numbered arrows. First, there is a Ca^{++}-dependent aggregation of protein at the top of the gel (arrow 1). This aggregate can also be observed by chromatography in dodecyl sulfate on Sepharose 4B. The effect does not appear to be specific to a single membrane protein, since densitometry indicates that there is a disappearance of both compon-

ents I and III from the acrylamide gels. It is not possible to determine accurately whether other components also are decreased, since their staining densities are too low to yield reliable results. The second change noted (arrow 2) is an increase in a band (component II, molecular weight 174,000) just below component I. Third, there is a loss of component IVa (arrow 3) which is dependent on Ca^{++} concentration. These three effects are all calcium-specific as shown by the absence of similar changes in the membranes of cells hemolyzed in the presence of magnesium (5-20 mM).

The appearance of component II and disappearance of IVa can be explained by a consideration of the effects of low trypsin concentrations on erythrocyte ghosts (14). Figure 2 shows the effect of trypsin treatment at 0, 0.1 and 0.5 mg/ml. The gel electrophoresis patterns of membranes subjected to a limited trypsin treatment are quite similar to the patterns from membranes obtained after hemolysis in the presence of high concentrations of calcium (1-5 mM) with the exception of the aggregation phenomenon. This similarity suggests that the appearance of component II and disappearance of IVa result from proteolysis associated with the calcium treatment. The proteolytic effects can also be observed by adding low concentrations of trypsin to red cells during hemolysis. Attempts to eliminate the Ca^{++}-induced proteolytic effects by careful washing of the red cells to remove buffy coat contamination have not been successful. It therefore seems likely that the protease is associated with the erythrocyte membrane and is activated or released by Ca^{++}. Membrane bound erythrocyte proteases have been previously described (15,16). If the protease is associated with the red cell membrane, it may represent a mechanism for accelerating red cell destruction as the cell ages and its intracellular calcium increases. Regardless of the source of the protease, membrane protein digestion must be considered in evaluating any changes in erythrocyte membranes induced by Ca^{++} addition during hemolysis.

The effects of Ca^{++} on ghosts which were isolated by a one step hypo-

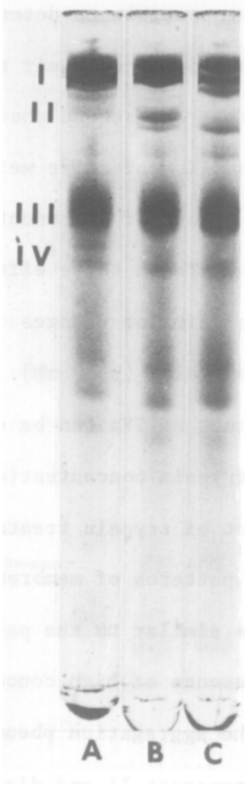

Fig. 2.

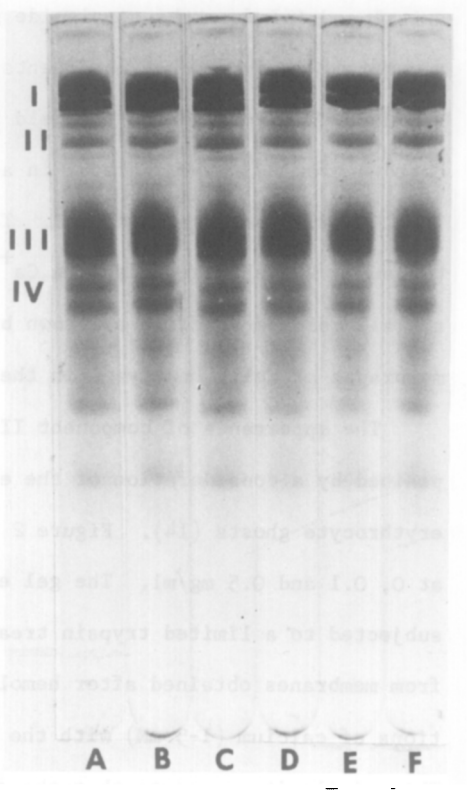

Fig. 3.

Fig. 2 - Trypsin digestion of human erythrocyte membranes at low enzyme concentrations in isotonic phosphate. Membranes (2.4 mg/ml) were treated with 0. - 0.5 μg/ml of trypsin in isotonic phosphate for one hour. Digestion was stopped by the addition of hot SDS. Trypsin concentrations were: Gel A, untreated control; Gel B, 0.1 μg/ml; Gel C, 0.5 μg/ml.

Fig. 3 - Effect of calcium addition on isolated human erythrocyte membranes. Experimental conditions are the same as those given in Figure 1, except an equivalent concentration of membranes was used in place of the erythrocytes. Gel A, untreated control; Gel B, 0.1 mM Ca^{++}; Gel C, 0.5 mM Ca^{++}; Gel D, 1.0 mM Ca^{++}; Gel E, 2.0 mM Ca^{++}; Gel F, 5.0 mM Ca^{++}.

---

tonic hemolysis plus hypotonic washes were also studied. Addition of Ca^{++} to a suspension of the ghosts in hypotonic buffer, followed by the addition of salt to isotonicity and incubation at 37°, had no significant effect on the protein patterns of the treated membranes (Figure 3).

As observed, the apparent aggregation simply reflects the inability

of dodecyl sulfate to disaggregate a portion of the membrane proteins after the Ca^{++} treatment. Thus the aggregate could represent a pre-existing component or structure of the membrane which is stabilized by the addition of Ca^{++}, or it could represent a new organizational form of the membrane which is induced by Ca^{++} and is resistant to disaggregation by the detergent. The failure of isolated, washed ghosts to show a response to Ca^{++} treatment suggests a reorganization of the membrane components during washing such that the aggregation no longer occurs. Similar differences in membrane organization between the isolated ghost and the intact erythrocyte have been noted from chemical modification (17,18) and lipolytic enzyme (19,20) studies. However, these effects were demonstrated only for the lipids of the membrane, while the changes observed in the present work apparently involve the membrane proteins as well.

The chemical nature of the aggregate and the forces involved in its stabilization are unknown and must be investigated in more detail. It is apparent that spectrin alone is not responsible for the aggregate. Component III is involved as well in the Ca^{++}-promoted aggregate, and its contribution to membrane structure and deformability must be considered in addition to previous suggestions that the fibrous proteins (e.g., spectrin) are responsible for calcium-dependent erythrocyte membrane structural changes (5).

Acknowledgements. Paper No. 2563 of the Oklahoma Agricultural Experiment Station. This work was supported in part by grants from the National Institutes of Health (GM 16,870), American Cancer Society (P-563, BC-77A and IN 99B) and by the Oklahoma Agricultural Experiment Station.

REFERENCES

1. J. F. Manery, Fed. Proc., 25, 1804 (1966).
2. P. L. LaCelle, Transfusion, 9, 238 (1969).
3. A. R. Haradin, R. I. Weed and C. F. Reed, Transfusion, 9, 229 (1969).
4. R. I. Weed, Am. J. Med., 49, 147 (1970).

5. P. L. LaCelle, Semin. Hematol., 7, 385 (1970).
6. J. Palek, W. A. Curby and F. J. Lionetti, Am. J. Physiol., 220, 19 (1971).
7. J. Palek, W. A. Curby and F. J. Lionetti, Am. J. Physiol., 220, 1028 (1971).
8. M. Chau-Wong and P. Seeman, Biochim. Biophys. Acta, 241, 473 (1971).
9. D. Kobylka, A. Khettry, B. C. Shin and K. L. Carraway, Arch. Biochem. Biophys., 148, 475 (1972).
10. C. M. Redman, J. Cell. Biol., 49, 35 (1971).
11. K. L. Carraway and D. Kobylka, Biochim. Biophys. Acta, 219, 238 (1970).
12. G. Fairbanks, T. L. Steck and D. F. H. Wallach, Biochemistry, 10, 2606 (1971).
13. K. L. Carraway and B. C. Shin, J. Biol. Chem., 247, 2102 (1972).
14. R. B. Triplett and K. L. Carraway, Biochemistry, 11, 2897 (1972).
15. W. L. Morrison and H. Neurath, J. Biol. Chem., 200, 39 (1953).
16. G. L. Moore, W. F. Kocholaty, D. A. Cooper, J. L. Gray and S. L. Robinson, Biochim. Biophys. Acta, 212, 126 (1970).
17. K. L. Carraway, D. Kobylka, J. Summers and C. A. Carraway, Chem. Phys. Lipids, 8, 65 (1972).
18. S. E. Gordesky and G. V. Marinetti, Fed. Proc., 31, 861 (1972).
19. B. Roelofsen, R. F. A. Zwaal, P. Comfurius, C. B. Woodward and L. L. M. van Deenen, Biochim. Biophys. Acta, 241, 925 (1971).
20. Y. Laster, E. Sabban and A. Loyter, FEBS Letters, 20, 307 (1972).