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CALCIUM-INDUCED ERYTHROCYTE MEMBRANE CHANGES

THE ROLE OF ADSORPTION OF CYTOSOL PROTEINS AND PROTEASES

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Summary

Changes in the membranes of human red cells similar to those of certain hemolytic anemias were produced by calcium in three model systems and found to result from membrane adsorption of cytosol proteins and from proteolysis. Proteins of the cytosol adsorbed to human erythrocyte membranes in the presence of calcium and extractable by EDTA were compared to those of the total cytosol by polyacrylamide gel electrophoresis and by isoelectric focusing. Catalase (EC 1.11.1.6) and band 8 were adsorbed to the membranes from the supernatant cytosol with calcium. Band 8 was a normal constituent of the cytosol, apparently a single chain of molecular weight 24 000 with a pI of 5.35. Other calcium-induced membrane changes could be demonstrated to be due to cytosol protease(s) adsorbed to the membrane in the presence of calcium and extractable with EDTA. When membranes were incubated with the proteases and calcium the decrease in bands 1, 2, 3 and 4.1 and the appearance of multiple low molecular weight peptides typical of calcium-induced membrane effects resulted.

Introduction

Increased intracellular calcium has been found in sickle cell anemia red cells [1], and may be present as well in red cells of hereditary spherocytosis [2]. Changes in erythrocyte membrane polypeptide composition in these hemolytic anemias have been ascribed to this increase in intracellular calcium, since they can be reproduced by hemolysing red cells with hypotonic buffers containing

Abbreviations: EGTA, ethyleneglycolbis(β-aminoethylether)-*N*,*N*'-tetraacetic acid; TLCK, *N*-α-tosyl-L-lysylchloromethane hydrochloride.

calcium, incubating red cells either for 24—48 h without glucose and with calcium [3—5], or for 1 h with the ionophore A23187 and calcium [6]. The effects of these three model systems on membrane polypeptides will be compared by electrophoresis in dodecyl sulfate using a discontinuous buffer system [7].

There is an increase in erythrocyte membrane non-hemoglobin protein as well as membrane hemoglobin when normal red blood cells are incubated without glucose and with calcium present [8,9]. Since increased membrane adsorption of non-hemoglobin proteins occurs when the red cells are incubated in buffers as well as serum [9], the origin of these proteins must be the red cell cytosol. Here we will characterize these non-hemoglobin proteins by isoelectric focusing and polyacrylamide gel electrophoresis with sodium dodecyl sulfate. We will confirm previous reports that hemoglobin, band 4.5 (catalase), and band 8 [3,4] are increased in the membranes and demonstrate that band 8 is a constituent of the normal cytosol.

While adsorption of cytoplasmic proteins to the inner surface of the red cell membrane will account for those new electrophoretic bands corresponding to cytoplasmic proteins, it will not explain the decrease in high molecular weight membrane polypeptides and increase in multiple new smaller polypeptides seen on electrophoresis after exposure of red cells to calcium [3—6]. Membrane and cytosol proteases have been described [10—13] whose role in these changes will be examined. Here we report proteolysis of isolated membranes and spectrin by cytosol protease(s) adsorbed to the membrane in the presence of calcium and released therefrom by EDTA.

Materials and Methods

Preparation of membranes and cytosol. Fresh whole blood was collected aseptically in heparin, EDTA or buffered citrate phosphate, and filtered through a cellulose column to remove white blood cells [14]. The red cells were then washed three times with isotonic saline. In certain cases red cells were incubated either in Dulbecco's phosphate-buffered saline containing calcium but without glucose for 48 h [4] or with $5 \cdot 10^{-5}$ M A21387 (courtesy of Eli Lilly Co.) with or without 1 mM calcium [15]. Red cells were hemolyzed with 20 volumes of 10 mM Tris, pH 7.4, and centrifuged at 80 000 × *g*, saving both the membranes and cytosol. The red cell membranes were then washed three times with the hemolyzing buffer. In certain experiments in which the hemolyzing buffer was also made 1 mM $CaCl_2$ the final wash did not contain $CaCl_2$.

Preparation of hemoglobin-free membrane extracts and cytosol. Membranes were extracted with an equal volume of 1 mM EDTA (pH 8, 16 h, 0°C). Spectrin extracted with EDTA from membranes was further purified on an Agarose A5M column with 10 mM Tris, 50 mM NaCl, 0.1 mM EDTA, pH 7.4 [16]. For removal of most of the hemoglobin and further analysis, extracts of membranes prepared with calcium and the cytosol were treated similarly. They were dialyzed against distilled water until the dialysate was free of chloride by $AgNO_3$, then absorbed with an equal volume of DEAE-cellulose preequilibrated with 1.5 mM phosphate buffer, pH 7.3, and the hemoglobin and carbonic

anhydrase removed by repeated washes of the same buffer. The DEAE-cellulose was then poured into a column, washed with more 1.5 mM phosphate, and developed with 50 mM phosphate. Eluted fractions were then concentrated by ultrafiltration and purified by gel filtration on a calibrated Biogel P-200 column in 50 mM phosphate buffer, pH 7.3.

Isoelectric focusing and electrophoresis. Analytical isoelectric focusing used 0.5 × 10 cm gels of 4—7% acrylamide with pH 3—10 ampholytes [17] and stained with Coomassie Blue G250 in 3.5% $HClO_4$ [18]. Polyacrylamide electrophoresis with dodecyl sulfate employed the discontinuous buffer system of Laemmli [7] with a 4% stacking gel and a 10, 12 or 15% running gel. Acrylamide gels were stained as usual [19] except that Coomassie Blue was omitted from the washing solutions.

Enzyme assay. Catalase [14], carbonic anhydrase (EC 4.2.1.1) [20], and superoxide dismutase (EC 1.15.1.1) [21], were measured by published methods. Amounts of enzymes present were estimated by comparison of the activity with standard enzymes obtained from Sigma. Protease was assayed by measuring the release of trichloroacetic acid-soluble peptides from [^{3}H]acetyl-labeled bovine hemoglobin (10^6 cpm/mg), in 10 mM Tris, 5 mM Ca^{2+}, pH 7.4, incubated 1 h at 37°C. Bovine hemoglobin (Sigma) was acetylated with [^{3}H]-acetic anhydride (New England Nuclear) [22].

Results

The complexity of the red cell membrane polypeptides and the changes produced by calcium are shown in a photograph of a slab gel employing the discontinuous buffer system of Laemmli (Fig. 1) [7]. Band 4.1 which appears homogeneous by phosphate gels [19] is actually a mixture of multiple components. The maximum effects of calcium on the red cells are seen when the cells are hemolyzed in 1 mM calcium (Fig. 1, wells 2, 9, 16) and when red cells are incubated 48 h with calcium and without glucose (Fig. 1, wells 3, 10, 17). Similar changes occur when cells are incubated for much shorter times (1 h) with the ionophore A23187 and calcium (Fig. 1, wells 4, 11, 18), but not with the ionophore alone (Fig. 1, wells 5, 12). Note that the increase in bands 4.5, 8 and hemoglobin and the decrease in bands 1, 2, 3 and 4.1 and the appearance of multiple bands in the region from 4.2 to 5 and from 6 to 8. These changes are present also in the patient with hereditary spherocytosis and glyceraldehyde-3-phosphate dehydrogenase (EC 1.2.1.12) deficiency (Fig. 1, wells 7, 14, 20) [23]. In this patient and a relative with this enzyme deficiency alone (Fig. 1, wells 6, 13, 19) a decrease in band 6 or glyceraldehyde-3-phosphate dehydrogenase is also present. Band 2.3 is increased when red cells are hemolyzed with 1 mM calcium (Fig. 1, wells 2, 9, 16) or treated with A23187 and calcium (Fig. 1, wells 4, 11, 18), but not after 48 h incubation with calcium (Fig. 1, wells 3, 10, 17). Note also that prolonged incubation differs in other respects as well; there is less change in the 4.1 complex, more bands between 4.5 and 5, and a new band just before 8 (Fig. 1, wells 3, 10, 17).

Assay for carbonic anhydrase of the EDTA extract of calcium-prepared membranes showed undetectable amounts. In the same preparation the ratio of superoxide dismutase to catalase was 0.035 whereas in the supernatant

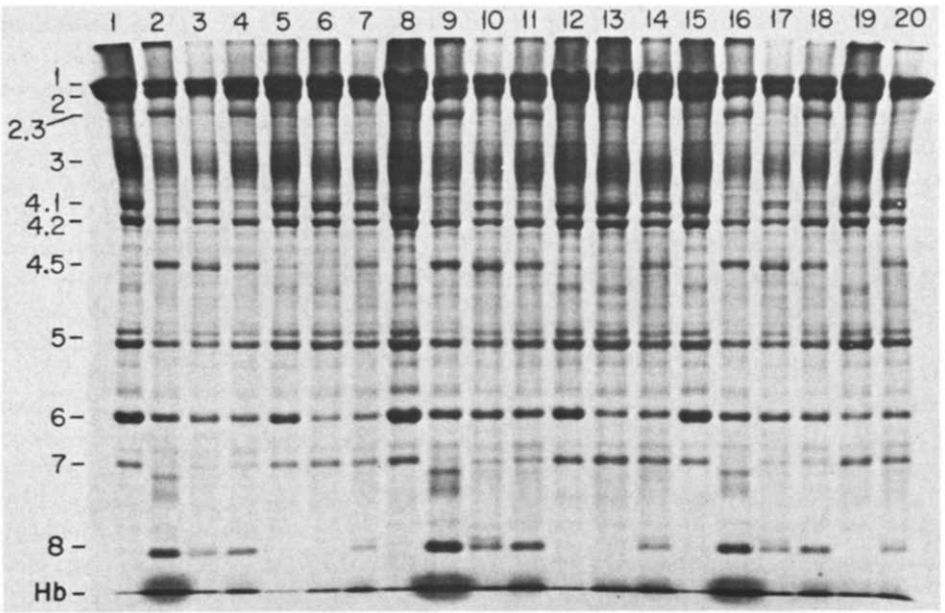

Fig. 1. Comparison of erythrocyte membrane polypeptides from red cells variously exposed to calcium, by electrophoresis with a 4% acrylamide stacking gel, and 10% acrylamide running gel [7]. On the left, polypeptides are identified by the system of Fairbanks et al. [19]. Across the top sample wells are numbered 1—20. Amount of samples was 50 μg in wells 1—7, 100 μg in wells 8—14, and 50 μg in wells 15—20. Wells 1, 8, 15, control membranes; wells 2, 9, 16, membranes from cells hemolyzed with calcium; wells 3, 10, 17, cells incubated 48 h with calcium; wells 4, 11, 18, cells incubated 1 h with A23187 and calcium; wells 5, 12, cells incubated 1 h with A23187 without calcium; wells 6, 13, 19, cells from patient with heterozygous glyceraldehyde-3-phosphate dehydrogenase deficiency; wells 7, 14, 20, cells from patient with hereditary spherocytosis and heterozygous glyceraldehyde-3-phosphate dehydrogenase deficiency.

cytoplasm it was 0.35. Thus, unlike hemoglobin and catalase [3,4,9], carbonic anhydrase and superoxide dismutase are not bound to membranes with calcium.

Fig. 2 shows the results of preparative isoelectric focusing on Sephadex G-75 of the EDTA extract of membranes prepared with calcium. Hemoglobin and any carbonic anhydrase have previously been partially removed by use of the DEAE column (see Materials and Methods). The concentrated sample was then frozen with 20% glycerol prior to preparative isoelectric focusing. When samples were focused without prior chromatography or freezing the catalase peak was single and sharp. The separation of band 8 from superoxide dismutase is evident. Band 8 extracted from calcium-exposed membranes with EDTA had a pI of 5.36; band 8 from supernatant cytoplasm had a pI of 5.33. Band 8, whether from calcium-prepared membranes or soluble supernatant had a subunit molecular weight of 24 000 ± 1000 in 10, 12 and 15% gels.

Fig. 3 shows the distribution of cytosol protease, band 8, and certain other proteins in DEAE-cellulose and Biogel P-200 column chromatography. Prior to elution of the peak from the DEAE column the hemoglobin and carbonic anhydrase had been washed off the column with 1.5 mM phosphate (see fraction 0, DEAE-cellulose column, Fig. 4). Fractions 30—41 from the DEAE column were then concentrated by ultrafiltration and placed on the Biogel

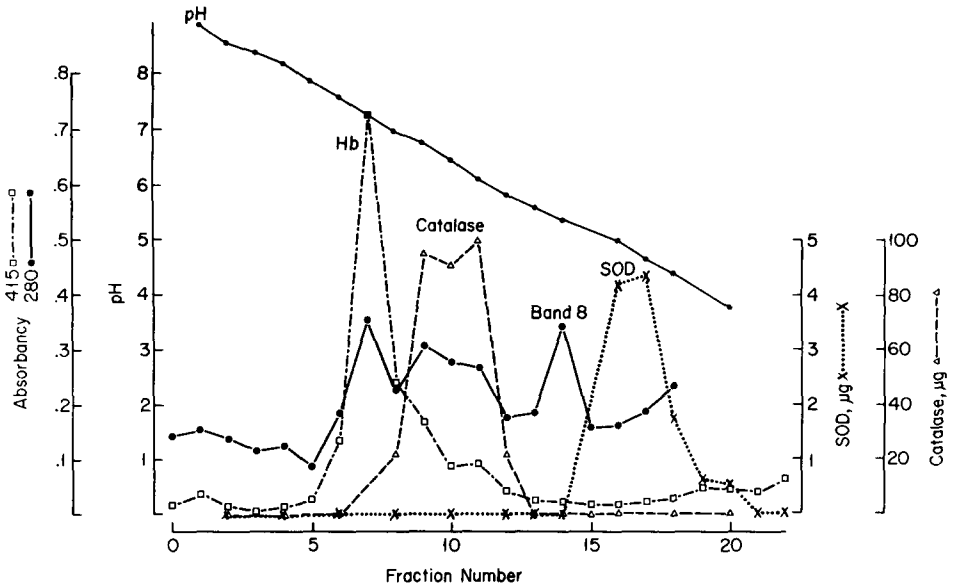

Fig. 2. EDTA extract of membranes from red cells hemolyzed with calcium, analyzed by preparative isoelectric focusing (20 h) lengthwise on a 0.5 X 10 X 23 cm slab of 5% Sephadex G-75, containing 5% pH 3—10 ampholyte. After determining the pH gradient 1-cm sections of the gel were eluted and analyzed as shown, and by dodecyl sulfate electrophoresis. Note the separation of band 8 and superoxide dismutase (SOD).

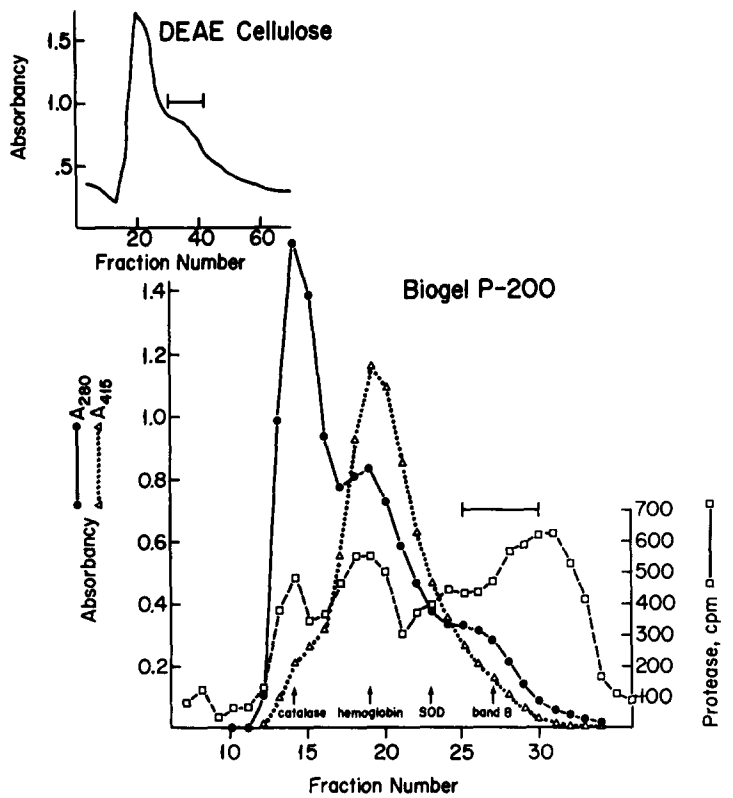

Fig. 3. Isolation of band 8 and protease from hemolysate by DEAE-cellulose (top) and gel filtration on Biogel P-200 (bottom). Fractions saved for further purification are shown: Fractions 30—41 from DEAE-cellulose column were concentrated by ultrafiltration and placed on Biogel P-200. In other experiments, fractions equivalent to 28—32 from the P-200 column were saved for protease.

P-200 column. Notice that catalase (240 000 daltons), hemoglobin (64 000 daltons), superoxide dismutase (32 000 daltons), and band 8 (24 000 daltons) are eluted serially from the column. Fig. 4 represents the gel electrophoresis of effluent fractions from the columns shown in Fig. 3. Band 8 occurs as the monomer on the Biogel P-200 column.

While in Fig. 3 it is evident that cytosol protease(s) occur at multiple molecular weights, in the following experiments we used preparations eluting at twice the hold up volume from P-200 columns which also contained band 8.

Protease assay showed 681 ng [^{3}H]hemoglobin solubilized by an EDTA extract of 10^9 control membranes and 4900 ng [^{3}H]hemoglobin solubilized by an EDTA extract of a like number of red cell membranes prepared with calcium. Thus there was more than seven times more extractable protease in membranes prepared with calcium than without. These proteases were 52% inhibited with 1 mM EDTA, 55% inhibited with 1 mM ethyleneglycolbis(β-aminoethylether)-*N*,*N*′-tetraacetic acid (EGTA), and 65% inhibited with 40 mM *N*-α-tosyl-L-lysylchloromethane hydrochloride (TLCK). Since most of the white cells had been removed by filtration, the protease content remaining originated from red cells and was independent of the white blood cell counts

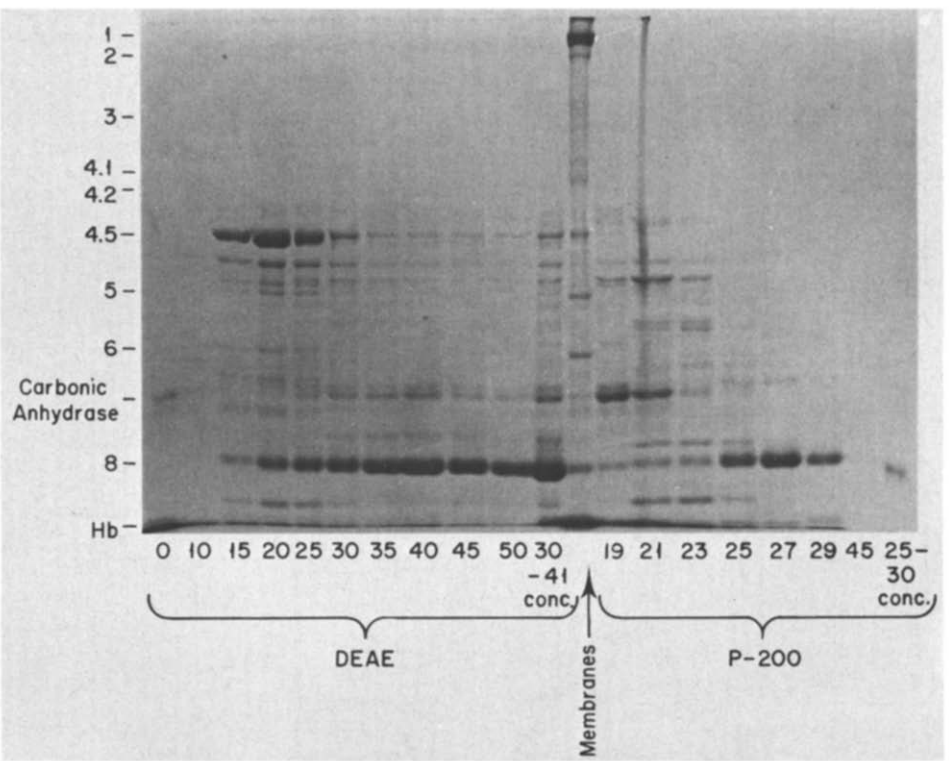

Fig. 4. Acrylamide gel electrophoresis of effluent fractions from columns shown in Fig. 5. Fraction 0 of the DEAE-cellulose column represents the first wash of this material and consists of hemoglobin and carbonic anhydrase. Membranes were electrophoresed simultaneously in well 12 as a reference. The wells are labeled at the bottom for ease in identification of fractions but samples were electrophoresed from above downward as before.

(0—20/μl of packed, filtered cells). Further removal of lymphocytes with Ficoll-Hypaque did not effect the protease content. Cytosol proteases also act on purified spectrin. The digestion of spectrin was estimated by the decrease in area of its peak on scan of the gel. Comparison of the activity of the protease(s)

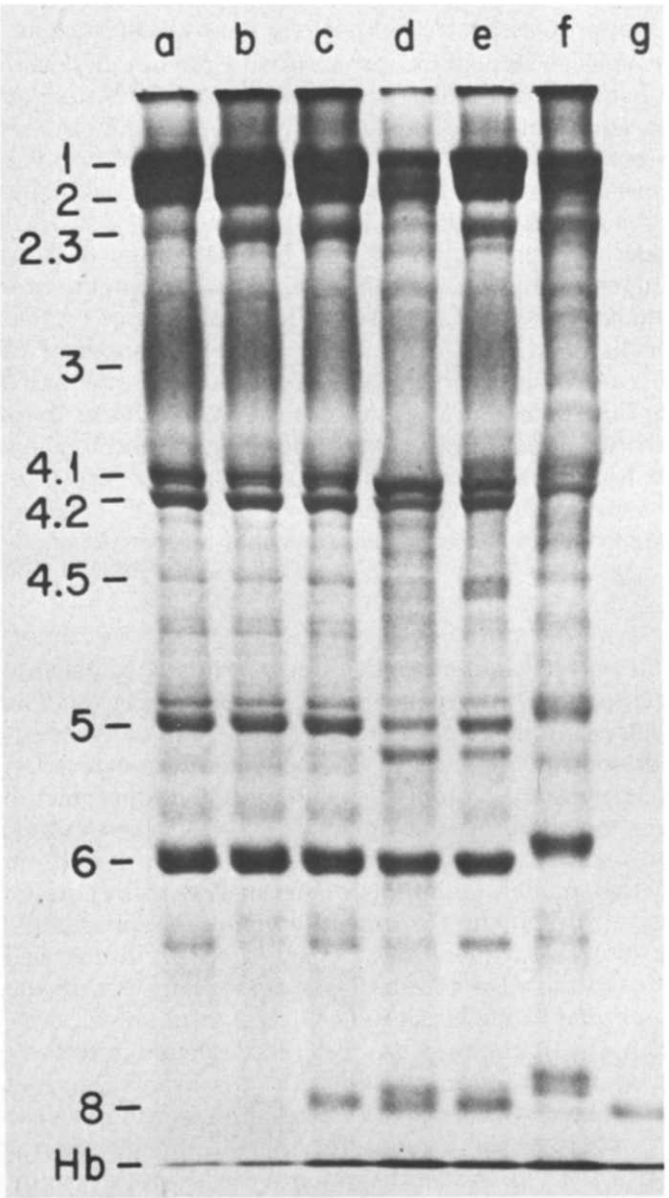

Fig. 5. Proteolysis of red cell membranes by cytosol proteases adsorbed to the membrane in the present of calcium. Bands are numbered as before [19], on the left, and well are identified by letters: a, unincubated control membranes; b, incubated (1 h, 37°, pH 7.4) control membranes; c, membranes and protease incubated separately until after boiling with dodecyl sulfate, 2 min; d, membranes, protease, and 5 mM calcium incubated together; e, membranes, protease, and 1 mM EDTA incubated together; f, membranes, protease, 5 mM calcium, and 40 mM TLCK incubated together; g, protease, unincubated.

on the two substrates, acetyl-hemoglobin and spectrin, shows 5 μg of the former was digested in 1 h, 7 μg of the latter in 2 h.

Fig. 5 shows the effect of cytosol proteases on the distribution of membrane polypeptides. Since incubation of the membranes alone (37°C, 1 h) with calcium (Fig. 5b) produced some changes from the control (Fig. 5a), most noticeably in the increases in band 2.3, there may be either intrinsic membrane proteases active or the cytosol proteases were not entirely removed by washing. Fig. 5c is a control experiment in which the protease and membranes were mixed but only after incubation and boiling with dodecyl sulfate to destroy proteases. Thus Fig. 5c is equivalent to the sum of incubated membranes (Fig. 5b) and the protease preparation (Fig. 5g). Fig. 5d shows the proteolysis present when the membranes and proteases are incubated together. Comparing Fig. 5d with Fig. 5c, note the decrease in bands 1, 2 (spectrin), 2.3, 3 and 4.1 and the multiple new bands between 4.2 and 5, and between 6 and 8. Thus cytosol protease(s) have increased the proteolysis of membrane proteins over the effect of any membrane proteases alone, and produced a decrease in 2.3 as with 48 h incubation of cells (Fig. 1, wells 3, 10, 17). In confirmation of previous results those changes due to adsorption of cytosol proteins (increased hemoglobin, 4.5 and 8) are not evident in comparing Figs. 5c and 5d as there are obviously no cytosol proteins available to be adsorbed. Fig. 5e and 5f show that EDTA and TLCK do not completely inhibit proteolysis. The protease-containing preparation is shown in Fig. 5g in which the contaminating catalase, band 8, and hemoglobin are evident, but the protease per se is not identified.

Discussion

Three in vitro model systems for examining the effects of increased calcium uptake on red cell membrane polypeptides have been compared (Fig. 1). The first, incubation of red cells without glucose and with calcium corresponds most directly with the incubation of red cells in their own defibrinated serum used by Weed et al. [8]. However, prolonged incubation without glucose results in other cell changes including effects on ATP [8], electrolytes [24], GSH, and sulfhydryl groups [25], any of which may be inappropriate to ascribe to calcium. The second model, hemolysis of red cells with hypotonic calcium-containing buffers, results in maximal, prompt, reproducible membrane changes [5,6]. However, since these changes result in red cell destruction, the system is not suitable for correlation of these changes with the physical changes of the membrane in the intact red cell. The third model, brief incubation of red cells with the ionophore A23187 and calcium results in similar membrane changes as the preceding without the disadvantages cited [6,15]. It is evident that the three models do not result in entirely equivalent membrane changes (Fig. 1), although the reasons for the differences are not clear. Band 2.3 is evident with the two models in which there is brief exposure to calcium, but not when the red cells are incubated 48 h. In the last case, band 2.3 may be itself digested by further proteolyisis as is the case with isolated membranes and cytosol proteases (see Figs. 5c and 5d). All three models however, produce changes in the membranes involving both adsorption of cytosol proteins and protease action.

The adsorption of hemoglobin [4,8,9] and catalase [4,26] to the membrane on exposure to calcium has been previously studied. Band 8 is a cytoplasmic protein similarly adsorbed, and not a digestion product as has been suggested [6]. We have been unable as yet to identify a possible enzymatic function for band 8, although we have distinguished it from carbonic anhydrase and superoxide dismutase. It is hoped that further characterization may enable its identification.

Incubation of proteases with intact membranes and spectrin, and the resulting proteolysis accounts for all those calcium-induced changes not ascribable to adsorption of cytosol proteins. At least some of these proteases, like the cytosol proteins, can be extracted with EDTA from membranes prepared with calcium. Their further characterization, including substrate specificity, should be undertaken to investate their possible physiological role. Such proteases could have functioned in immature red cells in the remodeling of intracellular organelles or precipitated hemoglobin chains [27] or could have some unidentified role in the mature erythrocyte. Regardless of their possible function, effective inhibition of such protease activity would be useful in preparation of unaltered membranes or membrane components.

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