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Lack of some Ca^{2+}-mediated processes in goat erythrocytes

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Ca^{2+} does not promote crosslinking of proteins nor stimulate proteolysis in goat and sheep erythrocyte membranes. Neither crosslinking nor proteolysis was observed even when the goat erythrocytes were loaded with calcium with the help of calcium ionophore A23187. Membrane-free human erythrocyte hemolysate, however, induced Ca^{2+}-dependent crosslinking in goat erythrocyte membranes.

Increase in the intracellular calcium causes alteration in shape and viscoelastic properties of the human erythrocytes [1–3]. The changes are attributed primarily to Ca^{2+}-mediated crosslinking between cytoskeletal matrix and membrane proteins, catalysed by transglutaminase [3]. Ca^{2+}-dependent transglutaminases are widely distributed and Lorand and Conrad [4] suggested that the enzyme mediated formation of large protein adducts linked via γ-glutamyl-ε-lysine bonds might represent a general paradigm for cells undergoing terminal differentiation in the process of aging and dying. Erythrocyte membranes derived from several mammalian species also exhibit Ca^{2+}-stimulated proteolytic activity [5]. Eaton et al. [6] reported that Ca^{2+}-mediated changes in shape and deformability are not exhibited by the sheep erythrocytes. $(Ca^{2+} + Mg^{2+})$-ATPase is very low in the sheep erythrocytes and almost absent from those of goat [7]. It was of interest, therefore, to investigate if Ca^{2+} stimulated proteolysis and crosslinking of the goat and sheep erythrocyte membrane proteins.

Freshly collected blood was used in all the experiments. Blood from various animals was collected either by cardiac or venipuncture into 0.33 vol. of acid/citrate/dextrose (ACD). Human blood in ACD was collected from apparently healthy donors. The erythrocytes were collected by centrifugation at $1000 \times g$ for 10 min. The cells obtained were washed three times with 154 mM NaCl. The buffy coat was removed by aspiration after each centrifugation. The ghosts were prepared from the washed erythrocytes by hemolysing in 40 vol. of 5 mM Tris-HCl buffer (pH 8.0) followed by washing with the same buffer as described by Fairbanks et al. [8]. In order to induce Ca^{2+}-mediated crosslinking, the packed erythrocytes were hemolysed by mixing with 10 vol. of 5 mM Tris-HCl (pH 8.0) containing 2.5 mM $CaCl_2$. The hemolysate was incubated at 37°C for 10 min, prior to separation of the membranes by centrifugation. Ionophore A23187 was used to load the erythrocytes with calcium [3]. A 5 mM stock solution of ionophore A23187 was prepared in DMSO and was diluted 5-fold with 5 mM Tris-HCl (pH 8.0) containing 0.06 M NaCl, 0.1 M KCl and 10 mM glucose (buffer A). 2 ml of the (50%) erythrocytes suspension in isotonic saline buffered with 5 mM Tris-HCl (pH 8.0), was mixed with the ionophore solution in buffer A to yield a final ionophore concentration of 20 μM. Calcium was

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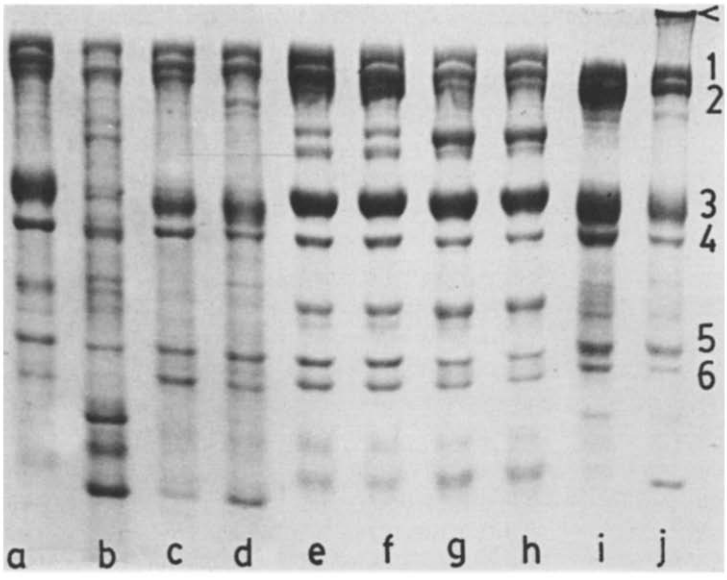
added to the suspension to a final concentration of 2.5 mM. The mixture was incubated for 2 h at 37 ° C. Following incubation, the erythrocytes were washed twice at 4 ° C with buffer A containing 1 mM EDTA and once with buffer A without EDTA. The cells were hemolysed, washed and the hemoglobin-free ghosts obtained were solubilised in 2% SDS. Staining and destaining of the gels were carried out as described by Fairbanks et al. [8]. Electrophoresis was carried out on a 3–15% polyacrylamide gradient gel with 0.1% SDS by using the method of Fairbanks et al. [8].

Several reports indicate that Ca^{2+}-dependent/stimulated processes, characteristic of several mammalian species, are absent from the sheep and goat erythrocytes. These include the deficiency of Ca^{2+}-sensitive phosphoinositide phosphodiesterase [9] and lack of membrane protein phosphorylation in response to Ca^{2+} and calcium ionophore treatment [10]. The latter effect is related to lack of diacylglycerol lipids in the outer layer of the membrane [11]. More recently, Farooqui et al. [12] have shown that the goat erythrocytes are also completely resistant to calcium and phosphate induced fusion. As shown in Fig. 1, the sheep and goat erythrocytes do not exhibit significant Ca^{2+}-stimulated crosslinking or proteolysis. In contrast, Ca^{2+} stimulated the formation of large molecular weight protein adducts, which was accompanied by 60–70% disappearance of band 3 and polypeptide of M_r 78000 was observed in case of the human erythrocytes as also reported earlier by Carraway et al. [13] and Lorand et al. [14]. In rat erythrocyte membrane, polypeptide bands corresponding to M_r 200000 and 95000 almost disappeared and the polypeptide of M_r 80000 was found to undergo very marked degradation. Some new polypeptide bands corresponding to M_r 55000, 50000, 28000, 25000 and 20000 were located in the gel. The band with an apparent molecular weight of 20000 in Ca^{2+}-treated membranes might represent the hemoglobin protomer, since Ca^{2+} are known to promote binding of hemoglobin with the membranes. It has been shown earlier that rat erythrocyte membrane contains highly active Ca^{2+}-stimulated proteinases [5]. Disappearence of M_r 190000 polypeptide and appearance of those of two new bands of M_r 170000 and 165000 were observed in case of rabbit erythrocyte membrane exposed to Ca^{2+} Ca^{2+}-stimulated degradation of membrane glycoproteins observed in case of human erythrocyte membrane [14] could not be observed in goat erythrocyte membrane (data not given). Evidently, the lack of Ca^{2+}-mediated effects are not related to the loss or dilution of essential factors since the calcium loading of the goat erythrocytes with the help of ionophore A23187 also produced no effects related to transglutaminase or proteinase action (Fig. 2). Under similar condition, Ca^{2+}-mediated effects were clearly evident in human erythrocyte membrane. Raval and Allan [10], however, observed significant degradation of band 4.1 in Ca^{2+}-treated sheep erythrocyte membrane.

The membrane-free hemolysate obtained from the goat erythrocytes treated with 2.5 mM $CaCl_2$ and ionophore was capable of inducing the crosslinking and proteolysis in partially washed human erythrocyte membranes (Fig. 3). This suggests that Ca^{2+} concentration is indeed elevated in the goat erythrocytes incubated with $CaCl_2$ and ionophore. The partially washed human erythrocyte membranes apparently contain adequate transglutaminase and could be crosslinked by incubation with Ca^{2+} alone. Incubation of washed goat erythrocyte membrane with membrane-free human hemolysate in presence of 2.5 mM $CaCl_2$ also induced crosslinking and loss of some membrane proteins (Fig. 4). Evidently, the goat erythrocyte membrane proteins are not refractive to the transglutaminase-mediated crosslinking and lack of Ca^{2+}-mediated response is the result of the enzyme deficiency. Absence of these and other Ca^{2+}-mediated responses like depletion of cellular ATP, increase in rigidity [15], loss of surface glycopeptides (our unpublished observation) that have been implicated in red cell aging and senescence suggest the operation of Ca^{2+}-independent processes in these cells. Ca^{2+}-mediated, transglutaminase catalysed crosslinking and proteolysis appear to be independent processes as the latter can be specifically inhibited with pepstatin [14]. The two processes may be, however, interconnected since inhibitors of transglutaminase with no known effects on proteinases, also restrict Ca^{2+}-activated proteolysis in human erythrocyte membrane [14,16]. It has been suggested that aggregation of membrane proteins resulting from



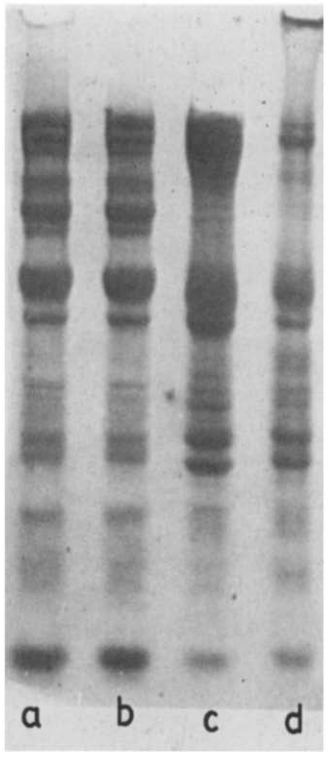


Fig. 1. (Left panel) Effect of Ca^{2+} on the membrane proteins of the erythrocytes derived from various mammals. Rat (a, b), rabbit (c, d), sheep (e, f), goat (g, h) and human (i, j) erythrocytes were hemolysed, incubated with or without 2.5 mM $CaCl_2$ for 10 min at 37 °C and were washed several times with 5 mM Tris-HCl (pH 8.0). The hemoglobin-free membranes were subjected to polyacrylamide gel electrophoresis in presence of 0.1% SDS as described in the text. Lanes (a, c, e, g and i) represent membranes incubated in absence of Ca^{2+}. The bands are numbered according to the system of Fairbanks et al. [8]. The arrow represents the top of the gel.

Fig. 2. (Right panel) Effect of Ca^{2+} loading on the membrane proteins of the human and goat erythrocytes. Human or goat erythrocytes were incubated with 2.5 mM $CaCl_2$ and 20 μM calcium ionophore A23187 for 30 min at 37 °C as described in the text. The cells were subsequently washed and hemolysed and the ghosts obtained were subjected to polyacrylamide gel electrophoresis. Lanes (a) and (c) represent membranes derived, respectively, from the goat and human erythrocytes incubated with $CaCl_2$ but without ionophore. Lanes (b) and (d) contained membranes derived from calcium and ionophore-treated goat and human erythrocytes, respectively.

transglutaminase action might produce conformations favourable for proteinase action [17].

Vincenzi et al. [7] have found very low (Ca^{2+} + Mg^{2+})-ATPase activity in the goat erythrocytes. We have also observed that the (Ca^{2+} + Mg^{2+})-ATPase activity of goat red blood cells membrane is about 200-times lower than that of the human erythrocyte membrane. Very low levels of (Ca^{2+} + Mg^{2+})-ATPase are likely to lead to high intracellular calcium concentration. Indeed our preliminary results show that goat erythrocytes contain 3–5-times more Ca^{2+}, as measured by atomic absorption spectroscopy, as compared to the human erythrocytes. Thus, unlike in human erythrocytes, Ca^{2+} may not play the role of regulatory ion in these erythrocytes. Apparently, not all the Ca^{2+}-mediated processes are absent in these cells since the goat and sheep erythrocytes have been shown to contain adequate calmodulin activity [7]. It is of interest to note that the erythrocytes of new born sheep unlike those of adult sheep [7] contain higher levels of (Ca^{2+} + Mg^{2+})-ATPase and Ca^{2+}-induced rapid ATP depletion and morphological alterations in these cells [14]. The

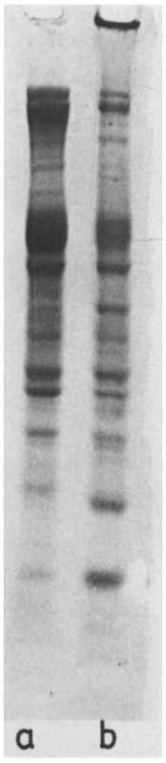


Fig. 3. (Left panel) Crosslinking of the human erythrocyte membrane proteins incubated with membrane-free hemolysate derived from the calcium-loaded goat erythrocytes. Goat erythrocytes were loaded with 2.5 mM $CaCl_2$ as described in text. The cells were hemolysed by mixing with three parts of 5 mM Tris-HCl (pH 8.0). The hemolysate was centrifuged at $11000 \times g$ to sediment membranes and three parts of the clear supernatant obtained was incubated with one part of the partially washed human erythrocyte ghosts (obtained by hemolysing the erythrocytes in 40 vol. of 5 mM Tris-HCl (pH 8.0) and subjecting them to only two washes with the same buffer) for 10 min at 37° C. The membranes were subsequently washed several times with 5 mM Tris-HCl (pH 8.0) buffer and subjected to polyacrylamide gel electrophoresis. Lanes (a) and (b) represent control and the hemolysate-treated human erythrocyte membranes, respectively.

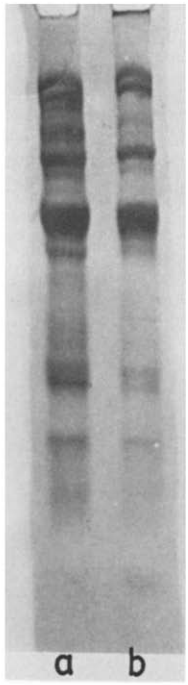


Fig. 4. (Right panel) Effect of Ca^{2+} and human erythrocyte hemolysate on the goat erythrocyte membrane proteins. The hemoglobin-free goat erythrocyte ghosts were treated with three parts of membrane-free human erythrocyte hemolysate prepared as described earlier, for 10 min at 37° C. Ca^{2+} was added to a final concentration of 2.5 mM in the incubation mixture. The ghosts were washed and subjected to electrophoresis (b). Lane (a) represents the control ghosts incubated under similar conditions but without Ca^{2+}.

Ca^{2+}-mediated effects, thus seem to be replaced by alternative mechanism during the maturation and growth of the animal. We are investigating these mechanisms in detail.

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