Biochimica et Biophysica Acta 904 (1987) 259–267
Elsevier

BBA 73745

Modulation of red cell vesiculation by protease inhibitors

Peter Bütikofer, Urs Brodbeck and Peter Ott *

Institut für Biochemie und Molekularbiologie der Universität Bern, Bern (Switzerland)

(Received 5 May 1987)

Key words Vesicle release, Proteinase inhibitor, Membrane penetrating drug, Shape change; Erythrocyte membrane, (Human erythrocyte)

Release of vesicles from human red cell membranes was induced either by ATP-depletion or by incubation of the cells in presence of sonicated dimyristoylphosphatidylcholine (DMPC) vesicles. Vesicles released from ATP-depleted red cells but not the DMPC-induced vesicles contained degradation products of band 3 protein. Furthermore, in ATP-depleted erythrocytes proteolytic breakdown products could be demonstrated that were not detected in cells incubated with DMPC. Proteolysis was neither significantly affected by the protease inhibitor *N*-α-tosyl-L-lysine chloromethyl ketone (TLCK) nor by other protease inhibitors tested in this study (diisopropylfluorophosphate, *N*-ethylmaleimide and phenylmethylsulfonyl fluoride). Both vesiculation processes were inhibited in a concentration dependent way by TLCK while other protease inhibitors did not significantly influence membrane vesiculation. Phase contrast microscopy showed that TLCK diminished the DMPC-induced formation of echinocytes which is known to precede vesicle release. These results suggest that the influence of TLCK on membrane vesiculation is not primarily due to inhibition of proteolysis but to a direct interaction of the inhibitor with the intrinsic domain of the erythrocyte membrane.

Introduction

Human red blood cells have been shown to release membrane vesicles under a variety of conditions [1–6] such as ATP-depletion or incubation of the cells in presence of dimyristoylphosphatidylcholine (DMPC) [1,6]. Generally, a shape change from discocytes to echinocytes and spheroechinocytes [7] is observed prior to vesicle release. The released structures contain all major transmembrane proteins to various extents but are usually devoid of spectrin [1–6,8]. The four major membrane phospholipid species are present in a similar quantitative distribution as in the red cell membrane. In membrane vesicles released from ATP-depleted red cells several low molecular weight bands are observed by sodium dodecyl sulfate polyacrylamide gel electrophoresis analysis that are usually not found in erythrocyte membranes and have to be considered as proteolytic breakdown products of membrane proteins [1,2,9–11].

Although the biochemical changes that occur during ATP-depletion of human red blood cells [12–17] have been discussed in some detail, the mechanism by which vesicles are finally released remains unknown. As previously suggested [18,19] vesiculation processes may have to some extent a common mechanism which involves as the final step a membrane fusion event.

Membrane fusion has been described to be

Abbreviations DMPC, dimyristoylphosphatidylcholine, TLCK, *N*-α-tosyl-L-lysine chloromethyl ketone

Correspondence: P Ott, Institut fur Biochemie und Molekularbiologie der Universitat Bern, Buhlstrasse 28, CH-3012 Bern, Switzerland.

mediated by changes in the organization of the lipid and protein components of membrane structures [20,21]. Recently, it has been proposed that proteolytic fragments of proteins are able to promote membrane fusion [22]. In line with this hypothesis it has been reported that chlorpromazine-induced fusion of erythrocytes and the associated proteolytic breakdown of the the membrane protein ankyrin were significantly impeded by the protease inhibitor *N*-α-tosyl-L-lysine chloromethyl ketone [23].

It is therefore reasonable to assume that fusogenic proteolytic fragments may also be of importance in the modulation of vesicle release from red blood cells. The present communication describes the effect of various protease inhibitors on red cell vesiculation, triggered by two different methods, ATP-depletion and incubation with DMPC.

Materials and Methods

Biological material Fresh human blood samples from healthy adult donors were obtained from the Central Blood Bank of the Swiss Red Cross. Erythrocytes were separated from plasma by centrifugation at 2500 rpm for 10 min at 4° C and washed three times with 10 mM Tris-HCl (pH 7.4) containing 144 mM NaCl. In each step the buffy coat and supernatant were carefully removed. The rabbit anti-human erythrocyte membrane antibody was obtained as immunoglobulin fraction in 0.1 M NaCl and 15 mM NaN_3 (Code A 104) from Dako-Immunoglobulins (Copenhagen, Denmark). Monoclonal mouse anti-human erythrocyte band 3 protein antibody was obtained as a culture supernatant and was a kind gift of Professor C. Bron from the Department of Biochemistry, University of Lausanne (Switzerland). Affinity purified goat anti-mouse IgG horseradish peroxidase-conjugate was from Bio-Rad Laboratories (Richmond, CA, U.S.A.).

Reagents. Dimyristoylphosphatidylcholine (DMPC) and bovine serum albumin were obtained from Sigma (St. Louis, MO, U.S.A.). 1,2-Di[1-^{14}C]myristoylphosphatidylcholine and glycerol tri[9,10(n)-^{3}H]oleate were supplied by Amersham International (Amersham, U.K.). Emulsifier scintillator solution Lipotron was from Kontron Analytical (Zürich, Switzerland). Penicillin was purchased from Novo Industri (Copenhagen, Denmark) and steptomycin-sulfate was from Grogg Pharmaceutical Products (Bern, Switzerland). Agarose A was obtained from Pharmacia Fine Chemicals (Uppsala, Sweden). Nitrocellulose sheets (0.45 μm) and all reagents for sodium dodecylsulfate polyacrylamide gel electrophoresis were purchased from Bio-Rad Laboratories (Richmond, CA, U.S.A.). Boehringer Monotest for cholesterol determinations was obtained from Boehringer (Mannheim, F.R.G.). Reagents for protein determinations were from Pierce Chemicals (Rockford, IL, U.S.A.). All other reagents were standard commercial products obtained either from Fluka AG (Buchs, Switzerland) or from Merck (Darmstadt, F.R.G.) and were of the highest purity available.

Assays. Acetylcholinesterase activity was determined according to Ellman et al. [24]. Lipids were extracted using the method of Rose and Oklander [25] and phospholipid phosphorus was determined according to Rouser [26]. Cholesterol was measured as described by Ott et al. [27]. Radioactivity was measured with a Kontron Betamatic II liquid scintillation counter. Protein was determined in microtiter plates using the bicinchoninic acid assay [28].

Erythrocyte vesiculation. ATP-depletion of erythrocytes and release of membrane vesicles was carried out as described by Lutz et al. [1]. Briefly, red cells (16% final hematocrit) were incubated at 37° C in a shaking waterbath in 10 mM Tris-HCl buffer (pH 7.4) containing 144 mM NaCl, and supplemented with $2 \cdot 10^5$ U/l penicilline and 1 5 10^5 U/l streptomycine (referred to as incubation buffer). Glucose (11.1 mM), adenine (0.54 mM) and inosine (12.7 mM) were added to the incubation buffer in experiments, where ATP levels were maintained. Protease inhibitors were added to the incubation mixtures from stock solutions in methanol (TLCK, 500 mM; phenylmethylsulfonyl fluoride, 500 mM), or isopropanol (diisopropylfluorophosphate, 50 mM), or buffer (*N*-ethylmaleimide, 100 mM). The respective solvents were added to control samples in each experiment in order to exclude an effect of the solvent itself on the vesiculation process. At appropriate times, red cells were pelleted by centrifugation (2500 rpm, 5 min., 4° C) and vesicle release was monitored in

the cell free supernatant. This was performed either (as previously described [6]) by measuring acetylcholinesterase activity, or by phospholipid quantitation. For purification and concentration of vesicles, the supernatant was centrifuged at 17000 rpm (22000 × g_{av}) for 12 min at 4°C in the Centrikon T 2070 ultracentrifuge equipped with an 8 × 38 ml angle rotor. After prolonged incubation the vesicle pellet was occasionally found to be overlaid with a fluffy layer of erythrocyte ghosts which was easily removed by aspiration without noticeable loss of membrane vesicles [8,29]. The vesicle pellet was resuspended in 10 mM Tris-HCl buffer (pH 7.4) containing 144 mM NaCl, and was either used immediately for quantitation of phospholipid or frozen and stored at −80°C until analyzed.

DMPC-induced release of vesicles was carried out as described previously [6,18,30]. Briefly, washed erythrocytes (8% final hematocrit) were incubated in presence of sonicated DMPC vesicles (0.5 mg/ml final concentration) in incubation buffer containing 1 mM EDTA and supplemented with glucose, adenine, inosine (as indicated above) at 30°C in a shaking water bath. Vesicle release was monitored by measuring acetylcholinesterase activity in the cell free supernatant. At the end of the incubation periods, remaining red cells were washed twice with incubation buffer and immediately frozen (−80°C)

To determine the rate of incorporation of DMPC into erythrocyte membranes, trace amounts of [^{14}C]DMPC and glycerol tri[^{3}H]oleate were added to the lipid dispersion before sonication, exactly as described before [18].

For characterization of cell morphology, erythrocytes were fixed in 2.5% glutaraldehyde in 0.03 M potassium phosphate buffer and subsequently examined by phase contrast light microscopy.

Protein analyses. Red cells for electrophoresis were lysed in 10 volumes of cold 5 mM Tris-HCl (pH 7.4) and the membranes pelleted for 10 min at 12000 rpm in a Hettich Microliter centrifuge. The pellet was resuspended in the above buffer and recentrifuged. This was repeated twice and the pink membranes were immediately used for protein analysis. Electrophoresis was carried out in 8-18% acrylamide slab gels using the Laemmli buffer system [31]. Unless otherwise indicated, 70–80 μg of protein were applied per lane. Electrophoresis followed by electroblotting was performed according to the method of Towbin et al. [32], using antibody concentration and staining procedures as described elsewhere [33].

Membrane solubilization and subsequent crossed immunoelectrophoresis were performed essentially according to Bjerrum and Bøg-Hansen [34] as described before [33,35]. Erythrocyte vesicles were solubilized by intermittent sonication for 2–5 seconds in 10 mM Tris-HCl buffer (pH 7.4) containing 144 mM NaCl and 1% (w/v) Triton X-100. Unsolubilized material which accounted for less than 5% of the total membrane protein [35] was removed by centrifugation for 90 min at 130000 × g and 4°C in an MSE Superspeed 65 centrifuge equipped with a 10 × 10 ml titanium fixed angle rotor. Samples were used for crossed immunoelectrophoresis immediately after centrifugation. Identification of proteins after crossed immunoelectrophoresis and electrotransfer to nitrocellulose membranes ('crossed immunoblotting') was carried out exactly as described before [33].

Results

Protein analysis of vesicles is shown in Figs. 1 and 2. Sodium dodecyl sulfate polyacrylamide gel electrophoresis of vesicles obtained by ATP-depletion of human red blood cells for 48 h showed several bands in the region between band 3 protein and band 5 (actin) and below band 6 (Fig. 1A, lane 2; see also Refs. 1, 2, 9–11). These bands are not observed with native red cell membranes and are usually indicative for band 3 degradation [36]. Electroblotting revealed that several of these bands reacted with a monoclonal anti-erythrocyte band 3 antibody (Fig. 1B, lane 2). In crossed immunoelectrophoresis – used as a second method for protein analysis – an unidentified precipitate was observed when a polyclonal anti-erythrocyte membrane antibody (Fig. 2A, indicated by the arrow) was included in the system This precipitate was further characterized by 'crossed immunoblotting' – a method which allows identification of immunoprecipitates after crossed immunoelectrophoresis and subsequent electro-

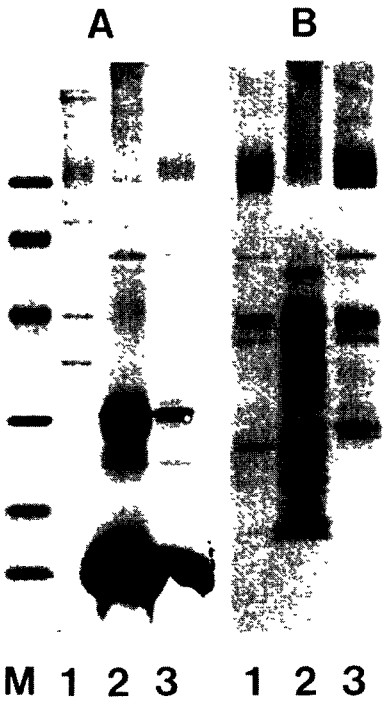


Fig 1 Protein analysis of released vesicles Membrane vesicles (approx 40 μg of protein) obtained from red cells either by ATP-depletion (lanes 2) or incubation with DMPC (lanes 3) were subjected to polyacrylamide gel electrophoresis and electroblotting as described in Materials and Methods After electrotransfer the nitrocellulose membranes were either stained immediately with amidoblack (panel A), or incubated with monoclonal anti-band 3 (panel B) antibodies and visualized with horseradish peroxidase-conjugate second antibodies. The control patters obtained with untreated cell membranes are shown in lanes 1 The gel was calibrated (lane M) with phosphorylase *b* (M_r 94000), bovine serum albumin (M_r 67000), ovalbumin (M_r 43000), carbamic anhydrase (M_r 30000), soybean trypsin inhibitor (M_r 20100) and α-lactalbumin (M_r 14400)

blotting [33] – and was shown to be immunological related to band 3 protein (Fig. 2B). DMPC-induced vesicles on the other hand, showed significantly less degradation products in electroblotting analysis (Fig. 1B, lane 3). By crossed immunoelectrophoresis no proteolysis was detected (result not shown; a crossed immunoelectrophoresis pattern of DMPC-induced vesicles is shown in Ref. 35).

The pattern observed in polyacrylamide gel electrophoresis of remnant cells is shown in Fig. 3. As compared to untreated control cells (lanes a and f) only minor differences were seen in the protein pattern of erythrocytes incubated for 48 h with maintained intracellular ATP levels (lane b). With ATP-depleted cells a significant number of breakdown products were observed (lane c), and a comparable pattern was observed when the incubations were carried out either in the presence of 1–5 mM EGTA or 0.1–10 mM EDTA (lane e). A similar protein pattern was also obtained when diisopropylfluorophosphate, phenylmethylsulfonyl fluoride and *N*-ethylmaleimide, respectively, were present during incubation. Only minor differences in the degradation pattern could be detected when TLCK was used as a protease inhibitor (lane d). The remnant cells obtained after DMPC-induced vesiculation revealed a similar membrane protein pattern as untreated erythrocytes (lane g)

Although no clear influence of protease inhibitors on the protein degradation was recognizable, there was a remarkable effect of TLCK on the vesiculation processes. Vesicle release as a consequence of ATP-depletion was significantly reduced and the observed effect was concentration dependent. After a total incubation time of 48 hours, vesiculation – determined with acetylcholinesterase as marker – was inhibited by 27–31% in presence of 1 mM TLCK and by 40–45% in presence of 2 mM TLCK. When phospholipid was used as marked for vesicle release the corresponding values were 30–34% and 37–42%, respectively On the other hand, diisopropylfluorophosphate, phenylmethylsulfonyl fluoride, and *N*-ethylmaleimide had no significant effect. Vesicle release after 48 h in absence of inhibitor was taken as 100% value.

In DMPC-induced vesiculation, TLCK treatment resulted in a similar decrease of vesiculation (Fig. 4) as in the case of ATP-depletion, although no significant proteolysis could be observed after the relatively short incubation periods used. The presence of 2 mM TLCK decreased the vesicle release – measured in terms of acetylcholinesterase [6] – by 46%, whereas 4 mM TLCK inhibited vesiculation by 80%. The vesiculation process was similarly inhibited (by 68% and 84%, respectively) when TLCK (4 mM and 6 mM, respectively) was added to red blood cells after 60 min of incubation in presence of DMPC (Fig. 5A). On the other hand, vesiculation was barely inhibited (by 18% and 23%, respectively, when erythrocytes were

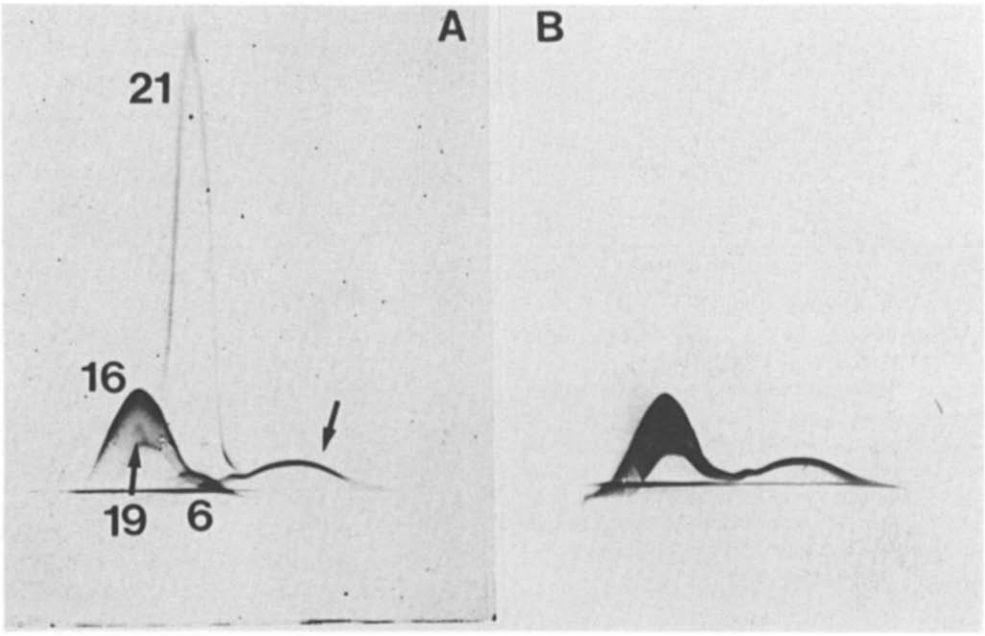
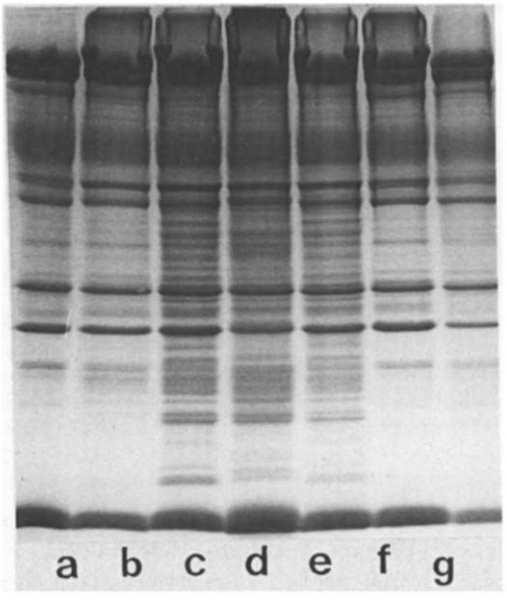


Fig. 2. Protein analysis of released vesicles Membrane vesicles were obtained by ATP-depletion of erythrocytes and subjected to crossed immunoelectrophoresis as described in Materials and Methods (A) The precipitates were stained and numbered according to Bjerrum and Bøg-Hansen [34], with No. 16 representing band 3 protein, No 21 glycophorin, and No 6 originating from minor amounts of spectrin that are present in vesicles obtained by ATP-depletion [1,2,36] Precipitate No 19 represents a yet unidentified protein. The arrow indicates a precipitation arc that is not observed in ghosts or untreated red cells, nor in DMPC-induced vesicles [36] (B) Proteins separated by crossed immunoelectrophoresis were transferred to nitrocellulose membranes ('crossed immunoblotting') and proteins immunologically related to band 3 protein were identified using monoclonal anti-band 3 antibodies [34]. For details see Materials and Methods. The procedure clearly identifies the precipitate in (A), indicated by the arrow, as being derived from band 3 protein



pretreated with TLCK (4 mM and 6 mM, respectively) in incubation buffer for 40 min at 30 °C, subsequently washed with buffer and then incubated with a DMPC suspension that did not contain TLCK (Fig. 5B). DMPC-induced vesicula-

Fig 3 Protein analysis of remnant red cell membranes. The control patterns obtained with untreated cell membranes are shown in lanes (a) and (f) Erythrocytes were incubated, washed and processed for polyacrylamide gel electrophoresis as described in Materials and Methods The pattern obtained from erythrocytes incubated for 48 h in presence of glucose, adenine, inosine, in order to maintain intracellular ATP levels, is shown in lane b. The patterns obtained after ATP-depletion are shown in lanes c through e. The cells were incubated for 48 h as described in Materials and Methods The incubations were carried out without protease inhibitor (c), or in presence of 2 mM TLCK (d), or 1 mM EGTA (e). The pattern obtained from erythrocytes incubated for 5 h in presence of DMPC is shown in lane g

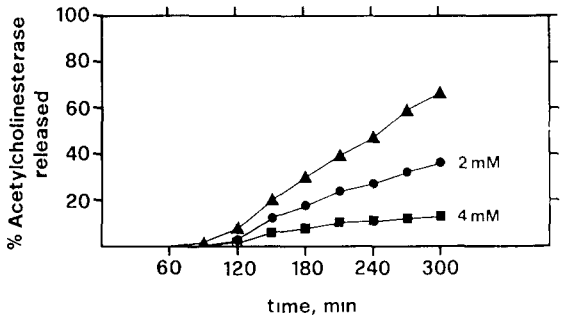


Fig 4 DMPC-induced vesiculation of human erythrocytes in the presence of TLCK. Red cells were incubated with sonicated suspensions of DMPC and release of membrane vesicles was followed by measuring acetylcholinesterase in the cell free supernatant as described in Materials and Methods Acetylcholinesterase activity in the vesicle fraction was expressed as % of total activity in the suspension The total activity remained constant during the entire incubation period TLCK was added at the begin of incubation in the concentrations indicated

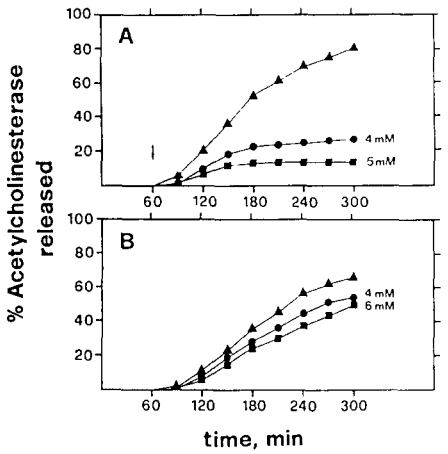


Fig 5 DMPC-induced vesiculation of human erythrocytes in the presence of TLCK Experimental conditions were as described in Fig 4, except that in (A) TLCK (4 mM and 6 mM, final concentration) was added to red cells after 60 minutes of incubation (indicated by the arrow) In (B) red cells were preincubated with TLCK (4 mM and 6 mM, respectively) for 30 min at 30 °C without DMPC The inhibitor was then removed by washing the red cells in 2×10 volumes of incubation buffer, and incubation of the cells was continued in the presence of sonicated DMPC as described in Materials and Methods

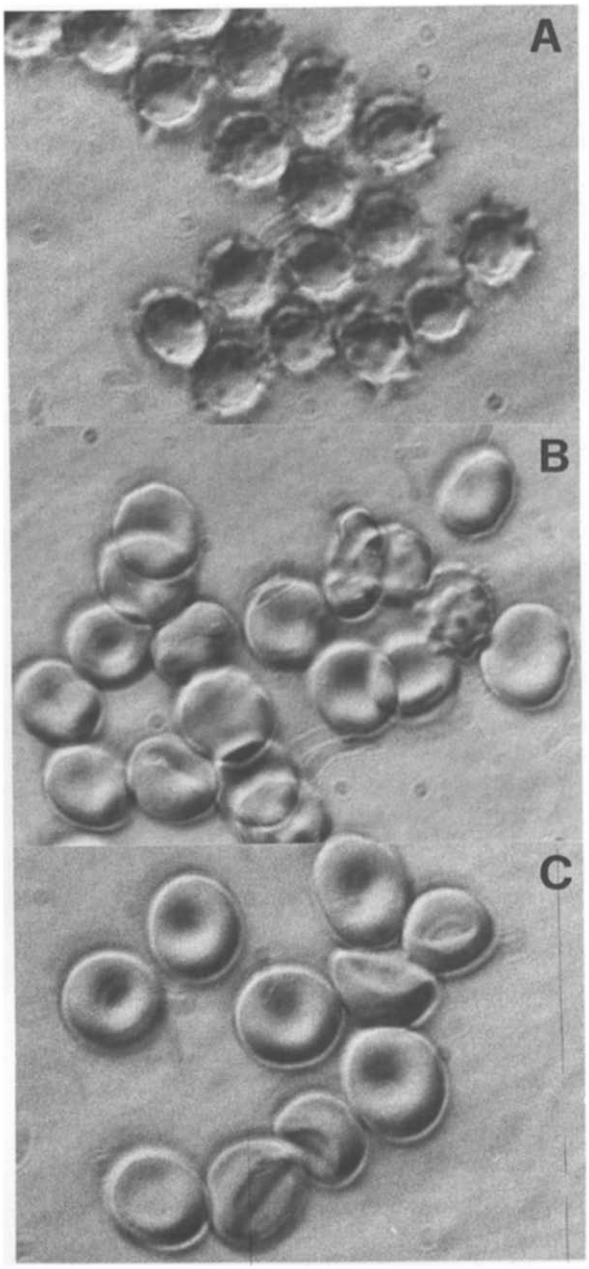


Fig 6 Phase contrast light microscopy of erythrocytes Red cells were incubated for 60 min at 30 °C with sonicated suspensions of DMPC in absence (A) or presence (B) of 4 mM TLCK (final concentration) or in absence of DMPC but with 4 mM TLCK (C) and prepared for microscopy as described in Materials and Methods

tion was not affected by 50 μM *N*-ethylmaleimide.

Phase contrast microscopy showed that TLCK had a clear influence on the shape of red cells (Fig. 6). The formation of echinocytes as a consequence of a one hour incubation with DMPC (Fig. 6A) was significantly reduced in presence of 4 mM

TLCK (Fig. 6B). Furthermore, a significant stomatocyte formation was induced by TLCK in absence of DMPC. After one hour of incubation in presence of 4 mM TLCK, the counting of a total population of 350 cells showed 58% of stomatocytes (Fig. 6C) while no stomatocytosis could be observed in absence of TLCK.

Discussion

The results presented in this paper show that ATP-depletion of red blood cells results in a significant formation of proteolytic breakdown products (Figs. 1–3). By two different methods, polyacrylamide gel electrophoresis and 'crossed immunoblotting' [33] proteolytic degradation products were demonstrated in vesicles released from ATP-depleted erythrocytes as well as in the corresponding remnant cells.

The mechanism by which such proteolysis may occur as a consequence of ATP-depletion is not yet elucidated. It should be noted that similar protein patterns have been described for erythrocytes incubated with high concentrations of calcium [37–39]. While the calcium concentrations used for protease activation in those studies were much higher than what would be expected in ATP-depleted cells, it should be noted that much longer incubation periods were used in the present study. Such prolonged incubation may well be responsible for a slow activation of endogenous proteases [39–41]. In line with this notion, protein degradation was not detected in vesicles and remnant red cells after the relatively short incubation for five hours in presence of DMPC (Fig. 1). Consequently, the generation of band 3 degradation products and the changes in membrane protein pattern are not a common feature of red cell vesiculation, but seem to be the consequence of ATP-depletion of erythrocytes combined with the long incubation periods of 48 h.

TLCK showed similar effects on the extent of vesiculation in both systems investigated (Fig. 4), in spite of its failure to block proteolysis effectively, and in spite of the differences in membrane protein degradation observed between the two incubation procedures. Hence, it appears that modulation of the vesiculation processes by TLCK is not primarily due to protection of membrane proteins from proteolysis but to its direct interaction with the intrinsic domain of the erythrocyte membrane. The influence of TLCK on red cell shape (Fig 6) supports this idea and suggests that its primary action is the inhibition of echinocyte formation. In line with this interpretation are previous reports which have shown that echinocyte shape change can be reversed by agents that promote stomatocyte formation [42–44] and that some of these agents have recently been shown to inhibit red cell vesiculation [45]. At present, however, the possibility that TLCK also prevents proteolysis of some minor protein component – that is not detected in electrophoresis – cannot completely be ruled out.

A variety of membrane penetrating agents that are known to affect red cell shape and shape change have been shown to act via expansion of either outer or inner monolayer of red blood cell membranes thus leading to the formation of echinocytes (creators) and stomatocytes (cup formers) [42–44]. The cup formers are believed to partition into the inner leaflet of the membrane bilayer due to their positively charged headgroups [42–44]. Accordingly, it can be assumed that TLCK, due to the positively charged lysine residue, will accumulate on the inner half of the red cell membrane and inhibit vesiculation by a similar mechanism as amphiphilic cationic drugs [45] An influence of TLCK on DMPC uptake into red blood cells [18,19] or on the extraction of membrane cholesterol – both prerequisites for DMPC-induced vesiculation [19] – can be excluded since these processes are comparable in presence and absence of TLCK (results not shown).

TLCK has been reported to be involved in regulation of erythrocyte shape [46,47] via an activation of the hexose monophosphate shunt. However, from the present study it appears that the inhibition of vesiculation by TLCK is not dependent on an active glucose metabolism of the cell, since the inhibitor decreases vesiculation both of ATP-depleted cells that are incubated for 48 h in absence of glucose and of DMPC-treated erythrocytes that are incubated for 5 h in presence of glucose. It seems unlikely that TLCK would affect ATP-depleted red cells in a similar way as metabolically active cells if its action were on a metabolic pathway. This interpretation is corrobo-

rated by two further observations. First, DMPC-induced vesiculation is reduced to a similar extent by TLCK when red cells are incubated in presence of the inhibitor throughout the experiment, or when the inhibitor is added after 60 min of incubation, i.e. only shortly before the onset of vesicle release (Fig. 5). Second, an only moderate influence on DMPC-induced vesiculation is observed when red cells are pretreated with TLCK and subsequently incubated with DMPC in a suspension devoid of inhibitor (Fig. 5). If the inhibitor would act in an irreversible and covalent mode on the hexose monophosphate shunt [46] just the opposite behavior would be expected.

Several effects of TLCK on cellular processes in different cell types have been described. These include the inhibition of calcium induced vesiculation of chicken erythrocytes [48] and suppression of protein secretion from liver cells [49]. Furthermore, the shape of virus transformed cells has been reported to be restored by TLCK [50,51] However, the mechanism of action remained largely unexplained. In light of our study, it is well possible that TLCK – besides being a protease inhibitor – acts via direct interactions with the intrinsic domain of the membrane structures involved.

Acknowledgements

We gratefully acknowledge the excellent technical assistance of Miss. A. Wegmüller and M. Boschung during part of this study as well as the photographic work of Miss S. Jungi. This work was supported by grant No. 3.251-0.85 of the Swiss National Science Foundation.

References

1 Lutz, H U , Liu, S C and Palek, J (1977) J Cell Biol 77, 548–560
2 Snyder, M L , Lutz, H U , Sauberman, N , Jacobs, J and Fortier, N L (1978) Blood 52, 750–761
3 Allan, D , Billah, M , Finean, J.B and Michell, R H (1976) Nature (London) 261, 58–60
4 Wagner, G M , Schwartz, R S , Chiu, D T -Y and Lubin, B H (1985) Clin Haematol. 14, 183–200
5 Herrmann, A , Lentsch, P., Lassmann, G , Ladhoff, A -M and Donath, E (1985) Biochim Biophys Acta 812, 277–285
6 Ott, P , Hope, M J , Verkleij, A J., Roelofsen, B , Brodbeck, U and Van Deenen, L L M (1981) Biochim Biophys. Acta 641, 79–87
7 Bessis, M and Mandon, P (1972) Nouv Rev Fr Hematol 12, 443–454
8 Allan, D , Thomas, P and Limbrick, A.R (1980) Biochem J 188, 881–887
9 Muller, H and Lutz, H U (1983) Biochim. Biophys Acta 729, 249–257
10 Shukla, S D , Berriman, J , Coleman, R , Finean, J B and Michell, R H (1978) FEBS Lett. 90, 289–292
11 Trautsch, C , Reimann, B , Gerber, G , Klatt, D , Ladhoff, A and Maretzki, D (1984) Haematology 17, 359–373
12 Patel, V P and Fairbanks, G (1981) J. Cell Biol. 88, 430–440
13 Palek, J , Liu, P A and Liu, S C (1978) Nature 274, 505–507
14 Clark, M R , Mohandas, N , Feo, C , Jacobs, M S and Shohet, S.B (1981) J Clin Invest 67, 531–539
15 Birchmeier, W and Singer, S J (1977) J Cell Biol 73, 647–659
16 Anderson, J.M and Tyler, J M (1980) J Biol Chem 255, 1259–1265
17 Feo, C J. and Mohandas, N (1977) Blood Cells 3, 153–161
18 Butikofer, P., Chiu, D T -Y , Lubin, B and Ott, P (1986) Biochim Biophys Acta 855, 286–292
19 Frenkel, E.J , Kuypers, F A , Op den Kamp, J A F , Roelofsen, B and Ott, P (1986) Biochim Biophys Acta 855, 293–301
20 Ahkong, Q F , Fisher, D , Tampion, W and Lucy, J A (1975) Nature 253, 194–195
21 Volsky, D J and Loyter, A (1978) Biochim Biophys Acta 514, 213–224
22 Lucy, J A (1984) FEBS Lett 166, 223–231
23 Lang, R D A , Wickenden, C , Wynne, J and Lucy, J.A (1984) Biochem J 218, 295–305
24 Ellman, G L , Courtney, D K , Andres, V and Featherstone, R M (1961) Biochem Pharmacol. 7, 88–95
25 Rose, H.G and Oklander, M (1965) J Lipid Res 6, 428–431
26 Rouser, G , Fleischer, S and Yamamoto, A (1970) Lipids 5, 494–496
27 Ott, P , Binggeli, Y. and Brodbeck, U (1982) Biochim Biophys Acta 685, 211–213
28 Sørensen, K and Brodbeck, U (1986) Experientia 42, 161–62
29 Wagner, G M., Chiu, D T -Y , Yee, M C and Lubin, B (1986) J Lab Clin Med 108, 315–324
30 Bütikofer, P. and Ott, P (1985) Biochim Biophys Acta 821, 91–96
31 Laemmli, U.K (1970) Nature 227, 680–685
32 Towbin, H , Staehelin, T and Gordon, J (1979) Proc Natl Acad Sci USA 76, 4350–4354
33 Butikofer, P , Frenkel, E J and Ott, P (1985) J Immunol Meth 84, 65–71
34 Bjerrum, O.J and Bøg-Hansen, T C (1976) Biochim Biophys Acta 455, 66–89
35 Weitz, M , Bjerrum, O J , Ott, P and Brodbeck, U (1982) J Cell Biochem 19, 179–191
36 Golovtchenko-Matsumoto, A M , Matsumoto, I and Osawa, T (1981) Eur J Biochem 121, 463–467

37 King, L E and Morrison, M. (1977) Biochim Biophys Acta 471, 162–168
38 Anderson, D.R , Davis, J L. and Carraway, K L (1977) J Biol Chem. 252, 6617–6623
39 Allan, D W and Cadman, S (1979) Biochim Biophys Acta 551, 1–9
40 Grasso, M , Morelli, A and De Flora, A (1986) Biochem Biophys Res Commun 138, 87–94
41 Corall, D E , Morrow, J S and DeMartino, G N (1986) Biochim Biophys. Acta 882, 287–296
42 Deuticke, B (1968) Biochim Biophys Acta 163, 494–500
43 Sheetz, M.P and Singer, S.J (1974) Proc Natl. Acad Sci USA 71, 4456–4461
44 Sheetz, M.P and Singer, S J. (1976) J Cell Biol. 70, 247–251
45 Butikofer, P , Brodbeck, U and Ott, P (1987) Biochim Biophys Acta 901, 291–295
46 Alhanaty, E and Sheetz, M.P (1984) Blood 63, 1203–1208
47 Alhanaty, E , Snyder, M and Sheetz, M P (1984) Blood 63, 1198–1202
48 Thomas, P , Limbrick, A R and Allan, D (1983) Biochim Biophys Acta 730, 351–358
49 Edwards, K , Nagashima, M , Dryburgh, H , Wykes, A and Schreiber, G. (1979) FEBS Lett 100, 269–272
50 Weber, M.J (1975) Cell 5, 253–261
51 Richert, N , Davies, P.J A , Jay, G and Pastan, I (1979) Cell 18, 369–374