Biochimica et Biophysica Acta 821 (1985) 91–96
Elsevier

BBA 72811

The influence of cellular ATP levels on dimyristoylphosphatidylcholine-induced release of vesicles from human erythrocytes

Peter Bütikofer and Peter Ott *

Medizinisch-chemisches Institut der Universität, P.O. Box, CH-3000 Berne 9 (Switzerland)

(Received July 10th, 1985)

Key words: Vesicle release; Dimyristoylphosphatidylcholine; ATP depletion; Echinocytosis; (Erythrocyte membrane)

Release of membrane vesicles from human erythrocytes was induced by modulation of red cell ATP levels, by incubation of erythrocytes with sonicated dimyristoylphosphatidylcholine (DMPC) suspensions, or by a sequential combination of both procedures. When red blood cell ATP levels were decreased prior to incubation with DMPC, the lag-time between addition of the lipid and beginning of vesiculation was reduced. Furthermore, the rate of vesicle release itself was accelerated. Experiments carried out with a rapid ATP depletion technique showed that the onset of vesiculation and the release were most evidently accelerated in those cases where echinocytes had been formed prior to the addition of DMPC. The results suggest that red blood cells with reduced cellular ATP levels or an altered cell shape are more susceptible to a further perturbation of the membrane by addition of exogenous DMPC.

Introduction

Shape changes of erythrocytes can be observed under a variety of conditions and have been related to changes in the structural properties of individual protein components of the membrane skeleton [1–5]. Likewise, membrane-penetrating agents such as lysophospholipids and amphipathic drugs have been shown to induce erythrocyte shape changes by triggering a perturbation of the lipid bilayer balance [6]. Either stomatocytes or echinocytes can be formed [7].

Echinocytes can release membrane vesicles as a consequence of prolonged incubation in glucose-free medium with concomitant ATP depletion [8], or by increasing the intracellular calcium levels [9]. On the other hand, incubation of red cells with sonicated suspensions of dimyristoylphosphatidylcholine (DMPC) or dilauroylphosphatidylcholine results in vesicle release without ATP depletion of the cells [10,11]. Vesicle release has also been observed as a consequence of repeated deoxygenation and reoxygenation of sickle cells [12].

While the phospholipid composition of erythrocytes and vesicles appears to be comparable, clear differences are observed with regard to their respective protein composition. Integral membrane proteins, such as acetylcholinesterase, band 3 protein and glycophorin are present in vesicles but only traces of cytoskeletal components can be discovered [8,9,12,13]. This strongly indicates that interactions within the membrane and the membrane skeleton [2,14] have to be changed or abolished prior to vesicle release.

The mechanisms by which red cells can be stimulated to release vesicles are still obscure. Both ATP depletion of erythrocytes or incubation of cells with sonicated suspensions of DMPC first results in formation of echinocytes and then in release of vesicles. Under conditions of metabolic

* To whom correspondence should be addressed.
Abbreviation: DMPC, dimyristoylphosphatidylcholine.

starvation, more than 20 h of incubation time are required to achieve ATP depletion and initiate vesicle release [8]. On the other hand, physiological ATP levels are maintained and only 4 h are required for vesicle release in the presence of DMPC [10]. These observations suggest that various mechanisms are effective in triggering a release of vesicles from red blood cells.

To obtain further information on the processes involved in vesiculation, combined incubation procedures were applied. Cell ATP levels were modulated by various preincubation protocols prior to addition of DMPC and the velocity of DMPC-induced vesiculation was analyzed in cells at different stages of metabolic depletion. The results show that factors known to influence the integrity of the membrane skeleton strongly affect the lag-phase between addition of DMPC and initiation of vesicle release, as well as the rate of vesicle release itself.

Materials and Methods

Materials

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 3000 rpm for 15 min at 4°C and washed three times with 10 mM Tris-HCl (pH 7.4) containing 144 mM NaCl. Penicillin was purchased from novo Industri (Copenhagen, Denmark) and streptomycin sulfate was obtained from Grogg Pharmaceutical products (Berne, Switzerland). ATP standards and ATP-monitoring reagent were obtained from LKB Wallac (Turku, Finland). 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.

Methods

Acetylcholinesterase activity was determined according to Ellman et al. [15]. The ATP content of red cell suspensions was measured using the bioluminescence assay reagent from LKB Wallac as follows: The red cell suspension was adjusted to a hematocrit of approx. 4% and protein was precipitated with an equal volume of a chilled solution containing 10% (w/v) trichloroacetic acid and 4 mM EDTA. The mixture was kept on ice for 10 min and then centrifuged for 5 min at 8000 rpm with a Hettich microliter centrifuge at 4°C. An aliquot of the supernatant was diluted 5000-fold with 100 mM Tris-acetate buffer (pH 7.75) containing 2 mM EDTA. 200 μl of this solution were mixed with 40 μl ATP-monitoring reagent and bioluminescence was immediately measured in an Packard Tri-Carb 3320 liquid scintillation counter in the off-coincidence mode. ATP solutions in the concentration range from $2.5 \cdot 10^{-11}$ to $2.5 \cdot 10^{-6}$ M were used to record a calibration curve.

DMPC-induced release of vesicles was carried out according to Ott et al. [10]. At appropriate times, the incubation mixture (or an aliquot) was chilled on ice for 5 min to stop the vesiculation process [10] and erythrocytes were removed by centrifugation at 3000 rpm for 10 min at room temperature. Alternatively, vesicles were obtained by ATP depletion of erythrocytes as described by Lutz et al. [8]. To modulate the red cell ATP levels prior to the addition of DMPC, washed erythrocytes (1 vol.) were preincubated at 37°C for 2, 4 and 22 h, respectively, with 4 vol. of a 10 mM Tris-HCl buffer (pH 7.4) containing 144 mM NaCl, and supplemented with 0.2 mg/ml streptomycin and 0.12 mg/ml penicillin to avoid bacterial growth. 5 vol. of a sonicated DMPC suspension were then added (0.5 mg/ml, final concentration) and the incubation was continued at 30°C. In control experiments, preincubation was carried out in buffer supplemented with 0.54 mM adenine, 12.7 mM inosine and 2 g/l glucose to maintain constant levels of red blood cell ATP [8]. Rapid ATP depletion of red blood cells was performed by addition of 6 mmol/l of iodoacetamide to the glucose-containing incubation buffer as described by Ferrell and Huestis [16]. The DMPC suspension was added after 2.5 or 5 h, respectively. Shape change of erythrocytes was followed by light microscopy using the Zeiss Nomarsky optic equipment. In all experiments, vesicle release was monitored by measuring acetylcholinesterase activity in the supernatant as previously described [8,10].

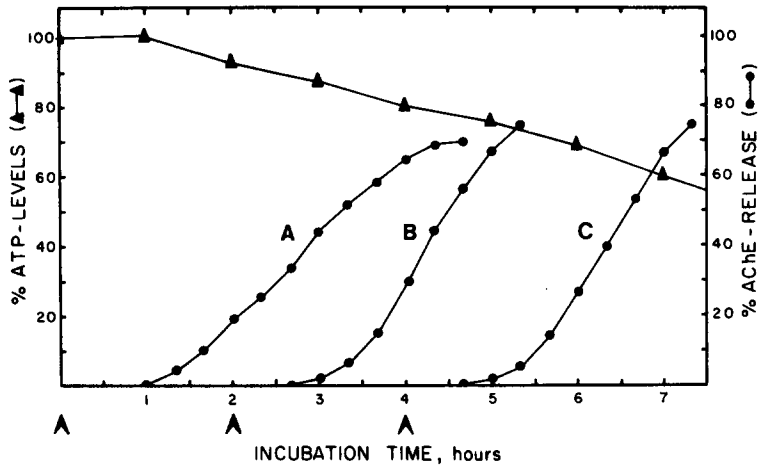

Fig. 1. Vesicle release as a function of incubation time and red cell ATP levels. Cells were incubated in a medium without glucose. ATP levels and vesicle release were measured. Circles represent acetylcholinesterase (AChE) activity in the supernatant obtained after removal of red cells by centrifugation. Enzyme activity was taken as a measure for vesicle release. Triangles represent the ATP levels of red blood cell suspensions. The arrows indicate the time of the addition of DMPC to the incubation medium. (A) Release curve obtained without preincubation; (B) release curve obtained after 2 h preincubation time; (C) release curve obtained after 4 h preincubation time.

Results

Incubation of red blood cells in glucose-free medium for more than 22 h resulted in ATP depletion and spontaneous vesicle release. In accord with previous reports [8], the release rate was very slow and acetylcholinesterase activity recovered in the supernatant reached 40–70% of the total amount only after 50 h (results not shown).

Red cells incubated with sonicated DMPC suspensions released vesicles after a lag period of approx. 60–90 min (Fig. 1, curve A). No significant hemolysis was observed and the ATP level of the cells was unchanged [10]. Approx. 70% of the total acetylcholinesterase activity was recovered in the supernatant within 4 h after initiation of vesiculation.

When cells were preincubated in glucose-free medium for 2 and 4 h, ATP levels were moderately decreased to 80–90% of the initial values, and the lag-period for vesiculation after addition of DMPC was slightly shortened to about 40 min (Fig. 1, curves B and C). The release rate was also accelerated so that acetylcholinesterase activity in the supernatant reached 70% of the total amount in less than 2.5 h.

Similar results were obtained when erythrocytes were preincubated for 22 h in a medium supplemented with adenine, inosine and glucose (Fig. 2). Microscopic examination of these cells revealed that more than 90% of the cells were discocytes before DMPC was added.

On the other hand, after almost complete ATP depletion of red blood cells by preincubation for 22 h in a medium without glucose, all cells were

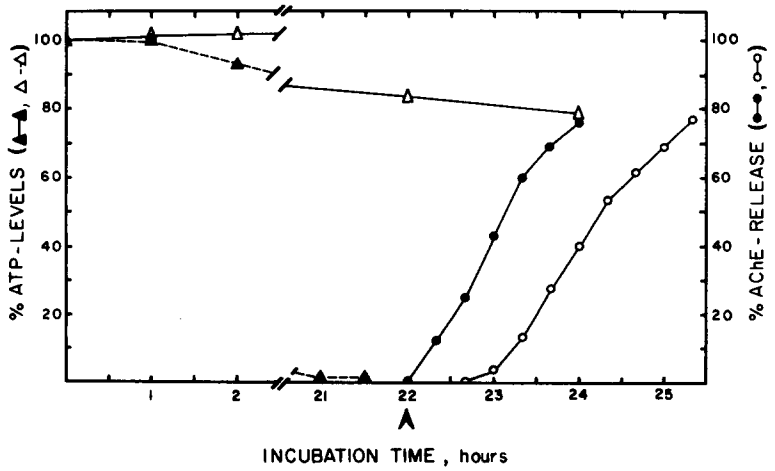

Fig. 2. Vesicle release as a function of incubation time and red cell ATP levels. Closed symbols represent experiments carried out in a medium without glucose and open symbols represent experiments performed in a medium supplemented with adenine, inosine and glucose. Other experimental details are as detailed in the legend to Fig. 1. AChE, acetylcholinesterase.

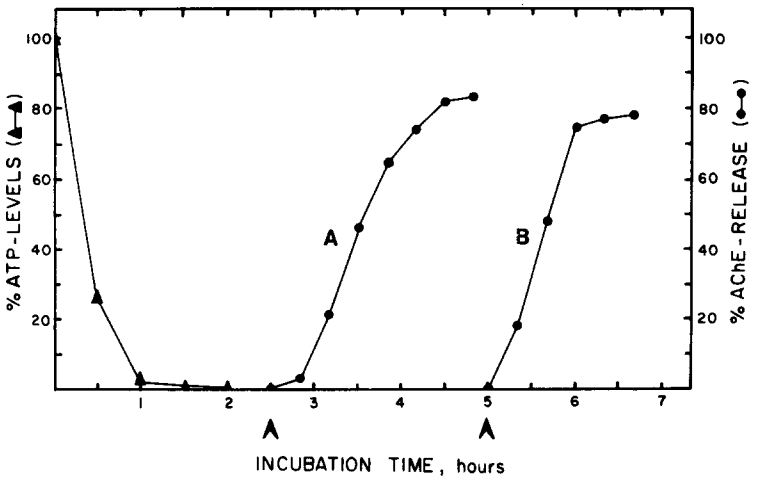

Fig. 3. Vesicle release after rapid ATP depletion of red blood cells. The symbols are as indicated in Fig. 1. (A) Release curve obtained after 2 h preincubation time; (B) release curve obtained after 5 h preincubation time. AChE, acetylcholinesterase.

echinocytic. Under these conditions, vesicle release started immediately upon addition of DMPC (Fig. 2) and acetylcholinesterase activity recovered in the supernatant was 70% of the total amount after about 1.5 h.

Addition of iodoacetamide to red blood cell suspensions resulted in rapid depletion of ATP (Fig. 3). After 2.5 h of preincubation, when ATP levels were less than 2% of the initial values, over 70% of the cells were still discocytic, in accord with a previous report [17]. Vesicle release started shortly after the addition of DMPC and acetylcholinesterase activity in the supernatant reached 70% within 2 h (Fig. 3). After 5 h of preincubation under the same conditions, all cells were transformed to echinocytes [17]. Release of vesicles started immediately after adding DMPC and the release rate was accelerated to such an extent that acetylcholinesterase activity in the supernatant reached 70% of the total amount in less than 1 h. In all of these experiments, hemolysis never exceeded 8%.

Discussion

Although some controversy exists in the literature concerning the influence of ATP on the red cell membrane and its cytoskeletal network [4,5,18,19], several effects of ATP depletion have been described. Besides a dephosphorylation of spectrin [18], metabolic depletion also results in a decreased protection against the oxidative damage that promotes cross-linking of spectrin [20] and possibly a change in membrane lipid asymmetry [21,22]. Furthermore, red cell shape recovery mechanisms appear to be energy dependent [23] and metabolic depletion has been shown to promote cell shape changes [17]. Kamada et al. [24] recently reported that the membrane-lipid phase was influenced by protein-lipid interactions which depended on the metabolic state of the cells and that a decrease of ATP levels was responsible for an increased membrane fluidity. Likewise, it was reported that diacylglycerol, a fusogenic lipid component, was formed in ATP-depleted red cells [25] and promoted to some extent membrane vesiculation processes which clearly involve local membrane fusion events.

The present study shows that after various preincubation protocols that induce echinocyte formation or reduce cellular ATP levels, erythrocytes release vesicles much sooner after the addition of DMPC than do discocytes with normal ATP levels (Figs. 1–3). In addition, the rate of vesicle release is accelerated. Apparently, alterations that occur in metabolically depleted red cells make their membranes more susceptible to a further perturbation by addition of exogenous DMPC.

At this point, a key question is whether it is the loss of ATP or the shape change (or both) which is responsible for the facilitated release of vesicles. Earlier work by Feo and Mohandas [17] has shown that after 2.5 h of fast ATP depletion, erythrocytes with no cellular ATP are obtained that still maintain their discocytic shape. Addition of DMPC to these cells results in rapid vesicle release

that is completed in approx. 2 h when 70% of the acetylcholinesterase activity are recovered in the supernatant (Fig. 3). On the other hand, after 5 h of preincubation with iodoacetamide, a complete transformation from discocytes to echinocytes takes place and the vesicle release is completed in less than 1 h after addition of DMPC. At that time, again 70% of the acetylcholinesterase activity are found in the vesicle fraction. These observations strongly suggest that it is the number of echinocytes rather than the cellular ATP level which determines the rate of vesicle release after addition of DMPC. The release pattern obtained after slow ATP depletion (Fig. 2) further supports this interpretation.

On the other hand, cellular ATP levels may still have a more direct influence on vesicle release. As mentioned above, shape recovery mechanisms of red cells have been shown to be energy dependent [23]. The present study shows that cells with reduced ATP levels but still a discoid shape, release vesicles more rapidly under the influence of DMPC (Fig. 1). Obviously, these cells respond more sensitively towards the crenating action of DMPC than do cells with normal ATP levels.

The results may also give a clue to the mechanism by which DMPC induces vesicle release. It has to be assumed that in a first step, incorporation of exogenous lipid into erythrocytes alters the phospholipid fraction of the cell membrane [26]. This in turn modifies interactions between the intrinsic domain and the membrane skeleton with a concomitant cell shape change. Such modifications have to be considered as a prerequisite for vesicle release. In erythrocytes with modified ATP levels, DMPC-induced vesicle release is facilitated. Hence, the interactions between skeleton and intrinsic domain must be changed in these cells. In sickle cells where spectrin-free vesicles are released as a consequence of repeated cycles of deoxygenation-reoxygenation, these interactions are believed to be disrupted mechanically by the polymerizing hemoglobin [12].

In conclusion, preincubation procedures that result in ATP depletion or echinocytosis of red blood cells strongly facilitate DMPC-induced release of vesicles. It has to be mentioned, however, that in the present study echinocytes are formed only after prolonged preincubation periods. Such treatment may also affect the protein components of the membrane. Therefore, further studies are underway to characterize the behavior of integral and peripheral membrane proteins during preincubation and subsequent DMPC-induced vesiculation.

Acknowledgements

We gratefully acknowledge valuable discussions with Drs. G.M. Wagner and D.T.-Y. Chiu. The authors are indebted to Mrs. Yvonne Binggeli for performing the ATP determinations and to Drs. D. Deranleau and E.J. Frenkel for carefully reading the manuscript. This work was supported by Grant No. 3.358-0.82 of the Swiss National Science Foundation.

References

1 Mohandas, N., Chasis, J.A. and Shohet, S.B. (1983) Sem. Hematol. 20, 225–242
2 Cohen, C.M. (1983) Sem. Hematol. 20, 141–158
3 Goodman, S.R., Steven, R. and Shiffer, K. (1983) Am. J. Physiol. 244, C121–C141
4 Sheetz, M.P. and Singer, S.J. (1977) J. Cell Biol. 73, 638–646
5 Birchmeier, W. and Singer, S.J. (1977) J. Cell Biol. 73, 647–659
6 Sheetz, M.P. and Singer, S.J. (1974) Proc. Natl. Acad. Sci. USA 71, 4457–4461
7 Weed, R.I. and Chailley, B. (1973) Red Cell Shape (Bessis, M., Weed, R.I. and Leblond, P.F., eds.), pp. 55–68, Springer-Verlag, New York
8 Lutz, H.U., Liu, S.C. and Palek, J. (1977) J. Cell Biol. 77, 548–560
9 Allan, D. and Thomas, P. (1981) Biochem. J. 198, 433–440
10 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
11 Takahashi, K., Kobayashi, T., Yamada, A., Tanaka, Y., Inoue, K. and Nojima, S. (1983) J. Biochem. 93, 1691–1699
12 Allan, D., Limbrick, A.R., Thomas, P. and Westerman, M.P. (1982) Nature (Lond.) 295, 612–613
13 Weitz, M., Bjerrum, O.J., Ott, P. and Brodbeck, U. (1982) J. Cell. Biochem. 19, 179–191
14 Haest, C.W.M. (1982) Biochim. Biophys. Acta 694, 331–352
15 Ellman, G.L., Courtney, D.K., Andres, V. and Featherstone, R.M. (1961) Biochem. Pharmacol. 7, 88–95
16 Ferrell, J.E. and Huestis, W.H. (1982) Biochim. Biophys. Acta 687, 321–328
17 Feo, C. and Mohandas, N. (1977) Nature (Lond.) 265, 166–168
18 Anderson, J.M. and Tyler, J.M. (1980) J. Biol. Chem. 255, 1259–1265

19 Patel, V.P. and Fairbanks, G. (1981) J. Cell Biol. 88, 430–440
20 Palek, J. and Lux, S.E. (1983) Sem. Hematol. 20, 189–224
21 Haest, C.W.M., Plasa, G., Kamp, D. and Deuticke, B. (1978) Biochim. Biophys. Acta 509, 21–23
22 Bergmann, W.L., Dressler, V., Haest, C.W.M. and Deuticke, B. (1984) Biochim. Biophys. Acta 769, 390–398
23 Alhanaty, E. and Sheetz, M.P. (1981) J. Cell Biol. 91, 884–888
24 Kamada, T., Setoyama, S., Chuman, Y. and Otsuji, S. (1983) Biochem. Biophys. Res. Commun. 116, 547–554
25 Müller, H., Schmidt, U. and Lutz, H.U. (1981) Biochim. Biophys. Acta 649, 462–470
26 Lange, L.G., Van Meer, G., Op den Kamp, J.A.F. and Van Deenen, L.L.M. (1980) Eur. J. Biochem. 110, 115–121