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Lipid and protein composition of exovesicles released from human erythrocytes following treatment with amphiphiles

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Abstract

Human erythrocytes were treated with different water-soluble amphiphiles (detergents) at sublytic concentrations, whereafter released exovesicles and treated cells were isolated. Lipid analyses showed that exovesicles had a lower cholesterol/phospholipid ratio and a higher phosphatidylserine/phospholipid ratio compared to parent cells. Protein analyses revealed that exovesicles were, relative to their total protein content, depleted in spectrin, actin and band 6 protein and enriched in band 3 protein and acetylcholinesterase. Exovesicles contained all major glycoproteins. By using a radiolabeled amphiphile ([^{14}C]cetyltrimethylammonium bromide) it was shown that the amphiphile/phospholipid ratio was similar in the vesicle membrane and in the parent cell membrane. This indicates that no significant segregation of the intercalated amphiphile between the exovesicle membrane and the parent cell membrane occurs during the vesiculation process. It is suggested that the redistributions of membrane lipids and proteins during the vesiculation process are secondary to the detachment of the cytoskeletal network from the membrane.

Key words: Amphiphile; Detergent; Erythrocyte; Vesiculation; Phospholipid; Cholesterol; Polypeptide; Glycoprotein

1. Introduction

A variety of treatments induce a release of exovesicles from human erythrocytes, indicating that membrane vesiculation is a general characteristic of the stressed erythrocyte membrane. Chemical characterization of exovesicles released from the erythrocyte membrane, have shown that they have largely a similar phospholipid composition and cholesterol/phospholipid ratio as the parent cells [1–3]. Exovesicles have, however, a lower protein/phospholipid ratio than parent cells and are, relative to their total protein content, depleted in cytoskeletal-anchored proteins and enriched in laterally mobile proteins [1,2,4,5]. The observed protein segregation has been ascribed to the detachment from the cytoskeletal network of those membrane domains from where the exovesicles are released [4,5].

A previous study indicated that cholesterol may play an important role in the exovesiculation process [6]. In this study of dimyristoylphosphatidylcholine (DMPC) induced vesiculation in human erythrocytes it was found that the DMPC vesicles induce cholesterol depletion of the membrane and that this depletion is essential for exovesiculation to occur. The cholesterol depletion was probably due to an extraction of cholesterol into the DMPC vesicles. Due to the slow transfer of DMPC molecules from the DMPC vesicles into the membrane and/or the slow extraction of cholesterol from the membrane into the DMPC vesicles, the DMPC-induced exovesiculation was preceded by a lag phase of about 60 min before exovesiculation started. Contrary to DMPC vesicles, the water-soluble amphiphiles (detergents) used in the present study can initiate exovesiculation within some minutes [7]. It was therefore of particular interest to see if such amphiphiles, at concentrations below cmc, may affect the cholesterol pool of the membrane and release exovesicles with a cholesterol level different from the remnant cells.

In the present study we have treated human erythrocytes with water-soluble amphiphilic compounds and compared the cholesterol level as well as the phospholipid and protein composition of released exovesicles and remnant cells. We also studied the distribution (cpm/phospholipid ratio) of a radiola-

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beled amphiphile between released exovesicles and remnant cells.

It is suggested that there exist a general trigger mechanism for chemically induced exovesiculation to occur. However, besides induction of vesiculation different treatments and perturbants may individually affect the exovesiculation process, leading to release of exovesicles with different chemical composition and morphological features.

2. Materials and methods

Chemicals. Dodecyltrimethylammonium bromide, cetyltrimethylammonium bromide (CTAB) and myristoyl L-α-lysophosphatidylcholine (lysoPC) were purchased from Sigma. 3-(Dodecyldimethylammonio)-1-propanesulphonate (dodecylzwittergent^R) was obtained from Calbiochem-Behring, sodium dodecyl sulphate from Merck and dodecyl D-maltoside from Fluka. Trimethyl[1-^{14}C]cetylammonium bromide ([^{14}C]CTAB) with a specific activity of 237 MBq/mmol (6.4 mCi/mmol) has been obtained from the Radiochemical Centre, Amersham, Bucks. This radiolabeled compound has been stored in our laboratory for several years. It was recrystallized twice from ethanol/acetone and thin-layer chromatography showed that the radiochemical purity was above 90%. All amphiphiles and chemicals were standard commercial products. All amphiphiles were dissolved in buffer. [^{14}C]CTAB was following recrystallization stored as a stock solution in ethanol.

Erythrocytes. Blood was drawn from healthy donors by vein puncture into heparinized tubes. The erythrocytes were washed three times in a buffer containing 145 mM NaCl, 5 mM KCl, 4 mM Na_2HPO_4, 1 mM NaH_2PO_4, 1 mM $MgSO_4$, 1 mM $CaCl_2$ and 10 mM glucose (pH 7.4). The erythrocytes were then suspended in the buffer at a cell density of $1.65 \cdot 10^9$ cells/ml and kept at 4°C until used. The blood was used within 30 h after drawing.

Incubation of erythrocytes. Aliquotes of a prewarmed (37°C) erythrocyte stock suspension were pipetted into polystyrene tubes or glass vials containing prewarmed (37°C) buffer with amphiphiles. The final cell density was $1.65 \cdot 10^8$ cells/ml (about 1.5% haematocrit) and the incubations were carried out in a shaking thermostat bath at 37°C. The amphiphiles were used at sublytic concentrations found to result in maximum protection against hypotonic haemolysis (cAH_{max}) following 60 min incubation at 37°C (Table 1). These concentrations were, as previously described [8,9], estimated from dose-response curves from experiments carried out in the buffer diluted to an osmolarity giving about 80% haemolysis of untreated samples.

Table 1
The concentrations of amphiphiles causing maximum protection against hypotonic haemolysis following 60 min incubation at 37°C (cAH_{max}) [8,9]

	(μM)
Dodecyl D-maltoside	40
3-(Dodecyldimethylammonio)-1-propanesulphonate (dodecylzwittergent^R)	263
Dodecyltrimethylammonium bromide	300
Myristoyl L-α-lysophosphatidylcholine (lysoPC)	13
Sodium dodecyl sulphate	50

Isolation of erythrocytes and exovesicles. Following incubation with amphiphiles at cAH_{max} for 60 min at 37°C, erythrocytes were pelleted by centrifugation at $1400 \times g$ for 10 min. Exovesicles were, after an additional centrifugation of the supernatant as above, pelleted by centrifugation of the resulting supernatant at $20000 \times g$ for 40 min. To remove eventually still remaining denser and lighter fractions than the exovesicle fraction, the pellet was suspended in buffer and centrifuged at $10000 \times g$ for 2 min in an Eppendorf tube. The supernatant (upper 95%) was then centrifuged at $20000 \times g$ for 40 min whereafter the resulting supernatant was completely removed to get rid of eventually remaining ghost and membrane fragments which, if present, occurred as a light, fluffy and easy removable layer on top of the tightly packed deep red exovesicle pellet.

Extraction of lipids. Lipid extraction was performed by adding chloroform/methanol/buffer (8:4:3, v/v) to the pelleted samples. The washed chloroform phase was taken to dryness and the lipids were stored in chloroform at −20°C.

Thin-layer chromatography separation and quantification of major phospholipids. Phospholipids were separated on Kieselgel 60 DC-Fertigplatten (Merck 5626) in chloroform/methanol/acetic acid/water (25:15:4:2, v/v). The plates were then dried and run in chloroform/diethyl ether/acetic acid (130:30:2, v/v) to avoid disturbances of cholesterol during the densitometry. Plates were stained with 3% cupric acetate in 8% orthophosphoric acid, burned and major phospholipid classes were densitometrically quantified. The method is not strictly quantitative.

Cholesterol / phospholipid ratio. Subsamples from the lipid extracts were taken for analyses of the cholesterol and phospholipid content. Cholesterol was gas chromatographically quantified as earlier described [10] using 5β-cholestan-3α-ol as internal standard. The phospholipid content was determined spectrophotometrically by using the Boehringer Mannheim GmbH phosphate/phospholipid test kit.

Intercalation of [^{14}C]CTAB into erythrocyte and exovesicle membranes. Erythrocytes were incubated as described above in a buffer containing about 14.2 μM

(cAH_{max}) [^{14}C]CTAB/CTAB. The specific activity of the [^{14}C]CTAB/CTAB mixture was about 48 MBq/mmol (1.3 mCi/mmol). Following 60 min incubation erythrocytes were pelleted at 1 400 × g for 10 min. After an additional similar centrifugation of the supernatant, exovesicles were pelleted from the resulting supernatant at 20 000 × g for 40 min, and washed twice in buffer. To minimize color quenching due to haemoglobin (Hb) in the determination of the radioactivity, erythrocytes were, following two washes in buffer, haemolyzed in water and washed three times in buffer. Subsamples from erythrocyte and exovesicle samples were taken for quantification of total phospholipid content and for determination of radioactivity. Optiphase^{R} 'Highsafe' 3 was used as scintillation cocktail and the radioactivity was measured in a liquid scintillation spectrometer. Total phospholipid content was quantified as described above.

AChE release. The acetylcholinesterase (AChE) activity in the exovesicle samples was determined as earlier described [7].

SDS-PAGE for polypeptide and glycoprotein separation. Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed in a Bio-Rad Minigel apparatus using 5% polyacrylamide stacking gel and 11% or 7.5% polyacrylamide separation gel and according to Laemmli [11]. Samples were prepared by adding Laemmli sample buffer to packed Dodge-ghosts [12], pelleted whole erythrocytes or pelleted exovesicles. Dodge-ghosts (and cytosol) were prepared at 4°C by using a lysis buffer containing 5 mM NaH_2PO_4 and 1 mM EDTA (pH 7.4). The polypeptide bands were visualized with Coomassie brilliant blue staining and glycoproteins with periodate acid schiff (PAS) staining [13]. Pharmacia low molecular weight calibration kit was used as a standard.

Statistical analyses. Student's t-test was used to detect statistically significant differences. A probability value ≤ 0.05 was considered to indicate a statistically significant difference.

3. Results

Phospholipid composition and cholesterol / phospholipid ratio of erythrocytes and exovesicles

Erythrocytes were treated with amphiphiles at cAH_{max} (Table 1) for 60 min at 37°C, whereafter cells and released exovesicles were isolated and analyzed. The release of exovesicle-phospholipid from the parent erythrocytes was 0.5–2% (data not shown).

Exovesicles released from erythrocytes following treatment with dodecyltrimethylammonium bromide (n = 4), dodecylzwittergent (n = 4) and dodecylmaltoside (n = 4) had a similar phospholipid composition. The data for the exovesicles were therefore pooled. The phospholipid composition of cells treated with different amphiphiles was also similar and these data were also pooled. The pooled data are presented in Table 2. The phospholipid composition of treated cells did not differ from that of untreated cells. The exovesicles, however, had a markedly (25% and 50%, respectively) higher content of phosphatidylserine compared to treated and untreated erythrocytes. A few experiments were also performed with lysoPC (n = 2) and sodium dodecyl sulphate (n = 2) and the results were similar to those above (data not shown).

The cholesterol/phospholipid ratio in exovesicles released by treatment with dodecyltrimethylammonium bromide (n = 2), dodecylzwittergent (n = 3) and dodecylmaltoside (n = 3) was similar. In comparison with untreated and treated cells the cholesterol/phospholipid ratio of the exovesicles (pooled data) was significantly lower (Table 2).

Table 2
Phospholipid composition and cholesterol/phospholipid ratio of untreated erythrocytes, amphiphile-treated parent cells and released exovesicles

	Untreated cells (n = 15) [a]	Treated cells (n = 10) [a]	Exovesicles (n = 12) [a]
Phospholipid composition			
phosphatidylethanolamine	32 ± 1	32 ± 2	30 ± 2
phosphatidylserine	10 ± 3	12 ± 2	15 ± 2 *
phosphatidylcholine	30 ± 2	29 ± 3	27 ± 2
sphingomyelin	27 ± 2	26 ± 2	27 ± 2
	Untreated cells (n = 5) [b]	Treated cells (n = 8) [b]	Exovesicles (n = 8) [b]
Cholesterol/phospholipid	0.99 ± 0.09	0.97 ± 0.08	0.80 ± 0.06 **

Erythrocytes were treated with dodecyltrimethylammonium bromide, dodecylzwittergent or dodecylmaltoside at cAH_{max} (Table 1) for 60 min at 37°C, whereafter cells and released exovesicles were isolated and analyzed as described in Materials and methods. Data for differently treated erythrocytes and for exovesicles released by different amphiphiles are pooled. Results are expressed as mean ± S.D.

[a] Results are given as relative percent.

[b] Results are given as molar ratios.

*, differs significantly from untreated and treated cells, P = 0.044.

**, differs significantly from untreated and treated cells, P = 0.002.

Recovery of [^{14}C]CTAB from erythrocyte and exovesicle membranes

Erythrocytes were treated with 14.2 μM (cAH_{max}) [^{14}C]CTAB/CTAB at 37°C for 60 min, whereafter cells and released exovesicles were isolated and analyzed.

No statistically significant difference between the cpm/phospholipid ratio of exovesicle membranes and of treated cell membranes, 331 ± 59 and 382 ± 43 (mean $\pm$ S.D., $n = 6$), respectively, was found. About 90% of the added radioactivity was found associated with the erythrocyte membrane following two washes and haemolysis of the erythrocytes. During three more washes of the Hb depleted erythrocyte membranes, about 7% of the membrane bound radioactivity was lost to the supernatant. These findings are in agreement with earlier studies of amphiphile incorporation into erythrocyte membranes [14,15]. The activity in the washed exovesicle pellet represents about 1.7% ($n = 6$) of the radioactivity in the washed membranes of their parent erythrocytes before exovesiculation.

Polypeptide and glycoprotein profiles of erythrocytes and exovesicles

Erythrocytes were treated with dodecylzwittergent, dodecylmaltoside, dodecyltrimethylammonium bromide and sodium dodecyl sulphate at cAH_{max} (Table 1) for 60 min at 37°C. The polypeptide and glycoprotein profiles of untreated cells, treated cells and released exovesicles were densitometrically scanned following SDS-PAGE and staining (Fig. 1). Untreated (control) and treated cells were sampled as Dodge-ghosts [12], while exovesicles were used as such.

It is difficult to analyze the polypeptide-profile of exovesicles with SDS-PAGE mainly because of the high Hb content of exovesicles [5,16,17]. Despite an application of sample in amounts resulting in a heavy Hb loading, other protein bands stay faint and may be distorted due to Hb overloading. The exovesicles are very stable and attempts to deplete exovesicles in Hb by hypotonic lysis or repeated freeze-thawing cycles

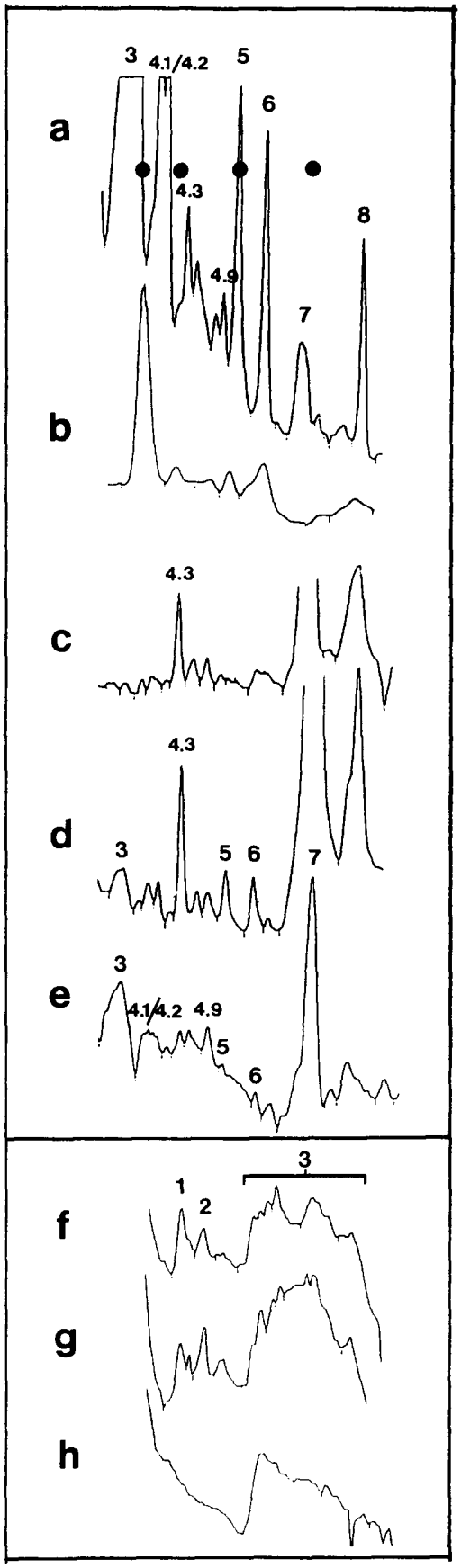

Fig. 1. Polypeptide and glycoprotein profiles of erythrocytes and exovesicles following SDS-PAGE and staining with Coomassie blue (scans a and c–h) or PAS (scan b), respectively. Scans a–e represent gels where 11% and scans f–h gels where 7.5% polyacrylamide separation gels were used. Erythrocytes were treated with amphiphiles at cAH_{max} (Table 1) for 60 min at 37°C, whereafter exovesicles, treated and untreated cells were isolated. Coomassie blue stained polypeptide bands are basically classified according to Steck [19]. (a) Dodge-ghost control; (b) exovesicles induced by dodecylzwittergent (PAS-stained); (c) cytosol; (d) whole-erythrocyte control; (e) exovesicles induced by dodecylzwittergent; (f) Dodge-ghost control; (g) whole-erythrocyte control; (h) exovesicles induced by dodecyltrimethylammonium bromide. The positions of the molecular mass markers 94 kDa rabbit muscle phosphorylase β, 67 kDa bovine serum albumin, 43 kDa ovalbumin and 30 kDa bovine erythrocyte carbonic anhydrase, are indicated with (•) in scan a.

were not successful. Furthermore exovesicles are present in minute amounts and may have a lower protein to lipid ratio compared to parent cells [1,2,4,18]. These circumstances made densitometric quantification of the bands in the profiles difficult. To have a control more comparable, in case of Hb content and handling, to the exovesicle samples than Dodge-ghosts we also used whole erythrocytes as control. Representative scans out of a large number of experiments are presented. The Coomassie blue banding of the Dodge-ghost control (Fig. 1, scan a) was used as reference and the protein bands were basically numbered according to Steck [19]. Scans a–e represent scans of gels where 11% polyacrylamide separation gels were used. The top of these scans is omitted because of high background staining (in exovesicle and whole-erythrocyte lanes) and a poor band separation in this region.

Coomassie blue stained SDS-PAGEs indicated no differences in major polypeptide bands between control and treated cells, or between differently treated cells (not shown). The lanes all showed the characteristic polypeptide profiles of Dodge-ghost (scan a).

The cytosol (scan c) is dominated by band 7 (dimeric Hb [5]), a narrow band 4.3 and a broad band in the band 8 (monomeric Hb [5]) region. A weak band 6 and two small bands in the band 4.5–4.9 region were also seen. The amount of Hb applicated on this lane is largely the same as that of the whole-cell control lane (scan d) and about two times that present in the exovesicle lane (scan e). Some of the 'cytosolic' proteins may be peripheral membrane proteins.

All major bands in the Dodge-ghost control lane (scan a) were present in the whole-erythrocyte control lane (scan d). The Hb overloading seemed not to severely affect the electrophoretic mobility of the major proteins but obstructed a quantitative comparison since the Hb amount allowed an application of only about one fifth of the amount of non-Hb membrane protein present in the Dodge-ghost lanes. The 'cytosolic' protein bands predominated the lane.

All major Dodge-ghost bands could also be identified in the exovesicle profiles (scan e, dodecylzwittergent). No differences were found between the polypeptide profiles of exovesicles treated with different amphiphiles (not shown). Exovesicles were, compared with whole-erythrocyte as well as the Dodge-ghost controls, enriched in band 3 and heavily depleted in band 5 (actin) and 6, relative to their total protein banding. Furthermore, depletion or lack of bands 1 and 2 (spectrin) in exovesicles (scan h, dodecyltrimethylammonium bromide) compared to Dodge-ghost controls (scan f) and whole-erythrocyte controls (scan g), was revealed when using 7.5% polyacrylamide separation gel. Traces of spectrin were detected in some exovesicle preparations but these traces are probably due to contamination (see Ref. 5). The membrane skeleton component band 4.9 protein was distinctly present in the exovesicle membrane lanes. Also band 4.1 was often present as a separate small peak top. Sometimes, as in the presented scan (scan e), bands 4.1 and 4.2 were represented by a common broad peak. The 'cytosolic' bands 4.3, 7 and 8 were less prominent compared to other bands in the exovesicle lanes than in the whole-cell control lanes. This is probably due to a higher membrane surface area to cytosol volume ratio in exovesicles compared with whole cells.

PAS stained SDS-PAGEs revealed that exovesicles (represented by scan b, dodecylzwittergent) released by treatment with different amphiphiles as well as Dodge-ghosts of untreated erythrocytes and erythrocytes treated with different amphiphiles (not shown) qualitatively had the same staining profile. The exovesicles, however, had a denser (by about 50%) PAS-stained band in the Coomassie blue band 8 region than untreated and treated erythrocytes. This band consists largely of 'cytosolic' components. The five detected PAS bands in exovesicles are from the top (average % density of scanned bands): the dominating band situated just underneath Coomassie blue band 3 (63%), a band coinciding with the albumin standard situated between Coomassie blue bands 4.2 and 4.3 (4%), a band in the Coomassie blue band 5 region (4%), a band in the Coommassie blue band 6 region (18%) and finally the broad band in the Coomassie blue band 8 region (11%).

The AChE activity/phospholipid ratio indicated that AChE was accumulated in the exovesicles 3–6 times (data not shown).

4. Discussion

The present study shows that the membrane of exovesicles released from amphiphile-treated human erythrocytes differs from that of parent cells. The exovesicles are depleted in cholesterol and enriched in phosphatidylserine, relative to their phospholipid content. Furthermore, exovesicles are depleted in bands 1 and 2 (spectrin), band 5 (actin) and band 6 proteins, and enriched in band 3 protein and AChE, relative to their total protein content.

Previous studies have shown that the overall phospholipid composition of exovesicles released following a variety of treatments of erythrocytes [3,17,20], including treatment with bile acids [18], DMPC vesicles [21] and merocyanine 540 [22], is similar to that of the parent erythrocytes. However, some differences in the phospholipid composition between exovesicles and parent erythrocytes have been reported in some studies [1,2,23,24]. These differences did not concern the relative phosphatidylserine content of the vesicle membrane.

Our results show that amphiphile-induced exovesicles are depleted in cholesterol by about 18% in comparison to parent cells. This result is in contrast to studies where exovesicles derived from ATP-depleted erythrocytes [2,3] and A23187-treated erythrocytes [1,3] were studied. These exovesicles were reported to have a cholesterol/phospholipid ratio similar to the parent cells. However, an altered cholesterol/phospholipid ratio in exovesicles released from erythrocytes has been reported upon storage [25], and in exovesicles released by hypertonic treatment it was found that the experimental temperature affect the cholesterol/phospholipid ratio of the exovesicles [23].

Whether the altered cholesterol/phospholipid ratio in exovesicles is a reminiscence of the events leading to vesicle release in amphiphile-treated erythrocytes is unclear. It has been shown by Frenkel et al. [6] that a decreased cholesterol level may play an essential role in the vesiculation process in DMPC-induced vesiculation in human erythrocytes. They showed that incubation with DMPC vesicles induce cholesterol depletion and echinocytosis in erythrocytes and that exovesiculation occur in these cholesterol depleted erythrocytes but not in erythrocytes where the cholesterol level was kept unaltered by incubation with DMPC-cholesterol vesicles. Thus it seems as if cholesterol depletion of specific domains in the erythrocyte membrane or of the whole membrane is a prerequisite for DMPC-induced exovesiculation to occur. A reduction in the cholesterol level is thought to increase the tendency of phosphatidylethanolamine to adopt nonbilayer configurations within the membrane. Non-bilayer phases, such as the hexagonal phase, have in turn been suggested to be essential as intermediates in the membrane fusion process [26,27], which is a prerequisite for a release of membrane vesicles. However, in the present study no difference in the cholesterol/phospholipid ratio between untreated and amphiphile-treated erythrocytes was found. Furthermore, while phosphatidylcholine vesicles are known to extract cholesterol from membranes, it is less probable that the water-soluble amphiphiles used in the present study do this since they are used at concentrations well below cmc [28]. Thus, a cholesterol depletion of the whole erythrocyte membrane seem not to be a prerequisite in vesiculation induced by water-soluble amphiphiles. At present we can only speculate upon the mechanisms leading to cholesterol-depleted exovesicles. It seems possible that water-soluble amphiphiles induce a segregation of cholesterol in the plane of the bilayer or out of the bilayer from those membrane domains that are subsequently released as exovesicles. It is also possible that preexisting cholesterol-poor membrane domains [29] are released as exovesicles. The changes in the cholesterol/phospholipid ratio of the parent cells that would accompany such processes may be too small to be detected.

According to the shape-structure concept [30,31] the molecular shape of bilayer lipids or amphiphiles intercalated into the bilayer is important for the stability of the bilayer. The amphiphiles used in the present study should have a destabilizing effect on the bilayer because they are cone-shaped and should therefore not easily accommodate into the bilayer structure. It is tempting to speculate that vesiculation could be a process by which compounds unsuitable for the bilayer structure could be removed from the membrane. However, the present study do not support this assumption since the [^{14}C]CTAB/phospholipid ratio was about the same in exovesicles and remnant cells, indicating that there is no preferential segregation of intercalated amphiphiles into the domains that are predestinated to become exovesicles.

In a previous paper we suggested that amphiphiles may due to their molecular shape affect the morphology of the released exovesicles [32]. Amphiphiles with a large and bulky polar head (decyl- and dodecylmaltoside and sucrose monolaurate) were found to induce mainly tubular exovesicles, and it was suggested that these wedge-shaped amphiphiles influence the shape of the exovesicles. This suggestion is supported by the findings in the present study, because the tubular exovesicles induced by dodecylmaltoside did not differ in their lipid and protein composition from spherical exovesicles induced by the other amphiphiles.

Like previous studies of exovesicles released following different treatments [2–5,33], the present study shows that amphiphile-induced exovesicles are depleted in spectrin (bands 1 and 2), actin (band 5), and enriched in band 3 protein and AChE, relative to their total protein content. The observed accumulation of band 3 protein and AChE seems to be a general feature of chemically induced erythrocyte exovesicles. It has been suggested that proteins like AChE and a pool of band 3 protein which are considered to be freely diffusible in the plane of the membrane can be segregated during the vesiculation process. Such proteins are thought to accumulate into the domains which are going to be released as exovesicles due to a decreased surface packing pressure in these domains [24]. The decreased surface packing pressure, in turn, may be due to removal of membrane skeleton-anchored non-diffusing proteins from these domains.

It seems reasonable to speculate that all chemical manipulations of erythrocytes that force vesiculation, act through a common mechanism. The shape changes of the erythrocytes that precede vesiculation may be due to a differential expansion of one monolayer of the bilayer as suggested in the bilayer couple hypothesis [34,35]. When the perturbation of the membrane in-

creases, in our study because of an increased incorporation of amphiphilic molecules, packing disturbances or increased packing pressure in the membrane or in one monolayer force an uncoupling of the cytoskeleton from the membrane surface in specific membrane domains. The uncoupling, in turn, enables and increases lateral movements of specific proteins and lipids from or into these domains that will be subsequently released as vesicles. Also transbilayer redistributions of membrane components may occur. The chemical composition and the high curvature of these membrane domains may lead to such a destabilization of the bilayer that favor membrane fusion and thus the creation of vesicles which are subsequently released from the membrane protrusions.

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