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Influence of band 3 protein absence and skeletal structures on amphiphile- and Ca^{2+}-induced shape alterations in erythrocytes: a study with lamprey (*Lampetra fluviatilis*), trout (*Onchorhynchus mykiss*) and human erythrocytes

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Abstract

Amphiphiles which induce either spiculated (echinocytic) or invaginated (stomatocytic) shapes in human erythrocytes, and ionophore A23187 plus Ca^{2+}, were studied for their capacity to induce shape alterations, vesiculation and hemolysis in the morphologically and structurally different lamprey and trout erythrocytes. Both qualitative and quantitative differences were found. Amphiphiles induced no gross morphological changes in the non-axisymmetric stomatocyte-like lamprey erythrocyte or in the flat ellipsoidal trout erythrocyte, besides a rounding up at higher amphiphile concentrations. No shapes with large broad spicula were seen. Nevertheless, some of the 'echinocytogenic' amphiphiles induced plasma membrane protrusions in lamprey and trout erythrocytes, from where exovesicles were shed. In trout erythrocytes, occurrence of corrugations at the cell rim preceded protrusion formation. Other 'echinocytogenic' amphiphiles induced invaginations in lamprey erythrocytes. The 'stomatocytogenic' amphiphiles induced invaginations in both lamprey and trout erythrocytes. Surprisingly, in trout erythrocytes, some protrusions also occurred. Some of the amphiphiles hemolyzed lamprey, trout and human erythrocytes at a significantly different concentration/membrane area. Ionophore A23187 plus Ca^{2+} induced membrane protrusions and sphering in human and trout erythrocytes; however, the lamprey erythrocyte remained unperturbed. The shape alterations in lamprey erythrocytes, we suggest, are characterized by weak membrane skeleton–lipid bilayer interactions, due to band 3 protein and ankyrin deficiency. In trout erythrocyte, the marginal band of microtubules appears to strongly influence cell shape. Furthermore, the presence of intermediate filaments and nuclei, additionally affecting the cell membrane shear elasticity, apparently influences cell shape changes in lamprey and trout erythrocytes. The different types of shape alterations induced by certain amphiphiles in the cell types indicates that their plasma membrane phospholipid composition differs.

Keywords: Red blood cell; Detergent; Surfactant; Resin acid; Shape alteration; Membrane vesiculation; Hemolysis

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1. Introduction

Alteration of the human erythrocyte shape, including membrane vesiculation, has been frequently used as an experimental model to study plasma membrane dynamics. Therefore, it can be of interest to study shape alteration and vesiculation in erythrocytes having different plasma membrane and intracellular properties than the human erythrocyte.

The recently characterized erythrocyte of the river lamprey, a primitive vertebrate, showed some unique properties among hitherto studied erythrocytes [1]. The nucleated lamprey erythrocyte predominantly has a non-axisymmetric stomatocyte-like resting shape. It has vimentin filaments, but lacks a marginal band of microtubules. A single high molecular weight spectrin band, but no band 3 protein or ankyrin, was detected. Furthermore, the lamprey erythrocyte plasma membrane seems to be enriched in phosphatidylcholine and depleted in sphingomyelin compared to the human erythrocyte plasma membrane.

The present study was undertaken to elucidate how lamprey erythrocytes respond to amphiphiles previously shown to induce echinocytic and stomatocytic shape alterations, as well as exovesiculation and endovesiculation, in human erythrocytes [2–5]. Band 3 protein, aided by ankyrin, provides the main anchoring sites of the membrane skeleton to the plasma membrane in human erythrocytes [6,7]. Since band 3 protein and ankyrin is deficient in the lamprey erythrocyte plasma membrane [1], we expected that the band 3 protein/ankyrin deficiency would significantly affect the amphiphile-induced shape alterations, because membrane skeleton–plasma membrane interactions are thought to be important for shape alterations in human erythrocytes [8–12]. We expected that a comparison of the responses of structurally and morphologically different erythrocytes to amphiphiles with different molecular characteristics would give further insight in the mechanisms important for plasma membrane stability and shape contour changes in erythrocytes. For similar reasons, the effects of elevated intracellular Ca^{2+} concentrations was investigated. The flat ellipsoidal trout erythrocyte, which has a nucleus and vimentin filaments [1], like the lamprey erythrocyte, but furthermore has band 3 protein [1,13] and carries a marginal band of microtubules, was used in a comparative purpose.

2. Materials and methods

2.1. Chemicals

Dodecylmaltoside and octaethyleneglycol mono-*n*-dodecyl ether were purchased from Fluka, 3-(dodecyldimethylammonio)-1-propanesulfonate (dodecylzwittergent) and ionophore A23187 from Calbiochem, chlorpromazine hydrochloride from Merck, dodecyltrimethylammonium bromide from Sigma and resin acids from Helix Biotech.

2.2. Isolation of erythrocytes

Blood from anesthetized (3-aminobenzoic acid ethyl ester, Sigma A5040, 1 g/l medium) lampreys was drawn by a needle (diameter 0.6 mm) into a heparinized syringe. Trout blood was drawn in a similar way, from fishes paralyzed by a hit in the scull. In some cases, blood was collected from unanesthetized decapitated lampreys. Blood was washed five times with the buffer (HEPES 10 mM, NaCl 128 mM, KCl 3 mM, $CaCl_2 \times 2H_2O$ 1.5 mM, $MgCl_2 \times 6H_2O$ 1.5 mM, pH 7.6). This buffer corresponds to the in vivo plasma composition of the lamprey erythrocyte [14]. The uppermost layer of the erythrocyte suspension (buffy coat) was discarded at every wash. Human erythrocytes were isolated from blood collected by venipuncture of the authors [2]. Cells were used the day they were drawn.

2.3. Cell density

Our attempt was to compare the effects of amphiphiles in lamprey, trout and human erythrocytes at the same amphiphile concentration/plasma membrane area of the erythrocyte. The surface area of the lamprey erythrocyte has been estimated to 283.5 μm^2 [15], the area of the trout erythrocyte to 265 μm^2 [16], and the area of the human erythrocyte to 140 μm^2. Erythrocytes were therefore suspended in the buffer at a cell density of: lamprey erythrocytes, 0.82×10^8 cells/ml; trout erythrocytes, 0.87×10^8 cells/ml; and human erythrocytes, $1.65 \times$

Table 1
Amphiphile concentrations inducing 50% hemolysis (release of hemoglobin) in lamprey, trout and human erythrocytes

Compound	Concentration (μM) inducing 50% hemolysis		
	Lamprey	Trout	Human
Dodecylmaltoside	166 ± 33	231 ± 19	122 ± 8
Dodecylzwittergent	1243 ± 172	869 ± 37	930 ± 28
Dodecyltrimethylammonium bromide	481 ± 46	546 ± 34	767 ± 37
Chlorpromazine	358 ± 26	427 ± 46	511 ± 49
Octaethylene glycol dodecyl ether	113 ± 17	104 ± 9	100 ± 6
Dehydroabietic acid	219 ± 16	149 ± 17	695 ± 36
Isopimaric acid	84 ± 9	68 ± 11	147 ± 8

The hemolytic activity of amphiphiles in lamprey, trout and human erythrocytes expressed as the concentrations inducing 50% release of hemoglobin following 60 min incubation at RT at a plasma membrane area in the samples of $\sim 2.31 \times 10^{10}$ μm^2/ml ($n = 3$).

10^8 cells/ml. In this way, the total area of erythrocytes in a sample is approximately similar (2.3×10^{10} μm^2/ml).

2.4. Determination of the hemolytic activity of amphiphiles

Incubation with amphiphiles was carried out at room temperature (RT ≅ 24°C) for 60 min under gently mixing. Following incubation, the amount of hemoglobin in the cell free supernatant was photometrically (545 nm) recorded. Amphiphile concentrations giving 50% hemolysis were compared (Table 1).

2.5. Determination of the shape altering effect of amphiphiles

Erythrocytes were incubated as above for 60 min (RT) with amphiphiles at concentrations ranging from low to high sublytic. With lamprey and trout erythrocytes, the high sublytic amphiphile concentrations (Table 2) were 75% of those inducing 2–10% hemolysis at 60 min incubation. With human erythrocytes, the high sublytic concentrations were those found to result in maximum protection against hypotonic hemolysis following 60 min incubation at 37°C, as previously described [2,17]. The high sublytic concentrations used for the three cell types are approximately equivalent. The concentration- and time-dependence of shape alterations were first studied by light microscopy of unfixed cells (in a hanging drop), before electron microscopy was applied.

2.6. Transmission electron microscopy (TEM)

Erythrocytes were suspension-fixed in 1% glutaraldehyde in the buffer for 30 min at RT, postfixed in 1% OsO_4 in 0.9% NaCl for 30 min at RT, dehydrated in a graded series of acetone/water (50 to

Table 2
Sublytic amphiphile concentrations used in the shape studies

Compound	Sublytic concentration used (μM) in experiments with erythrocytes from	
	Lamprey	Trout
Dodecylmaltoside	80	90
Dodecylzwittergent	720	560
Dodecyltrimethylammonium bromide	200	220
Chlorpromazine	150	190
Octaethylene glycol dodecyl ether	50	50
Dehydroabietic acid	70	60
Isopimaric acid	25	25

Concentrations are 75% of those giving 2–10% hemolysis.

100%, v/v) and finally embedded in Epon. The sections were stained with lead acetate and post-stained with uranyl acetate before examination in an JOEL 100SX electron microscope.

2.7. Scanning electron microscopy (SEM)

Erythrocytes, fixed, post-fixed and dehydrated as above, were critical-point dried, gold-sputtered, and finally examined in a Cambridge Instruments S360 microscope.

2.8. Confocal laser scanning microscopy (CLSM)

Erythrocytes were incubated in the presence of (10 mg/ml) fluorescein isothiocyanate (FITC)-dextran 70S (Sigma) for 60 min at RT with and without amphiphiles. Following washing, cells were fixed in paraformaldehyde (4%) and mounted between coverslips and slides in Mowiol 40-88 (Aldrich). Samples were studied (100×/1.4 aperture immersion oil objective, 10× ocular) and scanned (∼0.05 μm between scanned sections) using a Leica confocal laser scanning microscope. Scans were combined to images giving maximum projection.

Figures were composed from representative fields using Corel DRAW.

3. Results

3.1. Amphiphile-induced hemolysis

Concentrations giving 50% hemolysis of lamprey, trout and human erythrocyte samples are shown in Table 1. Each kind of amphiphile hemolyzed erythrocytes from the three species at a largely similar concentration, although some differences were seen. The most pronounced difference was seen with dehydroabietic acid, in which case, human erythrocytes were considerably less sensitive than lamprey and trout erythrocytes.

3.2. Shape alterations in human erythrocytes

The amphiphiles used in this study have previously been shown to induce, as demonstrated in Fig. 1, either echinocytic or stomatocytic shape transformations in human erythrocytes [2–4,18]. The shape alterations are concentration dependent. In the echinocytic shape transformation, human erythrocytes at low amphiphile concentrations rapidly (< 1 min) attain spiculated shapes with broad spicula (echinocytes) (Fig. 1B inset), and at high concentrations spherical shapes with small blebs/protrusions (sphero-echinocytes) (Fig. 1B), from which microex-

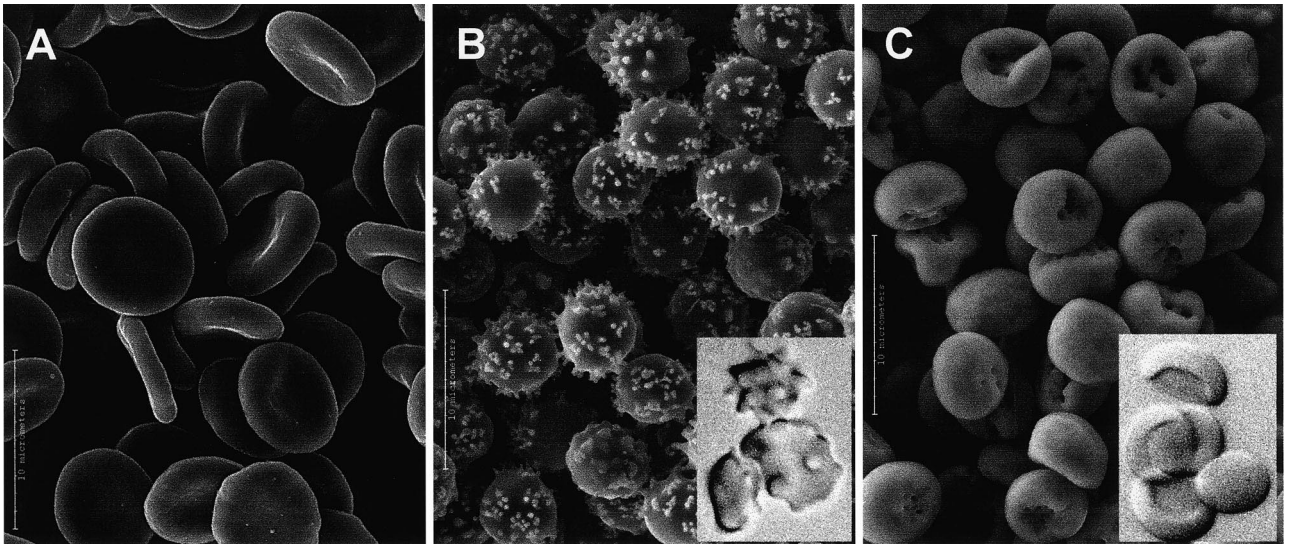


Fig. 1. Echinocytic and stomatocytic human erythrocytes. (A) Discocytes. (B) Sphero-echinocytes (dodecylmaltoside, 44 μM), in inset echinocytes (dodecylmaltoside, 4 μM). (C) Sphero-stomatocytes (chlorpromazine, 100 μM), in inset cup-shapes (chlorpromazine, 10 μM). A, B and C are SEM micrographs, while insets are interference contrast micrographs.

ovesicles are released. In the stomatocytic shape transformation human erythrocytes at low concentrations rapidly (< 1 min) attain a cup-like shape (stomatocytes) (Fig. 1C inset), and at high concentrations rounded non-axisymmetric poly-invaginated shapes (sphero-stomatocytes) (Fig. 1C). Endovesicles may be released from the stomatocytic invaginations.

It has been reported that the cell volume does not change in the early stages of amphiphile-induced echinocytic and stomatocytic shape alterations at low concentrations [19]. The results of theoretical considerations are consistent with the view that initial cell shape changes are not dependent from a cell volume change [11,20]. At higher amphiphile concentrations, erythrocytes irreversibly lose surface area and volume due to exo- or endovesiculation [21], which leads to a rounding up of cells. At high membrane concentrations of amphiphiles, the permeability properties of the membrane may be disturbed resulting in cell sphering due to inflow of water.

3.3. Shape alterations in lamprey erythrocytes

Lamprey (and trout) erythrocytes were treated 1 and 60 min with amphiphiles at sublytic concentrations. Erythrocyte shape was classified according to the nomenclature of Bessis [22], developed for classification of human erythrocyte shapes. In the present paper, echinocytic shape means echinocytes of type I–III.

Untreated resting lamprey erythrocytes have a non-axisymmetric stomatocyte-like shape (Fig. 2A,B) [1]. The 'echinocytogenic' amphiphiles dodecylmaltoside, dodecylzwittergent and dodecyltrimethylammonium bromide did not markedly perturb the overall lamprey erythrocyte shape, either at low or high sublytic concentrations, besides that a slight cell sphering was induced at high (Table 2) concentrations (Fig. 2C,D). No well-defined 'echinocytic' shapes with large broad spicula occurred. However, blebs and long (length up to 10 μm) thin tubular protrusions were rapidly (< 1 min) formed on the plasma membrane. Mainly spherical (diameter ~50–160 nm), but also tubular (diameter ~30–50 nm), exovesicles were found in the supernatant (not shown).

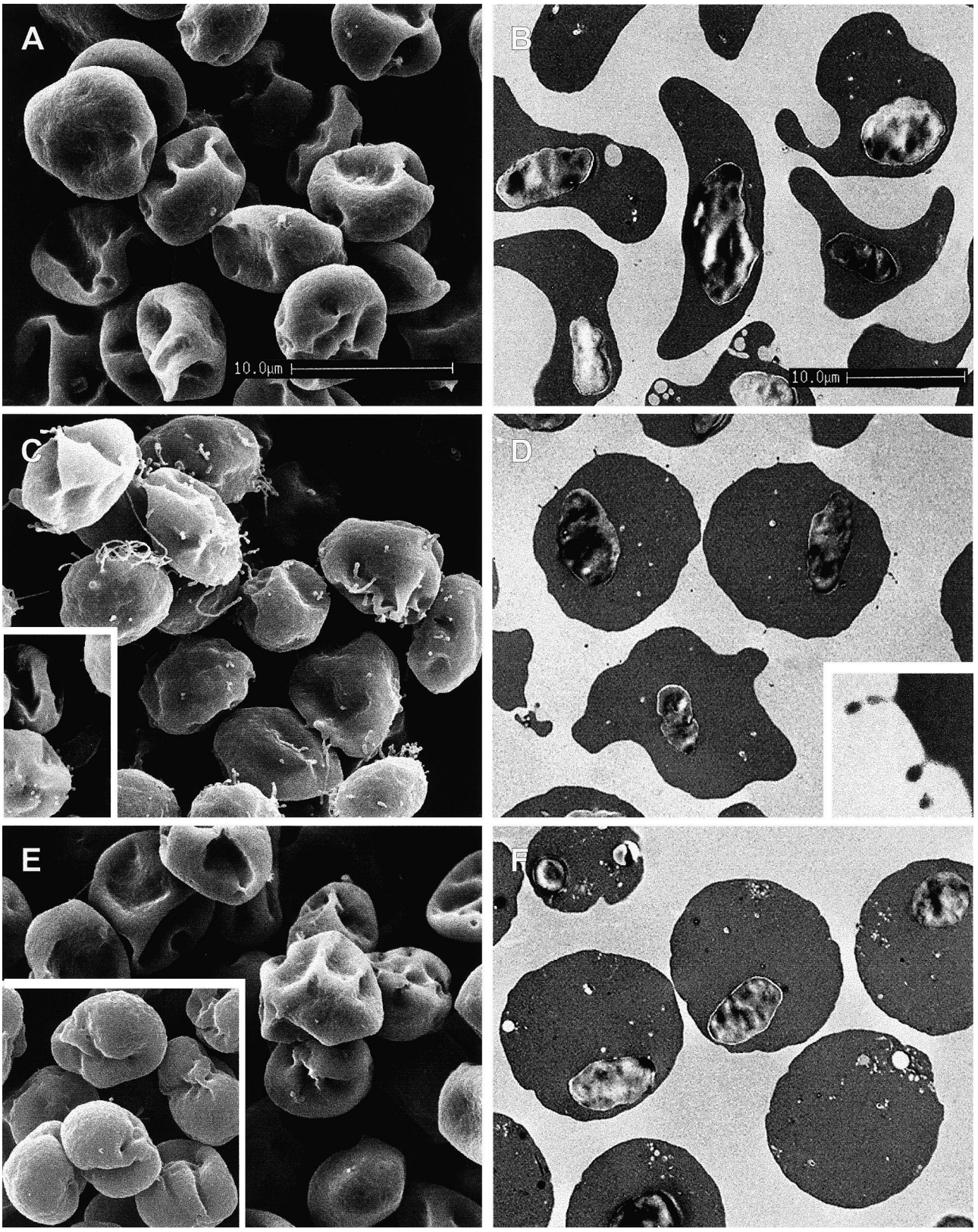
With the 'stomatocytogenic' amphiphiles chlorpromazine and octaethylene glycol dodecyl ether, no marked changes in the gross morphology were observed following 1 min incubation (Fig. 2E). Following 60 min incubation at high amphiphile concentrations, the erythrocytes were rounded, like in Fig. 2E inset (Fig. 2F). Notably, the resin acids dehydroabietic acid and isopimaric acid, which are weakly echinocytogenic in human erythrocytes [23], induced a rounding up and invaginations (Fig. 2E inset). The occurrence of membrane invaginations at high amphiphile concentrations was confirmed by CLSM using FITC-dextran as a non-specific fluid marker (Fig. 3C).

No obvious effect on the overall cell shape of amphiphile-treated cells following washing with 1% bovine serum albumin (BSA) was observed (not shown). Incubation of lamprey erythrocytes in 40% buffer (hypotonic swelling) revealed that an initial (at 1 min) sphering occurred, but this was reversed following 60 min incubation (not shown).

The resting shape of lamprey erythrocytes is stomatocyte-like [1]. It should be noted that the slightly wrinkled character of lamprey erythrocytes, and the presence of small invaginations, in SEM micrographs may be due to sample preparation.

3.4. Shape alterations in trout erythrocytes

Untreated resting trout erythrocytes have a flattened oval shape and a smooth membrane contour (Fig. 4A,B). At low concentrations, the 'echinocytogenic' amphiphiles dodecylmaltoside, dodecylzwittergent and dodecyltrimethylammonium bromide induced a corrugated appearance (Fig. 4C1), and echinocytic spicula occurred around the cell rim as in Fig. 4C2. At slightly higher concentrations, bent spiculated cells occurred (Fig. 4C3). At high sublytic amphiphile concentrations (Table 2), a rounding up of the spiculated cells occurred and erythrocytes attained a sack-like bent shape (Fig. 4C). Bouquets of tubular protrusions (length 500–700 nm) protruding from the plasma membrane occurred, like those seen in human erythrocytes incubated with echinocytogenic amphiphiles (Fig. 1B, see also [18]). Thin sections revealed a non-ellipsoidal (often seed-like with sharp ends) shape contour of the plasma membrane (Fig. 4D). Mainly spherical (diameter ~50–180 nm), but also tubular (diameter ~50 nm) exovesicles occurred in the supernatant (not shown).


A
10.0µm
B
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C
D
E
F

Fig. 2. Lamprey erythrocyte morphology (SEM) and ultrastructure (TEM). Untreated (A,B). Protrusions induced by (C) dodecylmaltoside (80 μM, 1 min), (C inset) dodecylmaltoside (10 μM, 1 min) and (D) dodecyltrimethylammonium bromide (200 μM, 1 min). (D inset) shows a higher magnification of D. Invaginations induced by (E) chlorpromazine (150 μM, 1 min), (E inset) dehydroabietic acid (70 μM, 60 min) and (F) chlorpromazine (150 μM, 60 min).
←

Following washing of cells with 1% BSA, no reversion from bent swollen shapes occurred, while a partial reversion from corrugated shapes occurred (not shown). Sack-like shapes initially occurred in erythrocytes hypotonically swollen by incubation in 40% buffer (not shown). As in the case with lamprey erythrocytes, the rounding up of trout erythrocytes at high amphiphile concentrations may be due to amphiphile-intercalation or/and membrane permeability increase. Similar to human erythrocytes [2], trout erythrocytes incubated with dodecyltrimethylammonium bromide reverted from shapes with echinocytic protrusions to nearly normal shapes following 60 min incubation (not shown).

Chlorpromazine and octaethylene glycol dodecyl ether, which are stomatocytogenic in human erythrocytes, did not induce marked shape alterations in trout erythrocytes (Fig. 4E). With chlorpromazine, at high sublytic concentrations, minor invaginations occurred in the plasma membrane around the nucleus. These invaginations appear as 'empty' structures around the nucleus in thin sections (Fig. 4F) and as fluorescent structures around the nucleus in CLSM (Fig. 3D). Following 60 min incubation, the nucleus had a more spherical shape and the erythrocyte was thinner (Fig. 4G). Notably, a few small protrusion occurred on the plasma membrane of trout erythrocytes treated with chlorpromazine and octaethylene glycol dodecyl ether for 60 min (Fig. 4C2). These protrusions were mainly located at the rim of the cell. Dehydroabietic acid and isopimaric acid did not perturb the trout erythrocyte shape (not shown).

Since acetylcholinesterase (AChE) is present in the

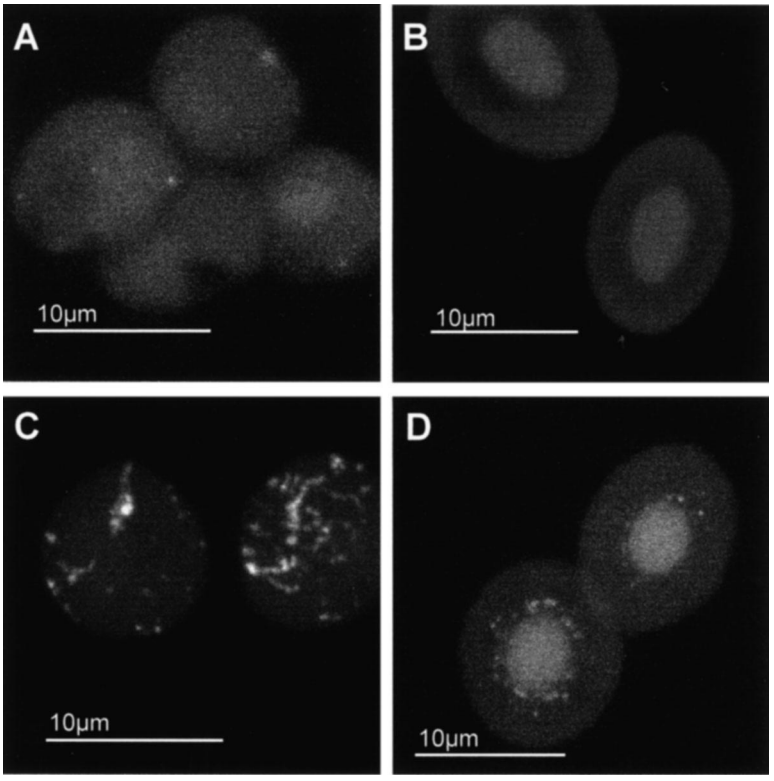

Fig. 3. FITC-dextran incorporation in lamprey (A,C) and trout (B,D) erythrocytes (CLSM). (A,B) Untreated cells. (C,D) Cells treated with chlorpromazine (150 and 190 μM, respectively, for 60 min).

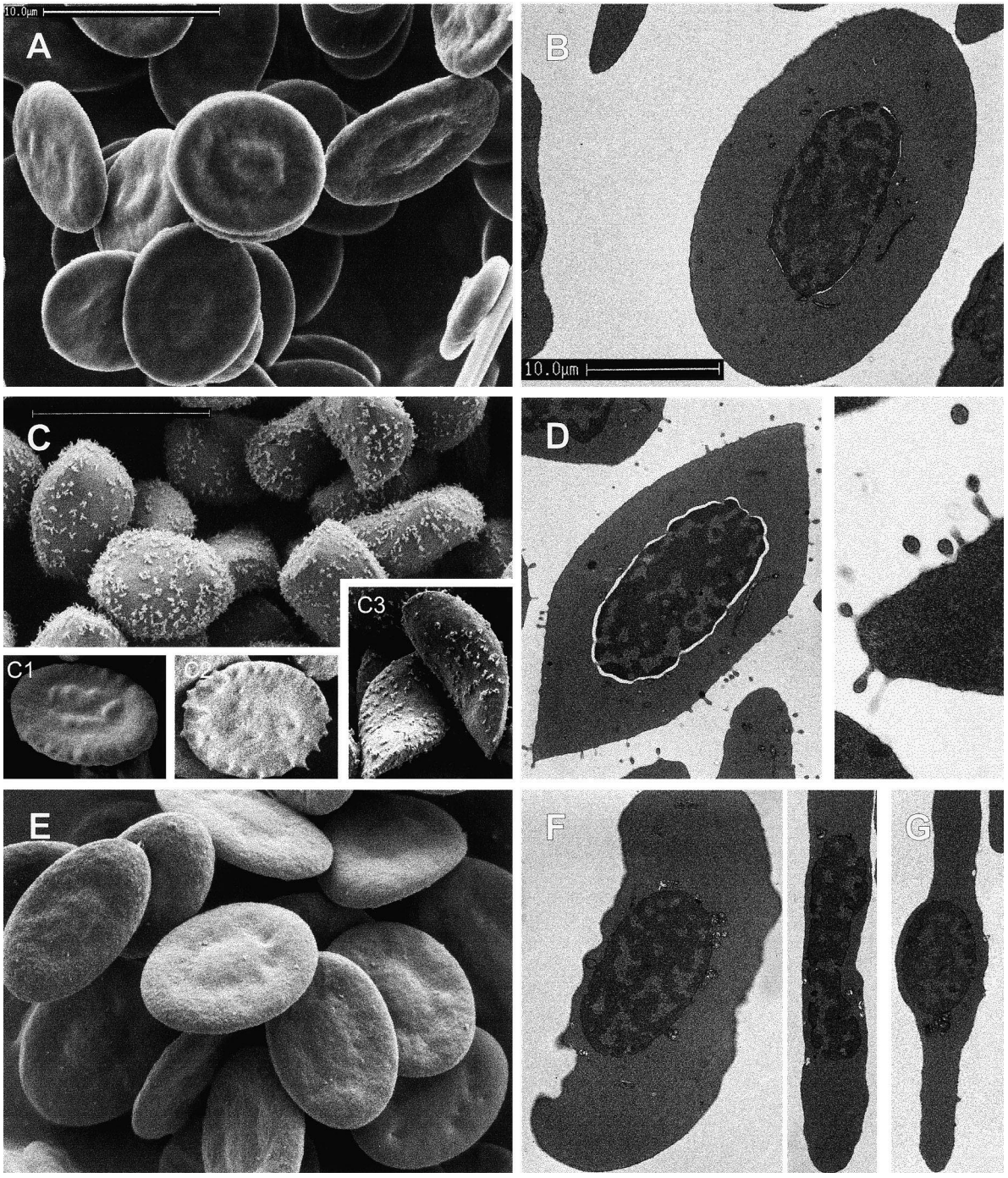

Fig. 4. Trout erythrocyte morphology (SEM) and ultrastructure (TEM). Untreated (A,B). Protrusions induced by (C) dodecylmaltoside (90 μM, 1 min), (C1) dodecylmaltoside (5 μM, 1 min), (C2) octaethylene glycol dodecyl ether (50 μM, 60 min), (C3) dodecyltrimethylammonium bromide (100 μM, 1 min), and (D) dodecylmaltoside (90 μM, 1 min). D inset shows a higher magnification of D. Invaginations induced by (E and F) chlorpromazine (190 μM, 1 min) and (G) chlorpromazine (190 μM, 60 min).

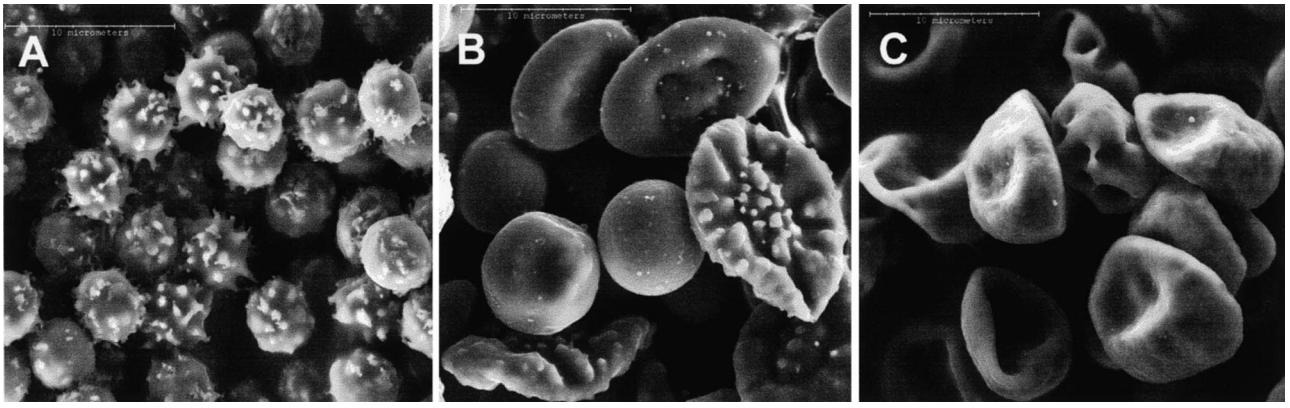

Fig. 5. Erythrocytes incubated with ionophore A23187 (5 μM) plus Ca^{2+} for 10 min. (A) human, (B) trout and (C) lamprey erythrocytes.

plasma membrane of human erythrocytes (where it has been frequently used to monitor exovesiculation), it can be noted that lamprey and trout erythrocytes did not show AChE activity as recorded by the method of Ellman et al. [24] (not shown).

3.5. Treatment with ionophore A23187

Human erythrocytes rapidly attained, as previously reported [25,26], an echinocytic shape upon incubation with ionophore A23187 (5 μM) plus Ca^{2+} (Fig. 5A, 10 min). In trout erythrocytes, ionophore A23187 (5 μM) rapidly induced wrinkled and spherical cell shapes (Fig. 5B, 10 min). The formation of wrinkled shapes as a result of both Ca^{2+} loading and ATP depletion has previously been observed in ellipsoidal goose erythrocytes [27]. It was observed that wrinkling precedes the formation of spherical shapes. The marginal band of microtubules had disappeared in Ca^{2+}-loaded trout erythrocytes (not shown). Surprisingly, lamprey erythrocytes did not respond with an altered morphology upon treatment with ionophore A23187 (5 μM) plus Ca^{2+} (Fig. 5C, 10 min).

3.6. Possible fixation artefacts

A marked influence of fixation on the amphiphile- and Ca^{2+}-induced overall lamprey and trout erythrocyte cell shape can be excluded since the cell shapes were similar in fixed and unfixed samples (not shown). The formation and shape of membrane protrusions also seem not to be fixation related, since it has been shown that the shape (spherical/tubular) of amphiphile-induced protrusions on fixed human erythrocyte is similar to the shape of exovesicles isolated before fixation [3,18].

4. Discussion

The present study shows that lamprey and trout erythrocytes respond to incubation with amphiphiles with more restricted and different alterations in the overall cell shape than human erythrocytes. Below, we first discuss how these shape alterations can be interpreted according to the bilayer-couple mechanism. Then, the impact of structural proteins on the shape alterations is discussed. Finally, Ca^{2+}-induced shape alterations are considered.

4.1. The bilayer-couple mechanism

Amphiphiles may induce either spiculated (echinocytic) or invaginated (stomatocytic) shapes in human erythrocytes [4,5,28,29]. These shape transformations are thought to mainly depend on the distribution of the amphiphile in the bilayer, i.e. whether the amphiphile is predominantly incorporated into the outer or inner plasma membrane leaflet, thereby expanding this leaflet relative to the other [30–36]. The distribution at equilibrium of charged amphiphiles, in case they can translocate to the inner bilayer leaflet, is thought to be dependent mainly on electrostatic interactions between the amphiphile and the negatively charged phospholipids at the inner leaflet. The mech-

anism whereby non-ionic detergents induce stomatocytosis is unknown [28]. It is unclear whether secondary amphiphile-induced rearrangements, involving endogenous membrane components [37], may contribute to the resulting erythrocyte shape.

The 'echinocytogenic' amphiphiles, except the resin acids, rapidly induced plasma membrane protrusions in lamprey and trout erythrocytes. However, no well-defined echinocyte shapes with large broad spicula, as is induced in human erythrocytes, were formed. Apparently, the protrusion-forming amphiphiles are readily intercalated into the outer plasma membrane leaflet of all three cell types.

The 'stomatocytogenic' amphiphiles, which induce invaginated shapes in human erythrocytes, showed similar characteristics in lamprey and trout erythrocytes. In trout erythrocytes, the invaginations were located on the flat parts of the cell around the nucleus, which indicates specific plasma membrane properties here. Surprisingly, some protrusions were induced by the 'stomatocytogenic' amphiphiles in trout erythrocytes, predominantly near the (curved) edge of the erythrocyte. It can be hypothesized that 'stomatocytogenic' amphiphiles like chlorpromazine prefer and laterally segregate into membrane areas having a negative curvature when located in the inner membrane leaflet, but oppositely prefer positive membrane curvature areas when in the outer leaflet. The occurrence of membrane protrusions may, according to the bilayer couple hypothesis [30,31], indicate that the equilibrium distribution of the 'stomatocytogenic' amphiphiles between the plasma membrane leaflets is slightly displaced to the outer leaflet in the trout erythrocyte plasma membrane, compared to the situation in the human erythrocyte. This interpretation implicates that there are differences between the trout, lamprey and human erythrocyte plasma membrane, presumably in the phospholipid composition and/or in the charge distribution between the outer and the inner leaflets of the plasma membrane. There are other indications supporting this interpretation. For example, the resin acids induced invaginations in lamprey and trout erythrocyte, while they were slightly echinocytogenic in human erythrocytes [23]. Furthermore, phosphatidylcholine was enriched and sphingomyelin depleted in the lamprey erythrocyte plasma membrane, compared to the phospholipid composition of the human erythrocyte [1]. The hemolysis experiments, showing that there are differences in the potency of some amphiphiles to hemolyze lamprey, trout and human erythrocytes, are in line with these findings.

4.2. Impact of structural proteins on erythrocyte shape

The membrane skeleton is thought to normally play a passive role in human erythrocyte shape alterations [38–40]. This can be at least partially explained by the fact that the area expansivity modulus of the membrane skeleton and its bending constant are in normal conditions a few order of magnitude smaller than the corresponding constants of the lipid bilayer [41]. However, the membrane skeleton shear elasticity is essential to stabilize the echinocytic shape where the shear energy of the skeleton is comparable to the membrane bending energy [9,10,42]. The membrane skeleton may also play a significant role in shape transformations when the normal physical state of the skeleton or the skeleton–bilayer interactions are seriously disturbed [8,43]. For example, it has been suggested that aggregation of the skeleton at low pH [44] and the abolishment of the skeleton–bilayer interactions at high pH [43] may cause a discontinuous erythrocyte shape transformations [12,45]. The membrane skeleton may probably also stabilize the erythrocyte shape by maintaining a homogeneous lateral distribution of membrane integral proteins [32,33,36,46].

It has been established that an increase of the membrane shear modulus stabilizes the cell shape and prevents major shape changes by echinocytogenic and stomatocytogenic agents [8]. However, budding (vesiculation) may still occur in such cells [44], presumably since it can be considered as a local process [21,47] not necessarily involving gross morphological changes and sphering of the cell. A local disruption of the skeleton [44] or a local detachment of the skeleton from the membrane bilayer [48,49] seems to be a necessary condition for the separation of daughter vesicles from the mother cell [47].

The effective membrane area shear modulus (μ) of nucleated fish, bird and turtle erythrocytes is usually 5–15 times greater than that of mammalian erythrocytes [50]. This is probably mainly because nucleated erythrocytes besides the two-dimensional membrane skeleton have an additional three-dimensional cyto-

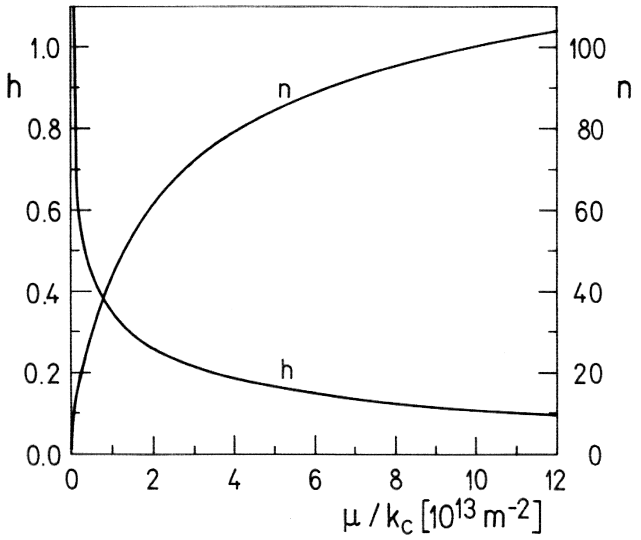

Fig. 6. Theoretically determined number of erythrocyte protrusions (n) and the corresponding length of a single protrusion (h) as a function of the ratio μ/k_C, where μ is the effective membrane area shear modulus and k_C is the local bending modulus of the membrane (adapted from [10,42]). The relative volume (v) is 0.6 and the relative difference between the outer and the inner lipid layer areas (Δa) is 2.4 [10]. The value of the ratio $\mu/k_C \cong 10^{13}$ m^{-2} was determined for human erythrocytes [51,52].

skeletal system (including the nucleus and the marginal band of microtubules). The three-dimensional cytoskeletal system may thus hinder major shape changes. For example, the bent cell shape of trout erythrocytes at high concentrations of echinocytogenic agents is probably mainly dictated by the marginal band of microtubules and the intermediate filaments. During cell rounding up/swelling of trout erythrocytes the presence of structures connecting adjacent plasma membrane segments around the nucleus was apparent.

The membrane shear modulus of lamprey erythrocytes has not been determined. It is, however, probably much lower than in trout erythrocytes because lamprey erythrocytes seem to lack band 3 protein/ankyrin, leading to weak membrane skeleton–lipid bilayer interactions, and a marginal band of microtubules [1].

Fig. 6 illustrates the effect of the ratio μ/k_C on the shape and the number of membrane protrusion of the theoretically determined erythrocyte shape [10,11,42], where μ is the effective membrane area shear modulus and k_C is the local bending modulus of the membrane [10,42]. In the theoretical prediction presented in Fig. 6 the equilibrium model erythrocyte shape at a given relative cell volume (v) and a given relative difference between the outer and the inner lipid bilayer area (Δa) [11,12] was calculated by minimization of the membrane elastic energy consisting of bending and shear contributions [9,11], as described in detail elsewhere [10,11,42]. It can be seen in Fig. 6 that at a given k_C, the number of protrusions (n) is an increasing function of μ, while the length of a single protrusion (h) decreases with an increasing value of μ. Since trout erythrocytes are assumed to have a much higher membrane area shear modulus (μ) than the human erythrocytes it could be anticipated from the theoretical results given in Fig. 6 that the protrusions in trout erythrocytes at high concentrations of echinocytogenic agents should be shorter, but more numerous, than those in human erythrocytes (Figs. 1B and 5C). On the other hand, in lamprey erythrocytes, the absence of band 3 protein/ankyrin-mediated membrane skeleton–bilayer interactions [1] should lead to a small effective membrane shear modulus μ, indicating according to our theoretical prediction (Fig. 6) the presence of only a few long protrusions at high concentrations of echinocytogenic agents. In accordance, the appearance of a few long protrusions on the lamprey erythrocyte membrane (Fig. 2C) is very different from the appearance of many short protrusions distributed all over the cell surface in trout erythrocytes (Fig. 4C).

4.3. Ca^{2+} and ionophore A23187

Treatment with ionophore A23187 plus Ca^{2+} is 'echinocytogenic' in human erythrocytes. In trout erythrocytes, elevated intracellular Ca^{2+} levels induced wrinkled and spherical shapes. Similar shapes have been observed in avian erythrocytes upon treatment with A23187 plus Ca^{2+} [53,54]. A Ca^{2+}-induced disassembly of the marginal band of microtubules, an effect also observed in frog [55] and chicken [56] erythrocytes, seems essential for such major shape alterations to occur. Surprisingly, lamprey erythrocytes showed no or a very restricted response to treatment with ionophore A23187 plus Ca^{2+}. A previous study indicated that lamprey erythrocytes lack band 3 protein and ankyrin [1], which together con-

stitute a major link between the membrane skeleton and the plasma membrane in human erythrocytes. Therefore, it is tempting to suggest that the Ca^{2+}-induced perturbation of the human and trout erythrocyte shape involves a primary effect of Ca^{2+} on the spectrin based skeleton, an effect which cannot be expressed on the lamprey erythrocyte morphology because of the lack of band 3 protein/ankyrin. In line with these suggestions, studies of Ca^{2+}/ionophore A23187 treated ankyrin- and spectrin-deficient mice erythrocytes indicate that the functional integrity of the membrane skeleton is essential for the maintenance and transformation of the erythrocyte shape [57]. Accordingly, it has been suggested that the impotency of Ca^{2+}/ionophore A23187 to induce shape alterations in Scott syndrome erythrocytes is due to an aberration in Ca^{2+}-regulated rearrangements of the membrane skeleton [58]. Preliminary results indicating that 4,4′-diisothiocyano-2,2′-stilbene disulfonate (DIDS) decreases Ca^{2+}/ionophore A23187-induced vesiculation in human erythrocytes further indicate an involvement of band 3 protein in Ca^{2+}-induced shape alterations (Hägerstrand, unpublished results). However, the precise mechanism for Ca^{2+}-induced shape alterations in erythrocytes remains unknown. The prevailing hypothesizes either involve a dephosphorylation (splitting) of phosphoinositides or a lipid scrambling [39,59]. Such processes may be influenced by skeleton–lipid bilayer interactions via the band 3 protein/ankyrin complex (see [60]).

To summarize, our study demonstrates that the shape altering character of an amphiphile is dependent on the properties of the target cell and its plasma membrane. Apparently, intracellular structural protein complexes, i.e. the membrane skeleton, the marginal band of microtubules and the intermediate filaments, as well as their interactions with the lipid bilayer and (or via) the nucleus, strongly influence shape alterations in erythrocytes.

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