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Vesiculation induced by amphiphiles in erythrocytes

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The ability of shape-transforming cationic, anionic, zwitterionic, and nonionic amphiphiles to induce vesiculation in human erythrocytes was studied. At concentrations where they exhibit maximum protection against hypotonic haemolysis (CAH_{max}) echinocytogenic amphiphiles induced a rapid release of exovesicles. Following 5 min of incubation, the vesicle release (acetylcholinesterase release) amounted from 4% (sodium alkyl sulphates) to 13% (zwittergents) of the total acetylcholinesterase activity of the erythrocytes. At concentrations corresponding to CAH_{50} the vesicle release was less than 15% of that released at CAH_{max}. The size and the appearance of the vesicles varied with the type of amphiphile. Stomatocytogenic amphiphiles which do not pass the erythrocytes through echinocytic stages, did not induce release of exovesicles. Electron and fluorescence microscopic observations of erythrocytes treated with stomatocytogenic amphiphiles strongly indicated that an endovesiculation had occurred. Amphiphiles which pass the erythrocytes through echinocytic stages before stomatocytic shapes are attained, induced a release of both exo- and endovesicles.

Introduction

A wide variety of treatments triggers vesiculation in human erythrocytes. Release of exovesicles has, for example, been reported following ATP depletion [1], increased intracellular calcium levels [2,3] and following incubation with sonicated dimyristoylphosphatidylcholine vesicles [4–7], while endovesiculation has been reported in erythrocytes treated with sphingomyelinase C [8] and in ghosts after ATP treatment [9,10]. These treatments also induce alterations of the erythrocyte shape.

It was recently stated [6] that no vesiculation has been observed after treatment of erythrocytes with membrane-penetrating agents, such as lysophospholipids and amphiphilic drugs, although these agents induce alterations in the erythrocyte shape. It has, however, been shown that several non-aliphatic amphiphilic compounds induce vesiculation in erythrocytes. Exovesiculation has been observed following treatment with glycocholate [11,12] and endovesiculation following treatment with some phenothiazines, including chlorpromazine [9,13,14]. Furthermore, release of vesicular fragments has been reported after treatment of erythrocyte ghosts with phenothiazines and decanol [15].

In a previous study on shape alterations induced by amphiphiles in erythrocytes, we observed that membrane components were released by some of the amphiphiles at sublytic concentrations [16]. In the present paper we show that amphiphiles with an aliphatic hydrocarbon chain (detergents), at concentrations where they exhibit maximum protection against hypotonic haemolysis, induce vesiculation in human erythrocytes.

Materials and Methods

Chemicals. Alkyltrimethylammonium bromides (C_{10}–C_{16}), chlorpromazine hydrochloride, acetylcholinesterase (human) and fluorescein isothiocyanate-conjugated dextran (FITC-dextran; M_r 70 000) were purchased from Sigma Chemical Co. Zwittergent® detergents (3-(alkyldimethylammonio)-1-propanesulphonates, C_{10}–C_{16}) and decyl β-D-glucopyranoside were obtained from Calbiochem-Behring, sodium alkyl sulphates (C_{10}–C_{14}), acetylthiocholine iodide and 2,2′-dinitro-5,5′-dithiobenzoic acid from E. Merck AG and dodecyl D-maltoside from Fluka. Octaethyleneglycol mono *n*-alkyl ethers (C_{10}–C_{16}) and pentaethyleneglycol mono *n*-dodecyl ether were obtained from Nikko Chemicals Co. All the amphiphilic agents used were of reagent grade and all other reagents were standard commercial products of the highest purity available.

Erythrocytes. Blood was drawn from healthy donors by vein puncture into heparinized tubes. The erythro-

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TABLE I

Concentrations of amphiphiles giving half-maximum (CAH_{50}) and maximum (CAH_{max}) protection against hypotonic haemolysis in human erythrocytes ((1.6–1.7)·10^8 cells/ml) following incubation for 1 h at 37°C.

Amphiphile	Concentration (μM)	
	CAH_{50}	CAH_{max}
Sodium alkyl sulphates		
C_{10}	153	708
C_{12}	20.8	50
C_{14}	8	23.3
Alkyltrimethylammonium bromides		
C_{10}	720	3300
C_{12}	76.7	300
C_{14}	15.3	40
C_{16}	7.5	14.2
Chlorpromazine hydrochloride	10	20
Octaethyleneglycol mono *n*-alkyl ethers		
C_{10}	113	300
C_{12}	9.9	43.8
C_{14}	7.9	24.4
C_{16}	6.4	20.5
Pentaethyleneglycol mono *n*-dodecyl ether	8.9	40
Decyl β-D-glucopyranoside	170	740
Dodecyl D-maltoside	16.6	40
3-(Alkyldimethylammonio)-1-propanesulphonates		
C_{10}	1430	3630
C_{12}	103	263
C_{14}	8	27.5
C_{16}	5	13.8

cytes 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 (1.6–1.7)·10^9 cells/ml and kept at 4°C until used. All the experiments were carried out within 48 h after the blood was drawn.

Incubation of erythrocytes. Aliquots of the erythrocyte suspension were pipetted into polystyrene tubes or glass vials containing buffer and various concentrations of the amphiphiles. The final cell density was (1.6–1.7)·10^8 cells/ml and the incubations were carried out in a shaking thermostat bath at 37°C. The amphiphiles were used at concentrations (Table I) previously determined to result in maximum protection (CAH_{max}) and half-maximum protection (CAH_{50}) against hypotonic haemolysis following 60 min incubation [17,18]. The concentrations of the amphiphiles used were well below their CMC values [19].

Release of acetylcholinesterase. Following incubation, samples were centrifuged twice at 10000 × *g* for 2 min, and the cell-free supernatant was monitored for acetylcholinesterase (AChE) activity according to Ellman et al. [20]. AChE activity in the supernatant was expressed as % of total activity in the erythrocyte suspension.

Processing of erythrocytes and vesicles for transmission electron microscopy. Erythrocytes were pelleted by centrifugation at 1000 × *g* for 7 min and vesicles were, after an additional centrifugation, pelleted by centrifugation of the supernatants at 40000 × *g* for 30 min. Vesicles and erythrocytes were fixed in 2% glutaraldehyde in buffer for 1 h at 4°C, and postfixed in 1% OsO_4 in buffer for 30 min at 4°C. After dehydration in a graded series of acetone/water, the samples were embedded in Epon. The grids were stained with lead citrate. In some experiments a small amount of fixed erythrocytes, pelleted from the same sample, was added to the vesicle suspension in order to facilitate the handling of the small vesicle pellet.

Internalization of FITC-dextran. Erythrocytes were incubated with amphiphiles in the buffer containing FITC-dextran (10 mg/ml) for 60 min at 37°C. Following incubation, the erythrocytes were rapidly washed five times with the buffer. The erythrocytes were then examined with a Leitz Orthoplan fluorescence microscope using a ×100 oil immersion objective. Photographs were taken with a Kodak Tmax p3200 film exposed for about 30 s.

Results

AChE release induced by amphiphiles

AChE is a membrane-bound enzyme frequently used to monitor the release of membrane vesicles from the erythrocyte membrane [4–7,11]. Fig. 1 shows the AChE activity in supernatants following incubation of erythrocytes with amphiphiles at concentrations corresponding to CAH_{max}.

All amphiphiles inducing echinocytes during the incubation (sodium alkyl sulphates, alkyltrimethylammonium bromides, dodecyl maltoside and zwittergents) caused a release of AChE from the erythrocytes. Some of the alkyltrimethylammonium bromides induce stomatocytes following 30–60 min of incubation but these amphiphiles first pass erythrocytes through echinocytic shapes [16]. The enzymatic activity released after 120 min of incubation amounted from 5% (sodium alkyl sulphates) to 20% (zwittergents) of the total AChE activity of the erythrocytes. Within one homologue series all the homologues induced releases of AChE to the same extent as the C_{12}-homologues shown in Fig. 1. The release of AChE started within 1 min of incubation and was almost complete within the first 30 min. At concentrations corresponding to CAH_{50} the echinocytogenic amphiphiles induced only a slight release of AChE. It was less than 15% of that induced at CAH_{max} (not shown).

Amphiphiles inducing stomatocytes (decyl β-D-glucopyranoside, pentaethyleneglycol dodecyl ether, oc-

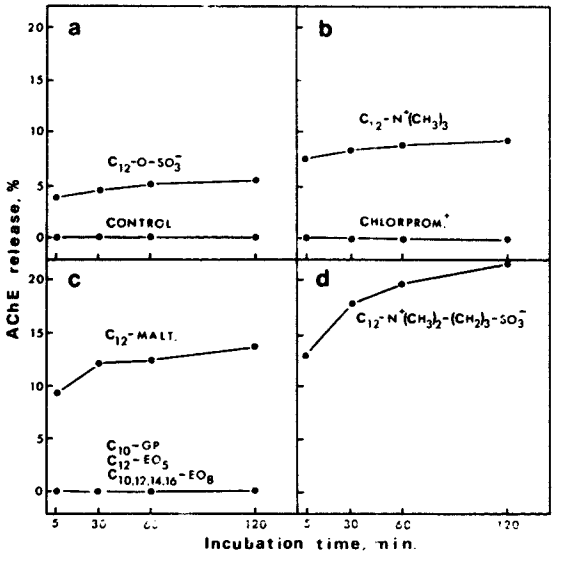

Fig. 1. Acetylcholinesterase (AChE) release from human erythrocytes during 120 min of incubation at 37°C with amphiphiles at concentrations corresponding to CAH_{max}. Cell density and amphiphile concentrations as in Table I. In the case of chlorpromazine a sublytic concentration (200 μM) higher than CAH_{max} was used. Each point represents the mean of 3–9 separate experiments. AChE activity in the supernatant is expressed as % of total activity in the erythrocyte suspension. CHLORPROM$^+$, chlorpromazine hydrochloride; C_{12}-MALT, dodecyl maltoside; C_{10}-GP, decyl glucopyranoside; C_{12}-EO_5, pentaethyleneglycol dodecyl ether; $C_{10,12,14,16}$-EO_8, octaethyleneglycol alkyl ethers.

taethyleneglycol alkyl ethers and chlorpromazine) did not induce a release of AChE at concentrations corresponding to CAH_{max} (Figs. 1b and 1c). The possibility that the amphiphiles inhibited AChE in the assay was checked by adding amphiphiles to AChE-containing supernatants and by measuring the activity of pure AChE in the presence of the amphiphiles. No decrease in the enzymatic activity was observed at concentrations corresponding to CAH_{max}.

It is important to note that a release of AChE was observed only with amphiphiles inducing echinocytic shapes. Thus, it appears that a transformation of erythrocytes to echinocytes is a prerequisite for a release of membrane vesicles to the external buffer. However, an induction of an echinocytic shape per se is apparently not sufficient to cause vesicle release, because when erythrocytes were crenated by elevating the pH of the buffer (pH 10) no release of AChE was observed (not shown).

Electron microscopic studies of vesicles and amphiphile-treated erythrocytes

In these studies, erythrocytes were treated with amphiphiles at concentrations corresponding to CAH_{max} for 30 min. Erythrocytes were first pelleted at 1000 × *g* and the supernatant was then centrifuged at 40000 × *g* to pellet vesicles. In order to make the processing of vesicles easier small amounts of fixed erythrocytes (from the same sample) were in some experiments added to fixed vesicles.

In Fig. 2A erythrocytes treated with the echinocytogenic amphiphile dodecyl maltoside are shown. As can be seen in the electron micrograph, the erythrocytes are crenated and many of the microvesicles have an elongated structure (Figs. 2a and 3a). Similar elongated microvesicles released from erythrocytes have previously been reported following an increased intracellular calcium level [21] and following treatment with glycocholate [11]. There was no sign of endovesicles in the erythrocytes. The other echinocytogenic amphiphiles (alkyl sulphates and zwittergents) gave essentially similar results.

Fig. 2b shows erythrocytes treated with the C_{12} homologue of the alkyltrimethylammonium bromides. This amphiphile first induces echinocytes and then, following 30–60 min of incubation, stomatocytes. As can be seen in the electron micrograph, the erythrocytes are cup-shaped and the exovesicles released are relatively homogeneous in size (diameter ≈ 200 nm). In some of the erythrocytes there are small vesicles indicating that an endovesiculation may have occurred (arrows). Interestingly, many of the vesicles had an open centre and an appearance indicating that they may be cup-shaped (Fig. 3b arrow). Some of the vesicles had a tail (Fig. 3b, inset). Similar tail-structures have previously been reported in erythrocyte vesicles induced by increasing the intracellular calcium level [21] and in vesicles released following in vitro ageing of erythrocytes [22]. The other homologues of the alkyltrimethylammonium bromides gave similar results.

Erythrocytes treated with the stomatocytogenic amphiphile octaethyleneglycol dodecyl ether are shown in Fig 2c. In these samples no exovesicles were seen. The erythrocytes frequently contained large empty spaces which may be interpreted as large endovesicles. All the other nonionic amphiphiles studied (C_{10}-GP, C_{12}-EO_5, C_{10}, C_{14} and C_{16}-EO_8) gave essentially similar results.

In Fig. 2d erythrocytes treated with the stomatocytogenic amphiphile chlorpromazine are shown. No exovesicles were seen in these samples but the electron micrographs strongly indicated that an endovesiculation had occurred. However, to induce vesiculation with this amphiphile, it was necessary to use a concentration which was 10-times higher (200 μM, sublytic) than that previously reported to induce maximum protection against hypotonic haemolysis [16]. This phenothiazine, however, differs from the amphiphiles with an aliphatic hydrocarbon chain in its ability to protect erythrocytes against hypotonic haemolysis. In our assay (erythrocytes were added to hypotonic solutions containing the amphiphiles) chlorpromazine induced only a slight re-

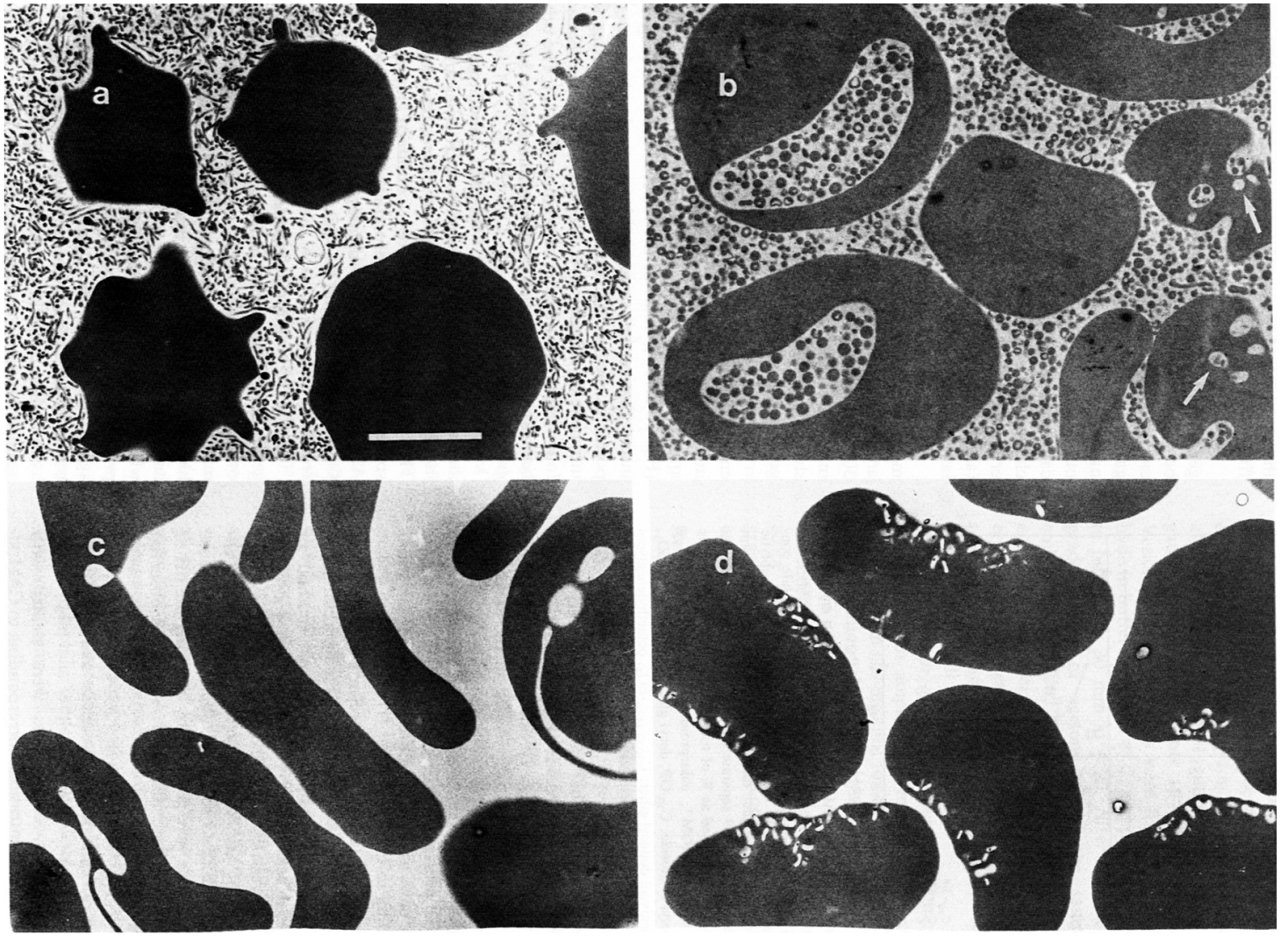

Fig. 2. Electron micrographs showing human erythrocytes following incubation with amphiphiles at 37 °C for 30 min at concentrations corresponding to CAH_{max}. Cell density and amphiphile concentrations as in Table 1. In the case of chlorpromazine a sublytic concentration (200 μM) higher than CAH_{max} was used. (a) Dodecyl maltoside, (b) dodecyltrimethylammonium bromide (arrows; probably endovesicles), (c) octaethyleneglycol dodecyl ether, (d) chlorpromazine. The bar represents 2 μm.

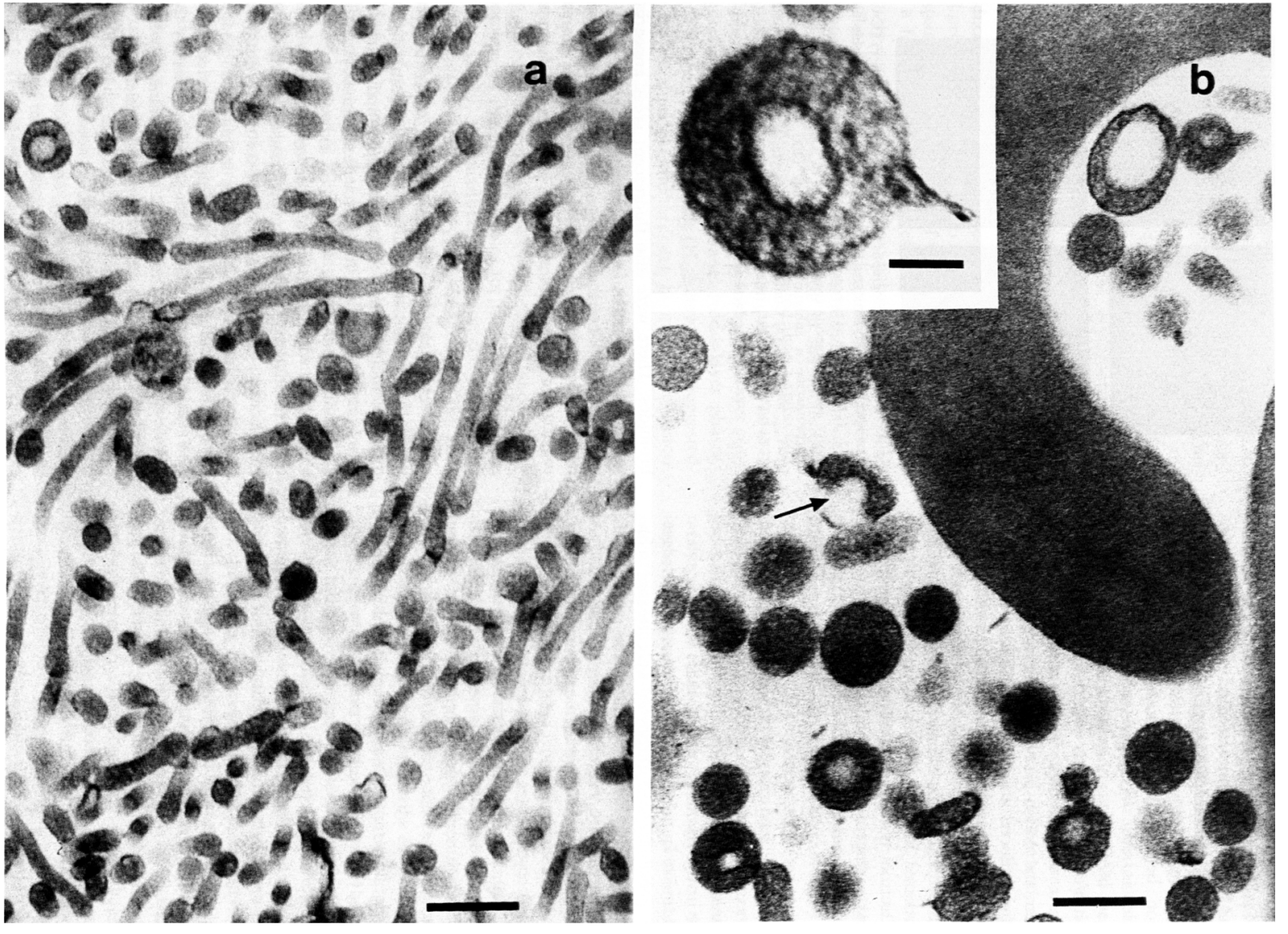

Fig. 3. Electron micrographs of exovesicles released from human erythrocytes following incubation with amphiphiles at concentrations corresponding to CAH_{max} for 30 min at 37 °C. Cell density and amphiphile concentrations as in Table I. (a) Dodecyl maltoside, (b) dodecyltrimethylammonium bromide (arrow; possibly a cup-shaped vesicle). The inset shows a vesicle with a tail and an open centre. The bars represent 200 nm, in the inset 50 nm.

duction in haemolysis over a broad concentration range (10–250 μM) which made it difficult to accurately determine CAH_{max}. The aliphatic amphiphiles, on the other hand, induced a pronounced reduction in haemolysis and a well-defined CAH_{max}. We therefore think that the difference in the concentration dependence for vesiculation between chlorpromazine and the other amphiphiles is more likely due to an inaccurate determination of CAH_{max} than to a difference in the interaction of the compounds with the membrane.

The electron microscopic studies thus support the results from the AChE assay. Echinocytogenic amphiphiles which induced a release of AChE to the buffer also induced a release of microvesicles to the buffer. With stomatocytogenic amphiphiles, which do not pass the erythrocytes through echinocytic stages, no AChE release occurred and no vesicles were released to the buffer. Electron micrographs of erythrocytes treated with stomatocytogenic amphiphiles, however, strongly indicated that an endovesiculation had occurred.

Internalization of FITC-dextran

The vesicles seen in electron micrographs of erythrocytes treated with stomatocytogenic amphiphiles may represent real endovesicles but they may also be membrane invaginations in open contact with the external environment. In an attempt to discriminate between these two possibilities erythrocytes were treated with amphiphiles (CAH_{max}) for 60 min in the presence of FITC-dextran (10 mg/ml). Following incubation, the erythrocytes were washed and then examined unfixed in a fluorescence microscope.

Untreated erythrocytes showed a week even fluorescence (Fig. 4a). The erythrocytes shown in the figure are slightly crenated due to the 'glass effect'. Erythrocytes treated with echinocytogenic amphiphiles had essentially the same appearance as control erythrocytes.

In erythrocytes treated with stomatocytogenic amphiphiles (alkyltrimethylammonium bromides, polyoxyethyleneglycol alkyl ethers and chlorpromazine) various types of fluorescent patches were seen but there were also cells without patches. Fig. 4b shows erythrocytes treated with dodecyltrimethylammonium bromide. These cells contained fluorescent patches and in many of these cells small distinct patches subjected to Brownian movements were seen. However, similar small 'moving' patches were in some cases also seen in untreated cells and cells treated with echinocytogenic amphiphiles. In Fig. 4c erythrocytes treated with an octaethyleneglycol alkyl ether (C_{10}) are shown. Erythrocytes treated with octaethyleneglycol ethers frequently contained a distinct and often ring-formed fluorescent region.

Erythrocytes treated with chlorpromazine (200 μM) contained fluorescent patches (Fig. 4d) resembling those following treatment with alkyltrimethylammonium bromides. There were also fluorescent areas appearing to consist of clusters of small patches.

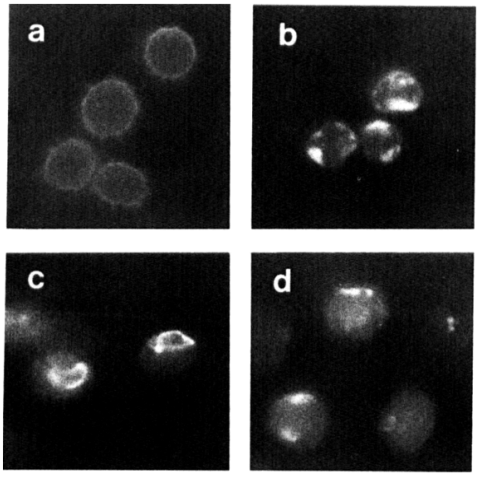

Fig. 4. Fluorescence micrographs showing incorporation of FITC-dextran into human erythrocytes following incubation with amphiphiles at 37 °C for 60 min at concentrations corresponding to CAH_{max}. Cell density and amphiphile concentrations as in Table I. In the case of chlorpromazine a sublytic concentration (200 μM) higher than CAH_{max} was used. (a) Control (the erythrocytes are slightly crenated due to the 'glass effect'), (b) dodecyltrimethylammonium bromide, (c) octaethyleneglycol decyl ether, (d) chlorpromazine.

Taken together, the electron and the fluorescence microscopic studies strongly indicate that the stomatocytogenic amphiphiles induce a release of endovesicles.

Discussion

Our results show that amphiphiles at concentrations where they exhibit maximum protection against hypotonic haemolysis may induce a rapid and extensive membrane vesiculation in erythrocytes. Echinocytogenic amphiphiles induced a release of exovesicles, while amphiphiles which induce stomatocytes without a transition through echinocytic stages, caused a release of endovesicles. Amphiphiles which pass the erythrocytes through echinocytic stages before stomatocytic shapes are attained, induced both exo- and endovesiculation. The shape induced by the amphiphiles and thereby the membrane curvature thus seems to determine to which side of the membrane the vesicles will be released.

It is generally agreed that a change in shape of the erythrocytes to either echinocytic or stomatocytic shape is a prerequisite for, respectively, an exo- or endovesiculation to occur and that vesicle release can be regarded as an ultimate state of these shape alterations [7, 23–26]. It is, however, thought that an attainment of a specific shape per se is not sufficient to induce vesiculation in

erythrocytes [6]. A partial replacement of native phosphatidylcholine species by foreign ones, without affecting the cholesterol level [6,27], has not been observed to induce vesiculation, even though the treatment transformed the erythrocytes to echinocytes. In our study no release of AChE was seen following crenation of erythrocytes by elevating the pH of the buffer, and in the case of chlorpromazine no sign of endovesicles was seen at 20 μM, even though the erythrocytes were transformed to stomatocytes. It thus appears that besides shape alterations, additional alterations within the membrane are needed to induce a release of membrane vesicles. In a study of dimyristoylphosphatidylcholine-induced exovesiculation in erythrocytes, Frenkel et al. [6] proposed that at least two independent processes are required to reach the stage of vesiculation. The first step is a formation of echinocytes which establishes an intimate contact between the two halves of the inner lipid leaflet. Another step is a destabilization of the bilayer to trigger the fusion process. This destabilization is thought to be brought about by a decrease in the cholesterol level of the membrane. It is suggested that a reduction in the cholesterol level results in an increased tendency of phosphatidylethanolamine to adopt non-bilayer configurations within the membrane. Non-bilayer phases, such as the hexagonal phase (H_{II}), have been suggested to be essential as intermediates in membrane fusion processes [28,29].

In a previous paper [16] we have suggested that transient non-bilayer phases are essential elements in the intercalation of amphiphiles, such as those used in the present study, into the erythrocyte membrane. Taking into account current views on the importance of the geometry of the lipid molecules for the stability of the bilayer [27,29–32], quantitative data on the binding of amphiphiles to the erythrocyte membrane [33–38], and the fact that erythrocytes immediately respond with an increased resistance against hypotonic haemolysis when added to hypotonic solutions containing the amphiphiles, we conclude that some kind of non-bilayer phase must be involved in the intercalation of the amphiphiles into the lipid bilayer in order to prevent a collapse of the bilayer structure. The amphiphiles used have the molecular shape of cones (large surface area of the polar head and a small hydrophobic volume) and they should therefore have a destabilizing effect on the lipid bilayer. Furthermore, due to the bulky polar head, the amphiphiles should be unable to rapidly diffuse from the outer to the inner monolayer. The outer monolayer should thus be subjected to a considerable stress, unless there are some events which can rapidly redistribute intercalated amphiphiles and some of the bilayer lipids between the two monolayers in such a way that a stable bilayer configuration can be attained. With the aid of transient intrabilayer non-bilayer phases, possibly the H_{II} phase, such a redistribution could occur without the bilayer loosing its integrity and barrier properties. Support for the idea that amphiphiles induce transbilayer redistributions was recently presented by Rosso et al. [39] in a study showing that chlorpromazine induced a sudden redistribution of spin-labeled phospholipids within the erythrocyte membrane.

The present study adds to our previous suggestion that when the concentration of intercalated amphiphiles in the membrane phase exceeds a certain level, the membrane rearrangements also involve a release of membrane vesicles. The critical concentration required to initiate vesicle release seems to be reached at a concentration corresponding to about CAH_{50}.

In this context it is significant to note that Maher and Singer [15] in a study using hygroscopic desorption filtration have shown that several small-molecule amphiphilic drugs, including chlorpromazine, at concentrations where they protect erythrocytes against hypotonic haemolysis induced a release of membrane components from the erythrocyte membrane. The membrane components released were suggested to be mainly in the form of vesicular fragments. They did conclude that amphiphiles, at sublytic concentrations, promote a gross redistribution of components in the plane of the membrane and that these rearrangements may have implications for the various physiological and biochemical effects exerted by different amphiphiles, including anaesthetics, on membranes. In previous studies [17,18] we have shown that amphiphiles at sublytic concentrations affect membrane transport in erythrocytes.

The picture emerging from our studies on the interaction of amphiphiles with the erythrocyte membrane is that the erythrocyte membrane can incorporate a vast amount of foreign amphiphiles without losing its integrity and barrier properties. The intercalation of amphiphiles into the membrane is, however, associated with gross rearrangements within the lipid bilayer. When intercalated into the membrane, the amphiphiles probably trigger a development of transient non-bilayer phases which bring about a rapid transbilayer redistribution of intercalated amphiphiles as well as of bilayer lipids. At high sublytic concentrations an additional process involving a release of membrane vesicles takes place. At these concentrations the composition and the function of the membrane may be strongly altered. At still higher concentrations the bilayer structure collapses and the cell will be lyzed. Finally, we want to stress that the alterations in shape resulting from treatment with amphiphiles are not simply due to a selective intercalation of differently charged amphiphiles into either of the two monolayers of the lipid bilayer, as suggested in the bilayer couple hypothesis [40,41], but are the consequences of all the rearrangements induced by the amphiphiles.

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