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Liposomes composed of a double-chain cationic amphiphile (Vectamidine) induce their own encapsulation into human erythrocytes

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Abstract

Vectamidine is a liposome-forming double-chain cationic amphiphile. The present work was aimed to microscopically study the interactions of Vectamidine liposomes with the human erythrocyte plasma membrane. Vectamidine rapidly induced stomatocytic shapes. Attachment of Vectamidine liposomes to the erythrocyte induced a strong local invagination of the membrane. This frequently resulted in a complete encapsulation of the liposome. Liposomes composed of phosphatidylcholine (neutral) or phosphatidylserine/phosphatidylcholine (anionic) did not perturb the erythrocyte shape. Our results indicate that besides an attraction of Vectamidine liposomes to the plasma membrane, there is a preference of Vectamidine for the inner bilayer leaflet. We suggest that cationic amphiphiles may transfer from membrane-attached liposomes to the plasma membrane and then translocate to the inner bilayer leaflet where they induce a strong local inward bending of the plasma membrane resulting in an encapsulation of the liposome.

Keywords: Human red blood cell; Cationic amphiphile; Liposome; Shape alteration; Membrane vesiculation

1. Introduction

Liposomes may serve as delivery vehicles for encapsulated, membrane intercalated and attached material (see [1]). Attachment of genetic material to liposomes may facilitate its entry into cells. Due to the high transfection efficiency of cationic liposomes the mechanisms of their action have been widely studied [2–6]. Although several parameters are known to affect the transfection efficiency of liposomes prepared from cationic amphiphiles [7–12], the reason for their high transfection efficiency is incompletely known. Endocytosis has been proposed as a possible pathway for the entry of cationic liposome–DNA complexes into cells [6,12,13]. It has also been suggested that the mechanism involves a amphiphile-induced destabilization of the plasma membrane [14].

We have previously learned that certain amphiphiles have the capacity to alter cell shape and to induce endovesiculation in human erythrocytes [15–19]. If cationic amphiphilic liposome components have a similar effect, this would suggest that a direct

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interaction between the amphiphile and the lipid bilayer might contribute to the carrier efficiency of the liposome. Vectamidine is a double-chain cationic amphiphile used for the preparation of liposome vectors with a high DNA transfection efficiency [20–22]. The aim of the present work was to study the interactions of Vectamidine liposomes with the plasma membrane of the human erythrocyte, a cell not normally undergoing endovesiculation.

2. Materials and methods

2.1. Chemicals

N-*t*-Butyl-*N*′-tetradecyl-3-tetradecylaminopropionamidine (Vectamidine), also described as diC_{14}-amidine (see [22]), was obtained from BiotechTools, and egg phosphatidylcholine (P2772), bovine brain phosphatidylserine (P6641) and fluorescein isothiocyanate (FITC)–dextran 70S from Sigma.

2.2. Isolation of erythrocytes

Human blood was drawn from the authors by venipuncture into heparinized tubes. Blood was washed three times with the buffer (mM: Hepes 10, NaCl 128, KCl 3, $CaCl_2$•$2H_2O$ 1.5, Mg_2Cl_2•$6H_2O$ 1.5; pH 7.4). The uppermost layer of the erythrocyte pellet was discarded following centrifugation. The erythrocytes were suspended in the buffer at a cell density of 1.65×10^9 cells/ml. Cells were used the day they were drawn.

2.3. Liposome preparation

Liposomes from Vectamidine, phosphatidylcholine and phosphatidylserine/phosphatidylcholine (ana parte) were prepared in the buffer at 1 mg/ml. Phosphatidylserine/phosphatidylcholine liposomes were prepared in the absence of divalent cations. Vectamidine liposomes were formed by adding Vectamidine from an ethanol stock solution to the buffer heated at 55–60°C for 10 min followed by vortexing. Phosphatidylcholine and phosphatidylserine were weighed, dissolved in chloroform, dried under a nitrogen stream and put under vacuum overnight. The lipid film was then hydrated with the buffer, vortexed and heated over the transition temperature. Each liposomal suspension was extruded through a 400-nm filter five times to standardize size.

2.4. Incubation

Incubation was started by adding erythrocytes to suspensions of liposomes composed of either Vectamidine, phosphatidylcholine or phosphatidylserine/phosphatidylcholine. The final cell density was 1.65×10^8 cells/ml ($\sim$1.5% hematocrit). Incubation was carried out at a room temperature of $\sim$24°C for 60 min under gently mixing.

2.5. Phase contrast and interference contrast microscopy (LM)

The morphology of isolated erythrocytes fixed with 1% glutaraldehyde was studied in a hanging drop by phase contrast microscopy. For photography (interference contrast microscopy) erythrocytes were fixed with 1% glutaraldehyde and applied between object and coverglass.

2.6. Transmission electron microscopy (TEM)

Erythrocytes were suspension-fixed in 1% glutaraldehyde in the buffer for 30 min at 22°C, postfixed in 1% OsO_4 in 0.9% NaCl for 30 min at 22°C, dehydrated in a graded series of acetone/water (50–100%, v/v) and finally embedded in Epon. Thin sections were stained with lead acetate and post-stained with uranyl acetate before examination in a JOEL 100SX electron microscope.

3. Results

Incubation was started by adding human erythrocytes to a suspension of liposomes composed of either Vectamidine, phosphatidylcholine or a mixture of phosphatidylserine/phosphatidylcholine. Sub-samples were microscopically examined at suitable time intervals.

3.1. LM

Erythrocytes (Fig. 1A, control) attained stomato-

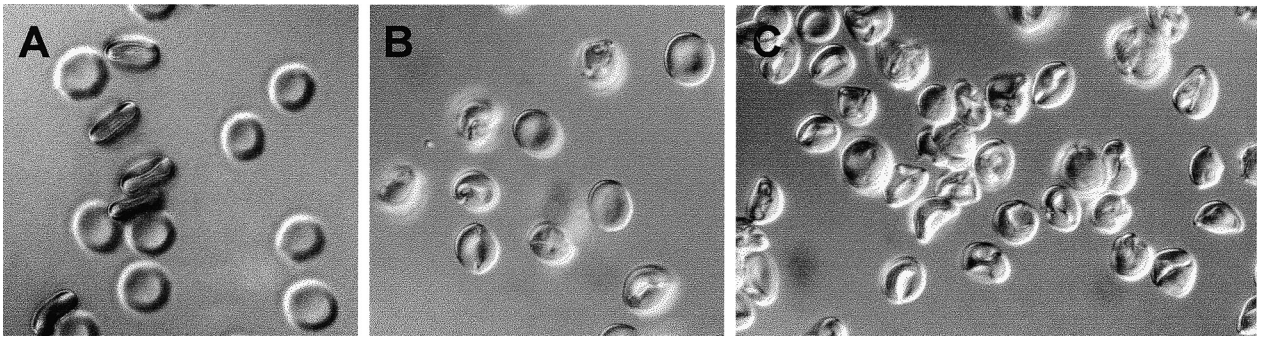

Fig. 1. Shape alterations in human erythrocytes (LM). (A) Control. (B) Incubation with a 10 μM Vectamidine liposome preparation for 10 min. (C) Incubation with a 50 μM Vectamidine liposome preparation for 1 min.

cytic (invaginated) shapes upon incubation with Vectamidine liposome preparations. Stomatocytic shapes occurred at Vectamidine concentration as low as 10 μM (Fig. 1B, 10 min). At higher Vectamidine concentrations erythrocytes rapidly attained a stomatocytic shape (Fig. 1C, 50 μM, 1 min). Aggregated erythrocytes were frequently seen in Vectamidine treated samples. Liposomes prepared from phosphatidylcholine or phosphatidylserine/phosphatidylcholine did not alter erythrocyte shape during 60 min incubation, although control experiments showed that free liposomes occurred in the sample (not shown). Similar results were obtained at 37°C.

3.2. TEM

Thin sections of erythrocytes (Fig. 2A, control) confirmed that the Vectamidine liposome preparation induced stomatocytic shapes (Fig. 2B, 50 μM, 60 min incubation). The stomatocytic erythrocytes had a few large invaginations. No marked sphering of cells occurred. High magnifications revealed liposomes attached to the plasma membrane (Fig. 2C). In many cases liposomes occurred at the base of plasma membrane invaginations (Fig. 2D–F). Vectamidine was shown to induce a close contact between plasma membrane segments, a phenomenon which could aggregate separate cells (Fig. 2G) or enclose a large invagination in a cell (Fig. 2H). Erythrocytes incubated with phosphatidylcholine or phosphatidylserine/phosphatidylcholine liposomes (at 5–10 μg phospholipid/ml) remained discoid and no attached or encapsulated liposomes were observed (not shown).

4. Discussion

Water-soluble amphiphiles rapidly induce either echinocytic (spiculated) or stomatocytic (invaginated) shapes in human erythrocytes [15,19,23,24]. The kind of shape induced is thought to depend on whether the amphiphile is mainly incorporated into the outer or the inner plasma membrane leaflet, thereby expanding this leaflet relative to the other [25–28]. The preferential location of a charged amphiphile is thought to be highly dependent on electrostatic interactions between the amphiphile and the negatively charged phospholipids at the inner bilayer leaflet. It is thought that cationic amphiphiles which may translocate to the inner bilayer leaflet accumulate there and induce stomatocytosis.

Vectamidine, a liposome-forming cationic double-chain amphiphile, induced a stomatocytic shape alteration (Fig. 1) somewhat similar to that induced by previously studied stomatocytogenic water-soluble single-chained amphiphiles (detergents) [15–19]. This similarity enforce us to suggest a similar mechanism of action, i.e., that stomatocytosis in both cases is induced due to a preferential intercalation of the exogenous amphiphile into the inner leaflet of the erythrocyte membrane. Since liposome-forming amphiphiles should have a relatively low water solubility, this suggests that Vectamidine enters the erythrocyte membrane mainly from attached liposomes. Accordingly, the membrane attachment of Vectamidine liposomes was shown to induce a strong local invagination of the cell membrane in the vicinity of the attached liposomes (Fig. 2C–F). The invagination process frequently resulted in a complete

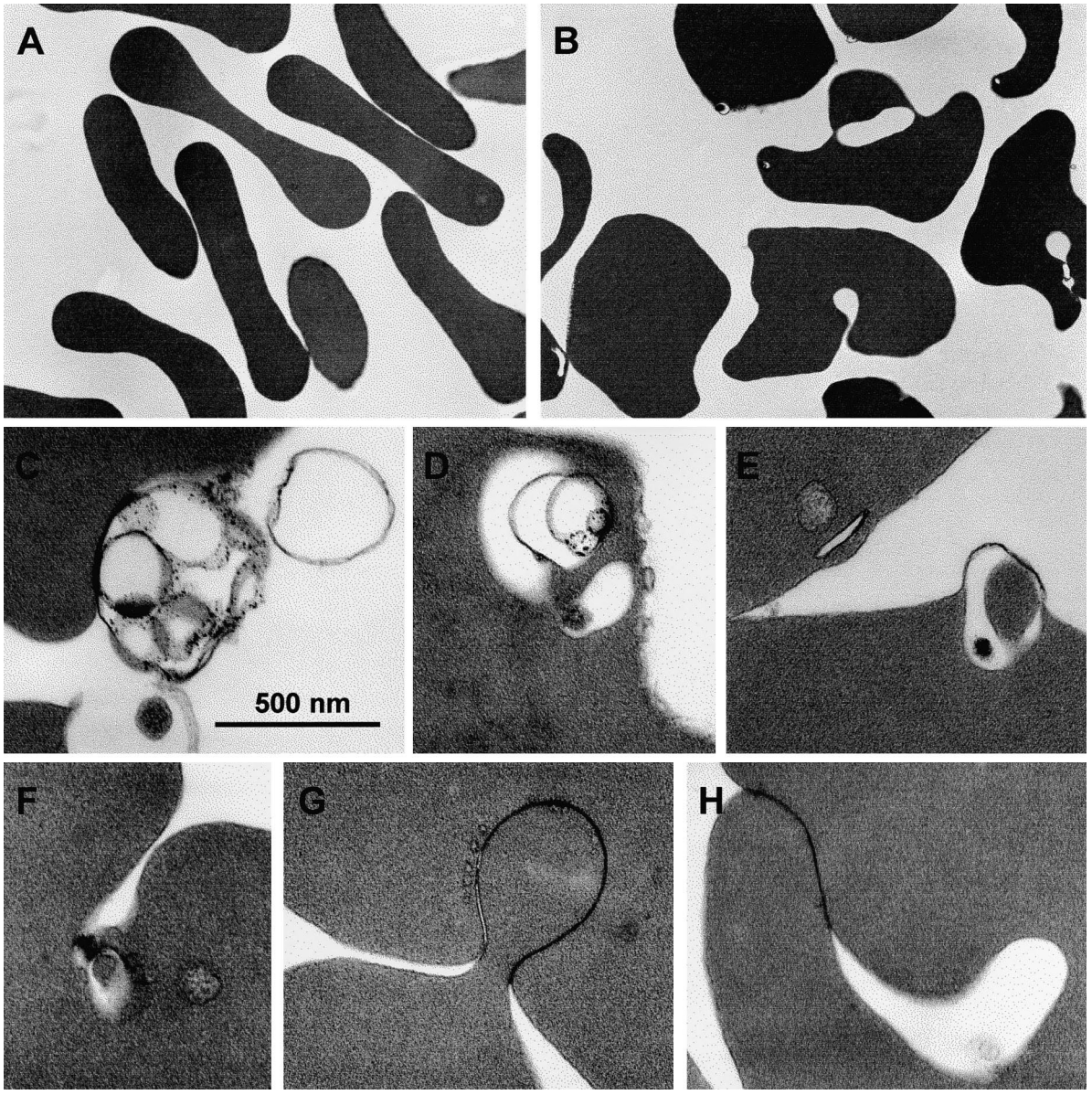

Fig. 2. Ultrastructure of human erythrocytes (TEM). (A) Control. (B) Incubation with a 50 μM Vectamidine liposome preparation for 60 min. (C–H) High magnification of erythrocytes treated in the same way as in B. (C) Liposomes attached to the plasma membrane. (D–F) Liposomes in plasma membrane invaginations. (G,H) Close contact between plasma membrane segments.

encapsulation of the liposome by the plasma membrane (Fig. 2D–F). These observations we take to indicate a transfer of Vectamidine molecules from the liposome to the plasma membrane followed by a translocation and accumulation of Vectamidine into the inner bilayer leaflet. The lateral density of membrane-intercalated Vectamidine molecules may remain high in the vicinity of the attached liposomes due to a low lateral redistribution of Vectamidine within the inner leaflet. This idea about a patchy distribution of Vectamidine in the erythrocyte membrane is supported by the observation that no sphero-stomatocytic limit shapes, i.e., spherical erythrocytes with small endovesicles [29,30], occurred. It is possible that a preference of Vectamidine molecules for a high membrane curvature, similar to that in liposomes, contributes to their accumulation in highly curved invaginated membrane regions (see [31]). It should be stressed that we cannot exclude that Vectamidine liposomes affect the erythrocyte

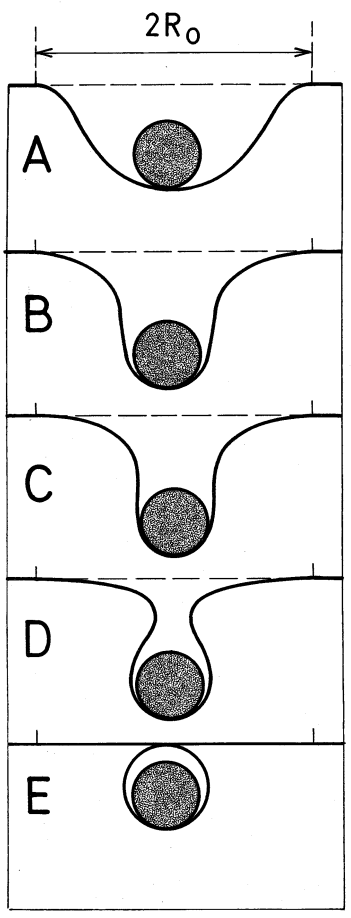

Fig. 3. The dependence of axisymmetric shapes of a spherical membrane segment with the radius R_o and the area A on the difference between the areas of the two leaflets (ΔA) of the segment for $A/\pi R_o^2 = 1.4$. In the model the values of the relative area difference of the segment ($\Delta A/\pi\delta R_o$) are: −0.368 (A), −0.945 (B), −1.178 (C), −1.897 (D), −2.530 (E). The symbol δ denotes the distance between the neutral surfaces of the bilayer leaflets [36]. The membrane-attached spherical liposome is shown schematically.

membrane by other mechanisms, e.g. by solubilizing membrane components or by altering the interactions between charges on the cell surface. Furthermore, an attraction between the positively charged liposome and the negatively charged membrane surface may possibly propagate liposome encapsulation (see [32–34]). However, the hemolytic activity of Vectamidine (not shown) and other highly transfection-efficient synthetic cationic amphiphiles [35] indicates that these amphiphiles are intercalated into the plasma membrane and do not only attach to it.

The development of a local liposome-induced invagination is illustrated in Fig. 3. A sequence of axisymmetric shapes of a spherical bilayer segment (with radius R_o and area A), induced by an increase of the area of the inner leaflet (A_{inner}) relative to the outer (A_{outer}) in the segment, is shown. The shape of the axisymmetric spherical segment at a given area difference (ΔA; defined as A_{outer} minus A_{inner}) is determined by minimizing the bilayer bending energy of the segment, as it is described elsewhere [36]. At each step of the liposome encapsulation (endovesiculation, endocytosis) process the area difference (ΔA) is decreased until the limit shape, composed of a spherical endovesicle and a flat membrane segment is reached (Fig. 3E). This final shape of the segment corresponds to the minimal possible value of the area difference (ΔA) of the segment. The decrease of the segment ΔA around the attached liposome (Fig. 3) may be driven by a progressive intercalation of Vectamidine molecules into the membrane segment from the attached liposome.

In Vectamidine-treated samples erythrocyte plasma membrane segments were frequently shown to attach to each other (Fig. 2G,H), a peculiarity not observed with those single-chained amphiphiles (detergents) which we have been previously studying [17,19]. Thus, Vectamidine or Vectamidine liposomes promote attraction between membrane segments and may have a fusogenic effect. Cationic liposomes have previously been reported to promote plasma membrane fusion [37].

To conclude, we suggest that a preference of cationic amphiphiles for the inner bilayer leaflet may be important for their efficiency as components of delivery vehicles. Cationic amphiphiles entering the plasma membrane from membrane-attached liposomes may locally induce or increase inward bending of the plasma membrane resulting in the encapsulation of the liposome. This property of cationic amphiphiles may possibly enhance or initiate endocytosis of the liposomes. Experiments revealing how attachment of DNA to Vectamidine liposomes modulate their above described cellular effects are under way.

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