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Shape changes in the erythrocyte membrane induced by the absorption enhancer didecanoylphosphatidylcholine

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Abstract

The phospholipid didecanoylphosphatidylcholine (DDPC) has been shown to promote the nasal absorption of peptide drugs in rabbits. The absorption enhancing effect has been related to the cytotoxicity of DDPC; however, the mechanism of action is not completely elucidated. Thus, the aim of this study was to further understand the absorption promoting effect of DDPC by elucidation of the interaction of DDPC with plasma membranes (i.e. erythrocyte membranes). Incorporation of DDPC (0.01–0.27 mg/ml) into erythrocyte membranes was estimated and correlated with DDPC-induced shape changes of the cells as well as with DDPC-induced hemolysis. Incubation of erythrocytes for 30 min with DDPC resulted in discocyte-to-spherocyte shape changes with loss of concavity, as well as formation of a small number of echinocytes. Echinocyte formation might suggest that DDPC was incorporated into the outer leaflet of the erythrocyte membrane resulting in a crenated form. Spherocyte formation might result from incorporation of larger amounts of DDPC. The highest DDPC concentration induced cell aggregation. At this concentration, the amount of phospholipids in the erythrocyte membrane was increased by 5.3 mol% and the concentration-dependent hemolysis was almost complete. In contrast to the effect induced by DDPC, no shape changes of the erythrocyte were observed when incubated with long chain phosphatidylcholine (PC) from egg (0.05 mg/ml, 0.50 mg/ml). In this case only slight hemolysis was observed. Thus, these results supported previous assumptions that incorporation of medium chain phospholipids into cellular membranes happens very quickly, resulting in membrane disorders that might, in part, be responsible for the absorption enhancing effect and the cytotoxicity of the enhancer.

Keywords: Absorption enhancers; Didecanoylphosphatidylcholine; Erythrocytes; Intranasal; Shape changes

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1. Introduction

The nasal mucosa is a potential delivery site for the systemic administration of peptide drugs, as it has a favourable construction for drug delivery: the vascularization is high, the surface area is large, first pass metabolism is reduced, the rate of absorption is high and delivery is easy (Chien and Chang, 1987, Zia et al., 1993, Zhou, 1994). An important drawback is, however, that the nasal permeability does not allow passage of drugs with a molecular size above 1000 Da (McMartin et al., 1987). In the case of peptide delivery, it might be necessary to co-administer an absorption enhancer in order to get a satisfactory and reliable blood concentration of the peptide.

The phospholipid absorption enhancer didecanoylphosphatidylcholine (DDPC) has been shown to enhance the nasal absorption of insulin and human growth hormone (hGH) in rabbits (Drejer et al., 1992, Vermehren et al., 1996). However, part of the enhancing effect might be related to the epithelial damage caused by the enhancer (Bechgaard et al., 1993, Vermehren et al., 1993, Agerholm et al., 1994, Vermehren et al., 1996). Due to the medium chain acyl groups, the rate of incorporation of DDPC into biological membranes is expected to be high, as the rate of incorporation of phospholipids into cellular membranes increases with decreasing length of the acyl chains (Silvius and Leventis, 1993). After intranasal exposure of 50 μg of [^{14}C]DDPC and [^{3}H]DDPC to rabbits in vivo, 4.4–7.5% of the administered radioactivity was immediately associated with the nasal epithelium, and this figure was not increased during the experimental period. The tissue associated DDPC was completely metabolized within 2 h after the administration (Vermehren et al., 1997). However, the enhancing effect of 0.96 mg DDPC on hGH absorption in rabbits in vivo was not reversed during the following 3 h after the enhancer administration (Vermehren et al., 1996), suggesting that this dose of DDPC may cause irreversible damage to the nasal mucosa. Another study employing DDPC plus α-cyclodextrin as enhancers suggested that the major route of hGH absorption was transcellular through damaged cells (Agerholm et al., 1994). In in vitro studies, low concentrations of DDPC were found to reversibly decrease the electrophysiological parameters of rabbit nasal mucosa whereas higher concentrations decreased these parameters in a toxic way (Bechgaard et al., 1993, Vermehren et al., 1993).

The aim of this study was to further study the interaction of DDPC with biological membranes using erythrocyte membranes as models. Thus, the amount of DDPC incorporated into the erythrocyte membrane and the following cell shape changes were investigated. The DDPC-induced hemolysis was measured as well, because the degree of hemolysis caused by DDPC might give an indication of the degree of enhancer cytotoxicity.

2. Materials and methods

2.1. Materials

1,2-Di[1-^{14}C]decanoylphosphatidylcholine (67 mCi/mmol) ([^{14}C]DDPC) was from Amersham International (UK). The initial radiochemical purity was $\geq 95\%$. The unlabelled DDPC powder was supplied by Novo Nordisk (Bagsværd, Denmark). Long chain phosphatidylcholine (PC) from egg was from Sigma (St Louis, MO).

2.2. Preparation of erythrocytes

Blood samples were obtained from healthy adult donors by venipuncture and collected into test tubes containing sodium citrate (1 vol. sodium citrate solution (3.15%. w/v) + 9 vols blood). The erythrocytes were immediately separated from plasma by centrifugation for 5 min at 2500 rpm (Heraeus Labofuge I), washed three times with 4 vols of Krebs Ringer buffer, and used immediately after collection.

2.3. Phospholipid-induced shape changes of the erythrocytes

One vol. of packed, washed erythrocytes corresponding to 5.6×10^{12} cells was incubated with 9 vols of dispersions of DDPC (0.03, 0.05, 0.11 or 0.27 mg/ml) or long chain PC (0.05 or 0.5 mg/ml)

in Krebs Ringer buffer, pH 7.0. Incubations of erythrocytes were carried out at 37°C for 30 min. For controls, erythrocytes were incubated with 9 vols Krebs Ringer buffer. At the end of the incubation period, the cell suspensions were centrifuged at 2500 rpm (Heraeus Labofuge I) for 5 min. The supernatant was used for spectrophotometric determination of hemolysis (Spectronic 601) by measuring the absorbance at 408 nm. Hemolysis induced by water was taken as 100%. The red blood cells were fixed for light microscopy with 2% glutaraldehyde in 0.05 M phosphate buffer, pH 7.0. Cytospin preparations were examined in a Leika differential interference contrast microscope at 800 × magnification.

2.4. Incorporation of [^{14}C]DDPC into the erythrocyte membrane

Erythrocyte incubations were performed as described above. DDPC concentrations investigated were 0.01 mg/ml (960 dpm), 0.03 mg/ml (2880 dpm), 0.05 mg/ml (4800 dpm), 0.11 mg/ml (10560 dpm) and 0.27 mg/ml (25920 dpm). Control incubations with DDPC in Krebs Ringer buffer (same concentrations as in the experiments) were performed to examine the degree of [^{14}C]DDPC adsorption to the test tube. Due to the observed adsorption of [^{14}C]DDPC to the test tube, the incorporation of [^{14}C]DDPC into the erythrocyte membrane was calculated from the difference in supernatant radioactivity between the erythrocyte samples and the controls.

3. Results

3.1. Phospholipid-induced shape changes of the erythrocytes

Incubation of erythrocytes for 30 min with DDPC (0.03, 0.05, 0.11 or 0.27 mg/ml) resulted in a discocyte-to-spherocyte shape change of the erythrocyte with loss of concavity (Figs. 1–3). A small amount of echinocytes was also observed, having an erythrocyte morphologic index score of 4.1 according to a scale of 0–5 for echinocyte formation (Daleke and Huestis, 1989) (Fig. 2).

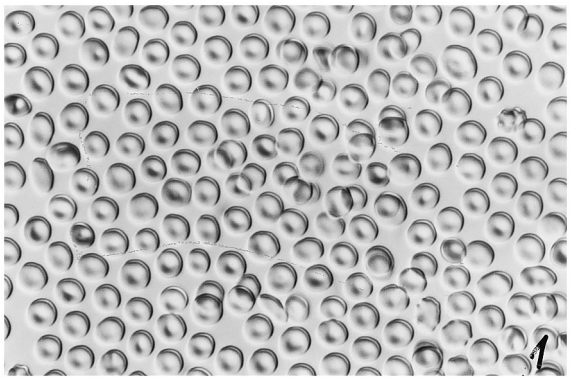

Fig. 1. Erythrocytes incubated for 30 min with Krebs Ringer buffer (control). The discocyte shape of the erythrocytes was preserved. Magnification: 800 × .

The highest concentration of DDPC (0.27 mg/ml) resulted in aggregation of the cells (Fig. 3), while the erythrocyte incubation control preserved the characteristic biconcavity (Fig. 1). Incubations with long chain PC (0.05 or 0.5 mg/ml) did not cause any changes in the erythrocyte morphology (Figs. 4 and 5).

3.2. Phospholipid-induced hemolysis

A concentration-dependent DDPC-induced hemolysis was observed (Fig. 6), reflecting membrane damage. At 0.27 mg/ml, DDPC hemolysis was nearly 100%. In contrast to this, hemolysis was not induced by the long chain PC (Fig. 6).

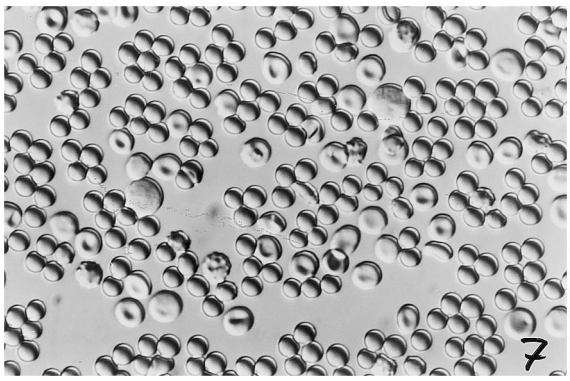

Fig. 2. Erythrocytes incubated for 30 min with 0.05 mg/ml DDPC at 37°C. The erythrocytes are mainly spherical with a small number of echinocytes. Magnification: 800 × .

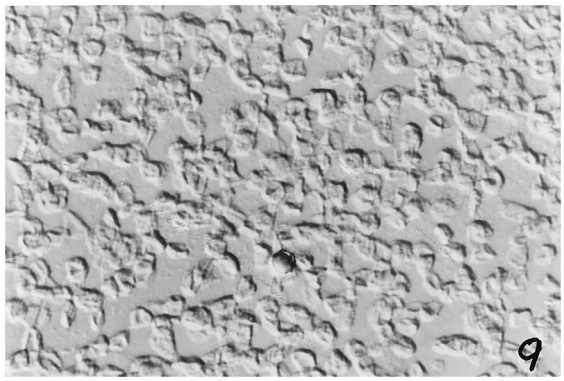

Fig. 3. Erythrocytes incubated for 30 min with 0.27 mg/ml DDPC at 37°C. The cells are aggregated. Magnification: 800 × .

The osmolarity of the incubation mixture was measured, and no measurable influence of DDPC on the osmolarity was found.

3.3. Incorporation of [^{14}C]DDPC into the erythrocyte membrane

Control experiments indicated that 68.5% of the radioactivity adhered to the test tube irrespective of the amount of radioactivity added. This figure was used for correction of the amount of radioactivity in the erythrocyte supernatant, and the amount of radioactivity incorporated into the membrane was estimated from the free supernatant radioactivity. For increasing concentrations

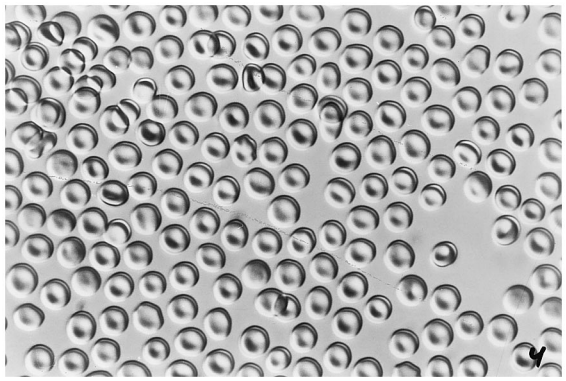

Fig. 4. Erythrocytes incubated for 30 min with 0.05 mg/ml long chain PC from egg yolk. Long chain PC did not induce shape changes of the cells. Magnification: 800 × .

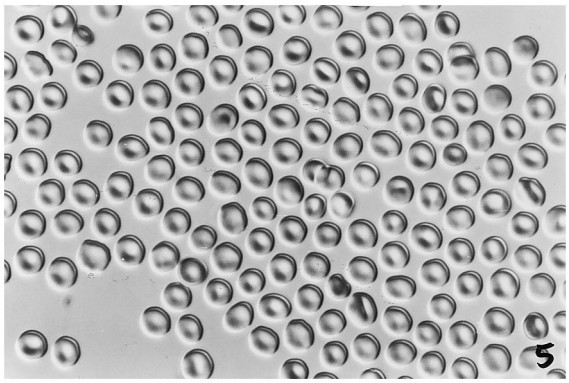

Fig. 5. Erythrocytes incubated for 30 min with 0.5 mg/ml long chain PC from egg yolk. Long chain PC did not induce shape changes of the cells. Magnification: 800 × .

of DDPC, the percentage of radioactivity incorporated into the erythrocyte membrane was decreasing as a result of increasing amounts of radioactivity added: 0.01 mg/ml (960 dpm), 85.9 ± 0.8%; 0.03 mg/ml (2880 dpm), 87.9 ± 0.3%; 0.05 mg/ml (4800 dpm), 71.0 ± 1.9%; 0.11 mg/ml (10560 dpm), 58.1 ± 0.9%; 0.27 mg/ml (25920 dpm), 43.0 ± 5.7% ($n = 2$). The incorporation of DDPC into the erythrocyte phospholipid bilayer

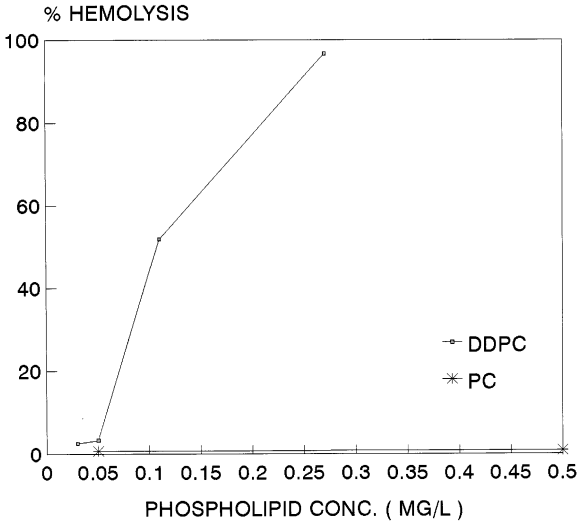

Fig. 6. The DDPC induced hemolysis was concentration dependent, while long chain PC did not induce hemolysis at the observed concentrations. The erythrocytes were incubated for 30 min with different concentrations of DDPC and long chain PC, respectively.

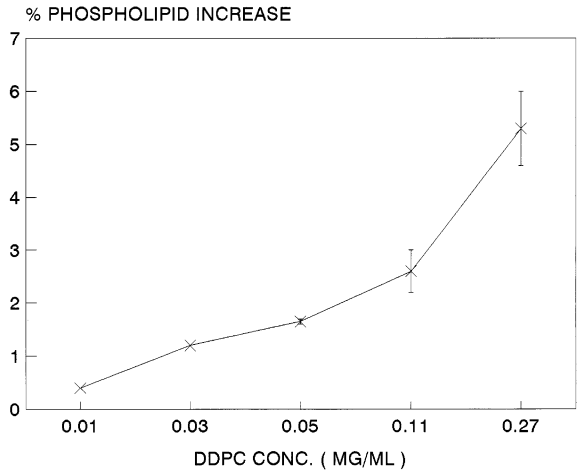

Fig. 7. The amount of erythrocyte-associated phospholipid increased when incubated with DDPC. The increase in phospholipid was concentration dependent. However, the highest concentration of DDPC caused considerable hemolysis.

was calculated to result in an increase in membrane phospholipid of 0.4–5.3%, when the DDPC concentration was increased from 0.01 mg/ml to 0.27 mg/ml (Fig. 7). These figures were calculated from a PC content of 1.8 μmol per 10^{10} erythrocytes (Pomfret et al., 1989), erythrocyte PC amounting to 25% of the total phospholipids (Mathews and van Holde, 1990) and a mean phospholipid molecular weight of 860 g/mol.

4. Discussion

Phospholipids have been introduced as potential absorption enhancers (Illum et al., 1989, O'Hagan and Illum, 1990, Drejer et al., 1992, Chandler et al., 1995). The fatty acid chains of DDPC are each composed of ten carbon atoms. Due to the saturated medium chain fatty acids, DDPC possesses a relatively high degree of hydrophilicity, high stability against degradation and an expected high degree of membrane reactivity. These properties make DDPC a potential absorption enhancer.

In this study, the DDPC membrane reactivity was investigated in erythrocytes as this property could be related to the absorption promoting effect.

The lipids of the erythrocyte membrane are asymmetrically distributed across the bilayer. PC and sphingomyelin are located mainly in the outer leaflet of the membrane, and the aminophospholipids are mainly located in the inner membrane leaflet (Devaux, 1991, Schroit and Zwaal, 1991). Supply of exogenous phospholipids to erythrocytes with discocyte shape may result in changes of the erythrocyte morphology, such as formation of crenated or cupformed erythrocytes (Sheetz and Singer, 1974). Exogenous phosphatidylcholine will, due to its polar head group and a low flip-flop rate across the membrane, initially preferentially accumulate in the outer layer of the mem-

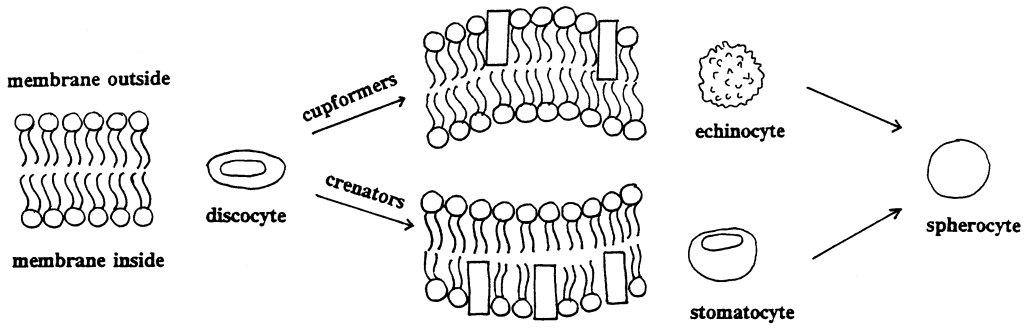

Fig. 8. Choline phospholipids are found mostly in the outer membrane leaflet and aminophospholipids or negatively charged phospholipids are found primarily in the inner leaflet. Incorporation of exogenous choline phospholipids (a crenator) into the outer leaflet of the erythrocyte membrane increases the exterior half of the bilayer producing the crenated echinocytes, whereas incorporation of serine phospholipids (a cupformer) results in accumulation in the interior leaflet generating stomatocytes (Moore et al., 1997). Both forms can be transformed to spherocytes by increasing the incorporations (Sheetz and Singer, 1974).

brane. The area of this layer will increase compared to the inner layer, thereby forming the crenated echinocytes (Fig. 8). However, at sufficiently large concentrations, exogenous PC causes the erythrocytes to become spherical and lyse (Sheetz and Singer, 1974). In this study, DDPC caused a dose-dependent shape change of the erythrocytes (Figs. 1–3), probably due to an initial accumulation in the outer leaflet. The highest concentration of DDPC (0.27 mg/ml) caused complete hemolysis and accumulation of cell debris (Fig. 3), while the lower concentrations mainly caused shape changes from discocyte to spherocyte with a minor occurrence of echinocytes (Fig. 2). The extent of erythrocyte shape changes induced by transfer of phosphatidylserine from liposomes to erythrocytes was previously found to be dose dependent and increased with increasing hydrophilicity of the phospholipid (Daleke and Huestis, 1985). Platelet shape changes induced by exogenous PCs were found to be dose dependent as well (Brunauer and Huestis, 1993). At sufficiently high concentrations, both crenators and cupformers cause the erythrocytes to become spherical and lyse (Sheetz and Singer, 1974). Our results suggest that large amounts of DDPC were rapidly incorporated into the outer cell membrane initially forming echinocytes turning into spherocytes. Replacement of native PC by long chain disaturated PC using a PC-specific transfer protein resulted in shape changes from discocyte to echinocyte and at larger PC concentrations to spherocyte (Kuypers et al., 1984). In this case, the shape change might be caused by an increase in the area of the membrane outer leaflet caused by a modified membrane packing. Generally, the PC molecules in the erythrocyte membrane contain one saturated and one unsaturated fatty acid (Kuypers et al., 1984). These PCs have a moderate cone shape. In diunsaturated PCs, the cone shape is more pronounced, while disaturated PCs such as like DDPC are cylindrical (Sheetz and Singer, 1974, Kuypers et al., 1984, Op den Kamp et al., 1985). In our study, it is most likely that the shape changes were mainly caused by an increase in the amount of phospholipids in the cell membrane. A decrease in the membrane content of cholesterol induced by DDPC could also be a contributing factor (Dwight and Hendry, 1995).

Shape changes might affect the cytoskeleton (Roelofsen et al., 1989), and it has been suggested that membrane proteins and cytoskeletal proteins might be involved in the expansion of erythrocytes since sulfhydryl agents were shown to modify the shape changes of erythrocytes (Tanaka et al., 1983). In the nasal mucosa, changes in the cytoskeleton might affect the tight junctions (Madara et al., 1986), resulting in increased epithelial leakiness. In previous studies, DDPC was shown to decrease the transepithelial resistance of rabbit nasal epithelium in vitro (Carstens et al., 1993, Bechgaard et al., 1993, Vermehren et al., 1993), which may suggest opening of the tight junctions. In a recent study, it was concluded that DDPC inhibited epithelial amiloride-sensitive Na^+ channels and increased paracellular tight junction conductance, which could influence non-specific lipid–protein interactions in the cell membrane (Røpke et al., 1997). Furthermore, DDPC has been shown to decrease the active transport of Na^+ across nasal tissue (Bechgaard et al., 1993, Vermehren et al., 1993), increase intracellular $[Ca^{2+}]$ and influence K^+ channels in nasal tissue (Røpke et al., 1996), and reduce the activity of the sodium pump in human erythrocytes (Dwight and Hendry, 1995).

To understand the mechanism of the interaction of exogenous phospholipids with membranes, it is important to obtain information on their accumulation into the membranes. Uptake of DDPC into erythrocytes was concentration dependent (Fig. 7). At the highest concentration of DDPC (0.27 mg/ml), the amount of phospholipids in the erythrocyte membrane was calculated to increase by 5.3 mol% (Fig. 7). A recent study showed an erythrocyte membrane incorporation of DDPC of 10–15 mol% of total membrane phospholipid by incubation of DDPC (0.05 mg/ml) with erythrocyte suspensions (hematocrit 20%) for 30 min at 37°C (Dwight and Hendry, 1995). In that study, incorporation of DDPC caused a reduction in membrane cholesterol and in content of polyunsaturated acyl chain (Dwight and Hendry, 1995) that may increase the permeability of the cells. Rabbit nasal epithelium showed an increase in tissue PC of approximately 4% upon incubation of isolated nasal mucosas

with DDPC (Vermehren et al., 1993) and uptake of dimyristoyl-PC in erythrocytes corresponded to 5% increase in the phospholipid content upon 2 h of incubation (Ott et al., 1981). If DDPC exclusively accumulate in the outer membrane leaflet of the erythrocytes, the present uptake corresponded to a 10 mol% phospholipid increase in the outer leaflet membrane. It was recently reported that incorporation of DDPC into dimyristoyl-PC bilayers resulted in a pronounced influence of DDPC on the structural phase behavior and the associated barrier properties of the bilayer (Risbo et al., 1997). Incorporation of DDPC into the bilayer may result in packing defects leading to leaky regions (Risbo et al., 1997). Accumulation of higher amounts of short chain PCs in the erythrocyte membrane cause membrane perturbation which may result in hemolysis. After an initial lag phase, the observed hemolysis was found to be dose dependent and hemolysis was initiated by increasing the membrane phospholipids above 1.6% by a concentration of 85 μM (0.05 mg/ml) DDPC (Fig. 6). It has previously been found that PCs with chain length C_8–C_{12} can lyse erythrocytes, and DDPC has been shown to induce hemolysis at concentrations of 6.3–12.5 μM when incubated with erythrocytes (10^7 cells/ml) for 30 min at 40°C (Mashino et al., 1983). Lysis was parallel to the rate of transfer. However the hemolytic activity of PCs may be dependent on both the rate of transfer and on the distribution coefficient (Mashino et al., 1983).

Incubation of erythrocytes with long chain PC from egg yolk (0.05 and 0.5 mg/ml) for 30 min resulted in neither shape changes nor hemolysis. Our results could indicate that long chain PC does not interfere extensively with the erythrocyte membrane within the time of the experiment. This is in agreement with other studies (Kuypers et al., 1984, Brunauer and Huestis, 1993, Haug et al., 1994) and may be a reason why long chain PC might be inapplicable as absorption enhancers.

In summary, this study showed that DDPC exhibited a high level of membrane activity compared to PC with long acyl chains. Secondary to the incorporation of DDPC into the erythrocyte membrane, the erythrocytes changed shape and this was followed by a concentration dependent hemolysis. A comparable shape change in epithelial cells exposed to DDPC might affect the tight junctions. Thus, both opening of tight junctions as well as cell lysis might contribute to the absorption enhancing effect of DDPC on rabbit nasal mucosa.

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