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International Journal of Pharmaceutics 114 (1995) 141–149

international journal of pharmaceutics

Drug delivery studies in Caco-2 monolayers. II. Absorption enhancer effects of lysophosphatidylcholines

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Received 12 February 1994; modified version received 8 July 1994; accepted 10 July 1994

Abstract

This paper concerns the mechanistic elucidation of a certain class of absorption enhancers within the group of phospholipids, lysophosphatidylcholines. The studies were performed in Caco-2 monolayers. Physico-chemical characteristics and effects on the integrity and viability of the Caco-2 monolayers were found to be correlated with the absorption promoting effects of the phospholipids. Comparing enhancers with varying size of the lipophilic moiety, the lipophilicity was shown to be of the utmost importance for all of the observed effects. As the chain length increased from 6 to 16 methylene groups, the effects measured on the monolayers were amplified. The acute effects were evaluated by various microscopic staining techniques as well as by transport studies. The transport rates of the vasopressin analogue 1-deamino-8-D-arginine-vasopressin (DDAVP) were determined as a measure of the monolayer integrity. Based on the apparent permeability coefficient, P_{app}, it could be concluded that phospholipid treatment increased DDAVP transport even under circumstances where the cell monolayer integrity was only slightly altered.

Keywords: Lysophosphatidylcholine; Phospholipid; Absorption enhancer; DDAVP; Caco-2; Cell culture; Peptide absorption; Intestinal absorption

1. Introduction

Absorption enhancers are compounds of varying origins being used for the purpose of increasing the transepithelial transport of macromolecules and poorly absorbable drugs (Fisher et al., 1991; Lee et al., 1991; Hovgaard et al., 1992; Murakami et al., 1992; Richardson et al., 1992; Gill et al., 1994). Due to the complexity of tissue models used for absorption enhancement studies, the interpretation of the effects has been difficult. The usage of cell culturing techniques has greatly improved our ability to elucidate mechanisms of action in this area. The Caco-2 monolayer model has recently been shown to be of value in such studies (Anderberg et al., 1992; Raeissi and Borchardt, 1993).

The Caco-2 monolayer model has been used widely in studies concerning both active and passive transport of drug molecules, nutrients and other chemicals (Hidalgo et al., 1989; Artursson, 1990; Hidalgo and Borchardt, 1990; Conradi et

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SSDI 0378-5173(94)00232-0

al., 1992; Eigtved et al., 1993; Gochoco et al., 1994; Hovgaard et al., 1994). It has been shown to be an excellent model for screening purposes and correlation has been made to bioavailability data from literature (Artursson and Karlsson, 1991). In mechanistic studies of various drug additives and absorption enhancers the Caco-2 monolayer model has been proposed to be a good model due to its simplistic nature (Anderberg and Artursson, 1993; Raeissi and Borchardt, 1993). In conventional in vivo and other in vitro models many, not well defined, variables can complicate the data interpretation. The Caco-2 monolayers consist of the actual epithelial barrier only. Therefore, the effects of the excipients on this layer are easily studied.

This study concerns the effects of lysophosphatidylcholines as absorption enhancers. They are of special interest, as they occur naturally in biological membranes as metabolites of phospholipids (Weltzien, 1979). Moreover, they have absorption enhancing effects at relatively low concentrations towards peptide drugs (Fisher et al., 1991), and after metabolism their metabolites are endogenous and presumably nontoxic. However, lysophosphatidylcholine itself may show toxic effects on the epithelia when applied as an absorption enhancer (Tagesson et al., 1985; Fisher et al., 1991) and the degree of this toxicity is an area of major concern, when the suitability of using this compound in peptide drug delivery is evaluated.

The enzymatically stable vasopressin analogue 1-deamino-8-D-arginine-vasopressin (DDAVP) has been shown to be absorbed orally in many species including man (Vilhardt and Lundin, 1986). The oral bioavailability, however, is only about 1%. In spite of its low bioavailability the compound is widely used as an oral antidiuretic drug (Zaoral, 1985). We used DDAVP as a model peptide drug molecule for transport studies in Caco-2 monolayers.

The aims of the studies presented in this paper were to study the absorption enhancing mechanism and toxicity of lysophosphatidylcholines as a function of chain length of the hydrophobic moiety, using Caco-2 monolayers, and further to investigate their effect on DDAVP transport in Caco-2 monolayers.

2. Materials and methods

2.1. Materials

Three lysophosphatidylcholines, caproyl lysophosphatidylcholine (LPC-C), lauroyl lysophosphatidylcholine (LPC-L) and palmitoyl lysophosphatidylcholine (LPC-P), with lipophilic chain lengths of 6, 12 and 16 methylene groups, respectively, along with dipalmitoyl phosphatidylcholine (PC) with two lipophilic chains of 16 methylene groups and thiazolyl blue (MTT) and trypan blue were purchased from Sigma Chemical Co. (St. Louis, MO). 1-Deamino-8-D-arginine-vasopressin (DDAVP) was a generous gift from Ferring AB, Sweden. Buffer substances and all other chemicals were analytical grade and were used as received without further purification.

2.2. Caco-2 cell cultures

The adenocarcinoma cell line Caco-2 was obtained from the American Type Culture Collection (Rockville, MD). The cells were cultured at 37°C in an atmosphere of 90% relative humidity, 90% air and 10% CO_2. The medium used was standard medium consisting of Dulbecco's Modified Eagles Medium (DMEM) containing 9% fetal calf serum (FCS), 1.0% nonessential amino acids and 1.0% L-glutamine to ensure optimal conditions for the cells. To the medium were added 100 U/ml benzylpenicillin and 10 U/ml streptomycin. All components of the medium were obtained from Gibco, U.K. The cells were grown as permanent cultures in 75 cm^2 culture flasks (Greiner, Austria), the medium was changed twice a week and the cells were trypsinized once a week. On a routine basis the cells were grown in small 25 cm^2 culture flasks without antibiotics for *Mycoplasma* testing. The tests were performed by Statens Veterinärmedicinska (Uppsala, Sweden). The cell cultures were at no time found to be infected.

2.3. Osmolarity and critical micelle concentration

The osmolarity of all test solutions was determined based on the freezing point depression on a Knauer semi-micro osmometer (Germany).

The critical micelle concentration, CMC, of the phospholipids was evaluated using the Wilhelmy plate method on solutions of enhancer in Hanks balanced salt solution (HBSS). For this study a Krüss, K10T digital tensiometer (Hamburg, Germany) was used.

2.4. MTT test

The MTT test utilizes the principle that thiazolyl-blue complex binds dehydrogenases in the mitochondria of cells (Freshney, 1987). If cells are affected by a treatment in such a way that their viability is affected, the dehydrogenase activity ceases. The test was performed as described by Anderberg and Artursson (1993). Approx. 4×10^4 suspended cells were seeded into each of 96 wells in ELISA plates (MicroWells, Nunk, Denmark). The cells were then cultured under standard conditions for 18–22 h before use. Just prior to the start of each experiment the medium was removed from the wells and 100 μl of enhancer solution in HBSS was added to each well. After exactly 10 min, 20 μl of 5 mg/ml MTT solution was added to each well and the cells were incubated for another 90 min. At this point complex bound MTT had precipitated and 100 μl of a solvent containing sodium dodecyl sulfate, butanol and hydrochloric acid was added to dissolve the crystals. After dissolution the visible absorption was measured on an ELISA photometer at 570 nm.

2.5. Trypan blue staining

For cytoplasmic staining, Caco-2 cells were cultured on transparent collagen coated polycarbonate filters of the type Transwell-Col® (Costar, U.S.A.) with a pore size of 0.4 μm and a surface area of 1 cm^2 as described for cells grown on filters for transport studies. Trypan blue staining was used to study the permeability of the cytoplasmic membrane. The Caco-2 monolayers were treated apically for 15 or 60 min with enhancer solutions in HBSS. The test solutions were discarded and 0.4% trypan blue solution was added. After exactly 1 min the cells were washed with HBSS. The filters were then investigated by light microscopy. The number of stained cells in a population of approx. 150 cells was then counted.

2.6. Transport studies

For transport studies the cells were cultured on porous polycarbonate filter membranes with a pore size of 0.4 μm and a surface area of 1 cm^2 in clusters of 12 wells (Transwell, Costar, U.S.A.). When the cells were trypsinized, 0.5 ml of the cell suspension containing approx. 10^5 cells/ml was seeded onto each filter. These cells were given fresh medium three times a week until the time of use. The filters were used between the 21st and 28th day on filters. For this series of studies passage numbers from 25 to 35 were used. The transport studies across Caco-2 monolayers were performed in a carbogen atmosphere at 37°C. The experiments were conducted in a Hitachi Merck AS-4000 intelligent autosampler, on a thermostated rotating plate at 300 rpm as previously described (Buur and Mørk, 1992). At the time −15 min, 0.5 ml enhancer solution in HBSS was added to the apical side of the cells and 1.5 ml HBSS to the basolateral side. At time zero the enhancer solutions were discarded and replaced with 0.6 ml of 10^{-4} M DDAVP solution. The inserts were then moved to wells containing 1.5 ml fresh HBSS. Samples of 100 μl were taken and replaced every 15 min for 105 min. The transepithelial electrical resistance, TEER, was measured prior to each experiment using a Millicell®ERS (Millipore, U.S.A.) to ensure the integrity of the monolayers formed on the filters. The average TEER in our laboratory was 953 ± 54.3 Ω cm^2. On a regular basis the monolayer integrity was assessed using the aqueous pore marker [^{14}C]PEG 4000 (New England Nuclear, U.S.A.). Permeation rates across untreated cell monolayers of up to 0.1%/h were acceptable.

Based on the transport profiles the apparent permeability coefficient was calculated using the following equation:

$$P_{app} = dQ/dt \cdot A \cdot C_0 \cdot 60$$

where dQ/dt is the slope of the penetration profiles of DDAVP across Caco-2 cell monolayer (% transported/min), A denotes the diffusional

area of the inserts (1 cm^2), C_0 represents the initial donor concentration (100%) and 60 is the conversion factor from min to s.

2.7. Analytical procedure

The quantitative analysis of DDAVP was carried out by HPLC. A reversed-phase system consisting of a Hibar LiChrosorb RP-8 (250 mm × 4 mm, 7 μm) column and a mobile phase of 28% acetonitrile, 0.1% phosphorous acid and 5×10^{-3} M triethylamine adjusted to a pH of 2.2 was used, the injection volume being 40 μl and flow rate 1.0 ml/min. An HPLC system consisting of a Hitachi-Merck L-6200 gradient controller pump, L-4000 UV detector and A-655-40 autosampler handled the analysis and data processing using the HPLC manager software D-6000. UV detection was carried out at 200 nm. Quantitation of DDAVP was performed by measuring peak areas in relation to those of a standard chromatographed under identical conditions. The retention time for DDAVP was 4.12 min.

3. Results and discussion

3.1. Osmolarity and critical micelle concentration

The lysophosphatidylcholines, LPC-C, LPC-L and LPC-P were the prime compounds in scope. The osmolarity of solutions ranging from 10^{-3} to 0.2% was measured. All LPC solutions as well as a 0.05% solution of PC were found to be isoosmotic and in an interval from 300 to 320 mOsm/l. The solutions could therefore be expected not to produce any abnormalities in the monolayers due to osmotic effects such as those recently described for caprate (Anderberg et al., 1993). The same solutions were used to measure the CMC of the enhancers (Fig. 1). Initially, the surface tension of the solutions decreases rapidly in a linear fashion, as the concentration of the enhancer is increased. At a certain point, no further change in the surface tension is observed. The concentration corresponding to this 'break point is taken as the CMC. Neither the shortest

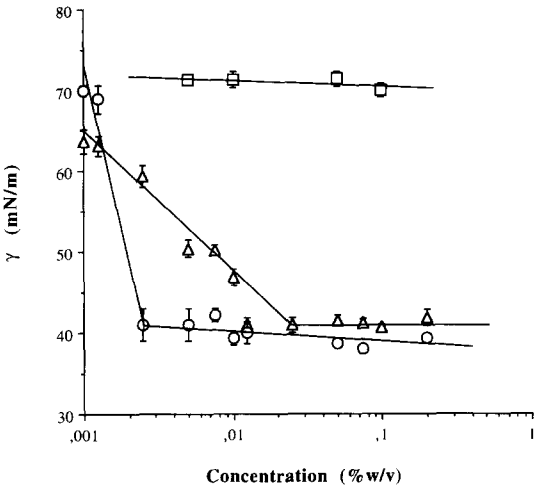

Fig. 1. Determination of CMC by measurements of surface tension as a function of concentration for LPC-C (□), LPC-L (△) and LPC-L (○).

member of the homologue series LPC-C nor PC showed any break point. This can be due to a high CMC for LPC-C, whereas for PC, the CMC can be assumed to be extremely low due to the high hydrophobicity of the dipalmitoyl moiety. Table 1 summarizes the CMC values. From the empirical equation:

$$\log (\mathrm{CMC}) = 1.4 - 0.25m$$

where m is the number of carbons in the hydrophobic chain, the CMC for LPC-C has been estimated (Matsuzaki et al., 1988). The CMC increases as the size of the hydrophobic part decreases. Our results are in accordance with data reported by Matsuzaki et al. (1988).

Table 1
Data for the lysophosphatidylcholines used as absorption enhancers

	Molecular weight	Chain length (No. of carbon atoms)	CMC (% w/v)
LPC-C	355.5	6	0.8 [a]
LPC-L	439.5	12	0.025 [b]
LPC-P	495.6	16	0.0025 [b]

[a] Estimated from log (CMC) = 1.4 − 0.25m.
[b] Found from Fig. 1.

3.2. MTT test

For the MTT test Caco-2 cells are seeded in ELISA wells, and the color intensity of the wells can be used as a measure of survival of cells after various treatments. The dehydrogenase activity was thus calculated and plotted against the logarithmic value of the concentration (Fig. 2). The graphs show a sigmoidal shape, starting at 100% enzyme activity for low concentrations of the enhancers. This indicates that the enhancers at low concentrations have no effect on cell viability. At higher concentrations, however, toxic effects are evident. The decline in dehydrogenase activity was found to depend on the size of the hydrophobic moiety of the enhancer. For LPC-P the enzymatic activity was affected at concentrations above 0.01% and reached a minimum at 0.1%. For LPC-L the region of change was found to be from 0.1 to 0.5%, and LPC-C did not have any effect on the cells in this test. The concentration giving 50% of the initial activity, LD-50, was found to be > 1, 0.20 and 0.07% w/v for LPC-C, LPC-L and LPC-P, respectively. Comparing the obtained LD_{50} values to the hemolytic activity of lysophosphatidylcholines, it was found that the toughness of the Caco-2 cells was much higher than that of human erythrocytes. In a review,

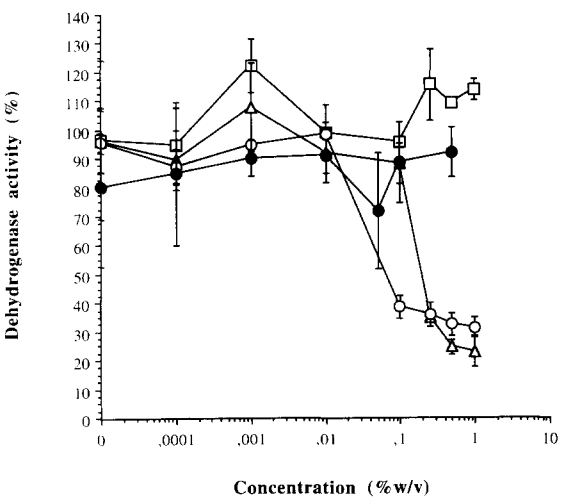

Fig. 2. Dehydrogenase activity in Caco-2 cells after treatment with phospholipids in various concentrations. LPC-C (□), LPC-L (△), LPC-P (○) and PC (●).

Table 2
Trypan blue staining of enhancer-treated Caco-2 monolayers

Enhancer	Concentration (% w/v)	Staining intensity [a]	
		15 min	60 min
LPC-L	0.25	18%	20%
LPC-P	0.05	5%	12%
	0.10	8%	27%

Staining measured as blue cells × 100/total cells %.
[a] For each treatment approx. 150 cells were counted.

Weltzien (1979) summarized LD_{50} values, given as the concentration of lysophosphatidylcholine, that produced lysis in 50% of erythrocytes, for lysophosphatidylcholines with 10–22 carbon atoms in the aliphatic chain. This activity was in all cases within a factor 4 above the CMC. Our data indicate that the LD_{50} found based on Caco-2 cells is at least a factor 8 above the CMC.

3.3. Trypan blue staining

The cytoplasmic membrane permeability was measured by trypan blue staining. Trypan blue staining is a vital stain counting method used to determine viability of cell cultures (Freshney, 1987). Trypan blue does not penetrate the unperturbed membrane of cells. If, however, the membrane is perturbed by any compound, penetration is induced. Other vital stain counting methods have been used for this purpose (Anderberg and Artursson, 1993). The monolayers were exposed to 0.05 and 0.10% LPC-P as well as 0.25% LPC-L for 15 and 60 min. The stain counts are given in Table 2. It was found that LPC-P at low concentration, below LD_{50}, only caused staining in 5% of the cells after 15 min treatment. However, if the exposure time was increased to 60 min, the degree of staining increased to 12%. This time dependency of the staining was also observed for LPC-P at a higher concentration, above LD_{50}, and for LPC-L. It was interesting to notice that LPC-L at a concentration of 0.25%, which is close to the LD_{50} determined by MTT test, exerted a considerable degree of membrane perturbation, around 20%. Moreover, 0.25% LPC-L treatment caused some cell detachment from the filters, which was not observed for LPC-P at the

tested concentrations. The results indicate that LPC-L is a more potent compound as measured by LD_{50} and vital stain counting than LPC-P. When concentrations relative to the CMC are compared, the CMC for LPC-L is 8-fold lower than LD_{50} and a concentration around LD_{50} gives 18% staining after 15 min, whereas the CMC for LPC-P is 28-fold lower than LD_{50} and concentrations around LD_{50} give only 5–8% staining after 15 min. For a series of acylcarnitines it was reported that no membrane perturbing effect was elicited for homologues with 10 or less carbon units (LeCluyse et al., 1991). From 12 carbon units and above significant perturbations occurred.

3.4. Transport studies

The lysophosphatidylcholines were all screened for their absorption promoting effect on DDAVP transport across Caco-2 monolayers. In Fig. 3 the transport profiles of DDAVP across untreated monolayers and filters without cells are shown. Transport basically does not exist when the cells are untreated. This is in full agreement with previous observations (Lundin and Artursson, 1990) and the low bioavailability of DDAVP after oral administration (Vilhardt and Lundin, 1986), and is close to the transport of PEG 4000. Moreover, Lundin and Folkesson (1993) recently reported that the oral bioavailability of DDAVP in rats was only 0.033%. However, this is a much lower transport rate than that presented here.

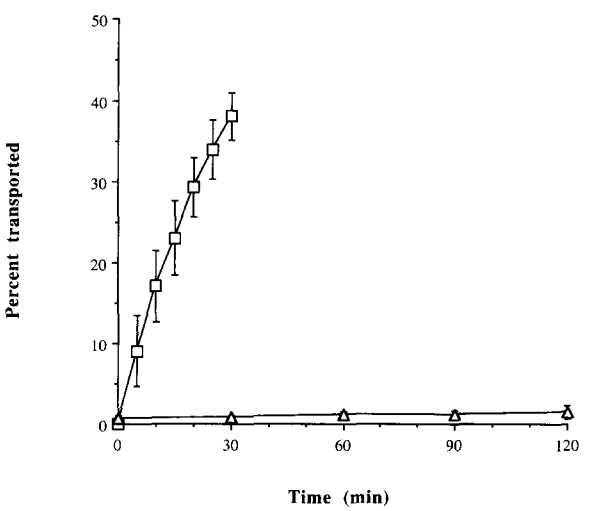

Fig. 3. Transport profiles of DDAVP across polycarbonate filters (□) and untreated Caco-2 monolayers (△).

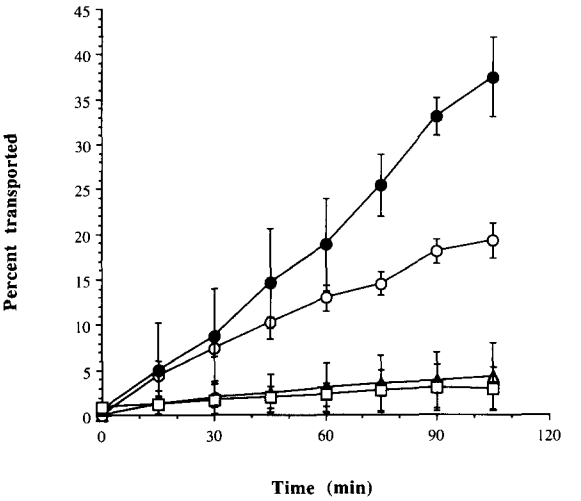

Fig. 4. Effects of lysophosphatidylcholines on DDAVP transport across Caco-2 monolayers. 1% LPC-C (□), 0.1% LPC-L (△), 0.25% LPC-L (○) and 0.5% LPC-L (●).

Fig. 4 shows the transport profiles of DDAVP after treatment with 1% LPC-C and various concentrations of LPC-L, all resulting in increased transport compared to untreated Caco-2 monolayers. Even 1% LPC-C, which did not have any toxic effects on the Caco-2 monolayers, as measured by the MTT test, increased the transport of DDAVP. As the concentration of LPC-L increased, the transport rate increased. This was also observed after treatment with various concentrations of LPC-P (Fig. 5). Application of 0.1% LPC-P produced a tremendous increase in the amount of DDAVP transported, but at the same time promoted 12% staining of cells on filters by trypan blue. Moreover, 0.1% was a concentration above the LD_{50}, so that the treatment may be considered relatively toxic. LPC-P in a concentration of 0.05% produced an intermediate transport increment. At the same time, it only stained 5% of cells by trypan blue and was below the LD_{50}.

Based on the linear parts of the transport profiles in Fig. 3–5, the apparent permeability coefficients have been calculated and are re-

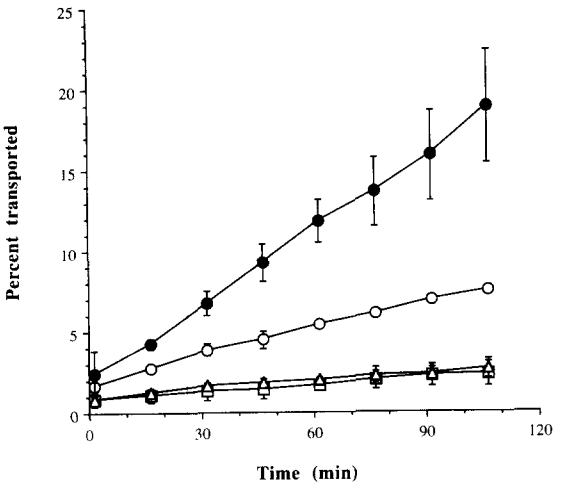

Fig. 5. Effects of lysophosphatidylcholines on DDAVP transport across Caco-2 monolayers. 0.01% LPC-P (□), 0.025% LPC-P (△), 0.05% LPC-P (○) and 0.1% LPC-P (●).

ported in Table 3. All treatments produced some increase in P_{app}. The effect depended on both the concentration and size of the hydrophobic moiety of the lysophosphatidylcholine. For LPC-P, the degree of enhancement was up to 30-fold. For a concentration of 0.05%, which in general was the most acceptable treatment based on toxicity, the absorption promotion was found to be 8-fold greater than control. This is a noteworthy increment, which would be expected to increase the oral bioavailability of DDAVP several-fold. PC produced a very variable absorption promotion of about 6-fold over control. However, the result was not significant because of the high degree of variation in this experiment.

Table 3
Apparent permeability coefficients for DDAVP transport across Caco-2 monolayers after various enhancer treatments

	Concentration (% w/v)	P_{app} [a] (cm/s) ($\times 10^7$)
Polycarbonate filters	–	1260.3 ±38.46
Untreated monolayers	–	6.37± 3.04
LPC-C	1.00	21.3 ±21.1
LPC-L	0.10	37.9 ±25.4
	0.25	210 ±19.5
	0.50	305 ±73.8
LPC-P	0.01	14.8 ± 3.35
	0.025	12.0 ± 0.55
	0.05	52.7 ± 3.61
	0.10	168 ±18.5
PC	0.05	34.66±33.90

[a] P_{app} values are given ±SD, $n = 3$.

Lysophosphatidylcholines have been used widely for nasal and vaginal absorption enhancement of peptides (Fisher et al., 1991; Richardson et al., 1992). Fisher et al. (1991) showed that nasal absorption of human growth hormone could be enhanced up to 72.8% of subcutaneous administration, when 0.2% lysophosphatidylcholine was used in rabbits. In the same study a concentration of 0.05% lysophosphatidylcholine produced a bioavailability of 3.7% in rats. We found that approx. 6% DDAVP was transported after 100 min in Caco-2 monolayers treated with 0.05% LPC-P. This concentration produced only minor staining in the trypan blue test and appears to be relatively nontoxic. Richardson et al. (1992) demonstrated that insulin absorption could be enhanced from the vagina by lysophosphatidylcholine. A concentration of 0.5% produced significant reductions in blood glucose levels. However, it was reported that this treatment not surprisingly caused damage to the outermost cell layers of the mucosa.

Anderberg et al. (1993) showed that sodium caprate exerts its action as an absorption enhancer by interacting with the intercellular complex. Peptide transport enhancement by caprate would therefore be due to paracellular transport. It appears that lysophosphatidylcholines, due to their amphiphilic nature, act on the cytoplasmic membrane as their primary effect. This is supported by our findings in MTT and trypan blue tests. However, this may affect the intercellular complexes resulting in an increased paracellular transport as a secondary effect, which may be utilized by high molecular weight compounds. This correlates with the observation, that TEER decreases after treatments with lysophosphatidylcholines. The decrease was found to be 50% for LPC-C, from 70 to 90% for LPC-L and from 30 to 90% for LPC-P. In a study concerning cyclodextrins as absorption enhancers, we recently found that cyclodextrins were able to extract membrane components, primarily cholesterol and lipids, from the Caco-2 monolayers (Brøndsted et al., manuscript in preparation). As a result of

this, the tight junctional complex appears to open up, allowing for paracellular transport of macromolecules.

In conclusion, lysophosphatidylcholines have been shown to perturb the cytoplasmic membrane in such a way that large molecules such as trypan blue can penetrate into the cells. The perturbation of the membrane was found to be dependent on concentration and length of hydrophobic moiety. A concentration of 0.05% of LPC-P gave rise to only slight perturbation, however, the transport of DDAVP was considerably enhanced. Even at concentrations below the LD_{50}, transport enhancement was observed, and monolayer integrity could be preserved. Lysophosphatidylcholines may be suitable as absorption enhancers for intestinal delivery of peptides.

Acknowledgements

The authors wish to acknowledge Dr Hans Vilhardt for valuable discussions. This work was supported in part by the PharmaBiotec Research Center, Copenhagen.

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