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Drug delivery studies in Caco-2 monolayers. III. Intestinal transport of various vasopressin analogues in the presence of lysophosphatidylcholine

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Abstract

The transport of a series of vasopressin and oxytocin analogues with varying lipophilicities was studied in Caco-2 monolayers. Transport was studied across the bare monolayer and after treatment with a phospholipid absorption enhancer, palmitoyl lysophosphatidylcholine. The range in lipophilicity of the analogues, estimated as the capacity factor, was found to be from 0.19 to 3.43. The intrinsic transport of the peptides across Caco-2 monolayers was found to be low. The apparent permeability coefficients, P_{app}, were in the range of 2×10^{-8}–6×10^{-7} cm/s. However, peptide transport was significantly greater (P_{app} in the range of 5×10^{-6}–2×10^{-5} cm/s) when facilitated by addition of palmitoyllysophosphatidylcholine. The results suggest that polypeptide transport across Caco-2 monolayers does not depend on lipophilicity, but that the facilitated transport does depend on the lipophilicity.

Keywords: DDAVP; Vasopressin; Intestinal transport; Absorption enhancer; Phospholipid; Lysophosphatidylcholine; Caco-2; Cell culture

1. Introduction

Oral peptide drug delivery is obstructed by the human body's natural defence and digestive mechanisms, and a number of factors are responsible for the low bioavailability experienced for oral peptide formulations. In general, peptide drugs have a higher molecular weight than conventional drugs and possess high degrees of hydrophilicity (Zhou, 1994). Moreover, they often have several intrinsic hydrogen bonding sites, which have been shown to hamper the transepithelial transport of peptides (Conradi et al., 1991). In order to overcome the low bioavailability, several approaches have been reported in the literature. The metabolic barrier to absorption has been proposed to be overcome by the use of enzyme inhibitors (Owens et al., 1988; Zhou, 1994) as well as by prodrug derivatization (Kahns et al., 1993) and analogue formation (Lundin and Folkesson, 1993). The physical barrier to absorption has been dealt with by the use of absorption enhancers (Watanabe et al., 1992; Gill et al., 1994) as well as by analogue (Brange et al., 1988) or prodrug formation (Møss et al., 1990). Analogue and prodrug formation have aimed at in-

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creasing the lipophilicity, lowering the molecular weight through minimizing self-association, and reducing the number of potential hydrogen bonding sites in the molecule. The vasopressin analogue desmopressin (DDAVP) is an example where the analogue formation principle has been used. DDAVP is a nonapeptide with a deaminated amino acid at the N-terminal and an L- to D- altered arginine at the C-terminal. It has a strong and specific antidiuretic effect and is used in the treatment of diabetes insipidus and nocturnal enuresis (Zaoral, 1985). Due to these modifications DDAVP has a higher, although still low, stability in the gut compared to vasopressin (Vilhardt, 1990). However, the permeability through the gut wall is not increased considerably (Vilhardt and Lundin, 1986).

Previously, Caco-2 monolayers were shown to be a valuable model in mechanistic studies of enhancers (Anderberg et al., 1993; Raeissi and Borchardt, 1993) and investigations of prodrug transport (Hovgaard et al., 1994). The transport of DDAVP has also been studied in Caco-2 monolayers and was found to be mediated by passive diffusion (Lundin and Artursson, 1990). The absorption enhancing effects of a series of lysophosphatidylcholines on Caco-2 monolayers were studied elsewhere and they were found to be of potential use as enhancers for DDAVP (Hovgaard et al., 1995). Earlier, the use of these enhancers was proposed for nasal and vaginal delivery of peptides (O'Hagan et al., 1990; Richardson et al., 1992; Gill et al., 1994).

A series of five vasopressin and oxytocin analogues was studied with respect to transport properties across Caco-2 monolayers in the presence and absence of palmitoyl lysophosphatidylcholine as an enhancer. The purpose of the study was to elucidate the importance of the lipophilicity of peptides and the effect of this property on their transport characteristics.

2. Materials and methods

2.1. Materials

The vasopressin analogue 1-deamino-8-D-arginine-vasopressin (DDAVP) was obtained as a gift from Ferring AB, Sweden. The DDAVP analogues, 2DTyr(Et)AVP (DTAVP), 2DTyr(Et)8-DAVP (DTDAVP), and 1Mpa2DTyr(Et)AVP (DDTAVP), and the oxytocin analogue, carbetocin, were kindly provided by Dr Hans Vilhardt, The Panum Institute, Denmark. The structures of the peptides are given in Scheme 1. Synthetic palmitoyl lysophosphatidylcholine (99%) (LPC-P) was purchased from Sigma Chemical Co. (St. Louis, MO). Buffer substances and all other chemicals were of analytical grade and were used as received.

Scheme 1
Structures of DDAVP and analogues

DDAVP (1-deamino-8D-arginine vasopressin)

Mpa-Tyr-Phe-Gln-Asn-Cys-Pro-DArg-GlyNH$_2$
S-S

DTAVP (2D-tyrosine(ethyl)-arginine vasopressin)

Cys-DTyr(Et)-Phe-Gln-Asn-Cys-Pro-Arg-GlyNH$_2$
S-S

DTAVP (2D-tyrosine(ethyl)-8D-arginine vasopressin)

Cys-DTyr(Et)-Phe-Gln-Asn-Cys-Pro-DArg-GlyNH$_2$
S-S

DDTAVP (1-deamino-2D-tyrosine(ethyl)-8D-arginine vasopressin)

Mpa-DTyr(Et)-Phe-Gln-Asn-Cys-Pro-DArg-GlyNH$_2$
S-S

Carbetocin (1-deamino-1-carba-2-tyrosine(methyl) oxytocin)

H$_2$C-Tyr(Met)-Ile-Gln-Asn-Cys-Pro-Leu-GlyNH$_2$
S-S

2.2. Caco-2 cell cultures

The colonic adenocarcinoma cell line, Caco-2, was purchased from the American Type Culture Collection (Rockville, MD). The cells were maintained in Dulbecco's Modified Eagle's Medium, DMEM, containing 9% fetal calf serum, 1% L-glutamine, 1% nonessential amino acids and antibiotics (10 U/ml streptomycin and 100 U/ml benzylpenicillin) (Gibco, U.K.) in 75 cm^2 culture flasks (Greiner, Austria). The cultures were kept

at 37°C in an atmosphere of 10% CO_2, 90% air and 90% relative humidity. The culture medium was renewed every other day and the cells were trypsinized once a week. To ensure the absence of *Mycoplasma* in the cultures the cells were grown in 25 cm^2 culture flasks (Greiner, Austria) without antibiotics for testing. Tests were carried out by Statens Veterinärmedicinska, Uppsala, Sweden. In all cases the cultures tested negative for infection.

2.3. Transport studies

For transport studies a suspension of approx. 5×10^4 Caco-2 cells per ml was seeded onto porous polycarbonate filter membranes with a pore size of 0.4 μm and a surface area of 1 cm^2 (Transwell, Costar, U.S.A.) 3 weeks prior to experiments. The cells were maintained under the same conditions as cells in flasks. Passage nos 28–34 were used for these studies. LPC-P was used at a concentration of 0.05% w/v in Hanks balanced salt solution (HBSS) for transport enhancement. The enhancing effect of LPC-P as well as the influence on the monolayer has been described in the preceding paper (Hovgaard et al., 1995). The monolayers were pretreated with 0.5 ml LPC-P solution on the apical side of the monolayers and 1.5 ml of HBSS on the basolateral side. This treatment was carried out for exactly 15 min after which the enhancer solution and HBSS was removed. Then 0.6 ml peptide solution at concentrations ranging from 10^{-4} to 10^{-5} M in HBSS was applied to the apical side (donor) and 1.5 ml of HBSS was applied to the basolateral side (receptor). Samples of 100 μl were collected from the receptor phase and replaced with fresh HBSS every 15 min for 1 h 45 min. During experiments the Caco-2 cells were kept at 37°C, supplied with 5% CO_2 and agitated on a plate shaker located in a Hitachi-Merck AS-4000 intelligent autosampler capable of sampling automatically as described by Buur and Mørk (1992). Similarly, transport of peptide analogues without pretreatment with enhancer was studied. However, in the latter case sampling was carried out every 30 min for 2 h 30 min. Transepithelial electrical resistance, TEER, was measured prior to each experiment to ensure the confluency of the monolayers. In our laboratory TEER for confluent monolayers was 953 ± 54.3 Ω cm^2. From transport profiles the apparent permeability coefficient was calculated from 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.

2.4. Analytical procedure

Quantitative analysis of the peptides was performed by reversed-phase HPLC. The HPLC system consisted of a Hitachi-Merck model L-6200 gradient controller pump, model L-4000 UV detector and model 655A-40 autosampler. Data acquisition and processing were performed using the Hitachi-Merck HPLC manager (model D-6000). The analytical column was a Hibar LiChrosorb RP-8 column (250×4 mm; 7 μm) protected by Hibar filters and injection volume was 40 μl. To optimize the analysis for each peptide the amount of acetonitrile in the mobile phase, the flow rate of the system and the wavelength for UV detection were individually adjusted. As mobile phase 27–33% v/v acetonitrile in 0.1% v/v phosphoric acid and 5×10^{-3} M triethylamine (TEA) adjusted to a pH of 2.2 was used. Table 1 gives the specific conditions and individual retention times.

Table 1
Analytical data for DDAVP and analogues

	Wave length (nm)	Mobile phase (% v/v AcCN) [a]	Flow rate (ml/min)	Retention time (min)
DDAVP	200	28	1.0	4.12
DTAVP	206	27	1.5	5.97
DTDAVP	207	27	2.0	6.23
DDTAVP	209	28.5	1.0	11.34
Carbetocin	207	33	2.5	15.27

[a] AcCN, acetonitrile.

2.5. Determination of lipophilicity

The relative lipophilicities of the analogues were evaluated from the respective capacity factors, k', determined by reversed-phase HPLC, as described under section 2.4. However, the mobile phase contained 28% v/v acetonitrile and a flow rate of 1.0 ml/min was used for all peptides. The respective retention times, t_R, for the five analogue peptides and t_M for the solvent, as a non-retained substance, were used to calculate the capacity factors according to Lindeberg et al. (1980) and Willard et al. (1981) from the following equation:

$$k' = (t_R - t_M)/t_M$$

3. Results and discussion

3.1. Determination of lipophilicity

Peptides are typically hydrophilic in nature. Therefore, they are poorly transported across mucosal barriers (Lee et al., 1991). It was then important to determine the hydrophilic/lipophilic relations between the vasopressin and oxytocin analogues used in the transport studies. The chemical composition is very similar for the vasopressin analogues; only a few variations can be pointed out. Carbetocin is an analogue of oxytocin and is thus quite different in its structure. However, the molecular weight is close to those of the vasopressin analogues. The relative lipophilicities for the compounds were calculated by the use of capacity factors determined by reversed-phase HPLC. The values are given in Table 2. The capacity factors for the compounds were found to vary from 0.19 to 3.43, corresponding to a relative increase of up to 18-fold that of DDAVP. DDAVP was found to be the least lipophilic peptide in the study. The octanol-water partition coefficient for DDAVP was reported by Kahns et al. (1993) to be less than 3.2×10^{-4}. The analogues DTAVP and DTDAVP possessed similar capacity factors, approximately a factor 4 higher than that of DDAVP. Both compounds are ethylated on D-tyrosine, but DTAVP has an L-arginine and DTDAVP has a D-arginine. This steric difference between the compounds does not affect the lipophilic character of the compounds. However, the D-amino acid analogues have been shown to be more stable against enzymatic attack of carboxypeptidases than L-amino acid analogues (Söderberg-Ahlm et al., 1993). Ethylation provides the molecules with an increase in lipophilicity compared to DDAVP. In DDTAVP the N-terminal is deaminated and the tyrosine is ethylated as well as altered from L to D. For this compound an increased lipophilicity as well as increased stability against enzymatic attack was expected. In agreement with these presumptions, the measured capacity factor was a factor of 12 larger than that of DDAVP. It has previously been shown that these factors affect stability and increase the transport rate across intestinal tissue relative to DDAVP (Lundin et al., 1991). The last peptide, carbetocin, is an analogue of oxytocin. It has a methylated tyrosine residue, contains isoleucine and leucine, which are nonpolar amino acids, and has a very lipophilic C-terminal. These factors provide it with an 18-fold greater capacity factor than that of DDAVP. Carbetocin has fewer possible hydrogen bonding sites than the other four peptides, due to the missing arginine residue at position 8 and owing to the methylation of the tyrosine residue (Conradi et al., 1992). In accordance with our results, Lundin and Folkesson (1993) recently found that the ethylation in DDTAVP led to a more than 10-fold increment in capacity factor compared to DDAVP, thus increasing the lipophilicity.

Table 2
Physical characteristics for DDAVP and analogues

	Molecular weight (g/mol)	Capacity factor [a]	Relative lipophilicity [b]
DDAVP	1069	0.19	1.0
DTAVP	1112	0.73	3.8
DTDAVP	1112	0.81	4.3
DDTAVP	1096	2.29	12.1
Carbetocin	979	3.43	18.1

[a] Capacity factors calculated according to Eq. 1.
[b] Lipophilicities relative to DDAVP.

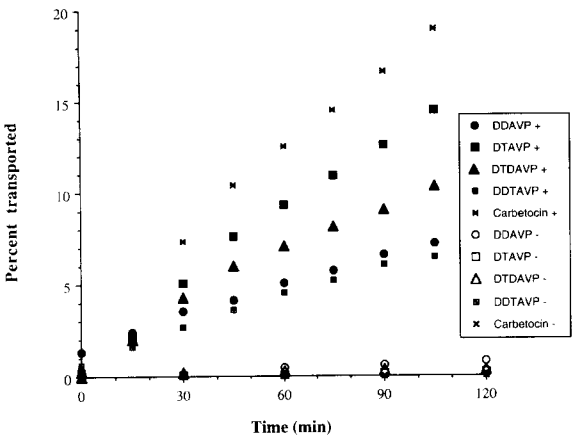

Fig. 1. Transport profiles of DDAVP and DDAVP analogues as well as carbetocin across palmitoyl lysophosphatidylcholine pretreated Caco-2 monolayers (+) and untreated monolayers (−).

3.2. Transport studies

The Caco-2 monolayers presented a massive diffusional barrier towards the epithelial transport of all the analogues studied. It is evident from Fig. 1 that none of the peptides were transported to extents greater than 0.5%/h. The apparent permeability coefficients ranged from 6.4 $\times 10^{-7}$ cm/s for DDAVP to 2.4 $\times 10^{-8}$ cm/s for carbetocin (see Table 3). Permeability coefficients for DDAVP have previously been reported to be in the range of 4×10^{-8}–1.2×10^{-7} cm/s by Lundin and Artursson (1990) and Kahns et al. (1993) found a permeability coefficient of 5.15–5.99 $\times 10^{-7}$ cm/s. Generally, for low molecular weight compounds, increased lipophilicity mediates a transcellular contribution to transport. For prodrugs and conventional drugs the passive transport rate has been shown to follow the order of lipophilicity within a series of homologue compounds (Artursson and Karlsson, 1991; Hovgaard et al., 1994). For peptides this relation is not valid. However, the tendency of oligopeptides to form hydrogen bonds has been found to correlate inversely with transport across human intestinal epithelium (Conradi et al. 1991). Therefore, a model tetrapeptide was studied as a prodrug in order to diminish the number of hydrogen bonding sites (Conradi et al., 1992). The peptides studied here exert high hydrophilicities and are thus restricted from transcellular transport (Lee, 1991). Hence, the transport mechanism is mostly mediated by paracellular transport (Lundin and Artursson, 1990; Lundin et al., 1991; Ungell et al., 1992), however, due to their high molecular weight they demonstrate very low transport. Kahns et al. (1993) studied transport of DDAVP and found no correlation with either lipophilicity or hydrogen bonding ability, even though one prodrug showed a higher transport rate than DDAVP. Fig. 1 also shows the enhancer facilitated transport profiles for the peptides. The absorption enhancer significantly increases the transepithelial transport of the peptides. The transport of DDAVP reaches approx. 3%/h. All analogues exceed this transport, reaching as much as 10%/h. P_{app} for peptides across enhancer treated monolayers range from 5×10^{-6} to 1.8×10^{-5} cm/s. The treatment with palmitoyl lysophosphatidylcholine was shown by Hovgaard et al. (1995) to have a strong absorption enhancing effect and at the same time a relatively low cytotoxicity. It was found to be able to perturb the membrane and further to increase paracellular transport. However, increased transcellular transport may also be involved.

Table 3
Apparent permeability coefficients for analogues in Caco-2 monolayers

Compound	P_{app} [a] (cm/s) ($\times 10^7$)	
	Without enhancer	With enhancer
DDAVP	6.37 ± 3.00	57.2 ± 3.60
DTAVP	1.54 ± 1.50	136.0 ± 32.0
DTDAVP	2.19 ± 0.62	94.0 ± 21.1
DDTAVP	0.66 ± 0.20	57.3 ± 15.3
Carbetocin	0.24 ± 0.05	181.3 ± 17.6

[a] P_{app} values given ± SD, $n = 3$–5.

The apparent permeability coefficients of the peptides across enhancer treated and untreated monolayers are plotted against the logarithm of the capacity factors as shown in Fig. 2. In the case of untreated monolayers, transport is low and controlled by the paracellular route. However, after pretreatment with palmitoyl lysophosphatidylcholine the more lipophilic peptides, as determined by capacity factors, are generally

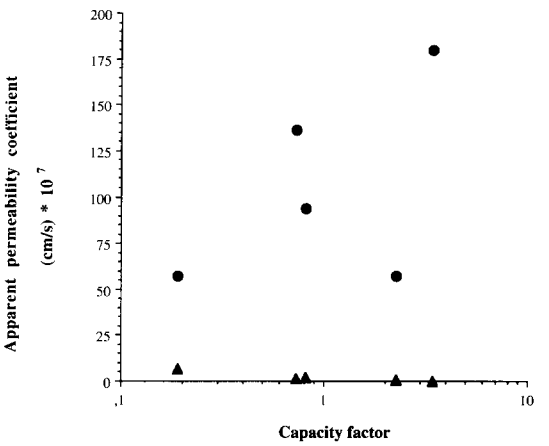

Fig. 2. Correlation between P_{app} determined on pretreated Caco-2 monolayers (●) and untreated monolayers (▲) and capacity factors of DDAVP and DDAVP analogues as well as carbetocin.

transported faster. Thus, the lipophilicity appears to be important for the transport of peptides after enhancer treatment. One would not expect to find any effect of lipophilicity on the transport, if the paracellular route were the only transport pathway. As a part of the enhancement mechanism by palmitoyl lysophosphatidylcholine the cytoplasmic membrane of the Caco-2 cells is perturbed (Hovgaard et al., 1995). Trypan blue, used in this study, does not penetrate the unperturbed membrane, therefore, the treatment should be expected to increase the transcellular transport of other nonabsorbable molecules, i.e., peptides. Anderberg and Artursson (1993) showed that a perturbation of the cytoplasmic membrane induced by SDS, measured in a vital stain count by propidium iodide, was reversible. The authors, however, did not discuss the possibility of increased transcellular transport as a function of this perturbation. DDTAVP deviates from the correlation found between P_{app} and the capacity factor. The reason for this is not understood, but a similar result was obtained by Lundin and Folkesson (1993). It was reported that generally an increase in lipophilicity increased the bioavailability of DDAVP analogues. However, DDTAVP deviated from this rule.

In conclusion, increasing the lipophilicity of peptides by analogue formation may be a method through which increments in enhancer facilitated transport of drugs can be achieved. It is possible to lower the amount of absorption enhancer needed to obtain a certain amount absorbed and thus reduce the toxic side effects caused by the enhancer compounds on the epithelial barrier.

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