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In vitro evaluation of the potential of thiomers for the nasal administration of Leu-enkephalin

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Summary. It was the aim of this study to evaluate the potential of thiolated polycarbophil for the nasal administration of Leucine-enkephalin (Leu-enkephalin). The enzymatic degradation of Leu-enkephalin on freshly excised bovine nasal mucosa was analysed qualitatively via thin layer chromatography and quantitatively via high performance liquid chromatography (HPLC). The potential of thiolated polycarbophil gels to provide a sustained release for the therapeutic peptide was investigated via diffusion studies. Permeation studies were performed in Ussing-type diffusion chambers with freshly excised bovine nasal mucosa. Results demonstrated that Leu-enkephalin is mainly degraded by the cleavage of tyrosine from the N-terminus of the peptide. Within one hour more than $63.5 \pm 2\%$ of this therapeutic peptide are degraded on the nasal mucosa. In the presence of 0.25% thiolated polycarbophil, this degradation process, however, could be significantly lowered. Diffusion studies demonstrated that Leu-enkephalin being incorporated in a 0.5% thiolated polycarbophil gel is sustained released out of it. The appearent permeability coefficient (P_{app}) for Leu-enkephalin on the nasal mucosa was determined to be $1.9 \pm 1.2 \times 10^{-7}$ cm/sec. Furthermore, in the presence of 0.5% thiolated polycarbophil and 1% glutathione, which is used as permeation mediator for the thiomer, the uptake of Leu-enkephalin from the nasal mucosa was even 82-fold improved. According to these results thiolated polycarbophil might be a promising excipient for nasal administration of Leu-enkephalin.

Keywords: Leu-enkephalin – Nasal permeation – Enzyme inhibition – Thiomers – Thiolated polycarbophil

Introduction

Leu-enkephalin is known to act as neurotransmitter or neuromodulator in pain transmission. Because of its non-additive morphine-like activity this peptide drug might have great potential in pain management. Non-invasively administered Leu-enkephalin, however, is rapidly degraded and poorly absorbed from mucosal membranes strongly limiting the therapeutic application of this drug. Among various routes of non-invasive administration, the nasal route seems to be most promising offering various advantages such as a rapid onset of the therapeutic effect, avoidance of liver first-pass effect and a simple and painless mode of application. Strategies to overcome the nasal membrane barrier include the use of protease inhibitors such as bestatin and puromycin (Agu et al., 2004), of permeation enhancers such as glycocholate and dimethyl-β-cyclodextrin (Agu et al., 2004) and mucoadhesive polymers providing a prolonged residence time (Tafaghodi et al., 2004) and a comparatively steeper concentration gradient for passive drug uptake.

Recently, a new type of polymeric excipients has been introduced in the pharmaceutical literature. Thiolated polymers – designated thiomers – were shown to exhibit strong permeation enhancing properties for peptide drugs (Clausen et al., 2000) and mucoadhesive properties (Bernkop-Schnürch et al., 1999). Leitner et al., for instance, could show in rats that the nasal uptake of hGH is even 3-fold improved in the presence of a thiomer (Leitner et al., 2004). In addition, thiomers exhibit peptidase inhibitory properties reducing the presystemic metabolism of peptide drugs on mucosal membranes (Bernkop-Schnürch et al., 2001a). Moreover, the residence time of Leu-enkephalin being incorporated in a thiomer on the nasal mucosa might be at least to some extent prolonged due to the mucoadhesive properties of these excipients (Bernkop-Schnürch et al., 1999).

Because of these properties thiomers might be promising excipients for the nasal administration of Leu-enkephalin. It was therefore the aim of this study to evaluate their potential for the nasal administration of Leu-enkephalin. Polycarbophil-cysteine was chosen as model thiomer as its permeation enhancing properties (Clausen et al., 2000), its inhibitory effect on membrane bound peptidases

(Bernkop-Schnürch et al., 2001a) and its mucoadhesive properties (Bernkop-Schnürch et al., 1999) are well-documented. The enzyme inhibitory effect on the degradation of Leu-enkephalin on freshly excised bovine nasal mucosa, the capability to provide a sustained release and the permeation enhancing effect of this polymer will be evaluated *in vitro*.

Materials and methods

Polymer synthesis

The poly(acrylic acid)-cysteine conjugate (PCP-Cys) was synthesized according to a method described previously (Marschütz et al., 2002). In brief, cysteine was covalently linked to crosslinked poly(acrylic acid) (polycarbophil; Noveon, Raubling, Germany) via the formation of amide bonds between the primary amino group of cysteine and a carboxylic acid group of the polymer. The reaction was mediated by a water-soluble carbodiimide. After purification of the conjugate via exhaustive dialysis against 1 mM HCl it was lyophilized by drying frozen aqueous polymer solutions at -30° at 0.01 mbar (Benchtop 2 K, VirTis, NY, USA). The polymer was stored at 4 °C until further use.

Determination of the thiol/disulfide content

The amount of thiol groups on the poly(acrylic acid)-cysteine conjugate was determined via Ellman's reagent (5,5′-dithiobis(nitrobenzoic acid)) (Bernkop-Schnürch et al., 2001b). Disulfide content was measured after reduction with $NaBH_4$ and addition of 5,5′-dithiobis(nitrobenzoic acid) (Habeeb 1973).

Metabolism on freshly excised bovine nasal mucosa

A glass cylinder with an internal surface area of 1.0 cm^2 was placed vertically on top of the mucosal side of freshly excised bovine nasal mucosa and clamped as described previously (Bernkop-Schnürch et al., 1997). A solution containing 0.2 mM Leu-enkephalin and optionally 0.25% (m/v) PCP-Cys and 1% glutathione in 50 mM phosphate buffer were added into the reaction cylinder. At predetermined time points samples of 200 µl were withdrawn and substituted by 200 µl of 50 mM phosphate buffer. To each sample 20 µl of 20% trifluoroacetic acid (TFA) (v/v) were added in order to stop any further enzymatic reaction. Samples were centrifuged at 13 000 rpm for 10 min.

The degradation of Leu-enkephalin was investigated via thin layer chromatography (TLC). Aliquot volumes of 30 µl were withdrawn and degradation fragments separated by TLC [layer: aluminium sheets silica gel 60 F_{254} (Merck, Darmstadt, Germany)]; layer thickness: 0.2 mm; mobile phase: n-butanol/acetic acid/H_2O (3 + 1 + 3.5); migration distance: 8.5 cm; detection: spraying with ninhydrin-reagent (0.3 g ninhydrin, 100 ml n-butanol, 3 ml acetic acid). Leu-enkephalin, des-Tyr-Leu-enkephalin and tyrosine were used as references.

In addition the amount of degraded Leu-enkephalin was also quantified via HPLC (Hitachi LaChromeElite series L-2130 pump, Hitachi LaChromeElite series L-2200 auto sampler and a Hitachi LaChromeElite L-2450 diode array detector). Remaining traces of polymer were hold back on a precolumn (Nucleosil 100-5C18, 40 mm × 4 mm). Leu-enkephalin and its degradation fragments were separated on a C_{18}-column (Nucleosil 100-5C18, 250 mm × 4 mm) at 20 °C. Gradient elution was performed as follows: flow rate 1.0 ml/min, 0–22 min, linear gradient from 90% A/10% B to 10.0% A/90.0% B (eluent A: 0.1% trifluoroacetic acid in water; eluent B: acetonitrile and 0.1% TFA). Peptides and amino acids were detected by absorbance at 220 nm as well as 280 nm with a diode array absorbance detector and were identified by comparison and comigration with authentic peptide standards. Degradation of Leu-enkephalin was evaluated by following the disappearance of Leu-enkephalin and the appearance of tyrosine and des-Tyr-Leu-enkephalin from the reaction mixture via HPLC. Peak areas were directly proportional to mass of standards injected and peptide hydrolysis was quantified from integrated peak areas and molar absorbance values calculated from standards for Leu-enkephalin, des-Tyr-Leu-enkephalin and tyrosine.

Diffusion studies

Drug diffusion studies were performed in Franz diffusion cells displaying an acceptor chamber volume of 2 ml and a diffusion area of 1 cm^2. First, 1 ml of a 1% Leu-enkephalin solution containing 0.5% PCP-Cys and 1% GSH in 50 mM phosphate buffer pH 5.4 were applied on a dialysis membrane with a molecular weight cut off of 12.5 kDa mounted in the diffusion cell. The lower chamber was filled with 2 ml of 50 mM phosphate buffer pH 5.4, used as receptor medium. The assembled cells were placed in an incubator (37 °C ± 0.5 °C). Sink conditions were maintained throughout the whole study. At set time intervals, aliquots of 200 µl were removed via the side arm and assayed for Leu-enkephalin via HPLC as described above. Removed samples were immediately replaced with an equal quantity of pre-warmed receptor medium. Cumulative corrections were made for previously removed samples.

In vitro nasal permeation studies

Tissue with nasal mucosa (conchae nasals) was excised from the noses of freshly slaughtered cattle and stored in Krebs-Ringer-Buffer (KRB; ion composition (mM): $MgCl_2 \cdot 6H_2O$ 0.492; KCl 4.56; NaCl 119.8; Na_2HPO_4 0.70; NaH_2PO_4 1.5; $NaHCO_3$ 15; D-glucose 10) at 37 °C during transport to the laboratory. Mucosa was separated from the underlying cartilage by blunt stripping using a pair of tweezers and mounted in Ussing-type diffusion chambers displaying a permeation area of 0.64 cm^2. The apical side of the tissue was facing the donor compartment. Thereafter, 1 ml of preheated (37 °C) KRB pH 5.4 was added each to the donor and acceptor chamber. For oxygenation and agitation a mixture of 95% O_2 and 5% CO_2 was bubbled through each compartment. The temperature within the chambers was maintained at 37 °C. After preincubation the buffer medium in the donor chamber was substituted by a 0.1% Leu-enkephalin solution in KRB pH 5.4 optionally containing 0.5% polycarbophil-cysteine/1% glutathione. Over a time period of 3 hours samples of 200 µl were withdrawn from the acceptor chamber every 30 minutes. The amount of intact peptide drug permeated was quantified via HPLC as described above.

Data analysis

Apparent permeability coefficients for Leu-enkephalin were calculated according to the equation $P_{app} = Q/(A*c*t)$, where P_{app} is the apparent permeability coefficient (cm/s), Q is the total amount of test substance permeated through the mucosa (µg), A is the diffusion area of the Ussing-type chamber (cm^2), c is the initial concentration of the marker substance in the donor compartment (µg/cm^3), and t is the total time of the experiment(s).

Statistical data analyses

Statistical data analyses were performed using a non paired Students *t*-test, and a p value of 0.05 or less was considered to be significant.

Results

Qualitative analyses of enzymatic degradation

In order to identify the cleavage sites of Leu-enkephalin, which are primarily the target of nasal mucosal peptidases,

the degradation products of Leu-enkephalin after incubation with the nasal mucosa were analysed via TLC. Results of this study demonstrated that the peptide is mainly degraded via cleavage of tyrosine. In Fig. 1 the target sites for enzymatic attack of Leu-enkephalin are illustrated. The TLC as shown in Fig. 2 demonstrated that within one hour of Leu-enkephalin incubation on the nasal mucosa almost exclusively des-Tyr-Leu-enkephalin and tyrosine are formed. Accordingly, the degradation of Leu-enkephalin on the nasal mucosa is mainly driven by aminopeptidases.

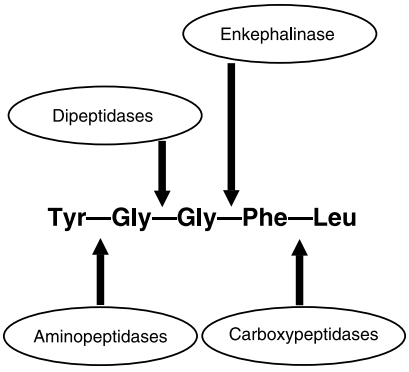

Fig. 1. Amino acid sequence of Leu-enkephalin and theoretical cleavage sites for membrane-bound peptidases

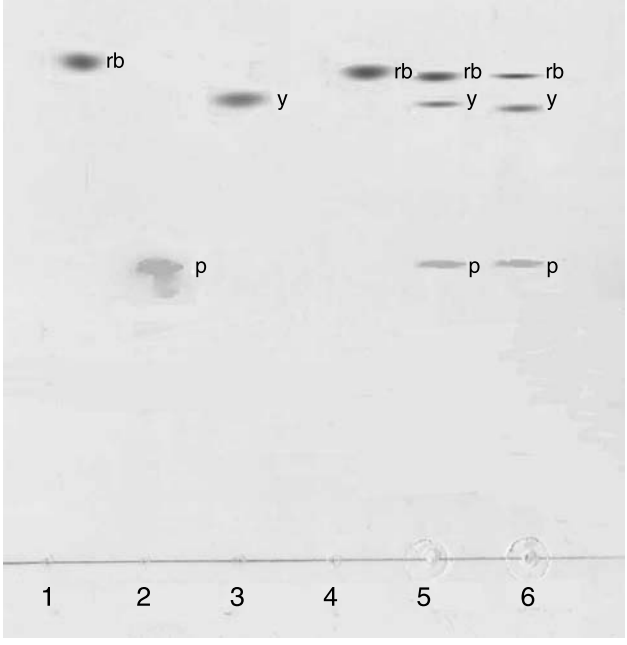

Fig. 2. Thin layer chromatogram of Leu-enkephalin and its degradation products; *1* Leu-enkephalin (*rb* red-brown); *2* tyrosine (*p* purple); *3* des-Tyr-Leu-enkephalin (*y* yellow); *4–6* Leu-enkephalin and degradation products gained by incubation of Leu-enkephalin with freshly excised bovine nasal mucosa within 0 min (*4*), 30 min (*5*) and 60 min (*6*)

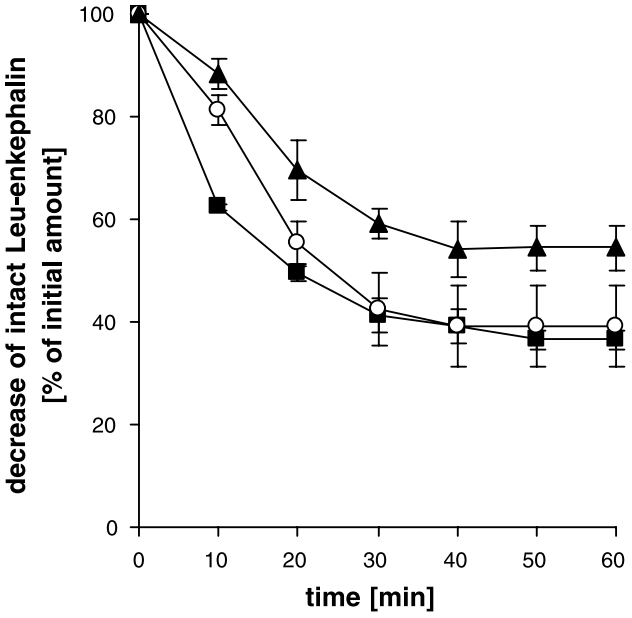

Fig. 3. Enzymatic degradation of Leu-enkephalin on freshly excised nasal mucosa (■) in the presence of glutathione (1%; m/v) (○) and polycarbophil-cysteine (0.25%; m/v)/glutathione (1%; m/v) (▲); indicated values are means of at least three experiments ±SD

Quantitative analyses of Leu-enkephalin degradation

The extent of enzymatic degradation of Leu-enkephalin was quantified via HPLC. As shown in Fig. 3 more than 60% of Leu-enkephalin were degraded on the nasal mucosa within an hour. In parallel the amount of tyrosine increased confirming that the Tyr-Gly bond is the main target of enzymatic cleavage. In addition, as shown in Fig. 4 also the concentration of des-Tyr-Leu-enkephalin increased as a function of time.

Inhibition of Leu-enkephalin degradation

In order to evaluate the inhibitory effect of polycarbophil-cysteine on the degradation of Leu-enkephalin on the nasal mucosa, degradation studies were also performed in the presence of this thiomer. Results as shown in Fig. 3 demonstrated a significantly lower degradation rate due to the inhibitory effect of the thiomer on aminopeptidases. An influence of the viscosity of the thiomer on the enzymatic degradation of Leu-enkephalin could be excluded, as substituting the thiomer by 0.15% hydroxyethylcellulose exhibiting an even higher viscosity than polycarbophil-cysteine in this experiment did not lead to a reduced degradation at all (data not shown). As glutathione has recently been shown to display an inhibitory effect on membrane bound peptidases (Langoth et al.,

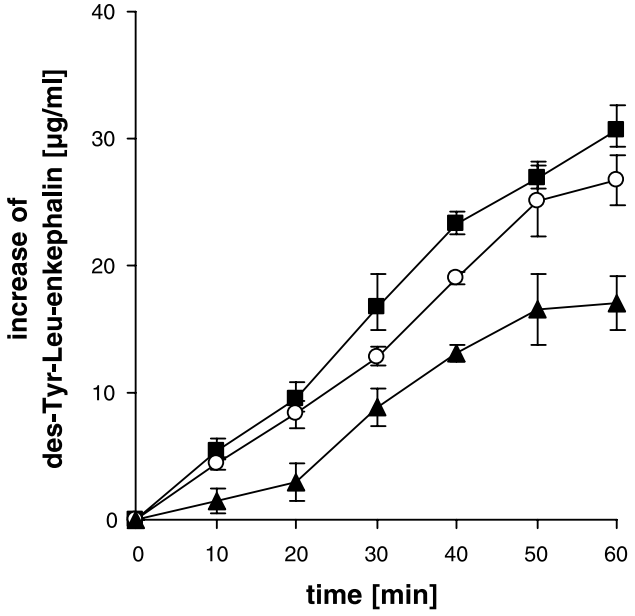

Fig. 4. Formation of des-Tyr-Leu-enkephalin during the degradation of Leu-enkephalin on freshly excised nasal mucosa (■) in the presence of glutathione (1%; m/v) (○) and polycarbophil-cysteine (0.25%; m/v)/glutathione (1%; m/v) (▲); indicated values are means of at least three experiments ±SD

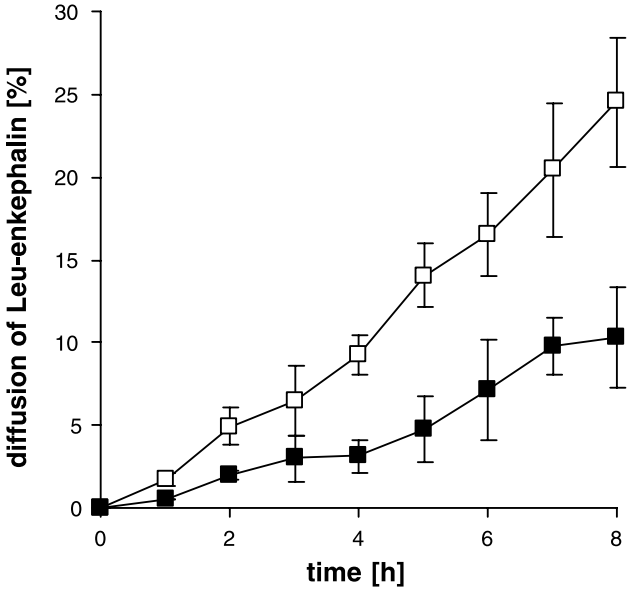

Fig. 5. Diffusion studies of Leu-enkephalin in buffer solution (□) and in the presence of 0.5% polycarbophil-cysteine/1% glutathione (■); indicated values are means of at least three experiments ±SD

2005a), its impact on Leu-enkephalin degradation was evaluated as well. Results of this study, however, revealed no significant effect of this tripeptide in the applied concentration. Results are shown in Figs. 3 and 4.

Diffusion studies

On the one hand a too rapid clearance of drugs from the nasal mucosa reduces the time period being available for absorption. On the other hand a too sustained release of poorly absorbed drugs reduces the concentration gradient on the membrane representing the driving force for passive drug uptake. According to these considerations the diffusion behaviour of Leu-enkephalin out of the polymeric network of the thiomer has been analysed. Results of this study are shown in Fig. 5. Due to the incorporation in 0.5% thiomer gel, the diffusion velocity of the drug was almost halved, demonstrating that the gel can guarantee a sustained drug release.

Permeation studies

The uptake of Leu-enkephalin from the nasal mucosa determined to be $1.9 \pm 1.2 \times 10^{-7}$ cm/s is high in comparison to other peptide drugs. The P_{app} of insulin displaying a molecular mass of approximately 6 kDa, for instance, was determined to be $3 \pm 1 \times 10^{-8}$ cm/s (Wadell et al., 2003). An explanation for this observation can be given by the comparatively lower molecular mass of Leu-enkephalin leading to a relative higher uptake. Nevertheless nasal Leu-enkephalin absorption is so far too low to reach the therapeutic level.

This poor membrane permeability of Leu-enkephalin, however, could be strongly improved by the addition of the thiomer. As listed in Table 1 the uptake of Leu-enkephalin from the membrane was even 82-fold improved, which is so far the most improved mucosal uptake of Leu-enkephalin to our notice.

In Fig. 6 the permeation of Leu-enkephalin through the freshly excised membrane is shown as a function of time. Focusing on the permeation behaviour of the main metabolite des-Tyr-Leu-enkephalin demonstrated that the

Table 1. Comparison of the apparent permeability coefficients (P_{app}) of Leu-enkephalin in the presence of polycarbophil-cysteine and glutathione

Test system	P_{app} [$*10^{-6}$ (cm/sec)]	Enhancement ratio
PCP-Cys/GSH (0.5%/1%)	15.61 ± 1.56	82
Buffer	0.19 ± 0.12	–

Improvement ratio = P_{app} (with polycarbophil-cysteine/glutathione)/P_{app} (without polycarbophil-cysteine/glutathione); indicated values are means of at least three experiments ±SD

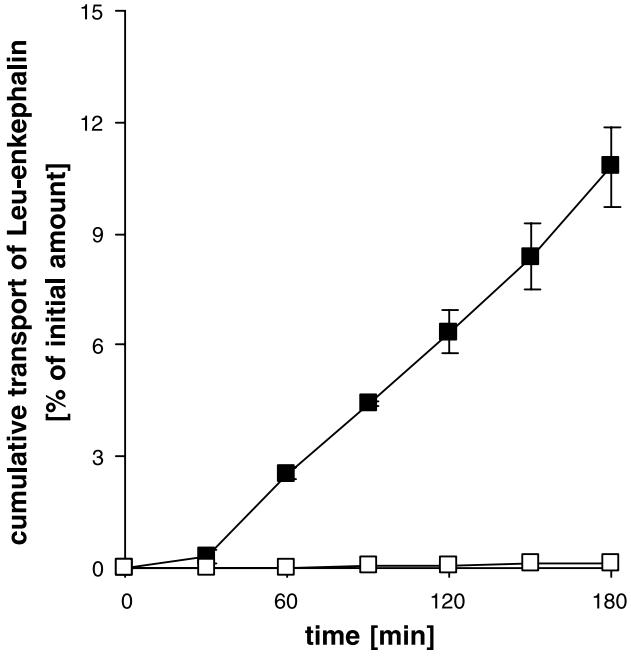

Fig. 6. Transport of Leu-enkephalin across freshly excised bovine nasal mucosa in buffer solution (□) and in the presence of PCP-Cys/GSH (0.5%/1%, m/v) (■); indicated values are means of at least three experiments ±SD

poor uptake of Leu-enkephalin is mainly based on the enzymatic degradation of this therapeutic peptide. As illustrated in Fig. 7, des-Tyr-Leu-enkephalin reached in significant quantities the acceptor chamber, whereas in the presence of the thiomer the concentration of des-Tyr-Leu-enkephalin was much lower. The comparatively poor permeation of Leu-enkephalin within the first 30 min as shown in Fig. 6 and the comparatively high amount of des-Tyr-Leu-enkephalin reaching the acceptor chamber within the same time period, suggests that under these test conditions inhibition of peptidases by the thiomer is a time consuming process. An explanation for the rapid onset of inhibition as shown in Fig. 3 and the retarded onset of inhibition as shown in Fig. 7 might be explained by the presence of Mg^{2+}-ions during permeation studies. As reported previously, the inhibitory effect of thiomers seems to be based on the extraction of divalent metal ions such as Zn^{2+}-ions out of peptidases leading to their inactivation (Bernkop-Schnürch et al., 2001a). The presence of Mg^{2+}-ions might slow down this mechanism to some extent.

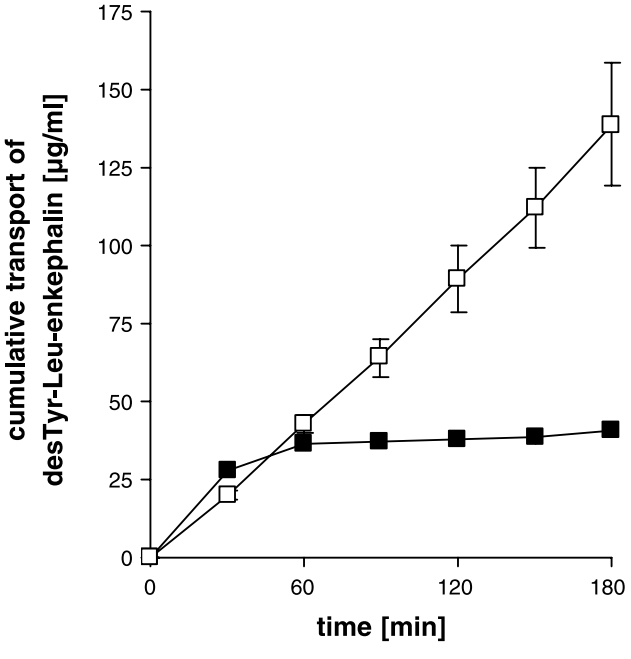

Fig. 7. Transport of the main metabolite des-Tyr-Leu-enkephalin across freshly excised bovine nasal mucosa in buffer solution (□) and in the presence of PCP-Cys/GSH (0.5%/1%, m/v) (■); indicated values are means of at least three experiments ±SD

Discussion

Within this study it could be demonstrated that enzyme inhibition as well as permeation enhancement are the key parameters in order to improve the bioavailability of nasally administered Leu-enkephalin. These results are in good agreement with previous studies demonstrating that the nasal uptake of enkephalins can be significantly improved by the co-administration of peptidase inhibitors and/or permeation enhancers. In the presence of the enzyme inhibitor puromycin, for instance, Met-Enkephalin absorption was even 22-fold improved (Agu et al., 2004). Due to the addition of permeation enhancers such as sodium glycocholate the degree of Met-enkephalin absorption from a nasal monolayer, for instance, could be 2-fold improved (Agu et al., 2004). The addition of such small molecular auxiliary agents, however, is from the toxicological point of view quite problematic. Apart from local toxic side effects such as severe cytotoxicity and ciliotoxicity (Remigius et al., 2003), small molecular auxiliary agents are in most cases rapidly and extensively absorbed from the nasal mucosa leading also to systemic toxic side effects. Furthermore, due to this rapid and extensive uptake from the nasal mucosa leaving the therapeutic agent unabsorbed on the membrane and additional dilution effects of these auxiliary agents in the nasal cavity, their efficacy *in vivo* seems to be quite limited. Out of these reasons the practical use of as severe and from toxicological point of view problematic permeation enhancers such as sodium glycocholate and small molecular enzyme inhibitors is not feasible. In contrast, dilu-

tion effects and absorption of thiolated polycarbophil can be excluded. Studies focusing on the toxic profile of thiolated polycarbophil showed that the cytotoxic potential of this thiomer is in the same range as that of unmodified polycarbophil (Langoth et al., 2005b). In addition, ciliary beat frequency studies revealed that thiolated polycarbophil shows an even less pronounced effect on the ciliary beat frequency than unmodified polycarbophil (Greimel et al., 2005). As polyacrylates were shown not to be absorbed from mucosal membranes at all (Riley et al., 2001), also systemic toxic side effects can be excluded.

Simple formulations for nasal Leu-enkephalin delivery systems containing a thiomer are over all gel formulations. Micro- and nanoparticulate delivery systems, however, might be more efficient. Utilizing thiomer micro- or nanoparticles as nasal delivery systems for Leu-enkephalin offers the advantage that the thiomer is brought in dry form into contact with the mucosa. Consequently, the concentration of the thiomer on the mucosa will be much higher than the concentration which has been tested regarding the permeation enhancing effect. In addition, Imam et al. could demonstrate that the interpenetration of mucoadhesive polymers and the mucus gel layer is much more pronounced, when the thiomer is applied on the membrane in dry form than in a prehydrated form (Imam et al., 2003). Because of this comparatively much higher concentration of the thiomer on the mucosa and this more tight contact with the epithelium, the permeation enhancing and enzyme inhibition effect should be much higher than shown within this study. Recently, Leitner et al. could demonstrate that the efficacy of thiomers in nasal peptide delivery is significantly higher utilizing particulate formulations. Using a microparticulate formulation instead of a gel formulation led to a 3-fold higher nasal bioavailability of hGH in rats (Leitner et al., 2004a, b). According thiomer nano- and microparticles can be prepared via emulsification solvent evaporation or coacervation techniques (Krauland et al., 2004, Albrecht et al., 2005).

In summary, within this study thiolated polycarbophil could be identified as highly efficient auxiliary agent for the nasal administration of Leu-enkephalin *in vitro*. Likely because of the permeation enhancing and enzyme inhibitory properties of thiolated polycarbophil, the mucosal uptake of Leu-enkephalin could be even 82-fold improved. Due to the comparatively safer toxicity profile of this thiomer in comparison to low molecular mass permeation enhancers and enzyme inhibitors and the strongly improved nasal absorption, it might be a promising tool for nasal dosage forms for this therapeutic peptide.

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