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TR146 cells grown on filters as a model of human buccal epithelium: V. Enzyme activity of the TR146 cell culture model, human buccal epithelium and porcine buccal epithelium, and permeability of leu-enkephalin

Hanne Mørck Nielsen, Margrethe Rømer Rassing *

Department of Pharmaceutics, The Royal Danish School of Pharmacy, 2 Universitetsparken, 2100 Copenhagen Ø, Denmark

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Abstract

The objective of the present study was to characterise the TR146 cell culture model as an in vitro model of human buccal mucosa with respect to the enzyme activity in the tissues. For this purpose, the contents of aminopeptidase, carboxypeptidase and esterase in homogenate supernatants of the TR146 cell culture model, and human and porcine buccal epithelium were compared. The esterase activity in the intact cell culture model and in the porcine buccal mucosa was compared. Further, the TR146 cell culture model was used to study the permeability rate and metabolism of leu-enkephalin. The activity of the three enzymes in the TR146 homogenate supernatants was in the same range as the activity in homogenate supernatants of human buccal epithelium. In the TR146 cell culture model, the activity of aminopeptidase (13.70 ± 2.10 nmol/min per mg protein) was approx. four times the activity of carboxypeptidase (3.73 ± 0.53 nmol/min per mg protein), whereas the level of esterase activity was significantly higher (223.39 ± 69.82 nmol/min per mg protein). In the TR146 cell culture model, the apical esterase activity was found significantly higher than the basal activity, and found comparable to the porcine buccal mucosa. However, the esterase activity on the serosal side of the porcine buccal mucosa was higher than in the TR146 cell culture model. Approx. 1.5% of leu-enkephalin permeated the TR146 cell layers within 5 h (P_{app} $7.38 \pm 0.83 \times 10^{-7}$ cm/s) and approx. 77% of intact peptide was still present in the donor phase after 5 h. The present study suggests that the TR146 cell culture model is a valuable in vitro model for permeability and metabolism studies with enzymatically labile drugs, such as leu-enkephalin, intended for buccal drug delivery.

Keywords: TR146 cell culture model; Human; Porcine; Buccal enzyme activity; Leu-enkephalin; Aminopeptidase; Carboxypeptidase; Esterase

* Corresponding author. Tel.: +45-35-306233; fax: +45-35-306030.
E-mail address: mrr@dfh.dk (M. Rømer Rassing)

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1. Introduction

Buccal delivery offers a feasible alternative to oral delivery of drugs, which are extensively metabolised after oral administration. Even though the bioavailability of such drugs, e.g. peptides, can be improved after buccal administration compared to oral administration, the bioavailability may still be significantly less than after s.c. or i.m. injection (Lee, 1988). With regard to peptide and protein drugs, the physical permeability barrier of buccal mucosa is one of the main reasons for the low bioavailability, but another important barrier is the biochemical barrier (Garren et al., 1989). Even though the enzyme activity in the buccal epithelium has been shown to be lower than in the intestine (Stratford and Lee, 1986; Lee, 1988; Zhou and Li Wan Po, 1990), this barrier often has to be circumvented in order to obtain therapeutically active plasma concentrations of the drug. Studies have shown that TRH is completely degraded after application to isolated buccal mucosa from rabbit (Dowty et al., 1992), and that leu-enkephalin is extensively degraded in homogenates of rabbit buccal mucosa (Kashi and Lee, 1986). However, it has also been shown that only 12–16% of the enzymes responsible for degradation of leu-enkephalin are located in the plasma membrane of buccal epithelial cells from rabbit (Lee, 1988), indicating that the extracellular degradation of leu-enkephalin administered to the intact whole tissue may be lower. The most widely studied peptidase in the buccal mucosa is the exopeptidase, aminopeptidase. Human oral mucosa homogenate supernatant shows aminopeptidase activity (Giannitis et al., 1972; Audus et al., 1991). By use of a range of different substrates, the aminopeptidase activity has been estimated in buccal mucosa from other species (Stratford and Lee, 1986; Cassidy and Quadros, 1988; Garren and Repta, 1988; Garren et al., 1989; Zhou and Li Wan Po, 1990, 1991) as well as in primary cultures of hamster cheek pouch epithelium (Tavakoli-Saberi and Audus, 1989; Audus et al., 1991). Carboxypeptidase is another exopeptidase, which has been studied in rat and hamster buccal mucosa homogenates (Garren and Repta, 1988).

Apart from peptidases, knowledge of the esterase activity is important with respect to local and systemic delivery of ester drugs or prodrugs (Yamahara and Lee, 1993). Esterase activity is widely distributed in mammalian tissue (Leinweber, 1987) and the non-specific esterase activity in homogenates of buccal mucosa from dog and rodents has been measured (Garren and Repta, 1988; Zhou and Li Wan Po, 1990 and 1991).

The TR146 cell culture model has been proposed as a model of human buccal epithelium (Jacobsen et al., 1995). TR146 cells originate from a human neck metastasis of a buccal carcinoma (Rupniak et al., 1985), and after submerged culturing on permeable inserts for 3–4 weeks, a stratified nonkeratinized epithelium is formed (Jacobsen et al., 1999). The physical permeability barrier of the cell culture model has been evaluated with regard to M_w (Nielsen et al., 1999) and lipophilicity (Jacobsen et al., 1995; Nielsen and Rassing, 2000) of test substrates. The effect of pH, osmolality and bile salt enhancers on the integrity of the epithelium has been described (Nielsen and Rassing, 1999). In order to validate the in vitro model as a model of the human buccal epithelium for permeability studies with enzymatically labile drugs, it is necessary to characterise the enzyme profile of the model.

The aim of the present study was to determine the enzyme activity of aminopeptidase, carboxypeptidase and esterase relative to the protein content in the TR146 cell culture model. The results were correlated to human buccal epithelium, and since the porcine buccal epithelium is used as an in vitro model of human buccal epithelium that tissue was included in the present study. Further, the extracellular esterase activity was estimated. Finally, the objective was to determine the permeability and the metabolism of a peptide, leu-enkephalin, in the TR146 cell culture model.

2. Materials and methods

2.1. Materials

Hippurylphenylalanine (HPA), hippuric acid (HP), *o*-phthaldialdehyde (OPA), dithiothreitol

(DTT), *p*-nitrophenylacetate (NPA), *p*-nitrophenol (NP) and Folin & Ciocalteu's phenol reagent were obtained from Sigma Chemical Co. (St. Louis, MO). D-(1-^{14}C)-mannitol was from New England Nuclear (Boston, MA). Leu-enkephalin and Gly-Gly-Phe-Leu (GGPL) was obtained from Bachem Ltd. (Essex, UK). The chemicals for the protein determination, for the glucose-Ringer (GR) and for the mobile phase for RP-HPLC were from Merck (Darmstadt, Germany). Hanks balanced salt solution (HBSS) and phosphate buffered saline (PBS) were from Gibco BRL (Paisley, UK). Acetonitrile (AcCN) (Sigma-Aldrich, Dorset, UK) and triethylamine (TEA) (Merck, Darmstadt, Germany) were used for the chromatographic analysis, and Ultima Gold™ scintillation cocktail from Packard Instrument BV (Groningen, The Netherlands) was used for the scintigraphic analysis. All chemicals were of analytical grade and used without further purification. Spectrophotometric analysis was performed using a Cary 1 Bio spectrophotometer (Varian Australia Pty Ltd., Mulgrave, Australia) with Cary software.

2.2. Methods

2.2.1. Cell culture

The TR146 cell line was kindly provided by Imperial Cancer Research Technology and cultured as previously described (Jacobsen et al., 1995; Nielsen and Rassing, 1999) on 4.2 cm^2 Falcon® terepthalate inserts with a pore size of 0.4 μm. Eight mycoplasma free passages were cultured for 30 days before use. The cell layers were rinsed with 37°C PBS, the cells were removed from the inserts with a cell scraper and homogenised manually (Kontes Duall homogenisator, Struers KEBOLab, Albertslund, DK) in 1.5–5.0 ml ice cold PBS. After centrifugation (19 000 × *g*, 5°C, 5 min), the supernatant was used for the enzyme and protein assay. For determination of protein in the whole tissue homogenate, centrifugation was omitted. The apical and basal degradation of the esterase substrate in the TR146 cell culture model was determined using a temperated (37°C) plate shaker (150 rpm). The volume of each half-chamber was 2.5 ml. The permeability study with leu-enkephalin was carried out under the same experimental conditions.

2.2.2. Human and porcine buccal mucosa

Tissue samples from healthy human volunteers (non-smokers) were removed under local lidocaine-noradrenaline anaesthesia by otorhinolaryngological surgeons (approved by the local ethics committee, project no. (KF) 01-128/96, Denmark). The samples were placed in ice cold HBSS and immediately frozen at −80°C. Buccal mucosa from pigs was removed immediately after sacrificing the animal, maintained in ice cold GR (pH 7.4), and the epithelium was isolated within 2 h. The human and porcine buccal epithelium was isolated mechanically with scissors and a tissue slicer (Thomas Scientific®, Swedesboro, NJ). The epithelium was weighed (approx. 80 mg), cut into smaller pieces, homogenised and centrifuged as for the studies with the TR146 cell culture model. For measuring the degradation of the esterase substrate on the mucosal and the serosal side of the mucosa (epithelium, basal lamina and a minimum of connective tissue) the porcine mucosa was mounted in the Ussing chamber with an exposed surface area of 0.5 cm^2. The volume of each half-chamber was 1 ml. The experiments were performed at 37°C and a gas-lift of 95% O_2/5% CO_2 provided stirring.

2.2.3. Aminopeptidase assay

Aminopeptidase activity was measured by use of a diagnostic kit for determination of leucine aminopeptidase (Sigma Chemical Co., St. Louis, MO). In the assay, 0.5 ml of supernatant was used.

2.2.4. Carboxypeptidase assay

Carboxypeptidase activity was determined as the rate of degradation of HPA to HP. The degradation was stopped by the addition of a mixture of DTT and OPA. The HP–OPA-complex, which could be quantitated spectrophotometrically at 340 nm, was formed. The supernatants (0.25 ml) were incubated for 10 min in 0.25 ml of 10^{-3} M HPA in PBS at 37°C, and 1.5 ml of 6.5×10^{-3} M DTT and 6×10^{-3} M OPA in Milli-Q was added. After $1\frac{1}{2}$ min, the absorbance was measured. The blind samples consisted of HPA in PBS, and DDT and OPA in PBS with supernatant. Standards consisted of phenylalanine and HP in PBS.

2.2.5. Esterase assay

The esterase activity was monitored by the amount of NP formed as a result of esterase degradation of NPA. The amount of NP was quantitated by spectrophotometric measurement at 400 nm. For determining esterase activity in tissue homogenates, the supernatant (0.5 ml) was incubated in 0.5 ml freshly made 10^{-3} M NPA in PBS in the thermostated (37°C) detector cell. The absorbance was measured $1\frac{1}{2}$ min after addition of the substrate. The blind samples were NPA in PBS and PBS with supernatant. For measurements of the degradation of NPA after application to the apical and the basal side of the TR146 cell culture model and the mucosal and serosal side of the porcine buccal mucosa in vitro, freshly made 10^{-3} M NPA in buffer was applied to the relevant side of the tissue and buffer was applied to the other side. At fixed time intervals, 0.1 ml was withdrawn from both sides of the tissues for 60 min. Immediately, the samples were placed on ice, diluted with PBS, and the absorbance was measured.

2.2.6. Protein assay

The total amount of protein was determined by a modification of the method described by Lowry et al. (1951). Homogenate or homogenate supernatant (0.5 ml) was incubated for 1 h with 0.5 M NaOH, and for 10 min in 10^{-3} M copper sulphate in 2.15×10^{-3} M tatrate and 0.85 M carbonate buffer. Folin & Ciocalteu's reagent diluted 1:10 in Milli-Q water was added and after 45–65 min the absorbance was measured. All experiments were performed at room temperature. With human and porcine buccal epithelium, the homogenate supernatant or the whole homogenate was diluted 1:5 with PBS.

2.2.7. Leu-enkephalin permeability and metabolism in the TR146 cell culture model

Leu-enkephalin (1.1×10^{-3} M) in HBSS was applied to the apical surface of the TR146 cell culture model, and HBSS was applied to the basal side. Samples of 100 μl were withdrawn from the basal side every 30 min for 5 h and frozen until analysis. The withdrawn volume was replaced with HBSS. At the beginning and at the end of the study, samples were collected from the apical side of the cell layers as well. As a control of the integrity of the TR146 layers, the permeability of ^{14}C-mannitol (120 nCi/ml) in HBSS across the cell culture model was determined. Before and after the experiments, the transepithelial electrical resistance (TEER) was monitored by use of an Endohm™ culture cup (World Precision Instruments Ltd., Herts, UK), and microscopic inspection was conducted. The experiments were performed in triplicate.

The samples were analysed by RP-HPLC by use of a Merck Hitachi system consisting of a L-7100 pump, a 655A variable wavelength UV monitor and a 655A-40 autosampler. The D-7000 HPLC software system was used for data collection. A Waters Spherisorb S5ODS2 column (250×4.6 mm; 5 μm) (HiChrom Ltd., Berkshire, UK) was used. The wavelength was 215 nm, the flow rate 1.0 ml/min, the injected volume 20 μl. The mobile phase consisted of 20% (v/v) AcCN, 0.1% (v/v) H_3PO_4 and 5×10^{-3} M TEA in Milli-Q adjusted to pH 2. The retention of leu-enkephalin was 17.0 min and for GGPL it was 9.0 min. For the limit of detection, a signal-to-noise ratio of 4 was acceptable. The limit of detection of leu-enkephalin and GGPL was 10^{-6} M. Quantitative determination of ^{14}C-mannitol was done by liquid scintigraphy.

2.2.8. Data analysis

The enzyme activity of the assays was measured at V_{max}. The amount of substrate metabolites was corrected for reference values and expressed the enzyme activity in homogenates. This value was related to the protein content of the same sample, and expressed as nmol/min per mg protein, i.e. non-specific enzyme activity values are presented. This was done under the presupposition that 1 mol formed metabolite corresponded to 1 mol of enzyme. The results of the protein assay were used with the assumption that the relative amount of the aromatic amino acids, tryptophan and tyrosine, in the samples was comparable.

Data are expressed as mean $\pm$ SD (n), where n is the number of replicates. The mean value is calculated according to Eq. (1).

Table 1
Content of protein in the TR146 cell culture model, human buccal epithelium and porcine buccal epithelium[a]

	TR146 cell culture model	Human buccal epithelium	Porcine buccal epithelium
Supernatant	33.06 ± 5.59 ($n = 12$)	41.59 ± 9.44 ($n = 6$)	31.82 ± 7.75 ($n = 12$)
Whole homogenate	54.39 ± 10.71 ($n = 12$)	48.24 ± 13.50 ($n = 6$)	42.75 ± 12.10 ($n = 12$)

[a] The results are presented as μg protein per mg epithelium. Mean ± SD.

$$\text{mean}_n = (A_1/B_1 + A_2/B_2 + \ldots\ldots\ldots + A_n/B_n)/n \quad (1)$$

A is the enzyme activity in a sample and B is the protein content in the same sample or A is the protein content in a sample and B is the weight of the tissue.

The apparent permeability coefficient (P_{app}) for the permeability of leu-enkephalin and ^{14}C-mannitol across the TR146 cell culture model was calculated according to Eq. (2):

$$P_{app} = dQ/dt \times 1/A \times C_0 \quad (2)$$

Where dQ/dt (mol/s or dpm/s) is the steady state rate of permeability, A (cm^2) the diffusion area and C_0 (M or dpm/ml) the initial donor concentration.

For significance testing, the Student's t-test is used at a 95% level.

3. Results

3.1. Protein content and enzyme activity in homogenates and supernatants

In Table 1, the contents of protein in the supernatant of homogenates as well as in the whole homogenate are presented. There is no significant difference between the values obtained with either supernatants or homogenates from the three different epithelia. The amount of membrane bound protein in the human buccal epithelium, in the TR146 cell culture model, and in the porcine buccal epithelium is approx. 12, 39 and 24%, respectively.

Table 2 contains the values of aminopeptidase, carboxypeptidase and esterase activity relative to the protein content in the homogenate supernatants. The aminopeptidase activity in the TR146 cell culture is significantly higher than in the human homogenates ($0.01 < P < 0.05$), but not different from the porcine homogenates. The carboxypeptidase activity in homogenates of the TR146 cell culture model and in homogenates of human buccal epithelium is not statistically different, whereas the carboxypeptidase activity in homogenates of porcine buccal epithelium seems to be slightly higher than in the TR146 cell culture model ($0.01 < P < 0.05$). The esterase activity in homogenates of the TR146 cell culture model is not statistically different from the activity in either human or porcine buccal epithelial homogenates ($P > 0.05$). It was shown that there is no significant difference in the carboxypeptidase activity in porcine buccal epithelium, which has been frozen in -80°C (4.70 ± 0.55 nmol/min per mg protein) and fresh porcine buccal epithelium

Table 2
Homogenate supernatant enzyme activity of aminopeptidase, carboxypeptidase and esterase relative to protein contents in the TR146 cell culture model, human buccal epithelium and porcine buccal epithelium[a]

	TR146 cell culture model	Human buccal epithelium	Porcine buccal epithelium
Aminopeptidase	13.70 ± 2.10 ($n = 12$)	8.82 ± 3.59 ($n = 5$)	12.26 ± 3.24 ($n = 16$)
Carboxypeptidase	3.73 ± 0.53 ($n = 6$)	4.44 ± 0.87 ($n = 6$)	4.34 ± 0.21 ($n = 6$)
Esterase	223.39 ± 69.82 ($n = 9$)	173.73 ± 90.35 ($n = 6$)	221.72 ± 97.82 ($n = 6$)

[a] The results are presented as nmol product formed per min per mg protein. Mean ± SD.

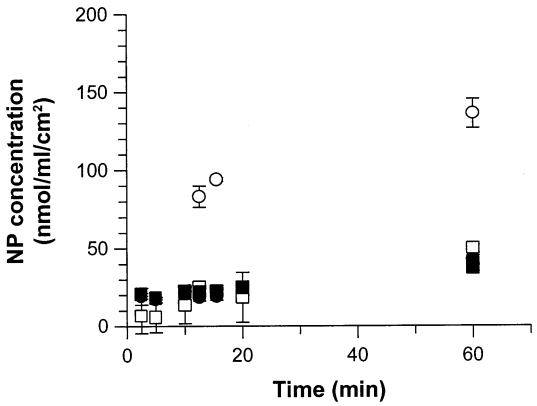

Fig. 1. Appearance of the esterase product NP on the apical side and on the basal side of the TR146 cell culture model after application of the substrate NPA to either of the sides of the epithelium. Mean ± SD, $n = 3$. ○, apical NP after apical application, □, basal NP after apical application, ●, apical NP after basal application, ■, basal NP after basal application.

(4.34 ± 0.21 nmol/min per mg protein) ($P > 0.05$, $n = 6$). The supposition that the enzyme activity in the human buccal epithelium was not decreased significantly due to storage at −80°C was taken.

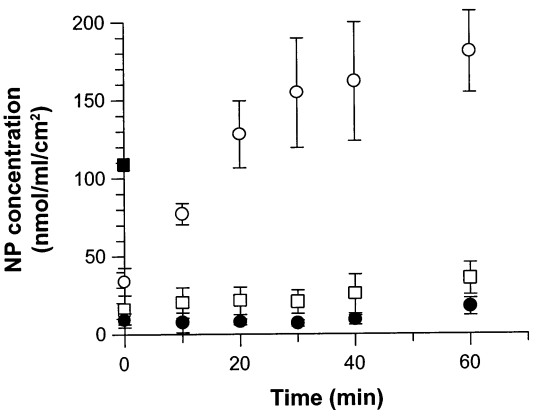

Fig. 2. Appearance of the esterase product NP on the mucosal side and on the serosal side of the porcine buccal mucosa after application of the substrate to either of the sides of the mucosa. Mean ± SD, $n = 4$. ○, mucosal NP after mucosal application, □, serosal NP after mucosal application, ●, mucosal NP after serosal application, ■, serosal NP after serosal application.

3.2. Esterase activity in the in vitro models

In Fig. 1, the formation of NP as a measure of the esterase activity on the apical and on the basal side of the TR146 cell culture model is presented. It is seen that the highest level of esterase activity is on the apical side of the cell culture model. Fig. 2 shows results from the study with porcine buccal mucosa and it is observed that the mucosal degradation of NPA is in the same range as the apical degradation in the TR146 cell culture model. However, the serosal degradation of NPA after application to the porcine mucosa is much faster than expected from the study with the TR146 cell culture model.

3.3. Leu-enkephalin permeability and metabolism

In Fig. 3, the appearance of leu-enkephalin on the basal side after apical application is presented along with the appearance of GGPL, which is the main product formed after aminopeptidase degradation of leu-enkephalin (Kashi and Lee, 1986; Bak et al., 1999). From the curve, the P_{app} value for the permeability of leu-enkephalin was calculated to $7.38 \pm 0.83 \times 10^{-7}$ cm/s. The figure also shows the estimated permeability curve for leu-enkephalin corrected for degradation to GGPL.

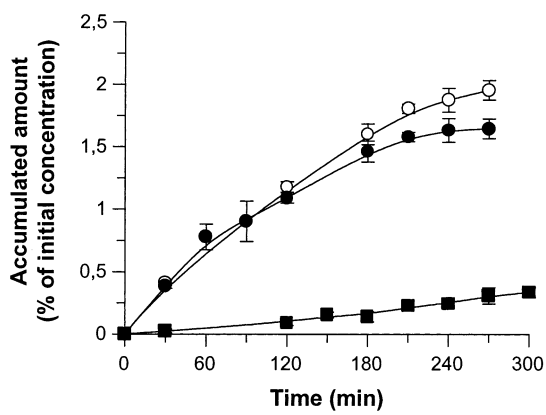

Fig. 3. The appearance of leu-enkephalin (●), GGPL (■) and leu-enkephalin corrected for degradation to GGPL (○) on the basal side of the TR146 cell culture model after apical application of leu-enkephalin. The permeated amount is expressed relative to the applied concentration of leu-enkephalin. Mean ± SD, $n = 3$.

Table 3
The molar amount of leu-enkephalin and GGPL after 5-h permeability study in the TR146 cell culture model[a]

Sample side	Time (min)	Enkephalin (%)	GGPL (%)
Apical	300	76.77 ± 13.19	5.10 ± 0.93
Basal	300	1.50 ± 0.22	0.34 ± 0.04

[a] The percentages are relative to the applied molar amount of leu-enkephalin. Mean $\pm$ SD, $n = 3$.

Table 3 shows the molar percentage of leu-enkephalin and GGPL on the apical and the basal side after the 5-h experiment. It is observed that approx. 80% of the leu-enkephalin is recovered, of which 1.5% had permeated the cell layers. The amount of leu-enkephalin that has not been recovered is probably located in the cell layers or degraded to products, which have not been determined in the experiment. The integrity of the TR146 cell culture model was maintained throughout the 5-h study, as the initial TEER value ($288 \pm 43\ \Omega \times cm^2$, $n = 6$) was not decreased after 5 h ($P > 0.05$). Further, the P_{app} value for the ^{14}C-mannitol permeability across the TR146 cell culture model was $1.60 \pm 0.16 \times 10^{-6}$ cm/s, which is similar to previous reported P_{app} values (Jacobsen et al., 1995; Nielsen and Rassing, 2000).

4. Discussion

4.1. Protein content and enzyme activity in homogenates and supernatants

The data presented in Table 1 show that with regard to the total amount of protein in the epithelium, the TR146 cell culture model and the porcine buccal epithelium are comparable to human buccal epithelium. The indication that the fraction of membrane bound protein is lower in the human buccal epithelium than in the two other models, may be explained by the fact that the human epithelium used for that part of the study might have changed characteristics during storage at -20°C. The results are comparable to values obtained with supernatants of homogenised rabbit buccal mucosa (Stratford and Lee, 1986).

The epithelial aminopeptidase activity presented in Table 2 is in the range of data reported from studies with supernatant from homogenates of buccal mucosa from human, approx. 5.5 nmol/min per mg protein (Audus et al., 1991), dog, 2.0 nmol/min per mg protein (Cassidy and Quadros, 1988), rabbit, approx. 5 nmol/min per mg protein (Stratford and Lee, 1986) and hamster cheek pouch, 2.4 ± 0.5 nmol/min per mg protein (Tavakoli-Saberi and Audus, 1989). Primary cultures of hamster pouch epithelium have also shown aminopeptidase activity values of 2.6 ± 0.3 nmol/min per mg protein (Tavakoli-Saberi and Audus, 1989) and approx. 7 nmol/min per mg protein (Audus et al., 1991), respectively. However, Giannitis et al. (1972) found a 100-fold higher value using the supernatant of homogenates as well as whole homogenates of human buccal mucosa and triglycin as a substrate. This difference might be due to the use of a different substrate and assay.

The results of the present study suggest that the carboxypeptidase activity in homogenates of buccal epithelial cells is two to four times lower than the aminopeptidase activity. The carboxypeptidase activity has not been studied extensively in buccal epithelium or buccal mucosa, but the level of activity in rat and hamster buccal epithelium has been found comparable to the level of aminopeptidase activity (Garren and Repta, 1988). Unfortunately, the data from that study can not be used for direct comparison since the activity is expressed relative to the tissue weight, and not to protein content. However, if the protein content relative to tissue weight found in this study (Table 1) is used to estimate the enzyme activity relative to tissue weight, the carboxypeptidase activity found in the present study is comparable to the activity in rat and hamster buccal mucosa (Garren and Repta, 1988).

The data from this study show that for the TR146 cell culture model, the human buccal epithelium and the porcine buccal epithelium, the enzyme activity of esterase is 16–20 times the activity of aminopeptidase and 40–60 times the activity of the carboxypeptidase activity. A comparable range was found in homogenate supernatant of buccal mucosa from hamster and rat, as

the esterase activity was between 15 and 40 times the aminopeptidase activity, and between 30 and 55 times the carboxypeptidase activity (Garren and Repta, 1988). The discrepancy of the results in the present study and that study may likely be related to species differences (Zhou and Li Wan Po, 1991), as the same enzyme substrates were used.

4.2. Esterase activity in the in vitro models

The degradation of the esterase substrate after application to the apical or the basal side of the TR146 cell culture model and to the mucosal or serosal side of the porcine in vitro model is comparable, as seen from Fig. 1 and Fig. 2. In the TR146 cell culture model, degradation of NPA at the apical side is faster than at the basal side after application of the substrate on either one of the sides. In both models, the apical/mucosal curve bends after approx. 20 min, probably due to depletion of substrate, loss of enzyme and/or enzyme inhibition. After apical/mucosal application, the product NP is also observed on the basal/serosal side, but at lower concentrations than at the apical/mucosal side. This is explained by the fact that the substrate and/or the product formed on the apical/mucosal side permeate the cell layers. This probably also explains the detection of NP on the apical/mucosal side after basal/serosal application of NPA. However, the amount of substrate that permeates the cell layers is more likely to be degraded completely on the apical/mucosal side than at the basal/serosal side. In the Ussing chamber experiments, however, the degradation on the serosal side of porcine buccal epithelium is much faster than expected from the results obtained with the TR146 cell culture model. The slicing of the mucosa results in damaged cells and probably causes a release of intercellular esterases to the serosal side, and substrate that permeate from the mucosal side, is probably degraded instantly. Further, the serosal side of the mucosa is not epithelium, but connective tissue, which may represent an enzyme profile different from the epithelium. Therefore, overestimation of the degree of metabolism of enzymatically labile drugs after permeation through porcine buccal mucosa in vitro seems possible.

4.3. Leu-enkephalin permeability and metabolism

The P_{app} value for the permeability of leu-enkephalin across the TR146 cell culture model is half the P_{app} value for mannitol, which is probably due to the higher M_w (Nielsen et al., 1999). After 5 h more than 78% of intact leu-enkephalin was present on the apical and on the basal side of the cell culture model. This is much more than after incubation with a homogenate supernatant of rabbit buccal mucosa, where less than 20% remained after 25 min (Kashi and Lee, 1986). Aminopeptidases are expected to be the primary enzymes responsible for degradation of leu-enkephalin (Kashi and Lee, 1986; Bak et al., 1999). Further, leu-enkephalin is expected to permeate the TR146 cell culture model by the paracellular pathway, as shown for other large hydrophilic substances (Nielsen et al., 1999), so the reports showing that most of the buccal aminopeptidase activity is cytosolic (Giannitis et al., 1972; Lee, 1988) is strengthened by this study. Yet, the morphology of the TR146 cell culture model is different from the human buccal epithelium with regard to the integrity (Nielsen and Rassing, 2000), which is also indicated by the TEER-value measured in the present study. The permeability coefficient of leu-enkephalin across the human buccal epithelium would be expected to be lower than the P_{app} value determined in this study, which might result in a higher degree of metabolism. Still, the amount of remaining leu-enkephalin is quite high compared to a nasal rat perfusate study, where less than 10% leu-enkephalin remained after 2 h (Hussain et al., 1995) and a similar study with intestinal perfusates from rats that showed an immediate almost complete degradation of leu-enkephalin (Friedman and Amidon, 1991). Even though the enzyme activity in a rat in situ model may be different from the enzyme activity in the epithelial in vitro TR146 cell culture model, the results strongly suggest that the buccal route may be applicable for systemic delivery of leu-enkephalin. Improvement of the hydrolytic stability of leu-enkephalin can be done by preparing prodrugs (Bak et al., 1999) or ana-

logues (Friedman and Amidon, 1991; Hussain et al., 1995) of the peptide, which probably would increase the amount of absorbed drug further.

5. Conclusion

In conclusion, the enzyme activity of aminopeptidase, carboxypeptidase and esterase was found to be similar in homogenates of the TR146 cell culture model compared to homogenates of human and porcine buccal epithelium. The extracellular esterase activity in the TR146 cell culture model was found comparable to the activity in the porcine buccal mucosa. Further, the esterase activity was significantly higher on the apical side than on the basal side of the TR146 cell culture model. The esterase activity on the serosal side of the porcine mucosa was very high, which should be considered for the performance of metabolism studies, where the TR146 cell culture model might be a better choice of model of the human buccal epithelium. The data on the permeability and the metabolism of leu-enkephalin in the TR146 cell culture model also support the assumption that the TR146 cell culture model may be a valuable model for studies of buccal drug delivery of labile drugs, e.g. leu-enkephalin. Additional studies could evaluate the localisation of the enzymes in the cell culture model and in human buccal epithelium, which is of particular interest as peptides like leu-enkephalin is expected to permeate the epithelium primarily via the paracellular pathway.

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