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Peptidase activity on the surface of the porcine buccal mucosa

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Abstract

Peptide drugs in buccal bioadhesive delivery systems are exposed to the surface of the buccal mucosa at high concentrations over long periods of time. The peptidase activity on the surface of the buccal mucosa has not been evaluated as a barrier to peptide buccal delivery. The in vitro stability of various synthetic substrates on the surface of intact porcine buccal mucosa was determined. No carboxypeptidase or dipeptidyl peptidase IV activity was detected on the buccal mucosa, while aminopeptidase N activity was detected using Leu-*p*-nitroanilide. No endopeptidase activity was observed towards the peptide substrates. Insulin and insulin B-chain were intact at the 2 h time point at 37 °C, while the percent of parent Leu-enkephalin remaining was 18 ± 9 (mean $\pm$ S.D., $n = 9$). In the presence of aminopeptidase inhibitors, amastatin, sodium deoxycholate and EDTA, the degradation of Leu-enkephalin was dramatically reduced. This work suggests that the buccal route maybe advantageous for the delivery of peptides that are susceptible to such activities. The inclusion of aminopeptidase inhibitors in buccal bioadhesive delivery systems could improve buccal bioavailability of Leu-enkephalin. We suggest that compared with the existing in vitro metabolism methods, the analysis of peptide or protein metabolism on intact buccal mucosa could better predict the degradation of the drug as it crosses the tissue.

Keywords: Buccal route; Peptide drug delivery; Metabolic barrier; Peptidase inhibitors

1. Introduction

Recent advances in biotechnology have enabled the production of a large number of peptide drugs in therapeutic quantities. The most favoured route for the delivery of drugs is the oral route, however, peptide drugs have low oral bioavailability, typically less than 1%. Consequently, alternative routes have been investigated for the delivery of peptide drugs; one such route is the buccal mucosa. This route is growing in popularity because of its advantages, including ease of administration and removal of the dosage form, the drug directly enters systemic circulation avoiding first pass hepatic metabolism and there is potential for sus-

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tained or controlled drug release (Rathbone et al., 1994). The bioavailability of peptide drugs via the buccal route has been shown to be better than the oral route, but is still significantly less than subcutaneous or intra-muscular injection (Lee and Yamamoto, 1990). The two main obstacles for transport of peptides across mucosal membranes are the low permeability of the mucosa and the metabolic barrier (Lee and Yamamoto, 1990).

Proteolytic activities that have been identified in buccal tissue homogenates are shown in Table 1. A number of peptide drugs have been shown to be degraded in buccal tissue homogenates including insulin, proinsulin (Yamamoto et al., 1990), enkephalin analogues (Kashi and Lee, 1986), thyrotropin releasing hormone (Dowty et al., 1992), calcitonin (Nakada et al., 1987) and substance P (Lee and Yamamoto, 1990). However, as the peptidase activity of the cytosol and the membrane cannot be resolved in tissue homogenates the relevance of these activities during peptide buccal transport is uncertain. Particularly, as it is believed that peptides are transported between the epithelial cells and therefore, the cytosolic peptidase activity may not be in contact with the peptide during its transport (Rathbone and Tucker, 1993).

An alternative in vitro method to buccal homogenates is to determine the stability of the peptide after absorption across freshly-excised buccal mucosa mounted in diffusion chambers (Dowty et al., 1992; Johnston et al., 1998). However, in the receptor chamber the peptide is exposed to peptidases from various cellular components released during the removal of underlying connective tissue. For instance, carboxypeptidase activity was observed on the receptor side of mounted rabbit buccal but not on the mucosal side; the origin of this activity is uncertain (Dowty et al., 1992).

In this study, in order to avoid cytosolic degradation and determine the metabolic barrier of the mucosal surface, the proteolytic stability of the peptide drug was measured on the surface of the intact buccal mucosa excised from pig. Furthermore, the observed proteolytic activity on the surface of the buccal mucosa may be representative of the surface membrane-bound proteases of the underlying buccal epithelial cells.

The proteolytic activity of the buccal mucosa was evaluated by the hydrolysis of synthetic substrates and the model peptides, Leu-enkephalin, insulin B-chain and insulin. The model peptides were chosen for this study as their hydrolytic profiles are well characterised (Table 2). The participation of individual proteases in the cleavage of the peptides was determined by degradation experiments in the presence and absence of inhibitors.

Table 1
Peptidases identified in the buccal tissue in vitro

Peptidase	Substrate	Species	Test system	Reference
Aminopeptidase	L-Leucyl-beta-naphthylamide	Human	Homogenate	Nielsen and Rassing, 2000
	L-Leucyl-beta-naphthylamide	Pig	Homogenate	Nielsen and Rassing, 2000
	Leu-*p*-nitroanilide	Rat	Homogenate	Harris and Robinson, 1992
	Leu-*p*-nitroanilide	Hamster	Homogenate	Harris and Robinson, 1992
	Leu-enkephalin	Rabbit	Homogenate	Kashi and Lee, 1986
Carboxypeptidase(s)	Thyrotropin releasing hormone	Rabbit	Excised intact tissue	Dowty et al., 1992
Carboxypeptidase A	Hippurylphenylalanine	Human	Homogenate	Nielsen and Rassing, 2000
	Hippurylphenylalanine	Pig	Homogenate	Nielsen and Rassing, 2000
Dipeptidyl carboxypeptidase	Enkephalin analogues	Rabbit	Homogenate	Kashi and Lee, 1986
Serine endopeptidase(s)	Insulin	Rabbit	Homogenate	Yamamoto et al., 1990
	Proinsulin	Rabbit	Homogeante	Yamamoto et al., 1990
Dipeptidyl peptidase	Enkephalin analogues	Rabbit	Homogenate	Kashi and Lee, 1986

Table 2
Peptidases involved in the primary cleavage of the model peptide drugs

Peptide	Peptidase	Reference
Leu-enkephalin	Aminopeptidases	Ogiso et al., 1997
	Dipeptidyl aminopeptidase	Ogiso et al., 1997
	Enkephalinase	Ogiso et al., 1997
Insulin B-chain	Carboxypeptidases	Thiede et al., 1995
	Metallo-endopeptidases	
	Endopeptidase 24.11	Bond et al., 1986
	Endopeptidase 24.18	Price et al., 1991
Insulin	Metallo-endopeptidase(s)	
	Unknown	Ikesue et al., 1993
	Serine endopeptidases	
	Chymotrypsin	Schilling and Mitra, 1991; Ikesue et al., 1993
	Elastase	Ikesue et al., 1993
	Trypsin	Schilling and Mitra, 1991; Ikesue et al., 1993

2. Materials and methods

2.1. Materials

Leu-enkephalin, insulin-B chain, insulin, *p*-nitroaniline, deoxycholic acid sodium salt, amasatin, ethylenediaminetetraacetic acid disodium salt (EDTA), aprotinin (lyophilised powder from bovine lung), hippurly-L-Arg, hippuryl-L-Phe, Leu-, and Gly-Pro-*p*-nitroanilide were all obtained from Sigma Chemical Company (St. Louis, MO). For chromatography, HPLC grade solvents were used. All other chemicals were of analytical grade and were purchased from Merck (Darmstadt, Germany).

2.2. Excised porcine buccal mucosa

The buccal tissue was excised from domestic pigs (*Sus scrofula*) the age of the animals ranged from 3 to 5 months with an average weight of 90–110 kg. The buccal tissue was immediately placed in cold buffer; 50 mM sodium phosphate buffer pH 7, which contained 0.23% (w/v) NaCl to give a total ionic strength of 150 mM. The membranes were stored in an airtight container (6–10 °C) during transport and prepared for the study within 2 h of the recovery.

2.3. Metabolism on intact porcine buccal mucosa

Using surgical scissors excess fat and connective tissues were removed carefully from the porcine buccal tissue within 2–3 mm of the buccal mucosa. A plastic cylinder with an internal surface area of 1.77 cm^2 was placed vertically on top of the mucosal side of the buccal tissue and clamped (Bernkop-Schnürch et al., 1997). Substrates were prepared in control buffer 50 mM sodium phosphate buffer pH 7, which contained 0.23% (w/v) NaCl to give a total ionic strength of 150 mM. Control buffer (1 ml) with or without inhibitor equilibrated at 37 °C was added into the reaction cylinder. After 30 min incubation 1 ml of the substrate in control buffer at 37 °C was added to the incubate and mixed. The final substrate concentrations for hippuryl-L-Arg, hippuryl-L-Phe, Leu-*p*-nitroanilide, succinyl-Ala-Ala-Ala-*p*-nitroanilide and Gly-Pro-*p*-nitroanilide was 1 mM, while for Leu-enkephalin, insulin and insulin B-chain it was 0.1 mM. At various times after the start of the reaction samples of 300 μl were taken. Samples of the synthetic substrates were placed on ice, centrifuged at $20\,000 \times g$ at 4 °C for 5 min and a 200 μl sample of the supernatant was analysed. For the measurement of *p*-nitroaniline formation from the *p*-nitroanilide substrates 100 μl samples were transferred to a microtitre plate and the absorbance was measured at 405 nm using a Anthos reader 2001 (Anthos labtec instruments, Austria). For analysis of the formation of hippuric acid from the substrates hippuryl-L-Arg and hippuryl-L-Phe, supernatants were diluted in control buffer to give a final volume of 1 ml. Diluted samples were transferred to a 1 ml quartz cuvette and analysed at 254 nm using a Perkin–Elmer Lamda 16 spectrophotometer. The Leu-enkephalin, insulin and insulin B-chain samples

were added to 5 μl of 20% (v/v) trifluoroacetic acid (TFA) to stop the reaction. The resulting mixture was centrifuged (20 000 × *g* for 5 min) and the supernatants were analysed by HPLC.

2.4. HPLC of peptides

Analysis of peptide reaction samples by reverse phase HPLC was conducted using a Perkin-Elmer (Norwalk, CT, USA) series 200 LC pump, Perkin–Elmer 200 series auto sampler with a 20 μl injection loop and a diode array detector (Perkin–Elmer 235C). Samples were eluted from a Nucleosil 100–5 C18 column (250 × 4 mm), with mobile phase A-0.1% TFA, B-90% acetonitrile, 0.1% TFA; flow rate of 1.0 ml min^{-1}; detection was at 220 nm. A linear gradient was applied from 90% A to 10% A over a 22 min period.

3. Results and discussion

3.1. Metabolic activity towards synthetic substrates

Numerous studies have used low molecular weight synthetic substrates to evaluate specific peptidase activities of different mucosal preparations. Aminopeptidase and carboxypeptidase activities have previously been identified in buccal homogenates using synthetic substrates (Harris and Robinson, 1992; Nielsen and Rassing, 2000). In this study, the activities of aminopeptidase N and dipeptidyl peptidase IV were assayed using Leu- and Gly-Pro-*p*-nitroanilide substrates, respectively. Carboxypeptidase A and B activities were measured using hippuryl-L-Phe and hippuryl-L-Arg, respectively.

Each synthetic substrate was tested in triplicate on three buccal tissues obtained from different animals. After two hours incubation at 37 °C on the surface of the intact buccal mucosa the only synthetic substrate that was hydrolysed was the aminopeptidase N substrate, Leu-*p*-nitroanilide. Fig. 1 shows that the formation of *p*-nitroaniline followed pseudo-zero order kinetics, the mean aminopeptidase activity for three buccal tissues was 3.45 ± 1.6 μM min^{-1} cm^{-2}, (mean ± S.D., $n = 9$). The constant rate of Leu-*p*-nitroanilide hydrolysis indicates that there is no cellular changes resulting in the release of aminopeptidases from the cytosol during this experiment.

Kashi and Lee (1986) showed that dipeptidyl peptidase IV, a serine peptidase was involved in the digestion of enkephalins in rabbit buccal tissue homogenates. Using the synthetic substrate for dipeptidyl peptidase IV, Gly-Pro-*p*-nitroanilide, no activity was detected on the buccal mucosa suggesting the activity in the buccal homogenates is cytosolic. The absence of dipeptidyl peptidase IV activity on the mucosal surface suggests that the buccal site may be advantageous for the peptide substance P, which is known to be initially cleaved by this enzyme (Stephenson and Kenny, 1988).

The absence of carboxypeptidase activity on the mucosal surface suggests that the carboxypeptidase activity in buccal tissue homogenates (Table 1) and on the serosal side of mounted rat buccal (Dowty et al., 1992) originates in the cytosol.

3.2. Metabolic stability of model peptides

Insulin, insulin B-chain and Leu-enkephalin were applied onto the mucosal side of the intact buccal mucosa. Using HPLC the metabolism of the model peptides was monitored by measuring the decrease in peak area of the native peptide

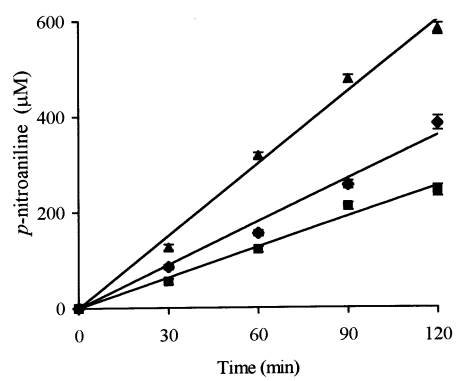

Fig. 1. Formation of *p*-nitroaniline from Leu-*p*-nitroanilide in 50 mM sodium phosphate buffer pH 7, at 150 mM ionic strength on intact porcine buccal mucosa obtained from three animals. Each point represents the mean ± S.D. ($n = 3$).

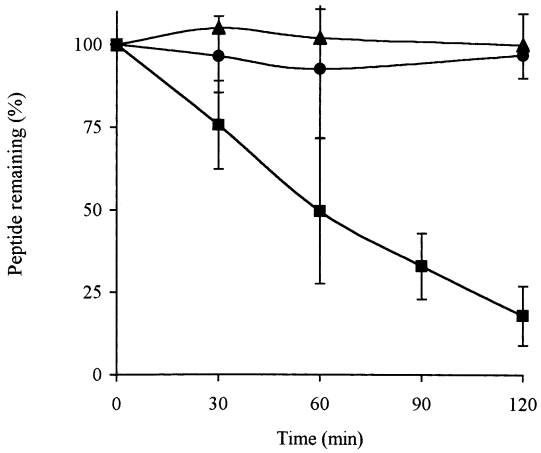

Fig. 2. Time-course of percentage remaining of insulin (●), insulin B chain (▲) and Leu-enkephalin (■) on intact buccal mucosa after incubation in control buffer, 50 mM sodium phosphate buffer pH 7, at 150 mM ionic strength. Each point represents the mean ± S.D. ($n = 3$).

over time. After a 2 h incubation at 37 °C Leu-enkephalin was metabolised while insulin and insulin B-chain remained stable (Fig. 2). The degradation of Leu-enkephalin was expected as the peptide is a substrate for aminopeptidases which are present on the mucosa (Fig. 1) and distributed in both the cytosol and the membrane of buccal epithelial cells (Lee and Yamamoto, 1990). Subcellular fractionation of rat buccal tissue homogenates showed that between 85 and 88% of the aminopeptidase activity was associated with the cytoslic fraction (Lee and Yamamoto, 1990).

The stability of insulin B-chain on the buccal mucosa indicates the absence of a number of peptidases known to hydrolyse insulin-B chain, including carboxypeptidases (Table 2). The stability of insulin B-chain in the presence of aminopeptidase activity is further support of the absence of endopeptidases, as aminopeptidases can only act on insulin B-chain after the initial cleavage by an endopeptidase (Song et al., 1986). The stability of insulin B-chain on the mucosa is in agreement with the observed inactivity towards the synthetic substrates of carboxypeptidase A and B.

The enzymatic stability of insulin on the buccal mucosa (Fig. 2) was in contrast to its rapid degradation in rabbit buccal tissue homogenates (Yamamoto et al., 1990). The lack of hydrolytic activity towards insulin on the mucosa indicates that the hydrolytic activity observed in the homogenates is also cytosolic. The stability of insulin indicates the absence of membrane-bound endopeptidases, which are known to hydrolyse insulin (Table 2). Insulin is not a substrate of aminopeptidases or carboxypeptidases (Stephenson and Kenny, 1988). The stability of insulin towards the exopeptidases was attributed to the highly ordered structure of insulin, including disulphide bridges, secondary, tertiary and possibly quaternary structures (Stephenson and Kenny, 1988).

Yamamoto et al. (1990) showed that the activity of the rabbit buccal homogenate towards insulin was strongly inhibited by the serine protease inhibitor aprotinin. In light of this, the enzyme inhibitor aprotinin was co-administered with insulin in vivo to improve the bioavailability of insulin across the buccal mucosa (Aungst and Rogers, 1988). However, aprotinin did not improve the in vivo transport of insulin across the rat buccal. This indicates that during the transport of insulin across the buccal mucosa the peptide does not come in contact with the serine protease identified in the homogenate. This in vivo observation is in agreement with the in vitro studies on the buccal mucosa, which show that insulin is not degraded by surface membrane-bound epithelial peptidases (Fig. 2). It is possible that the inability of aprotinin to promote the transport maybe due to the aprotinin itself not been absorbed with the insulin across these cell layers. However, insulin and aprotinin have similar molecular mass of 5.7 and 6.5 kDa, respectively. Furthermore, the addition of laureth-9 which is believed to increase permeability by extracting proteins and lipids from the membrane had no additional enhancing effect when compared with laureth-9 alone (Aungst and Rogers, 1988). Therefore, it could be speculated that the same membrane-bound epithelial peptidases on the surface of the mucosa are representative of the underlying epithelial cells, which are in the range of 40–50 cells thick (Rathbone et al., 1994).

3.3. Effect of peptidase inhibitors on the stability of peptides

To determine the participation of individual enzymes in the cleavage of Leu-enkephalin, degradation experiments were carried out in the presence of inhibitors. The aminopeptidase inhibitors, amastatin, (Sanderink et al., 1988), EDTA (Van Wart and Lin, 1981) and sodium deoxycholate (Bai, 1994) and the serine protease inhibitor aprotinin (Aungst and Rogers, 1988) were tested separately. Fig. 3 shows the time-course for the degradation of Leu-enkephalin by the intact buccal mucosa in the presence and absence of enyzme inhibitors. The aminopeptidase inhibitors dramatically inhibited the hydrolysis of Leu-enkephalin, thus indicating the importance of buccal epithelial aminopeptidases in its hydrolysis. The ineffectiveness of aprotinin to protect Leu-enkephalin was expected as this peptide is not a substrate for serine peptidases. The stabilisation of Leu-enkephalin on the buccal mucosa by these aminopeptidase inhibitors suggests that the co-administration of inhibitors in buccal bioadhesive systems could improve the bioavailability of the peptide. Protection against enzymatic attack by aminoeptidases may also be of value for other peptides susceptible to this activity, for instance the calcitonin analogues (Ogiso et al., 1997).

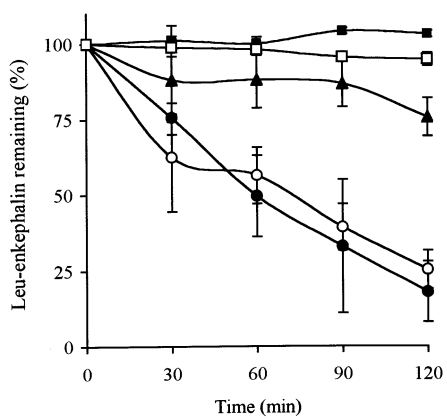

Fig. 3. Time-course of percentage remaining of Leu-enkephalin on intact buccal mucosa after incubation in control buffer, 50 mM sodium phosphate buffer pH 7, at 150 mM ionic strength, (●), control buffer containing aprotinin at 1 mg/ml (○), control buffer containing amastatin at 10 μM (▲), control buffer containing EDTA at 5 mM (□) and control buffer containing sodium deoxycholate at 2 mM (■). Each point represents the mean ± S.D. ($n = 3$).

4. Conclusion

In conclusion, aminopeptidases appear to be the exclusive peptidase activity on the buccal mucosa, therefore, representing a major metabolic barrier to the buccal delivery of peptide drugs. The hydrolysis of Leu-enkephalin was dramatically reduced by aminopeptidase inhibitors, therefore, the co-administration of such aminopeptidase inhibitors in a bioadhesive delivery system should improve the buccal bioavailability of the peptide drug. The absence of endopeptidase and carboxypeptidase activities will be advantageous for the buccal delivery of peptides, which are susceptible to these activities. The analysis of peptide or protein metabolism on intact buccal mucosa in vitro should approximate, to a greater degree, the degradation of the compound as it crosses the tissue. Future in vivo studies with peptidase inhibitors are required to validate this method for predicting peptide buccal metabolism in vitro.

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