Pharmaceutical Research, Vol. 22, No. 12, December 2005 (© 2005)
DOI: 10.1007/s11095-005-7894-4

Research Paper

In Vitro Evaluation of Various Buccal Permeation Enhancing Systems for PACAP (Pituitary Adenylate Cyclase-Activating Polypeptide)

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Received March 16, 2005; accepted August 9, 2005

Purpose. Buccal administration of pituitary adenylate cyclase-activating polypeptide (PACAP) could represent a new possibility for the treatment of type 2 diabetes. In this study the effect of various buccal permeation enhancers on PACAP and FD-4 was evaluated.
Methods. The permeation-enhancing properties of the well-established permeation enhancers sodium deoxycholate (Na DOC) and cetrimide on the permeation of PACAP were investigated on freshly excised porcine buccal mucosa in Ussing chambers. Furthermore, the effect of chitosan and that of chitosan-4-thiobutylamidine conjugate (chitosan–TBA) optionally in combination with reduced glutathione (GSH) on the permeation of PACAP across the buccal mucosa was studied.
Results. The apparent permeability coefficient (P_{app}) of PACAP in buffer only was $5.7 \pm 3.1 \times 10^{-8}$ cm/s. In the presence of 5% (m/v) Na DOC, the enhancement of the permeation was 18.6-fold, whereas due to the addition of 5% (m/v) cetrimide an enhancement ratio of 46.5 was obtained. In the presence of the chitosan–TBA conjugate (1%), a 38.9-fold increased permeation was achieved, whereas unmodified chitosan (1%) did not show any effect. The combination of chitosan–TBA conjugate (1%) with GSH (2%) led to an increase in P_{app} up to $441.7 \pm 89.9 \times 10^{-8}$ cm/s, which represents a 77.5-fold improvement. The P_{app} of GSH *per se* was only $1.0 \pm 0.2 \times 10^{-9}$ cm/s, showing that GSH remains concentrated on the surface of the buccal mucosa. Results were confirmed by additional permeation studies performed with FD-4 used as hydrophilic macromolecular test compound.
Conclusions. Based on their permeation-enhancing properties, chitosan–TBA conjugates represent a promising tool for the buccal administration of peptide drugs, e.g., PACAP.

KEY WORDS: buccal drug delivery; chitosan–TBA conjugate; glutathione; PACAP; permeation enhancement; thiolated polymers.

INTRODUCTION

Patients with type 2 diabetes are commonly treated with diet and oral antidiabetic agents (sulfonylureas, biguanides, thiazolidinediones) (1). However, sulfonylurea therapy, for instance, may cause hypoglycemic reactions and high primary and secondary failure rates (2). Patients treated with biguanides risk the development of lactic acidosis (3). Furthermore, thiazolidinediones can cause weight gain and liver toxicity (4). Finally, the majority of diabetics will require insulin therapy. Due to the insulin resistance that accompanies the disease, usually high doses of insulin are needed, which in turn lead to hypoglycemia. Unfortunately, neither insulin nor combined treatment with insulin and oral antidiabetics provide long-lasting, satisfactory metabolic control.

Recent studies have shown that pituitary adenylate cyclase-activating polypeptide (PACAP) is a promising alternative antidiabetic agent for the treatment of type 2 diabetes. PACAP augments glucose-dependent insulin secretion both *in vivo* and *in vitro* with an outstanding potency (5). It behaves as a superior insulin because it combines the potency of insulin without the risk of overdose and hypoglycemia (6). To use PACAP in the treatment of type 2 diabetes, it is necessary to develop a suitable drug delivery system. From the drug delivery point of view, implantable pumps or inserted depot formulations are needed, because of its relatively short elimination half-life period (a few minutes) on one hand, and the need for a permanent blood level of the drug on the other (7,8). The proposed dose for PACAP is 3 pmol/min kg when administered subcutanously (7). In all previous experiments, PACAP has been administered intravenously or subcutaneously with rapid absorption from subcutaneous tissue. However, parenteral administration is associated with pain, formulations need to be sterile, and it is a time-consuming process for both physicians and patients. Buccal mucosa offers an alternative to conventional parenteral administration, because of its comparatively higher patient tolerance. The buccal tissue is well vascularized with venous blood draining the buccal mucosa and reaching the heart directly via the internal jugular vein (9).

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Unfortunately, the permeability of the buccal mucosa is usually too low to allow therapeutic plasma levels. One approach to overcome this barrier is the use of surfactants. Bile salts have been extensively employed to enhance the absorption of drugs across the buccal mucosa (10). The increase in permeability of the oral mucosa by the cationic surfactant cetrimide is also well known (11,12). The disadvantage of these effective permeation enhancers is the cause of cell damage (13,14). Use of polymers with permeation-enhancing properties could be an alternative to avoid irritation at the site of administration caused by surfactants (15).

The mucoadhesive biopolymer chitosan is known to exhibit such permeation-enhancing properties. Its favorable biological properties such as nontoxicity, biocompatibility, and biodegradability make chitosan a promising candidate for safe buccal drug delivery systems (10). In addition, a sustained release of peptide drugs released from this polymer can be guaranteed (16).

It was recently shown that the thiolation of chitosan, by the covalent attachment of 2-iminothiolane, leads to a strongly improved mucoadhesion compared to unmodified chitosan (17). The chemical structure of chitosan–TBA is shown in Fig. 1.

The purpose of the present study was to evaluate and compare the influence of two different auxiliary agents, Na DOC and cetrimide, and the polymers chitosan and chitosan–TBA on the permeation of PACAP across the buccal mucosa. As it was demonstrated by our group (18) that the immobilization of thiol groups on polymers also leads to an improved permeation-enhancing effect being mediated by GSH (L-γ-glutamyl-L-cysteinylglycine), drug uptake in the presence of chitosan–TBA conjugate and GSH on the buccal tissue was investigated as well.

MATERIALS AND METHODS

Materials

Fluorescein isothiocyanate (FITC)-labeled dextran (FD-4) [molecular mass (MM) = 4.3 kDa], glutathione (GSH; γ-Glu-Cys-Gly), chitosan (MM = 400 kDa), Ellman's reagent [5,5′-dithiobis(2-nitrobenzoic acid)], L-cysteine hydrochloride hydrate, Bis-Tris buffer (bis[2-hydroxyethyl]imino-tris[hydroxymethyl]methane), sodium deoxycholate, and cetrimide were all purchased from Sigma (St. Louis, MO, USA); 2-iminothiolane HCl (Traut's reagent) was purchased from Pierce (Oud Beijerland, Netherlands); PACAP was kindly donated by Bayer AG (Leverkusen, Germany).

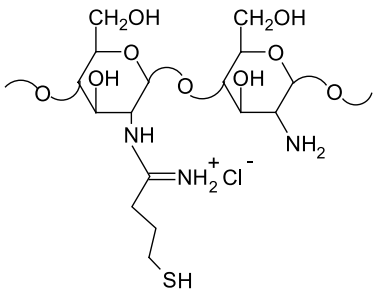

Fig. 1. Presumptive substructure of chitosan–TBA conjugate.

Synthesis of Chitosan–TBA Conjugates

Chitosan (500 mg; MM = 400 kDa) was dissolved in 50 mL of aqueous 1% acetic acid by stirring the mixture for 1 h. 5 M NaOH was added dropwise to raise the pH to 6.5, and 200 mg of 2-iminothiolane HCl (Traut's reagent) was added. After 24 h of incubation at room temperature under continuous stirring, the resulting polymer conjugates were dialyzed against 5 mM HCl, two times against 5 mM HCl containing 1% of NaCl, against 5 mM HCl, and finally against 0.4 mM HCl. Thereafter, samples and controls were freeze-dried at −30°C and 0.01 mbar (Christ Beta 1-8K, Osterode, Germany), and stored at 4°C until further use.

Determination of the Thiol Group Content

Ellman's reagent was used to quantify the amount of thiol groups on the modified chitosan and to determine its degree of modification. First, 5 mg conjugate was dissolved in 2.5 mL demineralized water. To aliquots of 250 μL conjugate solutions, 250 μL 5 M phosphate buffer (pH 8.0) and 500 μL of Ellman's reagent [3 mg of 5,5′-dithiobis(2-nitrobenzoic acid) dissolved in 10 mL of 0.5 M phosphate buffer, pH 8] were added. The reaction was allowed to proceed for 2 h at room temperature. After removing the precipitated polymer by centrifugation (24,000 *g*; 5 min), 300 μL supernatant fluid was transferred to a microtritation plate and the absorbance was immediately measured at 450 nm (microtritation plate reader; Anthos Reader, Salzburg, Austria). The amount of thiol moieties was calculated based on a standard curve obtained from solutions with increasing concentrations of L-cysteine hydrochloride hydrate.

Tissue Preparation

In these studies porcine buccal mucosa was used, because porcine and human buccal epithelia are similar in certain important parameters such as permeability, barrier lipid composition, histology, and ultrastructural organization (19). Buccal tissue from pigs weighing 70–100 kg, obtained freshly from a local slaughterhouse, was used at least within 4 h after the animals were slaughtered. The porcine buccal tissue has not been in the 60°C water bath before being excised from the animals. Most of the underlying tissue was removed from the mucosa with surgical scissors. The epithelium was separated from most of connective tissue with an electrodermatome (Aesculap® Accu Dermatome, Germany). The buccal tissue was dermatomed to a thickness of 500 μm.

Permeation Studies

Dermatomed buccal mucosa was mounted in Ussing chambers with a diffusion area of 0.64 cm^2 and a compartment volume of 1 mL. All experiments were performed in an atmosphere of 95% O_2 and 5% CO_2 at 37°C. Prior to the experiment, the acceptor chamber was filled with 40 mM Bis-Tris buffer (bis[2-hydroxyethyl]imino-tris[hydroxymethyl]-methane), pH 6.8, and the donor chamber with either chitosan–TBA (1% m/v) containing 2% (m/v) of the reduced form of GSH, or chitosan–TBA (1% m/v), or unmodified

chitosan (1% m/v). The polymers were dissolved in 40 mM Bis-Tris buffer pH 6.8. For studies on low molecular mass permeation enhancers, sodium deoxycholate and cetrimide were dissolved in buffer in a concentration of 5% (m/v). After an equilibration period of about 20 min, the 100-μL buffer on the donor side was replaced with a stock solution of PACAP and FD-4. The final concentration of PACAP was 1 mg/mL, whereas FD-4 was used at a concentration of 7.5 mg/mL. Every 30 min, 150-μL samples were withdrawn from the acceptor chamber and replaced by 150 μL buffer, equilibrated at 37°C. The amount of permeated PACAP or FD-4 was determined using high-performance liquid chromatography (HPLC) as described below. Cumulative corrections were made for the previously removed samples. The apparent permeability coefficients (P_{app}) were calculated according to the equation $P_{app} = Q/(Act)$, where P_{app} is the apparent permeability coefficient (cm/s); Q is the total amount permeated within the incubation time (μg); A is the diffusion area of the Ussing chamber (cm^2); c is the initial concentration of the marker in the donor compartment (μg/cm^3); and t is the total time of the experiment (s).

Viability Studies

After the permeation studies, the medium was removed from the donor chamber and incubated in 1 mL Trypan blue dye. Microscopic investigations were performed after a staining time of 15 min. Viability studies were performed with fresh tissue as control.

HPLC Analysis of PACAP

Samples analysis was performed via reversed-phase HPLC using a Perkin-Elmer series 200 LC pump (Norwalk, CT, USA), Perkin-Elmer 200 series autosampler with a 20-μL injection loop and a diode array detector (Perkin-Elmer 235C). PACAP and its degradation products were separated on a precolumn (Nucleosil 100-5C18, 40 × 4 mm) and a C_{18} column (Nucleosil 100-5C18, 250 × 4 mm) at 40°C. Gradient elution was performed as follows: flow rate 1 mL/min; 0–22 min; linear gradient from 90% A:10% B to 10% A:90% B (eluent A: 0.1% trifluoroacetic acid in water; eluent B: 90% acetonitrile and 10% of 0.1% trifluoroacetic acid in water). Absorbance of the peptide was detected at 220 nm. Amount of PACAP was calculated by interpolation from an appropriate external standard curve. The limit of quantification for the HPLC analysis method of PACAP was 1 μg/mL.

HPLC Analysis of FD-4

The permeated amount of FD-4 was determined by an HPLC system using a Perkin-Elmer series 200 LC pump, Perkin-Elmer 200 series auto sampler with a 20-μL injection loop and a fluorescence detector (Perkin-Elmer LS 40) with an excitation wavelength of 491 nm and an emission one of 520 nm (20). The mobile phase consisted of 5 mM phosphate buffer (pH 7.4) and acetonitrile (88:12), and the flow rate was 1 mL/min. For the calculation an appropriate external standard curve was used.

Data Analysis

Statistical data analysis was performed using *t*-test with $p < 0.05$ as the minimal level of significance.

RESULTS

Synthesis of Chitosan–TBA Conjugate

The resulting chitosan–TBA (=4-thio-butyl-amidine) conjugate displayed 291.2 ± 28.0 μM sulfhydryl groups per gram of polymer. The presumptive substructure of chitosan–TBA is shown in Fig. 1. The lyophilized polymer appeared as white and odorless powder of fibrous structure. It was easily swellable in acidic solutions and formed transparent gels of high viscosity. Mucoadhesive properties were in good correlation with previous results (17).

Studies with Low Molecular Mass Permeation Enhancers

Permeation studies were performed on freshly excised buccal mucosa. Permeation enhancers Na DOC and cetrimide were used in a concentration of 5% (m/v), which is reported to be sufficiently high to achieve significant enhancing effects (21). The permeation-enhancing effect of these two different low molecular mass enhancers is depicted in Fig. 2. Using sodium deoxycholate, an enhancement ratio of 18.6 was obtained (Table I). A higher flux rate was achieved by using the cationic permeation enhancer cetrimide (Fig. 2). A maximum P_{app} of 264.8 ± 39.3 cm/s was reached, which represents an enhancement ratio of 46.5.

Studies with Polymeric Permeation Enhancers

Subsequently, the permeation-enhancing effects of unmodified chitosan and chitosan–TBA conjugate with or without GSH on the transport of PACAP and FD-4 were evaluated. Studies were performed with the model drug FD-4, which was chosen as a model compound for high

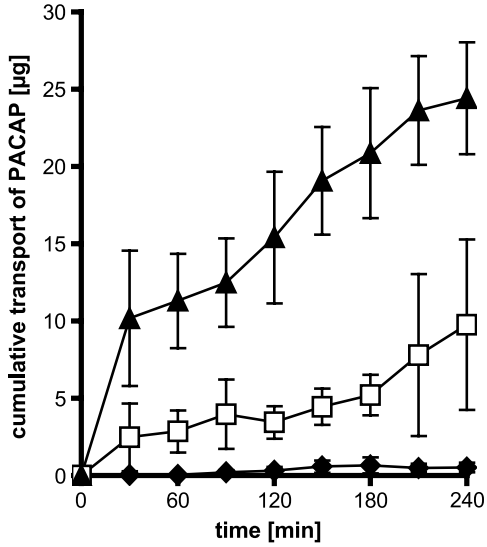

Fig. 2. *In vitro* permeation profile of PACAP across the buccal mucosa of pigs. Control without enhancer (♦); 5% (m/v) Na-deoxycholate (□); 5% (m/v) cetrimide (▲); (means ± SD; n = 4–6).

Table I. Effect of Chitosan, Chitosan–TBA, and Chitosan–TBA + GSH, Respectively, on the Buccal Permeation of PACAP and the Corresponding Enhancement Ratios

Test system	Permeability coefficient (±SD) ($\times 10^{-8}$ cm/s)	Enhancement ratio (P_{app} enhancer system/ P_{app} control)
Control	5.70 ± 3.31	1
Chitosan 1% (m/v)	8.51 ± 1.34	1.49
Na–DOC 5% (m/v)	105.98* ± 59.86	18.59
Chitosan–TBA 1% (m/v)	221.45* ± 35.5	38.85
Cetrimide 5% (m/v)	264.82* ± 39.27	46.46
Chitosan–TBA 1% (m/v) + GSH 2% (m/v)	441.67* ± 89.86	77.49

Indicated values are means (±SD, $n = 4$–6).
*Significantly different from the control ($p < 0.05$).

molecular weight hydrophilic drugs. Although the extent of transport of FD-4 across the buccal epithelium is rather low, buccal permeation could be enhanced by the addition of chitosan–TBA conjugate in combination with GSH (Fig. 3). Chitosan and chitosan–TBA conjugate showed an increase in permeation of FD-4. Their apparent permeability coefficients ($2.8 \pm 5.9\times10^{-9}$; $3.0 \pm 1.0\times10^{-9}$) were significantly higher compared to that of control ($0.6 \pm 0.4\times 10^{-9}$). No significant differences between the permeation-enhancing effect on FD-4 of chitosan and chitosan–TBA could be observed. In contrast, as a result of the addition of chitosan–TBA/GSH, a P_{app} of $5.7 \pm 2.6\times10^{-9}$ cm/s was achieved, which represents a 10.5-fold enhancement in comparison to control.

Results of the permeation studies with PACAP are shown in Fig. 4. Unmodified chitosan showed no significant increase in the permeation of PACAP across the buccal tissue in comparison to control, whereas chitosan–TBA led to a 38-fold increase in permeation. With chitosan–TBA in the presence of GSH, a very strong increase in permeation was achieved. A maximum P_{app} of $441.7 \pm 89.9\times10^{-8}$ cm/s was reached, which represents an enhancement ratio of 77.5.

Studies with Combinations of Permeation Enhancers

We also investigated whether the chitosan–TBA/GSH system and cetrimide in combination are acting synergistically. The enhancement ratio was found to be only 50.5 (data not shown). The combination of the chitosan–TBA/GSH system and Na DOC was not used for permeation studies because, after Na DOC was added to the polymer solution, the chitosan–TBA conjugate precipitated.

Absorption of GSH

The permeation of GSH *per se* was investigated as well (Fig. 5). The flux of GSH was rather low—only 33.8 ± 8.1 µg of GSH could be detected in the acceptor chamber after 4 h. This means that only 0.2 ± 0.04% of the applied amount were able to permeate across the buccal mucosa. This result indicates that GSH remains concentrated on the surface of the buccal mucosa, which guarantees a permeation-enhancing effect over a long period.

Viability Studies

After the permeation studies buccal tissues were taken out of the Ussing chambers for histological analysis. A strong swelling of the mucosa was macroscopically observed after exposure to low molecular mass permeation enhancers. No swelling was observed when the tissue was incubated with the polymeric permeation-enhancing systems. Microscopic inves-

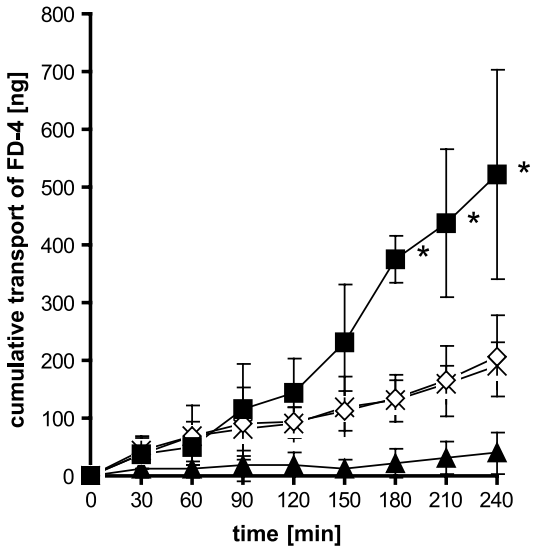

Fig. 3. *In vitro* permeation profile of FD-4 across the buccal mucosa of pigs. Control without polymer (▲); 1% (m/v) chitosan (×); 1% (m/v) chitosan–TBA (◇); 1% (m/v) chitosan–TBA and 2% (m/v) GSH (■); (means ± SD; $n = 4$). *Significant in comparison to chitosan–TBA ($p < 0.05$).

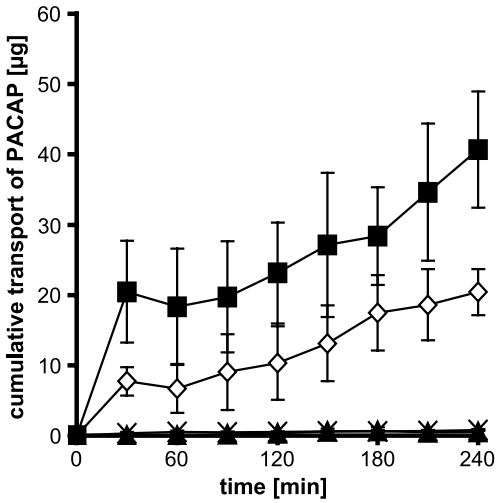

Fig. 4. *In vitro* permeation profile of PACAP across the buccal mucosa of pigs. Control without polymer (▲); 1% (m/v) chitosan (×); 1% (m/v) chitosan–TBA (◇); 1% (m/v) chitosan–TBA and 2% (m/v) GSH (■); (means ± SD; $n = 4$–6).

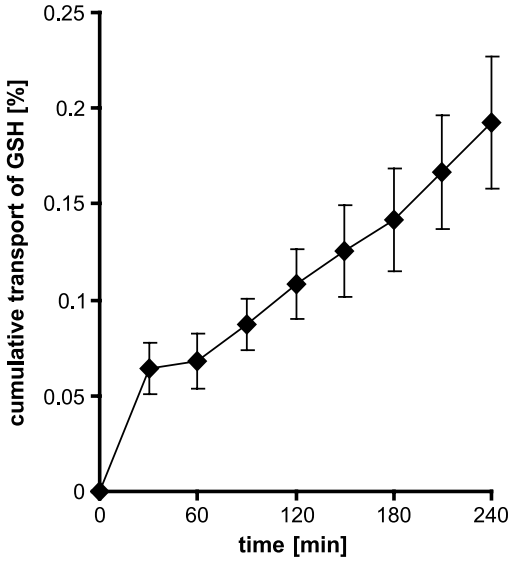

Fig. 5. *In vitro* transport of GSH (20 mg/mL) across the buccal mucosa of pigs. Each point represents the mean ± SD ($n = 5$).

tigations demonstrated the viability of the tissue as no dead cells could be found in any sample.

DISCUSSION

Synthesis of Chitosan–TBA Conjugate

2-Iminothiolane is a common reagent used to immobilize thiol groups to primary amino groups of proteins (22). In this study, it was used for modification of chitosan (Fig. 1). So far, the described pathway for chitosan–TBA conjugate synthesis is the easiest way to obtain polymer derivatives containing thiol groups. This method leads to desired derivatives via a simple one-step reaction without the need to add any other reagent. In contrast, for the synthesis of polymer–cysteine and polymer–TGA conjugates, it was necessary to add 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDAC) to activate the caboxylic acid moieties of the sulfhydryl ligands (Langoth *et al.*, personal communication, 2005).

Studies with Low Molecular Mass Permeation Enhancers

Within this study, two well-established permeation enhancers were evaluated concerning their permeation-enhancing effects on PACAP. Anionic sodium deoxycholate and cationic cetrimide were chosen. It can be assumed that ionic interactions between the positively charged PACAP and the anionic bile salt are influencing the permeation-enhancing properties of Na DOC. Cetrimide has a cationic net charge. Hence, ionic interactions between the peptide drug and the permeation enhancer can be excluded. Generally, low molecular mass permeation enhancers have the disadvantage of causing mucosal irritation (10). Furthermore, they are only effective for a short period because of the rapid absorption by the buccal mucosa. Hence, there is a need for permeation enhancers that are reversible in action, nonirritating for the oral mucosa, and effective for a long time.

Studies with Polymeric Permeation Enhancers

Chitosan was shown to have a significant enhancing effect on the permeation of drugs across the buccal mucosa without having any deleterious effects on the buccal mucosa. An explanation for the permeation enhancement by chitosan may be a direct effect on tight junctions, increasing the permeability, thus allowing paracellular absorption of hydrophilic drugs (23). Recently, improved permeation-enhancing effects were observed via the use of thiolated chitosan in combination with GSH. A chitosan–thioglycolic acid conjugate was used in this study (24). The mechanism of the permeation-enhancing effect of GSH, in combination with thiolated polymers, was studied by Clausen *et al.* (18). To test the permeation-enhancing properties of chitosan–TBA in combination with GSH, *in vitro* studies were performed with the model drug FD-4. FD-4 with a molecular mass of 4.3 kDa was chosen, because 4 and 10 kDa FITC dextrans are able to penetrate the buccal mucosa, whereas the larger 20 and 40 kDa FITC dextrans are not (25). Using the chitosan–TBA/GSH system, enhancement ratios of 10.5 were obtained, and based on these promising results, permeation studies were performed with PACAP.

Results showed a dramatically enhanced permeation of PACAP achieved via the combination of chitosan–TBA and GSH. The amount of permeated PACAP in the presence of chitosan–TBA/GSH was 78-fold compared to the buffer (Table I). These are much higher permeation ratios of PACAP, as already demonstrated in former studies, which were performed with chitosan–thioglycolic acid conjugate (24). For FD-4, the permeation-enhancing effect of chitosan–TBA/GSH was less dramatic compared to results obtained with PACAP. These results can be explained by the higher molecular mass of FD-4 in comparison to PACAP. Hence, the transported amount of FD-4 was rather low and the permeation-enhancing effect was not as significant as for PACAP.

The proposed dose for PACAP is 3 pmol/min kg when administered subcutaneously. As 4% of the used PACAP (concentration: 1 mg/mL) can permeate the buccal mucosa, within *in vitro* studies, it seems feasible that therapeutic levels of PACAP can be reached with the proposed buccal delivery system. However, these results must be verified with *in vivo* studies.

Focusing on the permeation of GSH, only 0.21% of the used amount permeated across the buccal mucosa. This demonstrates that GSH is able to act on the surface of the buccal mucosa for longer than 4 h.

Further Advantages of the Chitosan–TBA/GSH Permeation Enhancing System

The enzyme-inhibiting properties of GSH are another important advantage of the chitosan–TBA/GSH system. Because of the addition of GSH, PACAP remains stable on the buccal mucosa for more than 5 h, whereas without GSH PACAP is degraded to a large extent (Langoth *et al.*, personal communication, 2005).

Because of its size, chitosan–TBA is also not absorbed by the buccal mucosa. Hence, systemic toxic side effects by the carrier matrix can be excluded and permeation-enhancing effect over a long period can be guaranteed.

Additionally, our research group showed that, as a result of the covalent attachment of 2-iminothiolane to the cationic polymer chitosan, the mucoadhesive properties are improved 100-fold in comparison to unmodified chitosan. So far, this represents the strongest improvement in the mucoadhesive properties of a polymer by the covalent attachment of thiol moieties (17).

CONCLUSION

Buccal drug delivery offers an alternative to conventional parenteral administration in the treatment of type II diabetes. The buccal mucosa represents an effective absorption barrier, and therefore new strategies must be found to overcome it. The low molecular mass permeation enhancers, Na DOC and cetrimide, and the chitosan–TBA/GSH system were evaluated for their permeation-enhancing properties. Results reveal that the chitosan–TBA/GSH system shows strong permeation-enhancing properties within the *in vitro* model. Compared to low molecular mass permeation enhancers, the chitosan–TBA/GSH system is effective for a longer period, because only a small amount of GSH and no polymer are absorbed from the buccal mucosa. Based on these features, chitosan–TBA, together with GSH, is a very promising candidate for a buccal permeation-enhancing system for PACAP, a peptide drug that could be used for the treatment of type 2 diabetes.

ACKNOWLEDGMENTS

This work was supported by Bayer AG, 51368 Leverkusen, Germany. The authors wish to thank Mr. Mayerhofer and co-workers for the supply of buccal mucosa of pigs.

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