Systematic Investigations of the Influence of Molecular Structure on the Transport of Peptides Across Cultured Alveolar Cell Monolayers

A. N. O. Dodoo, S. Bansal, D. J. Barlow, F. C. Bennett, R. C. Hider, A. B. Lansley, M. J. Lawrence, and C. Marriott

Received September 17, 1999; accepted October 15, 1999

Purpose. To determine how the structures of peptides influence their alveolar permeability.

Methods. The studies were performed using 14 synthetic ‘model’ peptides, labelled with a novel, non-intrusive amino acid fluorophore, and their transport studied using rat alveolar cell monolayers cultured on permeable supports.

Results. The passage of the peptides across the epithelial cell monolayers is shown to be primarily paracellular, with an inverse dependence on molecular size, and an enhanced flux observed for cationic peptides. The apparent permeability coefficients ($P_{app}$) for the peptides (together with those for other organic solutes, taken from the literature) are shown to be well-modelled assuming two populations of ‘pores’ in the monolayers, modelled as cylindrical channels of radii 15 Å and 22 nm. The former pores are shown to be numerically equatable with the monolayer tri-junctional complexes, and the latter are taken as monolayer defects.

Conclusions. The various monolayer $P_{app}$ values correlate well with the results from in vivo transport experiments, and the conclusion is drawn that the pulmonary delivery of peptide drugs is perfectly exploitable.

KEY WORDS: transport of peptides; molecular structure; molecular size; cultured alveolar cell monolayers; alveolar permeability.

INTRODUCTION

Over the last five years or so there have been many musings as to the potential to be afforded by using the pulmonary route of administration for the systemic delivery of protein and peptide drugs (1,2). There are no systematic, comprehensive studies yet reported, however, that demonstrate that the predicted potential is both real and achievable, although there have been several studies concerned with the alveolar permeability of specific peptides (cf., 3–5). In the following work we have endeavoured to rectify this deficiency, undertaking a systematic investigation of the alveolar cell permeability of ‘model’ peptides with defined physico-chemical characteristics.

In the design of the model peptides efforts were made to ensure that the compounds were chemically and metabolically stable, and had no propensity to form regular secondary structures. It was also deemed important to use peptides possessing a reasonable aqueous solubility and, as far as could be predicted, a lack of pharmacological activity.

The three series of model peptides described in this study are based on the sequence triplet Gly-Ala-Ser and are presented in Table 1. Series I peptides were designed for the purpose of exploring the relation between alveolar absorption and peptide size. Series II peptides were designed with the aim of exploring the effects on alveolar absorption of modest (single residue) changes in hydrophobicity. Series III peptides were designed to permit an investigation of the influence of peptide charge on alveolar absorption.

To facilitate their preparation, detection and quantitation, all of the model peptides were synthesized incorporating the novel fluorescent amino acid DL-(6,7-dimethoxy-4-coumaryl) alanine (DCA, single letter code X*):

This label has previously been shown to be both chemically and metabolically stable, allowing the detection of peptides at concentrations as low as 5 picomolar (even in biological media), giving results identical with those obtained using radiolabelled species (6–7).

The transport properties of the model peptides were investigated using primary cultures of rat alveolar cells (nominally, type II cells), grown as monolayers on permeable supports.

MATERIALS AND METHODS

Peptide Syntheses

Peptide synthesis was carried out using Fmoc chemistry on a Perseptive Biosystems 9050 Pepsynthesizer. A PEG-poly-styrene resin was used with a modified Rink amide linker. Fmoc amino acids were used in 4-fold excess and activation carried out using 2-(1H-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium tetrafluoroborate in the presence of diisopropyl ethylamine. Peptides were cleaved from the resin using TFA, water and triisopropylsilane (95:4.5:0.5).

Synthesis of the Peptide Label

DL-(6,7-dimethoxy-4-coumaryl) alanine (DCA) was synthesized according to Bennett et al. (6–7). 3,4-dimethoxyphenol was condensed with ethyl-4-chloroacetoacetate in the presence of Amberlyst 15 to afford 4-chloromethyl-6,7-dimethoxycoumarin. Diethylacetamidomalonate was treated with sodium hydride followed by the 4-chloromethyl-6, 7-dimethoxycoumarin, and subsequent acid hydrolysis gave the novel amino acid, DCA.

Transport Studies

Alveolar cell monolayers were prepared according to Kim et al. (8). Alveolar cells were isolated from specific pathogen-free Sprague-Dawley male rats by digestion with porcine pancreatic elastase (2 U/ml; Worthington Biochemicals, NJ) and
purified on discontinuous Percoll® gradients. The purified cells were cultured in a humidified atmosphere of 5% CO₂/95% air at 37°C, on tissue-culture treated polycarbonate filters (1.13 cm²; Transwell, Costar, MA), and seeded (day 0) at a density of 1.5 × 10⁶ cells per cm². The cells were cultured in a humidified atmosphere of 5% CO₂/95% air with 100 U/mL penicillin, 100 ng/mL streptomycin and 0.1 μM dexamethasone. The medium on both sides of the monolayer was changed on alternate days.

Transport experiments were carried out using a modified Ringer’s solution (transport medium) comprising 1.8 mM CaCl₂, 0.81 mM MgSO₄, 5.4 mM KCl, 116.4 mM NaCl, 0.782 mM NaH₂PO₄·2H₂O, 5.55 mM D-glucose, 0.075 M bovine serum albumin (fraction V), 25 mM NaHCO₃ in 15 mM HEPES buffer pH 7.4. The cell monolayers were washed twice with transport medium and the apical and basolateral compartments filled with 0.6 mL and 1.5 mL transport medium, respectively. The cells were equilibrated with transport medium for 2 h at 37°C in a humidified 5% CO₂ incubator. Approximately 1 mL stock solutions of the model peptides were prepared in transport medium and trace amounts of ¹⁴C-mannitol (DuPont Ltd, Herts, UK) added to each solution (to give a final concentration of 0.5 μCi/mL). At the start of each transport experiment, half of the medium from the donor compartment was removed and replaced with an equal volume of peptide-containing medium, giving a final concentration of peptide in the donor medium of ~0.5 mM. At selected time intervals, 150 μL samples (for fluorescence assays) and 50 μL samples (for liquid scintillation counting) were taken from the receptor compartment and replaced with equal volumes of fresh transport medium. At the end of each transport experiment the donor and receptor compartment media were routinely assayed following separation by HPLC, to check for peptide breakdown products. Transepithelial resistance across the monolayers was monitored from day 2 using a Millicell® ERS system (Millicell, MA).

For all of the transport experiments performed, the integrity of the layers was routinely checked by monitoring their transepithelial electrical resistance (TER) and apparent permeability coefficient (P_app) for ¹⁴C-mannitol.

Apparent permeability coefficients for the various solutes (P_app, in cm.s⁻¹) were calculated as:

\[ P_{app} = \frac{\Delta Q/\Delta t}{A \cdot c(0)} \]

where \( \Delta Q/\Delta t \) is the linear rate of appearance of solute in the receiver solution, A is the cross-sectional area of the polycarbonate filter (i.e., 1.13 cm²) and \( c(0) \) is the initial concentration of solute in the donor compartment at \( t = 0 \). The \( P_{app} \) values were not corrected for the \( P_{app} \) of the matrix-free filters (because