Gly-L-Phe Transport and Metabolism Across Primary Cultured Rabbit Tracheal Epithelial Cell Monolayers

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INTRODUCTION

Primary culture models derived from different regions of the respiratory tract, that vary in morphological and physiological characteristics, are attractive tools for delineating the permeability and metabolism characteristics of the respiratory tract for systemic drug delivery (1–4). Recently, we have developed primary cultured rabbit tracheal epithelial cell monolayers at an air-interface, which exhibit a phenotypic appearance resembling the native tissue and retain a similarly high level of active ion transport capacity (5). Moreover, we have evaluated their transport characteristics using model compounds of varying lipophilicities and molecular weights (6). We found that the apparent permeability coefficient (P_{app}) for β-blockers increased with increasing log octanol/pH 7.4 buffer partition coefficient (log P) in a sigmoidal fashion where the log P at the half-maximal P_{app} was 2.08, and that the P_{app} for hydrophilic solutes decreased with increasing molecular weight, with a molecular cut off at about 20,000 daltons (6). The aim of this study was to delineate peptide transport and metabolism characteristics of the aforementioned tracheal epithelial cell monolayers for comparison with the alveolar epithelial cell monolayers. Towards that end, glycyl-L-phenylalanine (Gly-L-Phe), was chosen as a model peptide.

MATERIALS AND METHODS

Materials

Gly-L-Phe, L-phenylalanine (L-Phe), atenolol, actinonin, sodium p-hydroxymercuribenzoate (PHMB) were purchased from Sigma Chemical Co. (St. Louis, MO). Cell culture reagents and supplies were obtained from GIBCO (Grand Island, NY). PC-1 medium used as a cell-growth medium was obtained from Hycor Biomedical (Portland, ME). Collagen-treated Transwells (Transwell-COL, 0.45 μm, 12 mm O.D.) were obtained from Costar Co. (Cambridge, MA). All other chemicals were of the highest purity available commercially.

Primary Culture of Rabbit Tracheal Epithelial Cells

The primary cell culture procedure for routinely generating tracheal epithelial cell monolayers was published elsewhere (5). Briefly, upon excision from the rabbits, the trachea was cut into small pieces, and incubated in 0.2% protease XIV (Sigma Chemical Co., St. Louis, MO) at 37°C for 90 min. Epithelial cells were gently scraped off, suspended in a minimum essential medium (S-MEM) containing 10% fetal bovine serum (FBS) and 0.5 mg/ml deoxyribonuclease I (Sigma Chemical Co., St. Louis, MO), and centrifuged at 200× *g* for 10 min at room temperature. The cells were washed twice with S-MEM containing 10% FBS, filtered through a 40 μm cell strainer, pelleted at 200× *g* for 10 min, and resuspended in PC-1 medium supplemented with 2 mM l-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, 50 μg/ml gentamicin, and 1 μg/ml fungizone. The isolated tracheocytes were plated on collagen-pretreated Transwells at a density of 1.3×10^6 cells/cm^2 (day 0) and cultured in a humidified atmosphere of 5% CO_2 and 95% air at 37°C. The volumes of apical and basolateral culture media were 0.5 and 1.5 ml, respectively. From day 2 onward, the cells were cultured with their apical surface exposed directly to the air by removing the apical fluid, while 0.8 ml culture medium bathed the basolateral side. The 5–7 days old cell monolayers were used for transport experiments.

Transport Studies

All transport experiments were performed at 37°C in an incubator with a humidified atmosphere of 5% CO_2 and 95% air. Prior to each experiment, the cell monolayers were washed with bicarbonated Ringer's solution containing 0.2% bovine serum albumin, and allowed to equilibrate with the new bathing medium for 30 min in the incubator. The Ringer's solution comprised 1.8 mM $CaCl_2$, 0.81 mM $MgSO_4$, 5.4 mM KCl, 116.4 mM NaCl, 0.782 mM NaH_2PO_4, 5.55 mM glucose, 15 mM HEPES, 25 mM $NaHCO_3$ (pH 7.4 at 37°C). The apical and basolateral volumes were 0.5 and 1.5 ml, respectively, which afforded a zero hydrostatic pressure gradient across the monolayer. Following equilibration, a known concentration of the penetrant, with or without protease inhibitors, was added to either the apical or basolateral donor fluid to yield a final concentration of 10 mM. Two hundred microliters of the receiver fluid were taken at 30, 60, 120, 180, and 240 min for assay and immediately replaced with an equal volume of fresh Ringer's solution. The spontaneous potential difference (PD; in reference to apical side) and transepithelial electric resistance (TEER) of the monolayer were monitored, using an EVOM voltohmmeter (World Precision Instruments, Sarasota, FL). The initial and final PD values were 67.9 ± 5.0 mV and 54.6 ± 8.8 mV (mean ± s.d., n = 22), respectively; and the corresponding TEER values were 1.28 ± 0.23 kohm.cm^2 and 2.21 ± 0.42 kohm.cm^2.

HPLC Assay

Gly-L-Phe and L-Phe were measured using a reverse-phase HPLC system (Shimadzu LC-6A, Shimadzu Co., Ltd.,

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Kyoto, Japan) on a Rainin Microsorb C18 column (4.6 × 250 mm, 5 μm; Rainin Instrument Co., Woburn, MA). Samples (200 μl) were mixed with 200 μl of acetonitrile containing 5 μM atenolol (the internal standard), and centrifuged at 2000× *g* for 10 min. The supertanant was concentrated by evaporation under nitrogen. Each dried sample was resuspended in 100 μl of water, vortexed, and injected into the HPLC. The mobile phase consisted of 9% acetonitrile in 0.1 M sodium perchlorate adjusted to pH 2.5 with phosphoric acid. The flow rate was 1.0 ml/min. L-Phe, atenolol, and Gly-L-Phe were eluted at 8, 15, and 21 min, respectively.

Data Analysis

The cumulative amount of Gly-L-Phe appearing in the receiver fluid was plotted as a function of time. From the observed steady-state flux (J, mol/cm^2/sec) of Gly-L-Phe, its apparent permeability coefficient (P_{app}, cm/sec) was estimated according to the equation:

$$J = P_{app} \times C_0 \quad (1)$$

where C_0 (mol/cm^3) is the initial concentration of the dipeptide in the donor fluid. Steady-state flux of L-Phe, a metabolite of Gly-L-Phe, appearing in the receiver fluid was also estimated. When exogenously applied L-Phe was added to the donor fluid, P_{app} for L-Phe was estimated using Eq. 1.

RESULTS

Figure 1 shows the profiles of Gly-L-Phe transport and those of L-Phe translocated as a metabolite across rabbit tracheal epithelial cell monolayers. The transport of intact Gly-L-Phe in the apical-to-basolateral (AB) direction was similar to that in the basolateral-to-apical (BA) direction (Fig. 1A). By contrast, a far greater amount of the metabolite L-Phe was detected in the basolateral receiver fluid during AB transport of Gly-L-Phe than in the apical receiver fluid during BA transport (Fig. 1B). At 4 hr of AB transport, 1.2 ± 0.4% of the initial Gly-L-Phe dose was in the form of L-Phe in the donor fluid, as compared with 0.75% of the initial dose being translocated in the form of L-Phe to the basolateral receiver fluid.

Table 1 summarizes the unidirectional fluxes and P_{app} for Gly-L-Phe and exogenously applied L-Phe at 10 mM. The P_{app} for intact Gly-L-Phe was statistically the same in both AB and BA directions ($P = 0.05$). The protease inhibitors, 0.5 mM PHMB and 3 μM actinonin, in the apical donor fluid significantly decreased the AB flux of the metabolite (L-Phe), while not appreciably affecting intact Gly-L-Phe flux (Table 1). In the absence of inhibitors, the unidirectional flux of L-Phe as a metabolite in the AB direction was about 8 times greater than that of intact Gly-L-Phe. The P_{app} for exogenously applied L-Phe in the AB direction was about 10 times greater than that in the BA direction (Table 1).

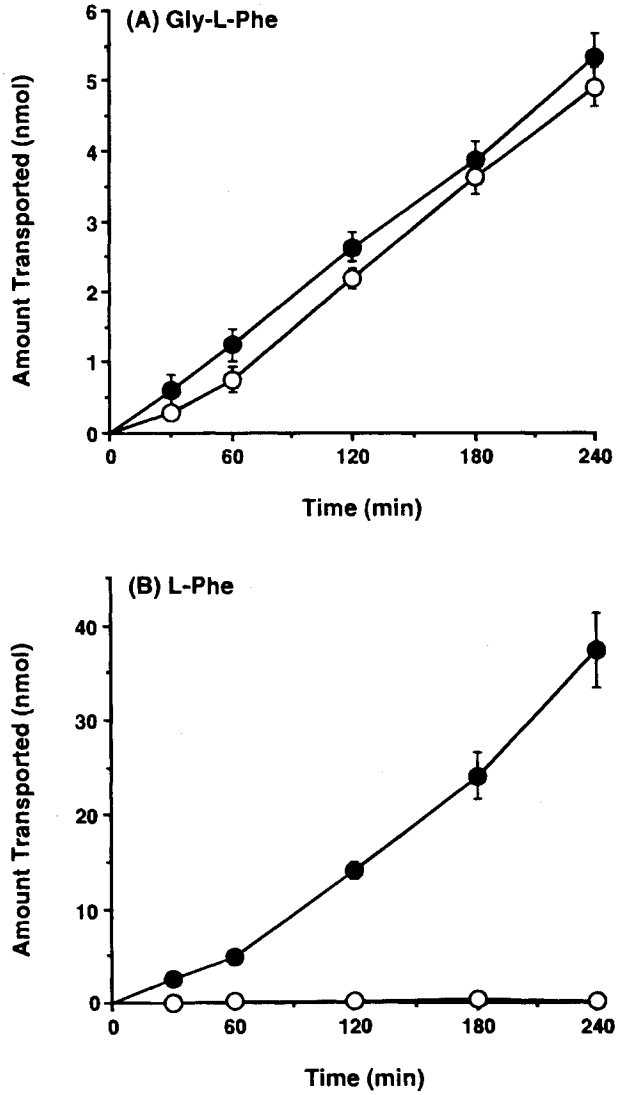

Fig. 1. Transport of intact Gly-L-Phe (Panel A) and Phe formed from Gly-L-Phe (Panel B) across rabbit tracheal epithelial cell monolayers in the apical-to-basolateral (closed symbol) and basolateral-to-apical (open symbol) directions. Each data point is expressed as mean ± s.e.m. of 7 or 8 observations.

DISCUSSION

In the present study, we demonstrated that apically-applied Gly-L-Phe was degraded during transport across the tracheal epithelial cell monolayers and that the transport of intact Gly-L-Phe in the AB direction was the same as that in the BA direction (Fig. 1 and Table 1). This finding is similar to that obtained in rat alveolar cell monolayers (1). The flux of the metabolite L-Phe in the AB direction in the tracheal cell monolayers (2.6 pmol/cm^2/sec) was comparable to that in alveolar cells (4.6 pmol/cm^2/sec (1)), so was the amount of formed L-Phe in the apical donor fluid at 4 hr (about 1% of the initially applied Gly-L-Phe). These findings suggest that the metabolic activities for Gly-L-Phe may be comparable in both cell types, species differences notwithstanding.

The protease inhibitors decreased the appearance of the metabolite L-Phe in the basolateral receiver fluid, without altering the transport of intact Gly-L-Phe. The inhibitory results afforded by treatment with 3 μM actinonin, an aminopeptidase M inhibitor with a Ki of 0.2 μM (7), suggests that this membrane-associated protease may partly be responsible for Gly-L-Phe metabolism. The larger inhibitory effect of a nonspecific protease inhibitor, PHMB, suggests the additional involvement of other proteases in Gly-L-Phe metabolism. Being polar,

Table 1. Transport Parameters (mean ± s.e.m.) for 10 mM Gly-L-Phe and L-Phe Across Tracheal Epithelial Cell Monolayers

		Gly-L-Phe		L-Phe		
Applied drug	Direction	Unidirectional flux (pmol/cm²/sec)	P_{app}[a] × 10^7 (cm/sec)	Unidirectional flux (pmol/cm²/sec)	P_{app}[a] × 10^7 (cm/sec)	n[b]
Gly-L-Phe	AB[c]	0.33 ± 0.03	0.33 ± 0.03	2.6 ± 0.3[e]	—	7
	BA[d]	0.33 ± 0.01	0.33 ± 0.01	0.016 ± 0.006[e]	—	8
+3 μM actinonin	AB[c]	0.33 ± 0.03	0.33 ± 0.03	1.8 ± 0.1[e]	—	3
+0.5 mM PHMB	AB[c]	0.34 ± 0.07	0.34 ± 0.07	0.33 ± 0.06[e]	—	3
L-Phe	AB[c]	—	—	8.0 ± 1.2	8.0 ± 1.2	3
	BA[d]	—	—	0.82 ± 0.03	0.82 ± 0.03	3

[a] Apparent permeability coefficient.
[b] Number of observations.
[c] Parameters measured in the apical-to-basolateral direction.
[d] Parameters measured in the basolateral-to-apical direction.
[e] Formed from Gly-L-Phe.

PHMB probably would not reach its effective concentration within the cell, implying that its inhibitory range is probably extracellular. Consequently, the metabolism of Gly-L-Phe is probably mediated mainly by membrane-associated proteases.

Considering its hydrophilicity, symmetric transport for intact Gly-L-Phe at 10 mM suggests paracellular diffusion as its translocation mechanism. Indeed, the P_{app} for intact Gly-L-Phe was comparable to that of sucrose (0.3×10^{-7} cm/sec (5)). The transport of 10 mM Gly-L-Phe was also symmetrical in alveolar cell monolayers, although the P_{app} values in tracheal epithelial cell monolayers were only one-fourth of those in the alveolar epithelial cell monolayers (1.38×10^{-7} and 1.29×10^{-7} cm/sec for AB and BA directions, respectively (1)). It is uncertain, however, whether Gly-L-Phe at 1 mM would also be asymmetrically transported across the tracheal cell monolayers as observed in the alveolar cell monolayers (5), since the amount transported across tracheal epithelial cell layers was below the u.v. detection limit.

Unlike Gly-L-Phe, the transport of exogenously applied L-Phe at 10 mM was asymmetric between AB and BA directions, implicating the involvement of a specialized amino acid transport process (Table 1). There have been several reports on the existence of amino acid transporters in the distal respiratory epithelium (8,9). Interestingly, the P_{app} for the exogenous L-Phe in the AB direction was approximately the same as that in alveolar epithelial cell monolayers (1).

The flux of L-Phe formed during AB transport of Gly-L-Phe was of the same magnitude as that of L-Phe applied at 10 mM, even though the concentration of L-Phe formed in the apical donor fluid was low (0.12 mM at 4 hr). It is likely that carrier-mediated transport of L-Phe might be saturated when applied exogenously at 10 mM. Alternatively, the concentration of L-Phe formed in the vicinity (the unstirred layer) of the cell surface may be higher than that in the bulk. Based on the unidirectional flux of L-Phe formed (2.6 pmol/cm²/sec), its apical concentration (0.1 mM), and the flux of exogenously applied L-Phe (8.0 pmol/cm²/sec at 10 mM), a Km value of 0.2 mM resulted. This is much smaller than that reported for the intestine (2.7–3.1 mM (10–12)). Underestimation of the Km would be due to the use of the bulk L-Phe concentration instead of its actual local concentration, which is probably higher.

In summary, the transport studies in primary cultured tracheal epithelial cell monolayers indicated (i) Gly-L-Phe at 10 mM might be transported mainly via paracellular diffusion, (ii) Gly-L-Phe is metabolized by proteases present in the apical cell membranes, and (iii) L-Phe formed from Gly-L-Phe appears to be efficiently transported via amino acid transporter(s). These transport characteristics of Gly-L-Phe and L-Phe in rabbit tracheal epithelial cell monolayers closely resemble those in rat alveolar epithelial cell monolayers. Thus, these primary cultured (tracheal and alveolar) epithelial cell monolayers allow us to evaluate both transport and metabolism of peptides in different regions of the respiratory tract.

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