Transepithelial Transport and Metabolism of Thyrotropin-Releasing Hormone (TRH) in Monolayers of a Human Intestinal Cell Line (Caco-2): Evidence for an Active Transport Component?

Elke Walter[1] and Thomas Kissel[1,2]

Received March 4, 1994; accepted May 13, 1994

Cell culture models for gastrointestinal transport and metabolism are important mechanistic tools. Our studies of Caco-2 monolayers demonstrate heterogeneity in transport characteristics depending on passage number and origin of the cells. In accordance with data obtained in animals and humans, TRH shows a carrier-mediated, saturable transport component, which operates parallel to a passive pathway in Caco-2 cells at passage number 89-99. At low TRH concentrations (<3 mM) active transport becomes prominent, as demonstrated by the temperature dependence of TRH transport and inhibition experiments. The Michaelis-Menten parameters of the active, saturable transport component are: K_m = 1.59 mM and V_{max} = 1.84 μM/min. The pH optimum was determined to be at pH 6.0. On the other hand an exclusively paracellular passive route was found with Caco-2 cells at passage number 30-34. These results are also in agreement with observations made by others in cell culture experiments. The aspect of rigorously characterizing the specific Caco-2 clone under investigation is emphasized, especially when active transport mechanisms are suspected.

KEY WORDS: thyrotropin-release hormone (TRH); cell-culture; Caco-2; metabolism; passive diffusion; carrier-mediated transport.

INTRODUCTION

Effective delivery of peptides via the peroral route is an ambitious goal of numerous research groups [1]. To optimize peroral peptide delivery, more insight into the transport mechanism on a cellular level is needed based on relevant models.

Thyrotropin-releasing hormone (TRH), a tripeptide produced by the hypothalamus, was frequently used as a model compound to study peptide absorption [2, 3] and has also some potential in the management of neurological disorders [4].

TRH absorption in rats, dogs, and humans was usually poor (<5%), dose-dependent and variable [2, 3]. These *in vivo* findings are compatible with a carrier-mediated transport of TRH, although no direct evidence was provided. In contrast to these observations it was reported, that TRH transport in Caco-2 monolayers is exclusively passive in nature, probably by a paracellular transport mechanism [5, 6]. These data cast some doubt on the suitability of Caco-2 cell culture techniques for the investigation of gastrointestinal absorption of drug candidates. Caco-2 cells are well known to express carriers for amino acids and dipeptides under culture conditions [7, 8]. In this study, we therefore reinvestigated TRH intestinal absorption using Caco-2 monolayers obtained from different laboratories at different passage numbers to elucidate the transport mechanisms involved.

MATERIALS AND METHODS

Materials

TRH (pyro-L-Glu-L-His-L-Pro-NH_2), TRH-OH (pyro-L-Glu-L-His-L-Pro), N-Benzyloxycarbonyl-glycyl-L-prolyl-L-alanine (Z-Gly-L-Pro-L-Ala) and N-Benzyloxycarbonyl-glycyl-L-proline (Z-Gly-L-Pro) were obtained from Bachem (Bubendorf, Switzerland). All other chemicals were purchased from E. Merck (Darmstadt, Germany) in analytical quality.

^{14}C-Polyethylene glycol 4000 (15 mCi/g) was from Amersham (Braunschweig, Germany) and ^{3}H-TRH ([L-proline-3,4-^{3}H(N), L-histidine-3-^{3}-H(N)] (83.0 Ci/mmol) was from New England Nuclear (Dreieich, Germany). Tissue culture reagents were obtained from Gibco (Eggenstein, Germany) except for fetal calf serum (FCS) from Biozol (Eching, Germany). Tissue culture articles were purchased from Nunc (Wiesbaden, Germany). Polyethyleneterephtalate (PETP) membrane cell culture inserts were from Falcon, Becton Dickinson GmbH (Heidelberg, Germany) and polycarbonate (PC) inserts were from Tecnomara (Fernwald, Germany).

Cell Culture

Caco-2 cells were routinely maintained in DMEM, containing 10% fetal calf serum, 1% nonessential amino acids, 1% L-glutamine and penicillin (100 U/ml) / streptomycin (100 μg/ml) in an atmosphere of 10% CO_2 at 95% (r.h.) at 37 °C. Cells grown in 100-mm-diameter plastic petri-dishes were passaged every 5 days at a split ratio 1:5 to 1:10. The mycoplasma-free cells were used between passage numbers 89 and 99 (MR, from University Hospital of Marburg, Germany), 30 and 34 (ATCC, from American Tissue Culture Collection, Rockville, MD USA).

For transport studies Caco-2 cells were seeded in cell culture inserts with polyethyleneterephtalate membrane (PETP, Falcon®, pores: 0.4 μm, area: 4.6 cm^2) or polycarbonate membrane (PC, Transwell®, pores: 0.4 μm, area: 4.71 cm^2) at a cell density of 6.5×10^5 cells/cm^2 and incubated in six-well culture plates with medium change every second day. The confluent monolayers were used between the 16th and 21th day (PETP) or 21th and 25th day (PC) after seeding.

Metabolic Stability of TRH and Z-Gly-Pro-Ala in Caco-2 Monolayer Homogenates

The stability studies were performed as described previously [9]. Briefly, the Caco-2 cell monolayers were homogenized in phosphate buffer pH 7.4 at a dilution of 1:10 (w/v) using an ultra turrax at 4 °C. After centrifugation (700 g, 4 °C, 10 min) the supernatant was used for the incubation of 10^{-4} M TRH and Z-Gly-Pro-Ala, respectively. Samples were

[1] Department of Pharmaceutics and Biopharmacy, University of Marburg, D-35032 Marburg, Germany.
[2] To whom correspondence should be addressed.

0724-8741/94/1100-1575$07.00/0 © 1994 Plenum Publishing Corporation

withdrawn, deproteinized with 0.1 M zinc sulphate, centrifuged and 50 μl of the clear supernatant was analyzed by HPLC.

Transport Studies

The transport studies were performed directly on the filter inserts. Filter inserts were rinsed with Hank's balanced salts containing 15 mM glucose (transport buffer) and Caco-2 cells were allowed to equilibrate at 37 °C for 15 min under a 10% CO_2-atmosphere. The integrity of the monolayer was checked at the beginning and at the end of each experiment by determination of the transepithelial electrical resistance (TEER, EVOM, WPI, Germany).

The TRH containing transport solution (1.5 ml) was added to the apical side of the monolayers, and transport buffer without TRH was added to the basal chamber (2 ml for PETP inserts and 2.5 ml for PC inserts to keep liquid levels constant). After 5, 10, 15, 30, 45, 60, 90, 120 min, 500 μl samples were withdrawn from the receiver chambers and replaced by fresh transport buffer. The pH-dependence of the transepithelial transport was measured using Dulbecco's PBS as medium, containing 5 mM D-glucose at different pH-values without a CO_2-atmosphere.

Data Treatment

Permeability coefficients (P_{eff}) were calculated using the following equation:

$$P_{eff} = \frac{dc*V}{dt*A*c_0} \quad [cm/s] \tag{1}$$

where dc/dt is the flux across the monolayer (μM/min), V the volume of the receiver chamber (ml), A the surface area of the monolayer (cm^2), and c_0 the initial concentration (mM) in the donor compartment. The flux across the monolayer was calculated from the slope of the regression line describing the amount transported versus time (which was linear up to 120 min) [10]. The nonpassive transport component is characterized by a Michaelis-Menten kinetic using following equation:

$$V = \frac{V_{max}*[S]}{K_m + [S]} + k_d*[S] \tag{2}$$

with the Michaelis-Menten constant K_m, the maximum transport rate V_{max}, the initial concentration of substrate [S] and the first order rate constant for passive transport k_d.

Statistical analysis was performed using two-sided independent t-test.

Sample Analysis

The radioactive samples were analyzed using a liquid scintillation counter (Hewlett Packard Tricarb 4660).

All other substrates and metabolites were determined by a reversed-phase HPLC procedure. The HPLC system consisted of a Merck Hitachi pump (L-6200A), an autosampler (AS-2000A) and a column oven T-6300 adjusted to 30 °C. The variable UV-detector from Kontron (UVIKON 720LC) operated at 220 nm for all substrates. Data acquisition and processing was performed on a Merck Hitachi HPLC-Manager (D-6000). The separation of TRH and TRH-OH was achieved on a 12.5-cm × 4-mm reversed-phase Superspher 100 RP-18 column (4 μm particles size, E. Merck, Darmstadt, Germany) using a gradient procedure with a mixture of acetonitril and 0.1% (v/v) trifluoroacetic acid as described previously [9]. The separation of Z-Gly-L-Pro-L-Ala and Z-Gly-L-Pro was accomplished on a reversed-phase Lichrospher RP-18 column (25 cm × 4 mm, E. Merck, Darmstadt, Germany) with a solvent system (acetonitril, 0.1% (v/v) phosphoric acid) under same conditions as TRH.

RESULTS

Metabolism of TRH in Caco-2 MR Homogenates (Passage 89-99)

TRH was rapidly deamidated in the Caco-2 homogenates at 37 °C, (Figure 1). TRH-OH was formed by an enzymatic reaction following first order kinetics, with a rate constant of 'k = 2.8×10^{-3} s^{-1}. Due to further degradation, mass balance of TRH-OH was not complete and the concentration decreased on prolonged incubation.

In Caco-2 cell homogenates the model substrate for prolyl-endopeptidase, Z-Gly-Pro-Ala, was cleaved to Z-Gly-Pro the anticipated metabolite. The rate constant for the deamidation was in the same range 'k = 1.9×10^{-3} s^{-1}. The reaction scheme is given in figure 2. Both TRH and Z-Gly-Pro-Ala were stable in transport buffer for more than 2 hours.

When TRH was incubated with intact Caco-2 cells no metabolic degradation products were detected by our HPLC method in neither the apical nor the basolateral compartment. In both cases only one peak was observed with a retention time of 6.52 ± 0.18 min, characteristic for TRH.

Transport of TRH in Caco-2 MR Monolayers (Passage 89-99)

The permeation rate of TRH transport through Caco-2 monolayer is linear over a period of at least 120 min. From the slope of the concentration time curve permeability coefficients were determined according to equation (1). At a concentration of 3 mM a permeability coefficient P_{eff} = 8.26×10^{-6} cm/s was obtained. There was a small, but a statisti-

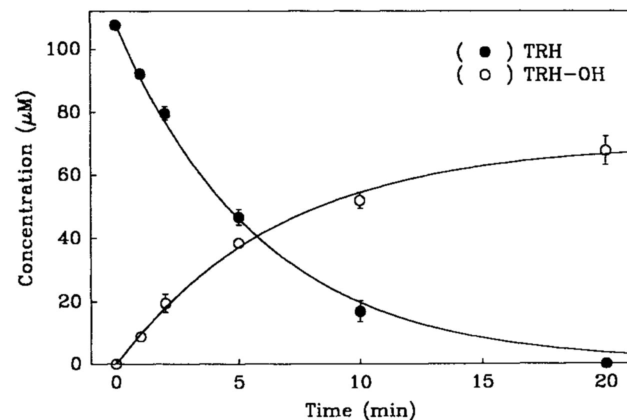

figure 1: Concentration time profiles of TRH and its metabolite TRH-OH in Caco-2 MR homogenates. Error bars represent standard deviation of the mean value for n = 3.

Z–Gly–L–Pro–L–Ala → Z–Gly–L–Pro

TRH (pyro–L–Glu–L–His–L–Pro–NH_2) → TRH–OH (pyro–L–Glu–L–His–L–Pro)

figure 2: Structures of TRH and Z-Gly-Pro-Ala and their respective metabolites in Caco-2 homogenates.

cally significant difference ($p < 0.001$) in transport from the basolateral to the apical compartment $P_{eff} = 6.43 \times 10^{-6}$ cm/s (Table I).

A large excess of the dipeptide Gly-Pro under isoosmotic conditions led to a decrease in the transport rate to 78%. Lowering the temperature from 37 °C to 4 °C reduced the flux to 54%. A large excess (18 ×) of the tripeptide Z-Gly-Pro-Ala, as well as a removal of D-glucose from the buffer, did not influence the transport rates. Moreover, supplementing the transport buffer with 1 mM sodium azide, a metabolic inhibitor of the oxidative phosphorylation, decreased flux of TRH. Permeation of TRH was dependent on sodium ions. Replacement of Na^+ by K^+ reduced P_{eff} by 74%. As further control experiments co-transport of Z-Gly-Pro and TRH as well as Z-Gly-Pro-Ala and TRH were investigated at a substrate concentration of 3 mM. Z-Gly-Pro is an inhibitor of TRH transport and led to a reduction of P_{eff} by 79%, whereas Z-Gly-Pro-Ala did not influence TRH permeability as expected.

Both peptides were stable under the conditions of the transport experiments and yielded permeabilities in the expected range with $P_{eff} = 9.06 \times 10^{-6}$ cm/s for Z-Gly-Pro and $P_{eff} = 7.10 \times 10^{-6}$ cm/s for Z-Gly-Pro-Ala.

The concentration dependence of the TRH transport rates in Caco-2 cells is demonstrated in figure 3. By comparing transport rates at 37 °C and 4 °C the active transport component can be quantitatively determined. At low concentrations TRH is mainly transported in an active manner, but at concentrations ≥3 mM the passive component clearly predominates. With increasing TRH-concentration the passive transport gains further importance. After subtraction of the rate constant for passive diffusion at 4 °C the values for the Michaelis-Menten constant K_m and V_{max}, the maximum transport velocity, were calculated from the slope of the regression line. As shown in figure 3 a satisfactory fit was obtained yielding a $K_m = 1.59 \pm 0.23$ mM and a $V_{max} = 1.84 \pm 0.12$ μM/min.

The influence of the pH-conditions on the permeability of TRH are shown in figure 4. Permeability reached an optimum at pH 6 and decreased considerably when transport was studied at pH 7.4.

Transport of TRH in Caco-2 ATCC Monolayers (Passage 30-34)

A permeability of $P_{eff} = 1.43 \times 10^{-6}$ cm/s for the hydrophilic marker PEG 4000 was obtained for Caco-2 ATCC cells (table II). These values correspond well to the flux of PEG 4000 through Caco-2 MR cells (not significantly different at $p < 0.05$). Nevertheless the transport of TRH is significantly different ($p < 0.001$). TRH yielded a $P_{eff} = 3.10 \times 10^{-6}$ cm/s for 3 mM TRH. Differences in TRH permeability due to different filter materials are unlikely since the hydrophilic marker PEG 4000 shows comparable transport rates.

A large excess of Gly-Pro and cephradine under isoosmotic conditions did not significantly influence TRH permeability as outlined in figure 5. There was only a weak statistically significant difference ($p < 0.05$) in transport from the basolateral to apical compartment, which may well be within the variability of transport experiments.

Table I. Permeability Coefficients of TRH in Caco-2 MR Monolayers (Passage 89–99) Under Different Transport Conditions at 37°C

Conditions	Permeability coefficients (*10^{-6} cm/s)[a]	Relative permeability (%)[b]	Significance[c]
control	8.26 ± 0.26	100	—
temperature 4°C	4.41 ± 0.20	53	***
basolateral to apical	6.43 ± 0.15	78	**
with Gly-Pro	6.49 ± 0.58	78	**
without glucose	8.14 ± 0.17	99	n.s.
without Na^+	2.14 ± 0.06	26	***
with 1 mM NaN_3	7.18 ± 0.22	87	*
with Z-Gly-Pro	5.89 ± 0.18	71	**
with Z-Gly-Pro-Ala	8.09 ± 0.28	98	n.s.
transport of Z-Gly-Pro	9.06 ± 0.17	110	*
transport of Z-Gly-Pro, basolateral to apical	7.10 ± 0.25	78[d]	**
transport to Z-Gly-Pro-Ala	5.68 ± 0.32	69	***

[a] Mean ± SD, n = 3
[b] Based on control experiment.
[c] Significant at p = 0.05 (*); p = 0.001 (**); p = 0.0001 (***)
[d] Based on transport of Z-Gly-Pro

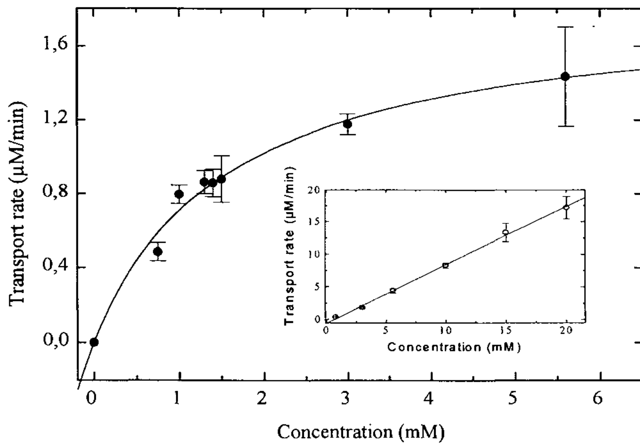

figure 3: Concentration dependence of TRH transport across Caco-2 MR cell monolayers after correction of the nonsaturable component. The inset shows TRH transport rates across Caco-2 MR cell monolayers at 4 °C. Error bars represent standard deviation of the mean value for n = 3.

As shown in figure 4 the transport of TRH across Caco-2 MR cells compared with ATCC cells is quite similar at pH 7.5. However an influence of the pH-conditions on the permeability of TRH as well as an optimum at pH 6.0 could not be confirmed with Caco-2 ATCC cells.

DISCUSSION

Metabolism of TRH in Caco-2 Cell Monolayers at Different Passage Numbers

Stability of TRH in Caco-2 homogenates was not very pronounced. We determined a half-life of ca. 4.1 min. It is well known that TRH is rapidly deamidated in human plasma yielding a half-life of ca. 9 min. The first metabolite TRH-OH shows a half-life of 27 min [11]. Enzymatic deamidation of TRH is catalyzed by a cytosolic prolyl-endopeptidase, cleaving the C-terminal prolyl-amid. A model substrate for prolyl-endopeptidase Z-Gly-Pro-Ala [12] was cleaved in Caco-2 homogenates comparable to rabbit gut homogenates, where a half-life of 7 min was observed [9].

Table II. Permeability Coefficients and Transepithelial Electrical Resistance in Differently Passaged Caco-2 Monolayers

	Caco-2 MR passage number 89-99 Falcon-Inserts	Caco-2 ATCC passage number 30-34 Transwell-Inserts
TEER (ohms * cm^2)	234 ± 102	250 ± 54
	P_{eff} (*10^{-6} cm/s)[a]	P_{eff} (*10^{-6} cm/s)[a,b]
TRH 3 mM	8.26 ± 0.26	3.10 ± 0.25**
^{3}H-TRH 0.1 mM	5.54 ± 0.32	3.56 ± 0.09**
^{14}C-PEG 4000	1.23 ± 0.04	1.43 ± 0.16[n.s.]

[a] Mean ± SD, n = 3–4
[b] Significant at p = 0.05 (*); p = 0.001 (**)

When transport experiments were carried out using confluent monolayers of Caco-2 MR and Caco-2 ATCC, TRH remained intact. In agreement with Artursson et al. [5] no metabolites could be detected using the HPLC method described above. Since prolyl endopeptidase is located intracellularly [13] these findings could be rationalized in terms of a transport by the paracellular route. On the other hand we deliberately selected pH conditions (pH~6) where the dipeptide carrier should operational (vide infra). In our opinion a transcytotic pathway would also be compatible with this metabolic behaviour of TRH.

Transport of TRH in Caco-2 MR Monolayers (Passage 89-99)

The intestinal cell line Caco-2 as a model in vitro system has been frequently employed to characterize carrier-mediated transport. Caco-2 cells are known to express carrier-mediated systems for: Na^+/H^+-antiporter [14], vitamin B_{12} [15], glucose [16], amino acids [17], and dipeptides [7, 8].

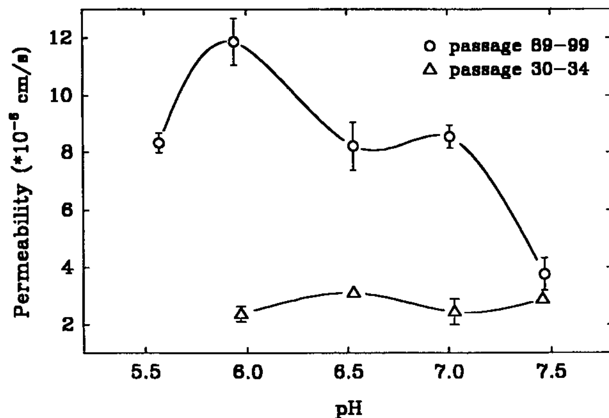

figure 4: pH-Dependence of transepithelial transport of 3 mM TRH by Caco-2 cell monolayers with different passage numbers. Each point represents the mean ± S.D. of three monolayers.

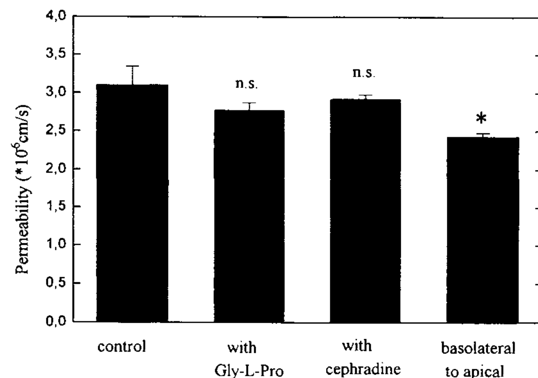

figure 5: Permeability of 3 mM TRH across Caco-2 ATCC cell monolayers under different transport conditions. Results are expressed as the mean of three determinations and t-test results expressed as differences related to the control value (n.s. not significantly different at $p < 0.05$; * $p < 0.05$). Each point represents the mean ± S.D. of three monolayers.

Available evidence indicates that peptidomimetic drugs can be transported by the di/tripeptide carrier [1].

TRH transport through Caco-2 monolayers is in good agreement with investigations in rat jejunum, where a permeability for TRH of $P = 8.9 \pm 1.1 \times 10^{-6}$ cm/s was found [9].

To characterize the transport system different factors were examined. Both temperature reduction to 4 °C and addition of a metabolic inhibitor significantly decreased the transport rate (Table I). The excess of Z-Gly-Pro, which has a higher affinity to the dipeptide carrier, inhibits the TRH transport. Transport was direction dependent and significantly higher from the mucosal to serosal side. In everted gut experiments from rat small intestine similar observations were made for TRH [18]. The sensitivity towards Na^+ replacement and effects of β-lactam antibiotics on TRH absorption are consistent with a carrier-mediated absorption mechanism. Similar results are obtained in Caco-2 monolayers (Table I); these findings in conjunction with a saturable component of TRH-transport (Fig. 5) would tend to suggest, that the dipeptide carrier system is also responsible for TRH absorption under in-vitro condition in Caco-2 monolayers.

The influence of sodium-ions on dipeptide transport is still a matter of controversy. While in experiments on cell layers [8, 19, 18] absorption seems to depend on Na^+, no effect was seen in studies with brush border membrane vesicles or uptake studies [20, 21, 7]. Our own results are showing inhibition of TRH transport on replacement of Na^+ ions by K^+, similar to Yokohama et al. [18]. Transport via the di- and tripeptide carrier is saturable and concentration dependent. Nonpassive and passive transport are independent from each other and can occur simultaneously. The nonpassive transport component is characterized by a Michaelis-Menten kinetic with the Michaelis-Menten constant K_m and the maximum transport rate V_{max} as representative parameters. In Caco-2 MR monolayers the following Michaelis-Menten parameters for TRH transport were $K_m = 1.59$ mM and $V_{max} = 1.8$ μM/min. The data obtained for K_m are in typical range for small peptide drugs transported by the dipeptide carrier [22, 23, 20, 7, 24].

At concentrations <3 mM active transport becomes prominent. In accordance with our findings of a saturable component, Nicklin et al. [25], reported that TRH crossed Caco-2 monolayers by passive transport involving a significant carrier-mediated component.

Lundin et al. obtained a $P_{eff} = 1.94 \pm 1.04 \times 10^{-6}$cm/s at pH 7.4 in Caco-2 monolayers. Taking into considerations a nearly 3-fold difference in permeability between pH 6.0 and 7.4 the data of both studies are in reasonable agreement.

TRH-transport was clearly pH-dependent with an optimum around pH 6.0. Previous transport data were obtained at pH 7.4, conditions which might favour the passive paracellular route. These conditions might be unphysiological, since the pH at the apical surface of small intestinal epithelia is estimated to be close to 5.5-6.3 [26, 27]. Cephalexin and cephradine are both transported by proton-dependent dipeptide transport carrier in-vitro and in Caco-2 monolayers with maximum transport at pH 6 [7, 8].

Moreover, the absorption characteristics of TRH under in-vivo conditions in rats, dogs [18] and humans [2] are reflected by our Caco-2 monolayer studies.

Transport of TRH in Caco-2 ATCC Monolayers (Passage 30-34)

Although the flux of the hydrophilic marker PEG 4000 through Caco-2 cells is quite similar in both Caco-2 cell clones, the transport of TRH is significantly different (Table II). The active component found in Caco-2 MR cells could not be confirmed in this case. Transport is not direction dependent, an excess of cephradine or the dipeptide Gly-Pro does not inhibit the TRH flux and there is no pH-dependence to be noted. Previous studies of other groups [5, 6], claiming TRH transport in Caco-2 to be exclusively passive in nature, could be confirmed with these ATCC cells in passage numbers 30-34.

GENERAL DISCUSSION

We have demonstrated striking differences in TRH transport characteristics when we compare Caco-2 cells from different sources. In both cases confluent monolayers with comparable transepithelial electrical resistances and ^{14}C-PEG 4000 permeability coefficients are obtained. These data are difficult to reconcile. One possible explanation is the heterogeneity of Caco-2 cells, a feature not unexpected for tumor cell cultures [28]. Isolated clones have shown significant differences in transepithelial electrical resistances and in the expression of the bile acid transporter [29]. It is also shown that enzyme activities in Caco-2 progressively change after passage 70 [28]. Moreover, different support membranes seem to influence cell densities and transport properties [30, 31]. More information on the expression and regulation of active transport systems in the human colon carcinoma cell line Caco-2 is clearly needed. TEER and permeability of hydrophilic marker substances, such as PEG or mannitol are valuable controls for defining passive transport characteristics. When active transport is suspected, these criteria are not sufficient. The Caco-2 MR cell clone would reflect more closely the in vivo absorption of TRH [2, 3], which is characterized by a carrier-mediated saturable absorption.

While in vitro cell culture systems for mechanistic studies of intestinal peptide transport and metabolism are clearly needed, their validation and standardization is an issue that deserves more attention.

REFERENCES

1. P. L. Smith, D. A. Wall, C. H. Gochoco and G. Wilson. (D) Routes of delivery: Case studies. (5) Oral absorption of peptides and proteins. *Adv. Drug Delivery Rev.* 8:253-290 (1992).
2. S. Yokohama, K. Yamashita, H. Toguchi, J. Takeuchi and N. Kitamori. Absorption of thyrotropin-releasing hormone after oral administration of TRH tartrate monohydrate in the rat, dog and human. *J. Pharmacobio-Dyn.* 7:101-111 (1984).
3. L. Duntas, F. S. Keck, U. Loos and E. F. Pfeiffer. Pharmakokinetik und Pharmakodynamik von Protirelin (TRH) beim Menschen. *Dtsch. Med. Wschr.* 113:1354-1357 (1988).
4. H. Bundgaard. (C) Means to enhance penetration. (1) Prodrugs as a mean to improve the delivery of peptide drugs. *Adv. Drug Delivery Rev.* 8:1-38 (1992).
5. S. Lundin, J. Moss, H. Bundgaard and P. Artursson. Absorption of thyrotropin-releasing hormone (TRH) and a TRH prodrug in a human intestinal cell line (Caco-2). *Int. J. Pharm.* 76:R1-R4 (1991).
6. D. T. Thwaites, B. H. Hirst and N. L. Simmons. Passive trans-

epithelial absorption of thyrotropin-releasing hormone (TRH) via a paracellular route in cultured intestinal and renal epithelial cell lines. *Pharm. Res.* **10**:674-681 (1993).
7. A. H. Dantzig and L. Bergin. Uptake of the cephalosporin, cephalexin, by a dipeptide transport carrier in the human intestinal cell line, Caco-2. *Biochim. Biophys. Acta* **1027**:211-217 (1990).
8. K. I. Inui, M. Yamamoto and H. Saito. Transepithelial transport of oral cephalosporins by monolayers of intestinal epithelial cell line. Caco-2: Specific transport systems in apical and basolateral membranes. *J. Pharm. Exp. Ther.* **261**:195-201 (1992).
9. J. Moss, A. Buur and H. Bundgaard. Prodrugs of peptides. 8. In vitro study of intestinal metabolism and penetration of thyrotropin-releasing hormone (TRH) and its prodrugs. *Int. J. Pharm.* **66**:183-191 (1990).
10. A. R. Hilgers, R. A. Conradi and P. S. Burton. Caco-2 cell monolayer as a model for drug transport across the intestinal mucosa. *Pharm. Res.* **9**:902-910 (1990).
11. J. Moss and H. Bundgaard. Kinetics and pattern of degradation of thyrotropin-releasing hormone (TRH) in human plasma. *Pharm. Res.* 7:751-755 (1990).
12. R. Walter and T. Yoshimoto. Postproline cleaving enzyme: kinetic studies of size and stereospecificty of its active site. *Biochemistry* 17:4139-4144 (1978).
13. S. Wilk. Minireview prolyl endopeptidase. *Life Sci* **33**:2149-57 (1983).
14. R. Wali, C. Baum, M. Sitrin and T. A. Brasitus. Regulation of protein kinase C, intracellular Ca^{2+} and Na^+/H^+ exchange in Caco-2 cells by $1,25(OH)_2D_3$. *Gastroenterology* **96**:A534 (1989).
15. C. J. Dix, H. Y. Orbay, I. F. Hassan and G. Wilson. Vitamin B_{12} transport through polarized monolayers of a colon carcinoma cell line. *Biochem. Soc. Trans.* **15**:443-440 (1987).
16. A. Blais, P. Bissonnette and A. Berteloot. Common characteristics for Na-dependent sugar transport in Caco-2 cells and human fetal colon. *J. Membr. Biol.* **99**:113-125 (1987).
17. M. Hu and R. T. Borchardt. Mechanism of L-methyldopa transport through a monolayer of polarized human epithelial cells (Caco-2). *Pharm. Res.* 7:1313-1319 (1990).
18. S. Yokohama, T. Yoshioka, K. Yamashita and N. Kitamori. Intestinal absorption mechanism of thyrotropin-releasing hormone. *J. Pharmacobio-Dyn.* 7:445-451 (1984).
19. M. Hu and R. T. Borchardt. Transport of a large neutral amino acid in a human intestinal epithelial cell line (Caco-2): Uptake and efflux of phenylalanine. *Biochim. Biphys. Acta* **1135**:233-244 (1992).
20. M. Das and A. N. Radhakrishan. Studies on a wide-spectrum intestinal dipeptide uptake system in the monkeys and in the human. *Biochem. J.* **146**:133-139 (1975).
21. V. Ganapathy and F. H. Leibach. Role of pH gradient and membrane potential in dipeptide transport in intestinal and renal brush-border membrane vesicles from the rabbit. Studies with L-carnosine and glycyl-L-proline. *J. Biochem. Chem.* **258**: 14189-14192 (1983).
22. D. I. Friedman and G. L. Amidon. Characterization of the intestinal transport parameters for small peptide drugs. *J. Contr. Rel.* **13**:141-146 (1990).
23. M. Muranushi, T. Yoshikawa, M. Yoshida, T. Oguma, K. Hirano and H. Yamada. Transport characteristics of cefibuten, a new oral cephem, in rat intestinal brush-border membrane vesicles: Relationship to oligopeptide and amino β-lactam transport. *Pharm. Res.* 6:308-312 (1989).
24. S. A. Adibi and M. R. Soleimanpour. Functional characterization of dipeptide transport system in human jejunum. *J. Clin. Invest.* **53**:1368-1374 (1974).
25. P. L. Nicklin and W. J. Irwin. Thyrotropin-releasing hormone transport across monolayers of human intestinal absorptive (Caco-2) cells in-vitro. *J. Pharm. Pharmacol.* 43:103P (1991).
26. T. Shimada and T. Hoshi, Na^+-dependent elevation of the acidic cell surface pH (microclimate pH) of rat jejunal villus cells induced by nucleotides and phorbol ester: possible mediators of the regulation of the Na^+/H^+ antiporter. *Biochim. Biophys. Acta* **937**:328-334 (1988).
27. M. L. Lucas, W. Schneider, F. J. Haberich and J. A. Blair. Direct measurement by pH-microelectrode of the pH microclimate in rat proximal jejunum. *Proc. R. Soc. Lond. B.* **192**:39-48 (1975).
28. A. Zweibaum. Differentiation of human colon cancer cells. In G. Wilson, S. S. Davis, L. Illum, A. Zweibaum (eds.), *Pharmaceutical applications of cell and tissue culture to drug transport,* Plenum Press, New York and London, 1990, pp. 27-38.
29. S. Woodcock, I. Williamson, I. Hassan and M. Mackay. Isolation and characterization of clones from the Caco-2 cell line displaying increased taurocholic acid transport. *J. Cell Sci.* **98**: 323-332 (1991).
30. P. Nicklin, B. Irwin, I. Hassan, I. Williamson and M. Mackay. Permeable support types influence the transport of compounds across Caco-2 cells. *Int. J. Pharm.* 83:197-209 (1992).
31. T. Sergent-Engelen, C. Halleux, E. Ferrain, H. Hanot, R. Legras and Y. J. Schneider. Improved cultivation of polarized animal cells on culture inserts with new transparent polyethylene terephthalate or polycarbonate microporous membranes. *Biotechnology Techniques* 4:89-94 (1990).