Passive Transepithelial Absorption of Thyrotropin-Releasing Hormone (TRH) via a Paracellular Route in Cultured Intestinal and Renal Epithelial Cell Lines

David T. Thwaites,[1] Barry H. Hirst,[1,2] and Nicholas L. Simmons[1]

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Transport studies using intestinal brush-border membrane vesicles isolated from rats and rabbits have failed to demonstrate proton- or Na^+-dependent carrier-mediated transport of thyrotropin-releasing hormone (TRH), despite a pharmacologically relevant oral bioavailability. To examine the hypothesis that reported levels of oral bioavailability reflect predominately a paracellular rather than transcellular route for transepithelial transport of TRH, we have studied TRH transport in cultured epithelial cell types of intestinal (Caco-2 and T_{84}) and renal (MDCK I, MDCK II, and LLC-PK_1) origin, whose paracellular pathways span the range of permeability values observed in natural epithelia. Transport of TRH across monolayers of intestinal Caco-2 cells was similar to the flux of mannitol (~1–4% per 4 hr), and unlike other putative substrates for the di-/tripeptide carrier, apical-to-basolateral transport was not increased by the presence of an acidic pH in the apical chamber. TRH transport did not show saturation, being uneffected in the presence of 20 m*M* cold TRH. In each cell type studied TRH and mannitol transport were similar and positively correlated with the conductance of the cell layers, consistent with a passive mechanism of absorption. This evidence suggests that, providing that a peptide is resistant to luminal hydrolysis, small but pharmacologically significant amounts of peptide absorption may be achieved by passive absorption across a paracellular route.

KEY WORDS: thyrotropin-releasing hormone (TRH); intestinal absorption; peptide transport; paracellular permeability; Caco-2 cells; MDCK cells; epithelial permeability.

INTRODUCTION

Thyrotropin-releasing hormone (TRH) exerts biological activity upon oral administration (1,2). In everted sacs of rat intestine *in vitro,* TRH absorption was suggested to be facilitated by a Na^+-dependent carrier-mediated mechanism located solely in the upper small intestine (3). In the preceding paper (4) we failed to find evidence for Na^+- or H^+-dependent carrier-mediated transmembrane absorption of TRH. An alternative possibility, therefore, is that intact TRH absorption is predominately passive and occurs through a paracellular route between the cells.

To examine the hypothesis that TRH transport is predominately paracellular in nature, we have chosen to study TRH transport using cultured epithelial cell monolayers. A range of epithelial cell lines (intestinal or renal in origin) that possess a wide and distinct variation in the properties (permeability and selectivity) of the paracellular route was chosen. These include two intestinal epithelial cell lines, T_{84} and Caco-2, derived from human adenocarcinomas of the colon (5–7). T_{84} cells have medium/high transepithelial resistance [circa 1000 $\Omega.cm^2$ (5)] typical of tight epithelia (and thus with a restricted paracellular route). However, some authors have found more variable transepithelial characteristics [300–1000 $\Omega.cm^2$ (6)] in T_{84} cell layers. Caco-2 cell layers have intermediate resistance values of circa 300–1000 $\Omega.cm^2$ (7). Three renal cell lines were also used: MDCK (Strain II) (8) and LLC-PK_1 cells (9), which are leaky epithelia but with cation-selective and nonselective paracellular pathways, respectively; and MDCK (Strain I) cells (8), which form epithelial layers of extremely high electrical resistance (>2000 $\Omega.cm^2$). In addition, to measurement of transepithelial TRH transport, we have made concurrent measurements of transepithelial mannitol transport. Mannitol is restricted to transport via extracellular routes and has proved to be of considerable utility in quantification of paracellular pathway modification in intestinal model epithelia such as the T_{84} cell line (6).

The possibility of transcellular transport of peptides by specific transcellular transport systems in the model epithelia is not excluded. For example, the human intestinal colorectal line Caco-2 expresses a number of brush-border membrane transporters including the intestinal di-/tripeptide carrier (10). These cells not only accumulate the orally active cephalosporin, cephalexin (10), but also show transepithelial transport of a number of cephalosporins in the presence of an inwardly directed H^+ gradient (11). The presence of a specific secondary active transport might result in net transepithelial transport. Net flux (if any) can be detected by measurements of bidirectional (apical-to-basal and basal-to-apical) TRH fluxes.

MATERIALS AND METHODS

Materials

[^{3}H]TRH ([L-proline-2,3,4,5-^{3}H(N)]-(L-pyroglutamyl-L-histidyl-L-proline amide); sp act, 127 Ci/mmol) was from NEN/DuPont (NET-577; Batch Nos. 2649-248, 2649-257, 2802-140, and 2818-113), and D-[1-^{14}C]mannitol (sp act, 60 mCi/mmol) from Amersham. TRH and TRH-OH were obtained from Bachem (UK), and His-Pro and cyclo(His-Pro) from Bachem Feinchemikalien AG (Switzerland). Acetonitrile (HPLC grade S) was supplied by Rathburn Chemicals (Scotland) and trifluoro acetic acid (TFA; HiPerSolv grade) by BDH. All other chemicals were from BDH and were of the highest quality available. Cell culture media, supplements, and plastic were all supplied by Life Technologies.

Cell Culture

Caco-2 cells were cultured in DMEM (with 4.5 g/L glu-

[1] Gastrointestinal Drug Delivery Research Centre and Department of Physiological Sciences, University of Newcastle upon Tyne, Medical School, Newcastle upon Tyne NE2 4HH, U.K.

[2] To whom correspondence should be addressed at Gastrointestinal Drug Delivery Research Centre, Medical School, Newcastle upon Tyne NE2 4HH, England.

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cose), with 1% nonessential amino acids, 2 m*M* L-glutamine, 10% (v/v) fetal calf serum, and gentamicin (50 μg/mL) (7). T_{84} cells were grown in a 1:1 mixture of Dulbecco's modified Eagle's medium (DMEM) and Ham's F12 with 5% (v/v) newborn calf serum, 40 IU/mL penicillin, and 40 μg/mL streptomycin (5,6). MDCK Strain I (passage 77) and Strain II (passage 143) cells were maintained in serial culture in Eagle's minimum essential medium containing Earle's salts and supplemented with fetal calf serum (2%, v/v), horse serum (8%, v/v), L-glutamine (4 m*M*), nonessential amino acids (2%, v/v), and kanamycin (200 U/mL) (8). LLC-PK_1 cells were cultured in Medium 199 supplemented with 1% (v/v) fetal calf serum and gentamicin (50 μg/mL) (9).

Epithelial monolayers of each cell type were prepared by seeding at a high density (5×10^5 cells/cm^2) onto 25-mm Anopore membrane (NUNC, Life Technologies) tissue culture inserts (4 cm^2) (12). The filters were coated with rat tail collagen for T_{84} cells. Epithelial confluence was assessed by microscopy and by determination of transepithelial resistance (measured at 37°C) using the WPI Evometer fitted with chopstick electrodes to allow current passage and potential sensing. Cells were maintained at 37°C in an atmosphere of 5% CO_2 in air for varying times until confluent layers were formed: MDCK I [2206 ± 197 Ω.cm^2 (X ± SE; n = 19; range, 960–3720] and MDCK II cells (503 ± 35 Ω.cm^2; n = 12; range, 280–640), 2–4 days; T_{84} cells (676 ± 72 Ω.cm^2; n = 10; range, 400–1040), 14 days; Caco-2 cells (951 ± 38 Ω.cm^2; n = 39; range, 640–1492), 18–19 days; and LLC-PK_1 cells (567 ± 39 Ω.cm^2; n = 11; range, 360–720), 5–8 days. In general, values of resistance grown on Anopore tissue culture inserts are high compared with Millipore cellulose nitrate [e.g., MDCK II (8) or LLC-PK_1 (9)]. This results from a more flattened appearance on Anopore membranes (13), thus reducing the effective junctional cross-sectional area. All layers were refed with appropriate media every 2–3 days.

Pilot studies with the Caco-2 cells were performed with 12-mm Millicell HA filters (Millipore). However, the binding to the cellulose filter membrane was equivalent to or greater than the transport. When the radiolabel was placed in the apical chamber, the filter binding of TRH was 0.03% (n = 6) and that of mannitol was 0.03% (n = 6). When the label was placed in the basolateral chamber, binding of TRH was 0.93 ± 0.12% (n = 6) and that of mannitol was 0.45 ± 0.06% (n = 6). TRH binding was greatest in the presence of acidic pH but pH had no effect on transport.

Transepithelial Transport Studies

Transepithelial flux determinations were made essentially as described previously (12). Most experiments were performed in Krebs buffer, pH 7.4 (137 m*M* NaCl, 5.4 m*M* KCl, 1.0 m*M* $MgSO_4$, 0.34 m*M* KH_2PO_4, 0.3 m*M* NaH_2PO_4, 2.8 m*M* $CaCl_2$, 10 m*M* glucose, and 10 m*M* HEPES/Tris), at 37°C. However, experiments with T_{84} cells were carried out in the appropriate culture buffer (see above). The effect of extracellular pH was also studied in Caco-2 cells. In these experiments 10 m*M* MES/Tris (pH 6.0) replaced 10 m*M* HEPES/Tris (pH 7.4). Functional epithelial layers in tissue culture inserts were washed in the serum-free Krebs buffer and placed in six-well plates. Aliquots of Krebs buffer were then placed in the upper filter cup (apical solution) and the tissue culture well (basolateral solution). Apical and basolateral buffer compositions were identical (except when stated otherwise). Radiolabeled TRH [0.5 μCi/mL; 1.0 μ*M* (except when stated otherwise)] and mannitol (0.5 μCi/mL; 4.6 μ*M*) were then added to the apical or basolateral chamber (to measure apical-to-basolateral flux and basolateral-to-apical flux, respectively). At the end of the 4-hr incubation period, 1 mL each of the apical and basal fluids was removed for determination of ^3H and ^{14}C activities (dpm) by liquid scintillation spectrometry using a Beckman LS 5000CE. Appropriate corrections were made for quench, channel spillover, and efficiency. Fluxes into the contralateral chamber are expressed as a percentage of the total activity initially placed in the apical or basal compartments. The stability and integrity of the layers were determined before and after the experiments by measurement of the transepithelial resistance (Evometer, WPI see above). The percentage of the radiolabel associated with the cells/filter at the end of the experiment was also determined. The filters were washed in 4 × 500-ml volumes of Krebs buffer (pH 7.4) to remove any loosely associated radiolabel. The filters were then removed from the tissue culture insert and processed accordingly.

HPLC Analysis of [^3H]TRH

Samples from tissue culture experiments were subjected to analysis by reversed-phase high-performance liquid chromatography (HPLC) as described for samples from membrane vesicle experiments (4). Two hundred microliters of fluid from each chamber was diluted with 800 μL of ice-cold 0.08% TFA (buffer A) to prevent peptide degradation and stored at −20°C for HPLC analysis. To control for any degradation during the periods of incubation and storage, control samples were treated similarly. Samples were adjusted to include 20 μg each of standards of TRH and its main degradation products [TRH-OH, His-Pro, and cyclo(His-Pro)]. The standard/sample mixture (50–900 μL) was separated using a Nova-pak C_{18} column (Waters). Samples were loaded at 0% B (70% acetonitrile in 0.08% TFA), 100% A and fractionated using a gradient (Waters Model 660 solvent programmer, program 7) of 0–20% B in 10 min (flow rate, 2 mL/min). Absorbance was measured at 214 nm. Fractions (1 mL) were collected and the radioactivity in each fraction was determined by scintillation counting.

Statistical Analyses

Results are expressed as mean ± 1 SE. Significance of difference between mean values was investigated by analysis of variance with significance levels set at $P < 0.05$.

RESULTS

Transepithelial Transport of TRH: Studies with Caco-2 Cells

The transport of TRH and mannitol was compared in confluent layers of Caco-2 cells. No evidence for a net (carrier-mediated) transport of TRH was observed, transport being linear up to 4 hr. Transport of TRH (using cell layers 10 days after seeding) was similar in both apical-to-basal and basal-to-apical directions and was not significantly different

from mannitol transport (Fig. 1). For the expression of some transport processes, longer cultivation of the cells on permeable supports is required. TRH and mannitol transport were also similar in both directions in Caco-2 monolayers used 19 days after seeding (Fig. 1). However, TRH and mannitol transport were significantly reduced, compared to transport across layers used 10 days after seeding, which may be explained by the higher transepithelial electrical resistance of layers cultivated for 19 days (Fig. 1).

Subsequent experiments, performed on separate sets of Caco-2 layers 19 days after seeding onto permeable supports, were performed to examine possible saturation of apical-to-basal TRH transport. The transepithelial electrical resistance for these Caco-2 layers was 888 ± 24 $\Omega.cm^2$. The concentration of radiolabeled peptide present in the apical compartment was 4.2 n*M*. In the control experiments 1.28 ± 0.07% (n = 4) of labeled peptide and 0.63 ± 0.09% of [^{14}C]mannitol crossed the layers during the 4-hr incubation period. In the presence of an excess of "cold" TRH (experimental concentration of 4.4 m*M*), no effect was observed on either [^{3}H]TRH (1.28 ± 0.14%; n = 6) or [^{14}C]mannitol (0.66 ± 0.17; n = 6) transport. Experiments were also performed with 20 m*M* excess cold TRH. [^{3}H]TRH fluxes in the absence (0.36 ± 0.04%; n = 3) and presence (0.26 ± 0.03%; n = 3) of 20 m*M* TRH were not significantly different, or different from the fluxes of [^{14}C]mannitol in the same circumstances [0.42 ± 0.04 and 0.26 ± 0.01% (n = 3), respectively]. In these experiments constant osmolarity was maintained by changes in the mannitol concentration.

Transport of [^{14}C]cephalexin, a substrate of the intestinal di-/tripeptide carrier, in Caco-2 cell layers, requires apical acidity (10). Transport of TRH and mannitol in both the apical-to-basal and the basal-to-apical directions across Caco-2 cell layers 19 days after seeding were equivalent in the presence of apical or basal acidity (pH 6.0), respectively (Fig. 2). Thus even when external conditions are manipulated to optimise di-/tripeptide transport, there is no evidence to suggest that TRH transport is by any route other than that shared by mannitol, i.e., TRH transport is restricted to the paracellular route.

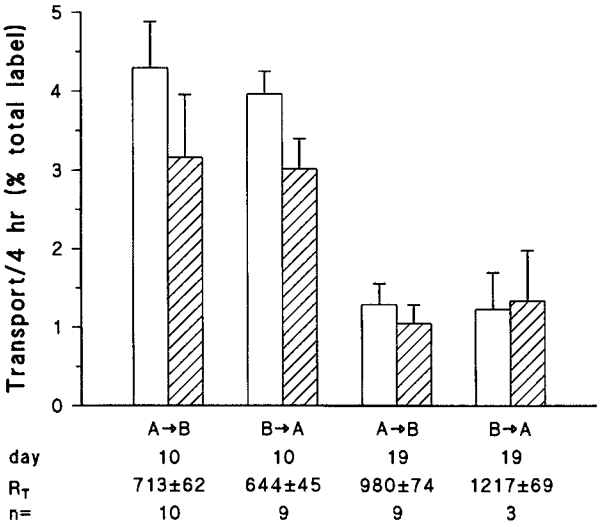

Fig. 1. Transport of [^{3}H]TRH (open columns) and [^{14}C]-mannitol (hatched columns) across epithelial cell monolayers of Caco-2 cells. Transport was compared in both the apical-to-basal (A → B) and the basal-to-apical (B → A) directions using layers 10 or 19 days after seeding. Results are expressed as X ± SE, with n values and electrical resistances (R_T) given.

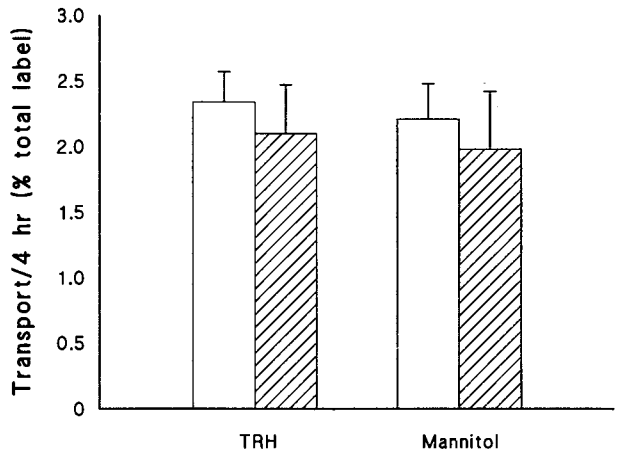

Fig. 2. The effect of an acidic gradient on the transport of [^{3}H]TRH and [^{14}C]mannitol across epithelial cell monolayers of Caco-2 cells, 19 days after seeding. The pH of the solution on the side on which the radiolabeled tracers were added was adjusted to 6.0. The pH in the contralateral chamber was held at 7.4. Transport was measured in both apical-to-basal (open columns) and basal-to-apical (hatched columns) directions. Results are expressed as X ± SE; n = 5–6 layers.

Transepithelial TRH Transport Is Correlated with the Magnitude of the Paracellular Pathway

The transport of TRH and mannitol was compared in a variety of other epithelial cell lines, covering a range of transepithelial resistances. In the intestinal cell line T_{84}, the transport of TRH across the layers was similar in both the apical-to-basal and the basal-to-apical directions and similar to the mannitol fluxes (Fig. 3a). The high flux of both substrates appeared to be related to transepithelial resistance (676 ± 72 $\Omega.cm^2$; n = 10).

In experiments with MDCK I (high-resistance) cells the transepithelial transport of both TRH and mannitol was low (<1%) (Fig. 3b) and showed no significant asymmetry. The low transport of both substrates reflected the "tightness" of the layers as measured by transepithelial resistance, which in MDCK I cells (2978 ± 135 $\Omega.cm^2$; n = 10) was the greatest of the cell lines used in this study. Transport across monolayers of MDCK II cells (low resistance) was greater than across MDCK I cells in both directions (Fig. 3c), a reflection of the "leakiness" of these cells (503 ± 35 $\Omega.cm^2$; n = 12). Transport of both TRH and mannitol tended to be greater in the apical-to-basal direction, a reflection of inter-filter variability (these differences were not significant). However, TRH and mannitol transports in any one direction, i.e., in the same filters, were always equivalent. In LLC-PK_1 cells, the results (Fig. 3d) were similar to those observed with MDCK II layers. TRH and mannitol transports were relatively large and were equivalent in any single filter. The similarity of TRH transport across LLC-PK_1 and MDCK II cell layers suggests that the cation selectivity of

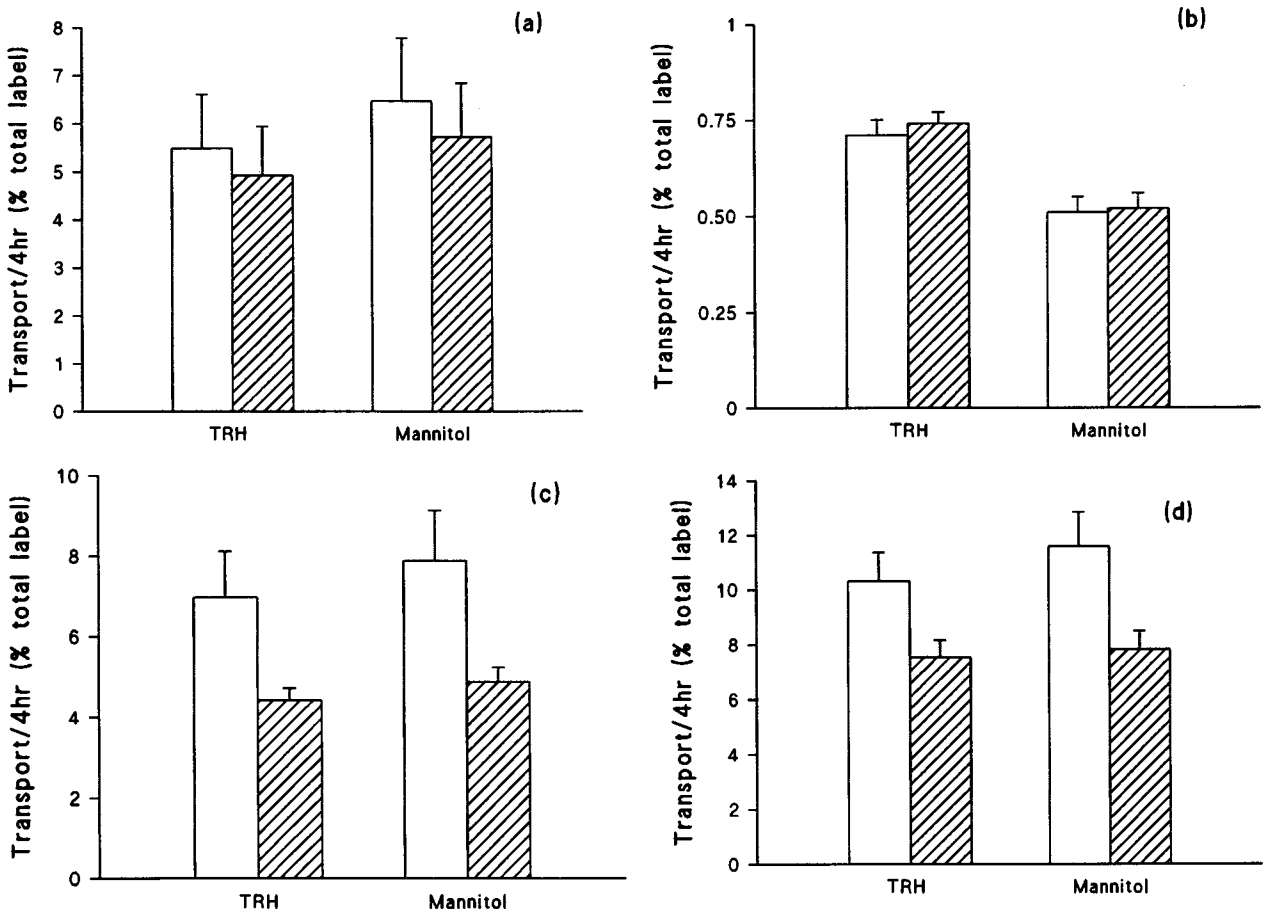

Fig. 3. Transepithelial transport of TRH and mannitol across (a) the human intestinal cell line T_{84}, the two canine renal cell lines (b) MDCK Strain I and (c) MDCK Strain II, and (d) the porcine renal cell line LLC-PK_1. Transport was measured in both the apical-to-basal (open columns) and the basal-to-apical (hatched columns) directions. Results are $X \pm SE$; $n = 4$–6.

the paracellular pathway of MDCK II cells does not play a significant role in determining transepithelial TRH transport.

The similarity between mannitol and TRH transport in all cell lines adds support to the suggestion that the paracellular route is important in TRH transport across these cell lines. The correlation between the transport of TRH or mannitol and the electrical conductance of the individual cell layers is illustrated in Fig. 4a. Below values of 1 $mS.cm^{-2}$ the magnitude of paracellular conductance is minimal and both TRH transport and mannitol transport are restricted. Above 1 $mS.cm^{-2}$ there is a linear correlation of TRH and mannitol transport with epithelial conductance (with high electrical conductance, mannitol and TRH transports are equally enhanced). Since electrical conductance is a direct measure (in these conditions) of the magnitude of the paracellular pathway, both TRH and mannitol transports are likely to be restricted to the paracellular route.

The percentage radiolabel associated with the cells/filters at the end of the incubation period was as follows: MDCK I (TRH, 0.02%; mannitol, 0.02%; $n = 9$), MDCK II (TRH, 0.02%; mannitol, 0.02%; $n = 12$), LLC-PK_1 (TRH, 0.07%; mannitol, 0.04%; $n = 11$), T_{84} (TRH, 0.08%; mannitol, 0.05%; $n = 10$), and Caco-2 (TRH, 0.06%; mannitol, 0.06%; $n = 12$). In each of the cell lines the cell-associated radioactivity was low and there was little difference between TRH and mannitol, suggesting that the TRH space is equivalent to the mannitol (extracellular) space.

Analysis of TRH After Transepithelial Transport

At the end of each incubation period, samples of apical and basolateral fluid were removed for analysis by reversed-phase HPLC. There appeared to be little or no degradation of [^{3}H]TRH placed in either the apical or the basolateral chambers during the 4-hr incubation period (Table I). Clearly, when incubated with each cell type, TRH did not undergo any substantial degradation. Although small amounts of radioactivity appeared in the fractions corresponding to His-Pro, c(His-Pro), and TRH-OH, there was little increase in the amounts over the duration of the experiment.

However, when the radiolabeled material, sampled from the contralateral chamber, was analyzed, a different pattern emerged. In the relatively "leaky" MDCK II cells ($500 \pm 74\ \Omega.cm^2$; $n = 4$), ~90.3% of the ^{3}H-material transported across the layers was in the form of intact TRH (this represents ~7.3% of the total ^{3}H label present in the apical chamber at the beginning of the incubation period). However, 6.2% of the transported ^{3}H-material, when analyzed by HPLC eluted in the void volume. The void volume-

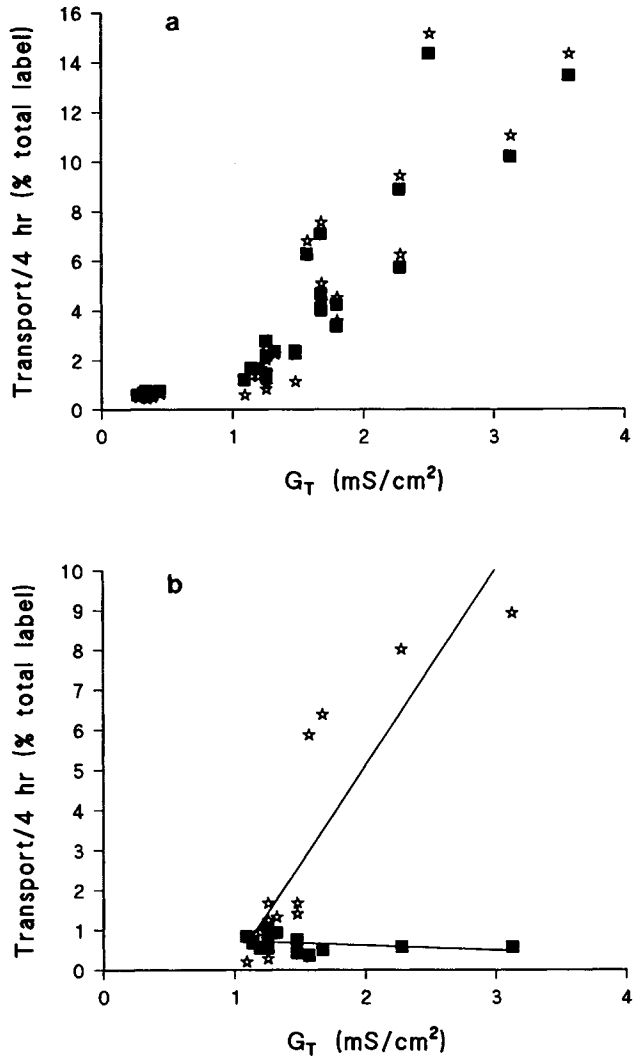

Fig. 4. (a) Transport of TRH (open stars) and mannitol (filled squares) across Caco-2, T_{84}, MDCK I, and MDCK II cells expressed as a function of the conductance (1/resistance) of the individual cell layers. Lines were fitted by linear regression analyses (not shown) for values greater than 1 mS/cm^2: TRH, $y = 5.945x - 5.482$, $r = 0.900$, $P < 0.0001$; mannitol, $y = 5.256x - 4.304$, $r = 0.897$, $P < 0.00001$. (b) The percentage of the total ^{3}H-material crossing Caco-2 and MDCK II monolayers in the form of "void volume material" (filled squares) or intact TRH (open stars), as determined by HPLC, plotted against the conductance of the individual layers. Lines were fitted by linear regression analyses: void volume material, $y = -0.118x + 0.844$, $r = -0.316$, $P = 0.25$; TRH, $y = 4.922x - 4.712$, $r = 0.885$, $P < 0.0001$.

associated ^{3}H represents only ~0.5% of the total ^{3}H label at the beginning of the experiment. Material eluting in the void volume may include low molecular weight amino acid degradation products and unidentified radioactive degradation products.

In experiments using Caco-2 cell layers showing higher transepithelial resistance (782 ± 22 Ω.cm^2; $n = 9$), the composition of the ^{3}H-material transported across the layers was quantitatively different. In these cell layers, of the transported ^{3}H-material, ~33.6 ± 6.7% ($n = 9$) eluted in the void volume, which represents 0.7 ± 0.2% of the radiolabel available for transport at the beginning of the experiment. Intact [^{3}H]TRH accounted for 57.1 ± 6.3% ($n = 9$) of the ^{3}H-material transported across these cells. This represents 1.2 ± 0.4% of the total ^{3}H-label available. In Caco-2 layers with a higher transepithelial resistance (920 Ω.cm^2) the percentages of the ^{3}H-material accounted for by void volume material increased (69.4 and 0.8% of the total label) and TRH decreased (15.8 and 0.2% of the total label). These results are illustrated in Fig. 4b, which demonstrates that the transport of HPLC-verified TRH, like mannitol (Fig. 4a), is linearly related to the electrical conductance of the layer. In contrast, the transport of the void volume material is independent of the conductance of the layer. These data suggest that, below a limiting value of epithelial conductance, TRH transport is restricted and only TRH metabolites/degradation products are able to negotiate the paracellular pathway or that TRH transport is via a cellular route in which degradation occurs. Unfortunately, the amount of radiolabeled material passing across the high-resistance MDCK I cells was too low to be accurately distinguished by this method of analysis.

DISCUSSION

There are two major pathways for transepithelial permeation: first, a transcellular route which is controlled by interfacial conditions, e.g., diffusion/solubility and/or specific membrane transporters; and second, a polar paracellular route whose anatomical location for small ions is the tight-junction lateral interspace (7,9,14,15). Epithelial transcytosis (16) (permeation across an epithelial barrier via a cellular vesicular route) is of minor importance for fluid phase ligands (see below).

Early investigations of intestinal peptide transport suggested that peptides are transported by an active Na^+-dependent mechanism (17,18). Similarly, studies of TRH absorption, using everted sacs of rat small intestine (3), indicated that TRH was also absorbed via a Na^+-dependent process. There exists several lines of evidence to suggest that the transmucosal absorption of TRH is not due to transcellular transport. In the preceding paper (4) we failed to find evidence that TRH may be accumulated within rat or rabbit proximal small intestinal brush-border membrane vesicles under either Na^+- or pH-gradient conditions. Also it is apparent that the rate of equilibration of TRH into an intravesicular space is neither accelerated under Na^+/H^+-gradient conditions nor reduced by high competing TRH concentrations, indicating that significant levels of a high-affinity facilitated diffusion pathway are unlikely to be present in the brush-border membranes of native intestinal epithelia. Finally, in the present data we fail to observe significant net TRH absorption, concentration-dependent TRH transport, or pH-accelerated TRH transport across human Caco-2 intestinal epithelial layers. Caco-2 cells express a range of specific transport systems including the proton-coupled dipeptide transporter (10,11). Proton-dependent transport of a number of cephalosporins has been reported (11). In unpublished studies, we have observed proton-dependent transport and pH-dependent cellular accumula-

Table I. Identification of [^{3}H]TRH After Incubation with Cell Cultures[a]

	TRH	TRH-OH	HP	c(HP)	Void
Standard (n = 2)	91.7	1.2	0.7	1.4	2.9
Caco-2[b] (n = 3)	93.2 ± 1.1	1.3 ± 0.2	0.9 ± 0.1	1.5 ± 0.3	0.9 ± 0.4
Caco-2[c] (n = 3)	91.0 ± 0.5	2.4 ± 0.6	0.7 ± 0.2	1.1 ± 1.4	2.1 ± 0.3
Caco-2[d] (n = 3)	92.9 ± 1.6	0.6 ± 0.3	0.3 ± 0.1	1.0 ± 0.2	1.7 ± 0.2
T_{84} (n = 3)	94.0 ± 0.5	0.7 ± 0.3	0.4 ± 0.1	1.7 ± 0.9	0.2 ± 0.1
MDCKII (n = 4)	94.9 ± 0.4	1.1 ± 0.3	0.1 ± 0.1	1.7 ± 0.4	0.9 ± 0.4

[a] The identity of the radiolabel remaining on the "drug" side after a 4-hr incubation (X ± SE).
[b] [^{3}H]TRH placed in the apical chamber (pH 6.0).
[c] [^{3}H]TRH placed in the apical chamber (pH 7.4).
[d] [^{3}H]TRH placed in the basolateral chamber (pH 6.0).

tion of the dipeptide Gly-Sar. While transport and cellular uptake of TRH were similar to those observed with mannitol, dipeptide transport and accumulation were up to 9- and 76-fold greater than mannitol. Excess cold TRH (20 mM) failed to reduce either uptake or transport of Gly-Sar.

Our findings with TRH are thus similar to those of Lundin and co-workers (19), who found no evidence of any carrier-mediated transport across Caco-2 cells under neutral conditions (pH_o = pH_i). In contrast, a recent abstract (20) suggests that a fraction (25%) of TRH transport across Caco-2 cells was carrier-mediated transport. However, it is unclear whether the latter data refer to net transport.

In our present studies we used mannitol to provide a simultaneous measurement of paracellular transport. Mannitol is restricted largely to extracellular transfer (6) but it may be taken up by cells by fluid-phase endocytosis and may be transcytosed. Measurements of transcytosis in intact MDCK Strain I epithelial layers (21) do not exclude paracellular leaks. Estimates of the magnitude of transcytosis using horseradish peroxidase (HRP) and labeled dextrans, circa 5000 daltons, give values of just 8–10 nL/cm^2.hr (21). This would comprise 0.02% of the initial dose transfer/4 hr for comparison with TRH and mannitol transport rates reported here. In MDCK epithelia (Strain I) transcytosis shows a small basal-to-apical polarity (21). In the present data mannitol transport shows no polarity in both MDCK cell strains used and the magnitude of mannitol flux exceeds that expected for transcytosis by at least 10-fold even in high-resistance epithelia. Thus though transcytosis may contribute to paracellular transport, it is quantitatively only a minor component in cultured epithelia. In natural intestinal epithelia, specialized M cells in gut-associated lymphoid tissue (GALT) may have enhanced rates of transcytosis, but their quantitative importance as compared to the whole epithelium is at present unknown (16).

Recently it has been suggested that human calcitonin may cross rat colon *in vivo* and that the route is transcellular on the basis of immunohistochemical visualization within colonocytes (22). It should be noted, however, that fluid-phase endocytosis exceeds transcytosis by an order of magnitude (21) and that calcitonin uptake was not correlated with extracellular marker uptake (see below).

Hydrophilic markers such as mannitol have been extensively used in studies of natural epithelia, and mannitol fluxes do correlate with junctional leak (6), although the epithelial barrier is heteroporous (23) and no single marker is without limitation. However, comparison of mannitol and TRH transport is consistent with TRH transport being passive, mediated by a paracellular route.

Epithelial tissues such as the jejunum and ileum are characterized by low epithelial electrical resistance (high ionic conductance) in which the majority of transepithelial ionic current flow is paracellular (24,25). The anatomical location of this paracellular shunt pathway is the tight junction/lateral space (14). In addition to high electrical conductance, the paracellular shunt may display cation selectivity as is the case in rabbit ileum (13,15). Observations made with molecules such as triaminopyrimidine [which reduce cation discrimination in intact epithelia (27)] suggest that the tight junction has an equivalent pore radius of 0.6 nm (27). Osmotic studies indicate that ileum is in fact a heteroporous barrier, cation-selective pores existing in parallel with wider neutral pores of equivalent radius 6.5 nm (23). Extrusion sites of cells from villous tips were considered a possible site for such a large equivalent pore but recent data suggest that tight junctions are maintained despite cell extrusion (28). In contrast, tissues such as colon are electrically tight and their paracellular conductance is extremely low compared to ileum. In such tissues the paracellular route is likely to be minimal (15). In the *in vitro* model systems used here the epithelial layers were confluent, as assessed by phase microscopy and by biophysical criteria, but epithelial contact with the sides of the culture inserts could provide a nonselective "wide-pore" polar route. In addition, cell mitosis, though at a very low frequency in confluent cultures, may provide a similar route. Whatever the physical basis of unrestrictive pores in natural and cultured epithelia, their existence is likely to determine transepithelial permeation of peptides such as TRH.

It is pertinent to examine how a definite conclusion of Na^+-dependent active transport could have been ascribed to TRH absorption (3). In everted sac experiments, Na^+-free high-K^+ media and metabolically compromised tissues were used (3). Under these conditions cell swelling will decrease lateral space width and increase tortuosity, thereby effectively decreasing paracellular absorption and hence reducing a small apical-to-basal TRH flux.

The ability of TRH to be transported intact across intestinal epithelia *in vivo* and *in vitro* (present data) is due largely to this peptide being relatively resistant to hydroly-

sis. There are two main pathways responsible for the degradation of TRH (29). Proline endopeptidase cleaves the Pro-NH_2 bond to release the deamidated peptide TRH-OH, while pyroglutamyl peptidase I cleaves the pGlu-His bond to release His-Pro-NH_2, which spontaneously transforms into the cyclic peptide, cyclo(His-Pro). These intracellular enzymes are both found at low concentrations in the adult rat intestine (30). Since TRH permeation is proposed to be mainly extracellular, TRH degradation during transport is limited. The amount of intact peptide that is found in the basolateral bathing solution is related to the magnitude of the paracellular conductance; when this is high, intact peptide comprises the majority of the transported peptide. Conversely, when paracellular conductance is restricted, the proportion of intact peptide is reduced. This might arise from two mechanisms. In the absence of a significant paracellular pathway TRH uptake will be restricted to a cellular (transcytotic) route in which peptide degradation occurs. Alternatively, low molecular weight contaminants in the original ^3H pool, contained in the void volume of HPLC analysis, will be less restricted in permeation compared to the intact peptide or its main degradation products [TRH-OH, cyclo(His-Pro), and His-Pro].

It is apparent from TRH transport data obtained using isolated membrane vesicles and cultured intact epithelial layers that TRH absorption is passive and occurs predominantly via a paracellular route. The location of such a paracellular route *in vivo* is where epithelia possess transepithelial resistances of 50–250 $\Omega.cm^2$, i.e., leaky epithelia such as the jejunum and ileum. Paracellular transport is minimal in so-called tight epithelia such as colon. An important conclusion arising from these studies is that pharmacologically relevant amounts of a biologically active peptide may be achieved with oral dosage despite small bioavailability via a paracellular route. Since jejunal and ileal transit is rapid compared to colonic transit, it would be anticipated that improved bioavailability might be achieved by formulations designed to achieve high local peptide concentrations close to the apical (brush-border) surface while delaying transit. Providing that pharmaceutical cost *per se* is not limiting, resistance to luminal hydrolysis may be sufficient to obtain limited oral bioavailability.

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