Thyrotropin-Releasing Hormone (TRH) Uptake in Intestinal Brush-Border Membrane Vesicles: Comparison with Proton-Coupled Dipeptide and Na^+-Coupled Glucose Transport

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The mechanism of thyrotropin-releasing hormone (pGlu-His-Pro-NH_2; TRH) uptake across the luminal membrane of intestinal enterocytes was investigated using brush-border membrane vesicles (BBMV) from rabbit duodenum and jejunum and rat upper small intestine. [^{14}C]Glucose accumulated within the intestinal vesicles (at 10 sec), in the presence of an inwardly directed Na^+ gradient, 7- to 14-fold higher than equilibrium values (65 min). The vesicles also accumulated the dipeptide [^{14}C]Gly-Sar. Dipeptide uptake was greatest in the presence of both an inwardly directed proton gradient and an inside negative membrane potential. The H^+-dependent Gly-Sar transport was not affected by the presence of an excess (46-fold) of cold TRH. In contrast to the observations with glucose and Gly-Sar, the uptake of [^{3}H]TRH after 10 or 60 sec (in each of the vesicle preparations) was not enhanced by either Na^+ or H^+ gradient conditions. The absence of vesicular accumulation of TRH was not due to peptide hydrolysis. For example, after a 60-sec incubation with rabbit jejunal BBMV no degradation of the tripeptide was evident. After 65 min, 6% of [^{3}H]TRH had undergone degradation, by deamidation, to form TRH-OH. These studies provide no evidence for the oral absorption of TRH by a Na^+- or H^+-dependent carrier system in the brush-border membrane. Previous observations of TRH absorption *in vivo* may be accounted for by passive absorption of the peptide combined with its relative resistance to luminal hydrolysis.

KEY WORDS: thyrotropin-releasing hormone (TRH); intestinal absorption; brush-border membrane; membrane vesicles; peptide transport; dipeptide transport; H^+-coupled dipeptide transport.

INTRODUCTION

Oral administration of thyrotropin-releasing hormone (TRH) in man and other animals is followed by an increase in plasma thyroid-stimulating hormone (TSH) concentrations (1–3). This indicated that sufficient TRH was absorbed intact to elicit a physiological response. An oral route of TRH administration might prove effective in a number of neuralgic conditions such as motor neurone diseases (4).

Experiments *in vitro*, using everted sacs of rat intestine to investigate transmucosal TRH transport, have suggested that TRH absorption is mediated by a Na^+-dependent carrier mechanism localized in the upper small intestine (5). These observations are consistent with early reports of dipeptide uptake into hamster jejunal rings *in vitro*, which suggested that di- and tripeptides were absorbed by a Na^+-dependent mechanism (6,7). However, recent studies using isolated purified brush-border membrane vesicles (BBMV) indicate that dipeptides are not absorbed by a Na^+-dependent process (8–10). It is now widely accepted that the brush-border proton-motive force energizes dipeptide transport (11). The differences between studies using intact tissue preparations and intestinal BBMV are reconcilable. *In vivo* there exists an acidic microclimate at the apical surface of the small intestine (12). This is maintained by the Na^+/H^+ antiport system present in the apical membrane (13,14). In intact tissue preparations this antiport system will be stimulated by the presence of Na^+ in the lumen, carrying H^+ out of the enterocytes in exchange for the inward movement of Na^+. The increase in H^+ concentration at the luminal surface enhances dipeptide transport across the apical surface via the H^+-coupled transporter. The intracellular Na^+ concentration is maintained by the (Na^+ + K^+)-ATPase at the basolateral membrane. Therefore, the Na^+ dependency of dipeptide transport detected in intact tissue preparations is an indirect effect. In BBMV, no interaction of Na^+ and H^+ on dipeptide transport is observed due to the small intravesicular volumes and the rapid dissipation of the Na^+ gradient in the absence of (Na^+ + K^+)-ATPase activity.

The aim of the present investigation was to identify and characterize directly the mechanism of TRH transport across the enterocyte apical membrane. In a comparable manner to the experiments characterizing dipeptide transport as a proton-coupled system, we used BBMV prepared from rabbit and rat small intestine. The vesicles demonstrated Na^+-coupled glucose and H^+-coupled Gly-Sar uptake. In contrast, our studies provide no evidence for either Na^+- or H^+-coupled TRH uptake across the luminal membrane of enterocytes. Alternative explanations for the mechanisms of oral TRH absorption are discussed.

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). [^{14}C]Glucose (D-[U-^{14}C]glucose; sp act, 304 mCi/mmol) and [^{14}C]Gly-Sar (L-glycyl[1-^{14}C]-sarcosine; sp act, 14 mCi/mmol) were 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 from Rathburn Chemicals (Scotland) and trifluoro acetic acid (TFA; HiPerSolv grade) from BDH. Valinomycin was from Sigma. All other chemicals were from BDH and were of the highest quality available.

Brush-Border Membrane Vesicle Preparation

Rabbits (2.75–3.50 kg) were killed by sodium pentobarbitone injection. Male Wistar rats (250–300 g) were killed by cervical dislocation. In the rabbit the proximal 40 cm (duo-

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denum) and mid 40 cm (jejunum) and in the rat the proximal 6 cm of small intestine were dissected immediately from the animal and placed in ice-cold 0.9% saline. The tissue was divided into 5- to 10-cm segments, flushed with 10 mL ice-cold saline, opened longitudinally, and blotted with filter paper to remove mucus. The mucosa was separated from the underlying muscle layers using glass slides. The mucosal scrapings from rabbit intestine were frozen in liquid N_2 and stored at −80°C until required. The rat tissue was used fresh.

Brush-border membrane vesicles were prepared using a modification of the method of Kessler *et al.* (15). Briefly, the mucosal scrapings were homogenized (1 mg/50 mL) in a buffer (pH 7.1) containing 50 m*M* mannitol and 2 m*M* Tris. After the addition of $MgCl_2$ (final concentration, 10 m*M*) the homogenate was stirred on ice for 20 min. Following sequential centrifugation steps the final pellet was resuspended in a buffer (Buffer V, pH 7.4) containing mannitol (100 m*M*), potassium gluconate (100 m*M*), $MgCl_2$ (5 m*M*), HEPES (50 m*M*), and Tris (18.1 m*M*). The protein concentration of the vesicle preparation was estimated using the method of Bradford (16).

The recovery and enrichment of the brush-border membranes during the isolation procedure were monitored using the activity of the brush border-associated enzyme alkaline phosphatase, measured with *p*-nitrophenol phosphate (pH 9.3) as substrate. The BBMV prepared from rat proximal small intestine were used in the uptake experiments at a final protein concentration between 13.3 and 16.0 mg/mL. During the isolation procedure the brush-border membranes were enriched 13.3 ± 3.3 (mean ± SE; n = 3)-fold compared with the initial tissue homogenate. The recovery of alkaline phosphatase activity in the rat BBMV was 21.0 ± 1.2% (n = 3). Rabbit BBMV were used at final protein concentrations of 9.3–28.0 and 14.5–34.0 mg/mL, for duodenal and jejunal BBMV, respectively. During the isolation, alkaline phosphatase activity in the duodenal preparation increased 25.7 ± 4.4 (n = 3)-fold, with a recovery of 51.0 ± 3.5% (n = 3). For jejunal BBMV, the enrichment was 22.1 ± 4.1 (n = 6)-fold, with a recovery of 51.3 ± 4.4% (n = 6).

Vesicle Transport Studies

Uptake experiments were performed using a rapid filtration technique. Briefly, vesicles were resuspended in buffer V (protein concentration, 9.3–34.0 mg/mL). The composition of the incubation buffers varied depending on the type of experiment (Table I). Buffer I was identical in composition to the intravesicular buffer (buffer V) and allowed study of uptake into the BBMV under control (in the absence of a gradient) conditions. Buffer II was similar to buffer I but sodium gluconate replaced potassium gluconate, allowing measurements to be made under Na^+-gradient conditions. Buffer III (pH 5.5) allowed study of uptake under H^+-gradient conditions. In some experiments, electrogenic uptake of substrate was optimized by imposing a K^+-diffusion potential (inside negative) on the vesicles. This was achieved by replacing the extravesicular Na or K gluconate with 200 m*M* mannitol and adding 69 μ*M* valinomycin (14). In competition experiments, high concentrations (30 m*M*) of cold di/tripeptides were used. In these experiments the osmolality of the incubation buffers was maintained by alteration of the mannitol concentration. Each incubation buffer also contained tracer quantities of either [^{14}C]glucose, [^{14}C]Gly-Sar, or [^{3}H]TRH. Transport was initiated by the addition of 90 μL of incubation buffer (containing radiolabeled substrate) to 10 μL of BBMV. At various time points, 20 μL of the reaction mixture was removed, placed directly onto a presoaked filter disk (Millipore GS, 0.22 μm) under vacuum, and washed with 10 mL of ice-cold wash buffer [containing 150 m*M* NaCl, 50 m*M* $MgCl_2$, and either 10 m*M* MES/Tris (pH 5.5) or 10 m*M* Tris/HEPES (pH 7.4)]. The wash buffer used in the glucose uptake experiments contained 0.5 m*M* phloridzin and 0.85% NaCl (w/v) and uptake of glucose was stopped by filtration through a nitrocellulose filter disk (Sartorius, 0.45 μ*m*). Each filter was placed in 5 mL scintillation fluid and radioactivity associated with the vesicles was determined. To correct for nonspecific filter binding of radiolabels, 18-μL samples of the appropriate incubation buffer (equivalent to 20 μL of the reaction mixture) were placed directly onto the filters and processed accordingly. All experiments were conducted at room temperature.

HPLC Analysis of [^{3}H]TRH

Incubation buffers were identical to those used in the uptake studies. After 60-sec and 65-min incubations with intestinal vesicles, samples (20 μL) of the reaction mixture were removed and placed into 1 mL of ice-cold 0.08% TFA (buffer A) to prevent further degradation. Separation of TRH from any products of hydrolysis was accomplished by reversed-phase high-performance liquid chromatography (HPLC) (17). Before analysis the samples were centrifuged for 1 min (10,000 rpm). Fifty microliters of the sample was placed in 880 μL of buffer A containing 20 μg each of standards of TRH and its main degradation products [TRH-OH, His-Pro and cyclo(His-Pro) (17)]. The standard/sample mixture was separated using a Nova-pak C_{18} column (Waters). Buffer A consisted of 0.08% TFA and buffer B was 70% acetonitrile in 0.08% TFA. Samples were loaded at 3% buffer A/97% buffer B and fractionated using a linear gradient of 3–30% B in 13.5 min (flow rate, 1 mL/min). Absorbance was measured at 214 nm. Fractions (500 μL) were collected and the radioactivity in each fraction was determined by scintillation counting. Protein concentrations used

Table I. Composition of Incubation Buffers Used in Uptake Experiments[a]

	NaGluc	KGluc	Mannitol	$MgCl_2$	MES	HEPES	Tris	pH
Buffer I	—	100.0	100.0	5.0	—	50.0	18.1	7.4
Buffer II	100.0	—	100.0	5.0	—	50.0	18.1	7.4
Buffer III	—	100.0	112.0	5.0	50.0	—	6.1	5.5

[a] Concentrations given as m*M*.

in these experiments ranged from 19.6 to 20.8 mg/mL for the rabbit jejunal BBMV to 13.3 mg/mL for rat intestinal BBMV.

During this study a number of batches of [^{3}H]TRH were used. The identity of the radiolabel in each batch was monitored. Preliminary studies using [^{3}H]TRH (NEN Batch No. 2649-201) suggested that TRH was accumulated in rabbit jejunal BBMV by a Na^+-dependent mechanism. However, this response was found to be a function of an unidentified contaminant. HPLC fractionation of this batch of [^{3}H]TRH resolved two areas of radiolabeled material, a peak corresponding to TRH (57%) and material (43%) contained within the void volume. When compared in rabbit jejunal BBMV the ^{3}H-void material was accumulated up to 10-fold more than TRH after 30 sec in the presence of an inwardly directed Na^+ gradient and reached a peak after 50 sec which was sixfold higher than equilibrium values (45 min). The uptake of this ^{3}H-void material was not competed for by 5 m*M* proline and its identity remains undetermined. [^{3}H]TRH isolated from batch 2649-201 behaved identically to the other batches of [^{3}H]TRH described in this report. The experiments described in this paper involved the use of NEN batches 2649-248, 2649-257, and 2802-140. The radiolabel in each batch was identified as [^{3}H]TRH (>95%), consistent with the supplier's data sheets.

Statistical Analyses

Results are expressed as mean ± SE (*n*). Significance of differences between means or groups of data was tested by ANOVA (significance was set at $P < 0.05$).

RESULTS

Substrate Transport and Accumulation by Na^+ and H^+ Cotransport

The time dependency and accumulation of glucose [a Na^+ cotransported substrate (13)] and the dipeptide Gly-Sar [a H^+ cotransported substrate (10)] into rabbit jejunal BBMV are shown in Figs. 1 and 2. As anticipated, glucose accumulated within the vesicles above equilibrium values in the presence of an inwardly directed Na^+ gradient. This "overshoot" phenomenon is a characteristic of carrier-mediated transport. Since Na^+-glucose cotransport is electrogenic, glucose transport is significantly enhanced in the presence of a negative intravesicular potential, generated by a K^+-diffusion potential; both the magnitude of the glucose accumulation above equilibrium and the initial rate of uptake were increased significantly (Fig. 1). In the absence of a Na^+ gradient, the initial rate of intravesicular glucose accumulation is slow and does not exceed equilibrium.

Rabbit jejunal BBMV also transport the dipeptide Gly-Sar (Fig. 2). The initial rate of accumulation is significantly greater in the presence of an inwardly directed H^+ gradient ($pH_o < pH_i$). However, only in the presence of both an inwardly directed H^+ gradient and a K^+-diffusion potential, was an overshoot detected. For example, Gly-Sar uptake after 30 sec under control or Na^+ gradient conditions was 27.4 and 29.6% of the final equilibrium uptake, but this was significantly increased to 106.5% when a H^+ gradient and K^+-diffusion potential were combined (Fig. 2).

The uptake of [^{14}C]Gly-Sar (0.643 m*M*) in rabbit jBBMV is competed for by excess (30 m*M*) unlabeled substrates for the dipeptide carrier, such as Gly-Sar and Gly-Pro but not by TRH (data not shown). Peak uptake (60 sec) of [^{14}C]Gly-Sar was reduced from 660 ± 54 pmol/mg (n = 3) to 178 ± 19 pmol/mg (n = 4) and 122 ± 81 pmol/mg (n = 3) in the presence of cold Gly-Sar and Gly-Pro, respectively. In contrast, an excess of unlabeled TRH had no significant effect on [^{14}C]Gly-Sar uptake [769 ± 138 pmol/mg (n = 4)].

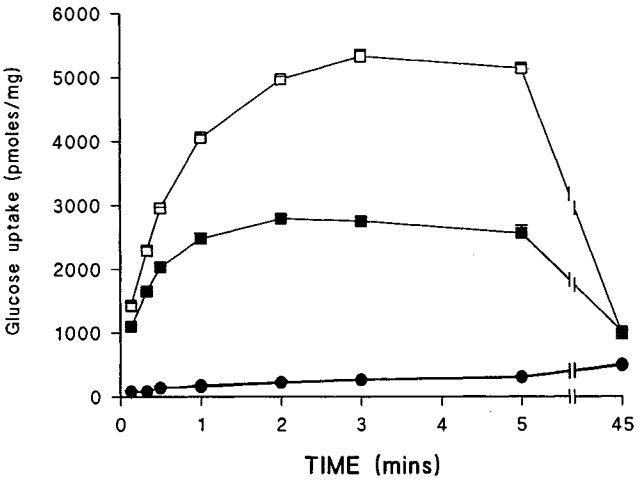

Fig. 1. Time course of [^{14}C]glucose (0.912 m*M*) uptake into rabbit jejunal BBMV. The uptake was determined under various gradient conditions (for full buffer compositions see Materials and Methods): inwardly directed Na^+-gradient (buffer II; filled squares); inwardly directed Na^+ gradient plus valinomycin (open squares); control conditions [absence of any ionic gradients (buffer I; filled circles)]; absence of ionic gradients plus valinomycin (open circles; superimposed on control conditions alone). Each point is the mean (±SE) of three to six determinations representative of experiments performed on two separate vesicle preparations.

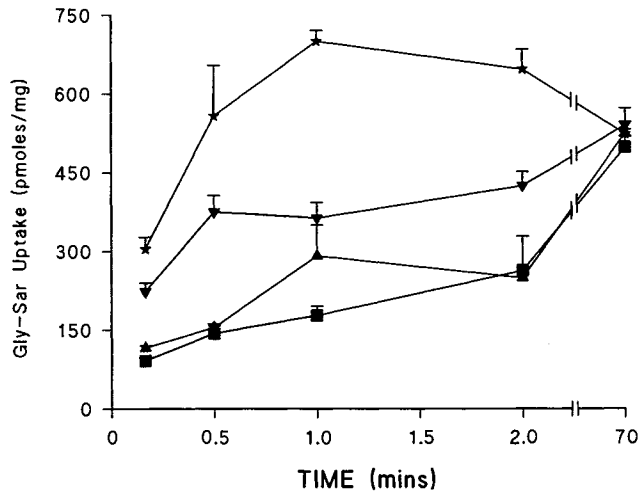

Fig. 2. Uptake of [^{14}C]Gly-Sar (0.643 m*M*) into rabbit jejunal BBMV. Time-dependent uptake under various gradient conditions: control (squares); inwardly directed Na^+ gradient (triangles); inwardly directed H^+ gradient (inverted triangles); inwardly directed H^+ gradient plus valinomycin-generated K^+ diffusion potential [inside negative (stars)]. Each point is the mean value (±SE) of three to six determinations, representative of experiments performed on two separate vesicle preparations.

Effect of Ionic Gradients on the Uptake of Glucose and TRH into BBMV

Uptake of [^{3}H]TRH and [^{14}C]glucose was compared in a series of vesicle preparations from rat proximal intestine, rabbit duodenum, and rabbit jejunum. The measurements were performed under control conditions (extravesicular = intravesicular ion concentrations) and also in the presence of inwardly directed Na^+ or H^+ gradients. Measurements were made at three time points, 10 sec (representing initial uptake into the vesicles), 60 sec (representing peak uptake), and 65 min (representing equilibration between the intra- and the extravesicular contents). In each tissue, evidence for Na^+-coupled glucose transport into the vesicles was observed, the uptake being significantly greater at 10 and 60 sec than 65 min (Fig. 3). In contrast, glucose uptake was not enhanced by the imposition of an inwardly directed proton gradient.

No evidence for carrier-mediated TRH uptake, neither Na^+- nor H^+-coupled, in rat proximal or rabbit duodenal and jejunal BBMV was obtained. TRH accumulation at 10 and 60 sec was minimal and always lower than that at equilibrium, 65 min (Fig. 3). In an attempt to unmask a quantitatively small electrolyte-coupled transport of TRH, experiments were repeated in the presence of a K^+-diffusion potential (inside negative). K^+-diffusion potentials significantly enhance the transport of glucose and Gly-Sar (Figs. 1 and 2). Such a K^+-diffusion potential, in the presence of an inwardly directed H^+ or Na^+ gradient, failed to provide evidence for carrier-mediated transport of TRH into either rat proximal intestinal or rabbit jejunal BBMV. In rat proximal BBMV, the initial rate of [^{3}H]TRH uptake (30 sec) in the presence of a H^+ gradient and a K^+-diffusion potential was 36.2% of the equilibrium value, compared with 27.7% with a H^+ gradient alone. In rabbit jejunal BBMV, uptake at 30 sec varied from 31.4 to 31.8% in the presence of an inwardly directed proton gradient with or without a K^+-diffusion potential. Similar results were obtained in the presence of an inwardly directed Na^+ gradient with or without a valinomycin-induced K^+-diffusion potential.

Hydrolysis of [^{3}H]TRH by Intestinal BBMV

The [^{3}H]TRH used in these experiments was labeled in the C-terminal proline residue. The stability of this peptide during the experiments was determined by reversed-phase HPLC (17). The separation of TRH from three common metabolites is illustrated in Fig. 4. The retention times after initiation of the gradient were as follows: His-Pro, 2.8 min; cyclo(His-Pro), 6.7 min; TRH, 8.6 min; and TRH-OH, 11.7 min.

The fractionation of the [^{3}H]TRH incubation mixture after incubation with rabbit jejunal BBMV is illustrated in Fig. 4. After 60 sec of incubation with BBMV, no appreciable degradation of [^{3}H]TRH had occurred. Similarly, after 65 min of incubation with BBMV, most of the radioactivity corresponded to TRH. However, although there was little or no radioactivity in the fractions corresponding to the void volume, or the two dipeptides, approximately 6% of the total radioactivity corresponded to the TRH-OH absorbance peak. Similar experiments were performed with rat proximal BBMV. The results from several incubations of [^{3}H]TRH

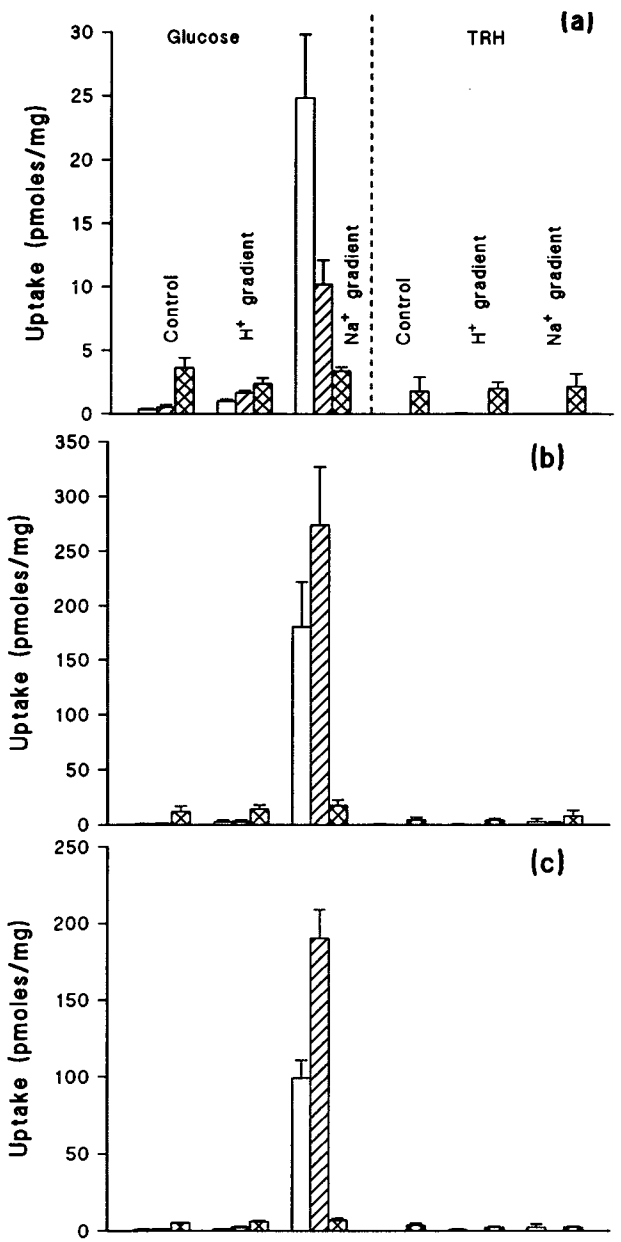

Fig. 3. Uptake of [^{14}C]glucose (12 μM) and [^{3}H]TRH (12 μM) into intestinal BBMV prepared from (a) rat proximal intestine, (b) rabbit duodenum, and (c) rabbit jejunum. Uptake was measured under control conditions or in the presence of inwardly directed Na^+ or H^+ gradients (see Table I). Uptake was determined at three time points: 10 sec (open columns), 60 sec (striped columns), and 65 min (cross-hatched columns). Each point is the mean (±SE) of three experiments where each measurement was determined three to six times. All experiments were performed at 25°C.

with BBMV are summarized in Table II. Analysis of [^{3}H]TRH before incubation with intestinal BBMV indicated that the radiolabel was approximately 95% pure. After 60 sec of incubation with either rabbit jejunal BBMV or rat proximal intestinal BBMV, little change was noted. However, after 65 min of incubation with rabbit jejunal BBMV, the radioactivity corresponding to TRH was reduced to 89.1%, which coincided with the appearance of [^{3}H]TRH-OH, accounting for 6.3% of the total radioactivity. No [^{3}H]TRH-

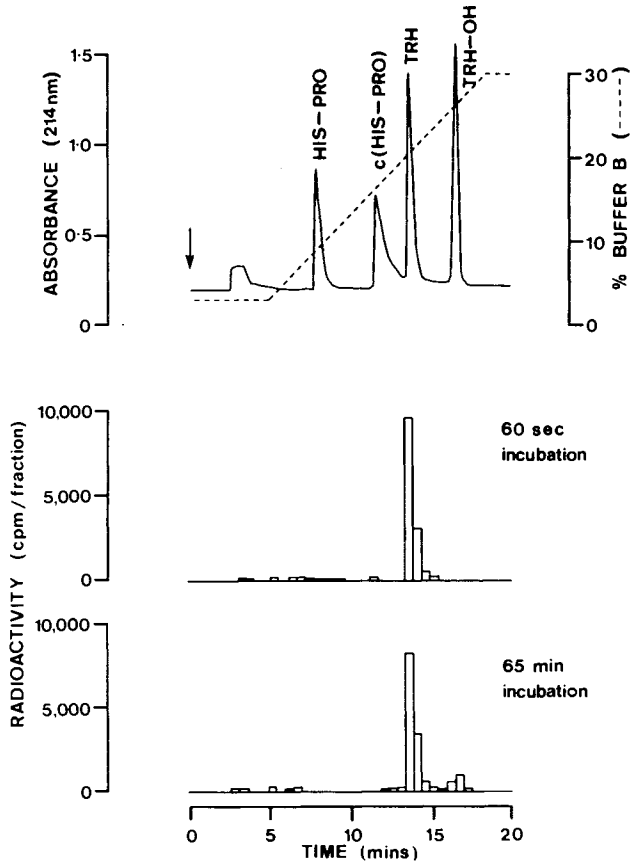

Fig. 4. Top: Separation of standards of TRH and three known degradation products by reversed-phase HPLC. The arrow indicates the point of injection. The dashed line represents the percentage buffer B (70% ACN in 0.08% TFA) in the elution mixture. Middle: Fractionation of [^{3}H]TRH and rabbit jejunal BBMV after a 60 sec incubation at 25°C. The radioactivity present in each fraction corresponds to the top elution profile. Bottom: Fractionation of a mixture of [^{3}H]TRH and rabbit jejunal BBMV after a 65-min incubation at 25°C.

OH was formed after a 65-min incubation with rat intestinal vesicles when incubated at either 25 or 37°C. Thus, although TRH is relatively stable during experiments with vesicles from both species, approximately 6% does undergo deamidation by proline endopeptidase-like activity to form TRH-OH (pGlu-His-Pro), when incubated with rabbit jejunal BBMV.

DISCUSSION

In membrane vesicles prepared from the brush border of rabbit and rat proximal intestinal enterocytes, we were unable to provide any direct evidence for carrier-mediated transport of TRH. TRH accumulation was not accelerated in the presence of either a Na^+ or a H^+ gradient and there was no evidence for accumulation above equilibrium. In the same vesicles, Na^+-coupled glucose transport and H^+-coupled dipeptide transport were clearly demonstrable. In addition, we provide evidence that TRH does not interact with the dipeptide carrier involved in Gly-Sar transport, since TRH failed to compete with transport of this dipeptide. Thus any carrier-mediated absorption of TRH must be via a transporter distinct from the intestinal di/tripeptide carrier (6–11,18). The tripeptide Gly-Gly-Pro appears to be transported in human jejunal BBMV, in part, by a nonconcentrating carrier-mediated process, independent of Na^+ or H^+ (19). The uptake of the latter tripeptide was enhanced by an intravesicular negative membrane potential. TRH accumulation was not significantly altered by the imposition of a K^+-diffusion potential under a variety of ionic gradients. Comparison of the vesicular filling rates (at 30 sec) of Gly-Sar (Fig. 2) and TRH indicates that the uptake of TRH under H^+-gradient/K^+-diffusion potential conditions (which would be predicted to maximize transport via a proton-coupled mechanism) is similar to the lowest rate of Gly-Sar transport (i.e., under control or Na^+ gradient conditions). This suggests that the uptake of TRH is mediated by passive transport only. These observations are supported, in part, by studies in renal BBMV where TRH failed to inhibit uptake of the structurally related dipeptide, pGlu-His (20).

The apparent lack of TRH transport was not due to peptide hydrolysis. There are two main pathways responsible for the degradation of TRH (21,22). The enzymes responsible for this degradation are the cytosolic proline endopeptidase (which cleaves the Pro-NH_2 bond to release the deamidated peptide TRH-OH) and pyroglutamyl peptidase I [this cleaves the pGlu-His bond to release His-Pro-NH_2, which spontaneously transforms into the cyclic peptide, cyclo(His-Pro)]. These enzymes are both found at low concentrations in the adult rat intestine (23). Experiments with both rabbit and rat have shown that although the peptide was stable when incubated with homogenates of rat intestine (5,24), the peptide was rapidly degraded by the action of proline endopeptidase during incubation with rabbit intestine (24). [^{3}H]TRH was stable when incubated with rat BBMV. Approximately 6% of the TRH was degraded, however, after 65 min of incubation with rabbit BBMV. The low rate of degradation of TRH by rabbit BBMV, as compared with rabbit intestinal homogenate, is consistent with the cytosolic nature of proline endopeptidase.

Our results from experiments using intestinal BBMV, providing no evidence to suggest that the oral absorption of TRH is mediated by a specific carrier located on the brush-border membrane, contrast with results obtained using intact tissue preparations. Based on data from everted sac preparations of rat intestine, Yokohama and co-workers (5) concluded that TRH is absorbed by Na^+-dependent transport system restricted to the proximal small intestine. Evidence for the Na^+ dependency came from replacement of Na^+ with K^+ in the bathing solution, which reduced the transport in a mucosal-to-serosal (absorptive) direction. However, in noneverted sacs, when Na^+ was replaced by K^+ on the serosal side, a reduction of transport of similar magnitude was seen in serosal-to-mucosal transport, suggesting nonspecific actions of the Na^+ replacement on mucosal permeability. The absorption of TRH in these everted gut sacs was reduced in the presence of both an oligopeptide mixture and some antibiotics, supportive of competition for a rate-limited carrier-mediated process, although nonspecific effects cannot be excluded. There was no concentrative accumulation of TRH in the everted gut sac experiments.

There are two routes for oral absorption of peptides:

Table II. Fractionation of [^{3}H]TRH and Degradation Products by RP-HPLC[a]

	TRH	TRH-OH	HP	c(HP)	Void	Unidentified
Rabbit jBBMV						
Standard (n = 3)	95.1 ± 1.3	0.0 ± 0.0	0.3 ± 0.1	1.3 ± 0.3	0.5 ± 0.1	2.8 ± 0.8
60 sec (n = 9)	94.1 ± 0.6	0.2 ± 0.1	0.4 ± 0.1	1.4 ± 0.2	0.4 ± 0.1	3.4 ± 0.5
65 min (n = 8)	89.1 ± 1.2	6.3 ± 0.2	0.7 ± 0.2	1.2 ± 0.2	0.5 ± 0.1	2.8 ± 0.2
Rat dBBMV						
Standard (n = 4)	95.7 ± 0.3	1.2 ± 0.4	0.4 ± 0.1	1.6 ± 0.3	0.4 ± 0.0	0.8 ± 0.4
60 sec (n = 4)	95.9 ± 0.7	1.1 ± 0.2	0.4 ± 0.1	1.6 ± 0.8	0.6 ± 0.1	0.7 ± 0.5
65 min (n = 3)	94.4 ± 1.0	1.2 ± 0.1	0.5 ± 0.2	1.6 (n = 1)	0.4 ± 0.0	0.7 ± 0.3

[a] Each column represents the percentage of total radioactivity contained within each peptide peak after incubation with intestinal BBMV (X ± SE).

either by a passive route mainly through the paracellular pathway or via a transcellular route involving facilitated diffusion mechanisms. Results from this study using BBMV preparations provide no evidence to suggest that TRH is absorbed via a H^+- or Na^+-dependent carrier-mediated mechanism. Some absorption via a H^+- and Na^+-independent low-affinity, low-capacity carrier would be difficult to detect, while the significance for such a pathway in the absorption of biologically active quantities of peptide is questionable. Rather it appears that the tripeptide is absorbed mainly by passive processes probably via the paracellular pathway (25). A paracellular route might be a preferable route for the absorption of a peptide such as TRH. It will reduce contact with intracellular enzymes such as proline endopeptidase and, combined with the relative resistance of this particular peptide to luminal hydrolysis, will enhance overall oral bioavailability.

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REFERENCES

1. B. J. Ormston. Clinical effects of TRH on TSH release after i.v. and oral administration in normal volunteers and patients with thyroid disease. *Front. Hormone Res.* 1:45–75 (1972).
2. E. D. Haigler, J. M. Hershman, and J. A. Pittman. Response to orally administered synthetic thyrotropin-releasing hormone in man. *J. Clin. Endocrinol. Metab.* 35(4):631–635 (1972).
3. 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. Pharm. Dyn.* 7:101–111 (1984).
4. G. Metcalf and I. M. D. Jackson. (eds.). Thyrotropin-releasing hormone: Biomedical significance. *Ann. N.Y. Acad. Sci USA* 553:1–631 (1989).
5. S. Yokohama, T. Yoshioka, K. Yamashita, and N. Kitamori. Intestinal absorption mechanisms of thyrotropin-releasing hormone. *J. Pharm. Dyn.* 7:445–451 (1984).
6. J. M. Addison, D. Burston, and D. M. Matthews. Evidence for active transport of the dipeptide glycylsarcosine by hamster jejunum *in vitro*. *Clin. Sci.* 43:907–911 (1972).
7. D. M. Matthews and S. A. Adibi. Progress in gastroenterology: Peptide absorption. *Gastroenterology* 71:151–161 (1976).
8. V. Ganapathy, J. F. Mendicino, and F. H. Leibach. Transport of glycyl-L-proline into intestinal and renal brush border vesicles from rabbit. *J. Biol. Chem.* 256(1):118–124 (1981).
9. 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. *J. Biol. Chem.* 258(23):14189–14192 (1983).
10. V. Ganapathy, G. Burckhardt, and F. H. Leibach. Characteristics of glycylsarcosine transport in rabbit intestinal brush-border membrane vesicles. *J. Biol. Chem.* 259(14):8954–8959 (1984).
11. V. Ganapathy and F. H. Leibach. Is intestinal peptide transport energized by a proton gradient? *Am. J. Physiol.* 249:G153–G160 (1985).
12. 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).
13. H. Murer, U. Hopfer, and R. Kinne. Sodium/proton antiport in brush-border membrane vesicles isolated from rat small intestine and kidney. *Biochem. J.* 154:597–604 (1976).
14. J. M. Wilkes and B. H. Hirst. Amiloride sensitivity of proton-conductive pathways in gastric and intestinal apical membrane vesicles. *J. Membr. Biol.* 126:115–122 (1992).
15. M. Kessler, O. Acuto, C. Storelli, H. Murer, M. Muller, and G. Semenza. A modified procedure for the rapid preparation of efficiently transporting vesicles from small intestinal brush border membranes. Their use in investigating some properties of D-glucose and choline transport systems. *Biochim. Biophys. Acta* 506:136–154 (1978).
16. M. M. Bradford. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* 72:248–254 (1976).
17. E. C. Griffiths, J. R. McDermott, and A. I. Smith. Mechanisms of brain inactivation of centrally-acting thyrotrophin-releasing hormone (TRH) analogues: A high-performance liquid chromatography study. *Regul. Peptides* 5:1–11 (1982).
18. T. Okano, K. Inui, H. Maegawa, M. Takano, and R. Hori. H^+ coupled uphill transport of aminocephalosporins via the dipeptide transport system in rabbit intestinal brush-border membranes. *J. Biol. Chem.* 261(30):14130–14134 (1986).
19. D. Wilson, J. A. Barry, and K. Ramaswamy. Characteristics of tripeptide transport in human jejunal brush-border membrane vesicles. *Biochim. Biophys. Acta* 986:123–129 (1989).
20. H. A. Skopicki, K. Fisher, D. Zikos, G. Flouret, R. Bloch, S. Kubillus, and D. R. Peterson. Carrier-mediated transport of pyroglutamyl-histidine in renal brush border membrane vesicles. *Am. J. Physiol.* 255:C822–C827 (1988).
21. E. C. Griffiths, J. A. Kelly, N. White, and S. L. Jeffcoate. Further studies on the inactivation of thyrotropin releasing hormone (TRH) by enzymes in the rat hypothalamus. *Acta Endocrinol.* 93:385–391 (1980).

22. C. Prasad and A. Peterkofsky. Demonstration of pyroglutamyl-peptidase and amidase activities towards thyrotropin-releasing hormone in hamster hypothalamus extracts. *J. Biol. Chem.* **251**:3229–3234 (1976).
23. Y. Fuse, D. H. Polk, R. M. Lam, A. L. Reviczky, and D. A. Fisher. Distribution and ontogeny of thyrotropin-releasing hormone degrading enzymes in rats. *Am. J. Physiol.* **259**:E787–E791 (1990).
24. 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).
25. D. T. Thwaites, B. H. Hirst, and N. L. Simmons. Passive transepithelial absorption of thyrotropin-releasing hormone (TRH) via a paracellular route in cultured intestinal and renal cell lines. *Pharm. Res.* **10**:674–681 (1993).