Different routes for the transport of α-lactalbumin in the rabbit ileum

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To identify the different pathways of proteins through the intestinal epithelium, the transport of [^{14}C]-radiolabeled α-lactalbumin was measured in the rabbit ileum mounted in Grass diffusion cells in both the presence and absence of glucose, galactose, or colchicine. The absorption of polyethylene glycol 4000 and the conductance of the tissue were not modified by the addition of 10 mmol/L galactose or 0.5 mmol/L colchicine, but were enhanced by the addition of 25 mmol/L glucose (by 37% and 75%, respectively). The total mucosal uptake of α-lactalbumin was not modified by the addition of 10 mmol/L galactose (3.09 ± 1.39 versus 3.46 ± 1.11 μg/h.cm² in Ringer) and significantly enhanced by the addition of 25 mmol/L glucose, which is known to dilate tight junctions (5.41 ± 1.34). Colchicine (0.5 mmol/L), a microtubule depolymeryzing agent, decreased the total uptake of α-lactalbumin by 35%. The fraction of intact protein (determined by high-pressure liquid chromatography and trichloroacetic acid precipitation) transported through the epithelium represented a small percentage of the total protein transported and this intact fraction was enhanced by the addition of 25 mmol/L glucose. The present results suggest that in control conditions α-lactalbumin is transported through a transcellular route, whereas in the presence of glucose an additional paracellular pathway is stimulated.

Keywords: protein absorption; intestine, α-lactalbumin; paracellular pathway

Introduction

Proteins and polypeptides are absorbed and processed by the intestinal epithelium of adult animals.[1-5] The major pathway in protein transport through the intestinal epithelium is a transcellular degradative route involving endocytosis, migration of the molecules in endosomes along microtubules, degradation mainly in lysosomal compartments by cathepsins, and exocytosis of the degraded products through the basolateral membrane. This transport has been described for different proteins including bovine serum albumin,[1] horseradish peroxydase (HRP),[6,7] insulin[4] and β-lactoglobulin (β-Lg).[8] These mechanisms have also been described in other cell systems, including the transport of ovalbumin in liver endothelial cells,[9] of insulin in cultured kidney cells,[10] and of the follicle-stimulating hormone in mouse sertoli cells.[11]

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A small fraction of proteins may also pass through the intestinal epithelium without being degraded, as demonstrated by the presence of intact protein in lymph or blood after oral administration or duodenal injection.[1,4,12] The transcytosis, which mainly exists in M cells of Peyer patches, is probably also present in enterocytes.[2] It is generally put forward to explain the passage of protein in its intact form. Another explanation is to take into account a paracellular pathway. This paracellular pathway is still much debated, but has been considered for BSA[1] and for HRP after surgical trauma[13] or under cholinergic stimulation.[14] Recently an 11-amino-acid hemepeptide (mol wt ≈ 1900) unlike HRP (mol wt ≈ 40,000) was shown to penetrate tight junctions in the presence of glucose.[15]

The present study was undertaken to see if proteins are transported through different routes in the intestinal epithelium. For this purpose, the transport of α-lactalbumin (α-La) (mol wt ≈ 14,100) was measured in the isolated rabbit ileum in Grass diffusion cells. Experiments were performed both in the presence and absence of 0.5 mmol/L colchicine (which affects microtubule assembly[16]), 25 mmol/L glucose (which has been shown to induce dilatation of tight junctions[15,17])

and 10 mmol/L galactose for comparison. The results indicate that the transport of α-La was mainly transcellular but may also be partially paracellular.

Materials and methods

Chemicals

Bovine milk α-La (type III) was purchased from Sigma Chemical Co. (St. Louis, MO USA) and ^{3}H-PEG from Dupont NEN (Boston, MA USA). Protein was [^{14}C]-radiolabeled by methylation with [^{14}C]-formaldehyde (Amersham Corp., Arlington Heights, IL USA) and $NaCNBH_3$.[18]

Diffusion cell experiments

Male New Zealand white rabbits weighing 2.3–2.8 kg were killed by an intravenous pentobarbital sodium injection (Sanofi). The ileum was rapidly removed and rinsed free of intestinal contents. After stripping and taking off Peyer patches, segments were mounted between two half Grass diffusion cells[19] (Precision Instrument Design, Los Altos, CA USA) and bathed on each side (exposed area 2.7 cm^2) with 6 mL of isotonic Ringer solution (containing the following in mmol/L: 140 Na^+, 5.2 K^+, 1.2 Ca^{2+}, 1.2 Mg^{2+}, 120 Cl^-, 25 HCO_3^-, 2.4 $H_2PO_4^-$, and 0.4 HPO_4^{2-}, pH = 7.4). The temperature was maintained at 37° C and the tissues were oxygenated by gas lifting (95% O_2-5%CO_2). Ringer was used as the control. When necessary, 25 mmol/L glucose (Prolabo, Paris, France), 10 mmol/L galactose (Sigma), 0.5 mmol/L colchicine (Sigma) were added into each reservoir. A short-circuit current (Isc) delivered by an automatic voltage clamp system (WPI, New Haven, CT USA) short-circuited the spontaneous transmucosal electrical potential difference (PD) to correct for fluid resistance. The electrical conductance (G) of the tissue was determined according to Ohm's law.

Transport studies

After a 30-min equilibration period, [^{14}C]-α-La (2 μCi, 1 mg/mL) and [^{3}H]-PEG 4000 (2 μCi) were added only to the mucosal reservoir. Aliquots were collected at different times from the mucosal and serosal reservoirs. After 140 min, the solutions were collected and used for high-pressure liquid chromatography or measurement of the trichloro-acetic acid (TCA) precipitable fraction. Cold TCA was added to solutions (10% final concentration). The test tubes were centrifuged at 1300*g* for 10 min, the pellet was rinsed 3 times with 10% TCA, resuspended in NaOH 1 N and then counted. The tissue was removed, rinsed three times with cold 0.3 M mannitol, dried on paper, incubated for 16 hr in 0.1 M nitric acid and the mixture was then directly used for radioactivity determination.

High-pressure liquid chromatography (HPLC)

Samples from the serosal reservoir of the Grass diffusion cells were injected in a Waters gradient system equipped with a C18 nucleosil (5 μm) (SFCC, Eragny, France) column (150 × 4.6 mm). The column was eluted at 30° C at a flow rate of 2 mL/min with a mixture of acetonitrile in 0.1% trifluoroacetic acid as follows: 4% acetonitrile from 0–15 min, a linear 4–15% gradient of acetonitrile in the 15–20 min period, and a linear 15–70% gradient of acetonitrile in the 20–40 min period. Fractions of 2 mL were collected and used for radioactivity determination.

Calculation

Results were expressed as means ± SE. The data were analysed by Student's *t* test and a probability of $P < 0.05$ was regarded as significant.

Results

The electrical parameters Isc, PD, and G, as well as PEG's permeability, were measured in the isolated rabbit ileum in each of the following conditions: control (Ringer), 25 mmol/L glucose, 10 mmol/L galactose and 0.5 mmol/L colchicine *(Table 1)*. Colchicine had no effect on the electrical parameters nor on PEG's permeability. Both 25 mmol/L glucose and 10 mmol/L galactose significantly enhanced the short-circuit current, Isc. The conductance, G, of the tissue and PEG's permeability were unaffected by 10 mmol/L galactose, but were significantly enhanced (+37% and 75%, respectively) by 25 mmol/L glucose.

One mg/mL [^{14}C]-α-La was added to the mucosal reservoir of the diffusion cells in the presence and absence of 25 mmol/L glucose, 10 mmol/L galactose, or 0.5 mmol/L colchicine. The radioactivity was measured as a function of time in the serosal reservoir of the chamber *(Figure 1)*. After an initial 30-min period, the serosal radioactivity linearly increased with time. In comparison with the control (Ringer), the rate of serosal accumulation of radiolabeled material significantly increased (+56%) in the presence of 25 mmol/

Table 1 Effects of the addition of 25 mmol/L glucose, 10 mmol/L galactose, or 0.5 mmol/L colchicine in Ringer on electrical parameters, short circuit current (Isc), transmucosal electrical potential difference (PD), conductance (G), and on PEG's permeability in isolated rabbit ileum

	Ringer	Glucose	Galactose	Colchicine
Isc (μA/cm^2)	30.5 ± 3.2	65.3 ± 6.7*	59.5 ± 1.9*	34.9 ± 2.5
PD (mV)	−2.02 ± 0.06	−2.44 ± 0.15*	−3.65 ± 0.05*	−2.22 ± 0.30
G (mS/cm^2)	15.2 ± 0.9	26.6 ± 2.8*	16.3 ± 0.3	15.7 ± 1.7
PEG (10-3 μCi/h.cm^2)	1.44 ± 0.28	1.97 ± 0.42*	1.32 ± 0.29	1.63 ± 0.32

Values are means ± SE for tissues from at least six rabbits. Isc, PD, and G were measured between 60–140 min after the addition of radiolabeled material.
*Significantly different from Ringer, $P < 0.05$.

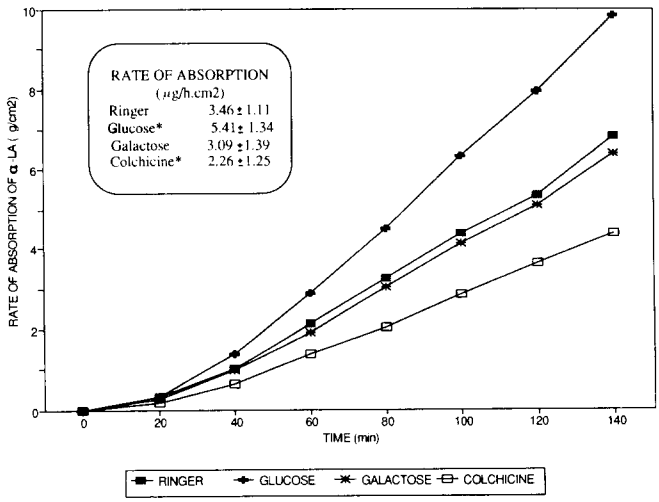

Figure 1 Time-dependent accumulation of ^{14}C-α-La equivalent in the serosal reservoir. At t = 0 min, 1 mg/mL α-La was added to the mucosal side of stripped rabbit ileum mounted in Grass diffusion cells (exposed area, 2.7 cm^2), in Ringer for control or in the presence of 25 mmol/L glucose, 10 mmol/L galactose, or 0.5 mmol/L colchicine. The rates of absorption of α-La determined in the linear part of the curves (between 30–140 min) are given in insets (mean ± SE). *Significantly different from control, $P < 0.05$.

L glucose, remained unaffected by 10 mmol/L galactose and significantly decreased (−34%) in the presence of 0.5 mmol/L colchicine. After 140 min, the concentrations of protein equivalent in the serosal reservoir were identical in the presence of Ringer (3.07 ± 0.88 μg/mL) and 10 mmol/L galactose (2.87 ± 1.23), significantly higher in the presence of 25 mmol/L glucose (4.41 ± 1.08), and significantly lower in the presence of 0.5 mmol/L colchicine (1.95 ± 1.02).

The TCA-precipitable radiolabeled fraction, both in the mucosal and serosal reservoirs, and the radioactivity bound and/or fixed to the tissues were measured 140 min after the addition of 1 mg/mL radiolabeled α-La to the mucosal reservoir *(Table 2)*. At time 0 min, the TCA-precipitable radiolabeled fraction in the mucosal reservoir never significantly differed from the total mucosal radioactivity and remained unchanged after 140 min in all conditions. In comparison, the serosal TCA-precipitable fraction represented 7–10% of the total serosal radioactivity and was significantly enhanced in the presence of 25 mmol/L glucose, but was unaffected by 10 mmol/L galactose and 0.5 mmol/L colchicine. The radioactivity bound and/or fixed to the tissues was not modified by 25 mmol/L glucose, 10 mmol/L galactose, or 0.5 mmol/L colchicine.

The serosal reservoir content was also analysed by HPLC 140 min after the addition of 1 mg/mL radiolabeled α-La in the mucosal reservoir in both the presence and absence of 25 mmol/L glucose and 0.5 mmol/L colchicine *(Figure 2)*. The radioactivity eluted from the column showed two main fractions, A and B. Fraction A, which had a short retention time and corresponded to degraded products, represented 90–98% of the eluted radioactivity. Fraction B had exactly the same retention time as native α-La and represented 3–6% of the eluted radioactivity. In comparison with the control (Ringer), 0.5 mmol/L colchicine reduced fraction A (−48%) but did not affect fraction B, whereas 25 mmol/L glucose mainly enhanced fraction B (+200%) and to a lesser extent fraction A (+67%).

Discussion

The present study indicates that α-La is mainly transported through the isolated rabbit ileum by a transcellular pathway, but that a paracellular route is also present and can be stimulated by the addition of glucose. These conclusions are supported by the following observations: (a) both degraded and intact α-La were found in the serosal side of the ileum; (b) 0.5 mmol/L colchicine, which affects intracellular structures, re-

Table 2. TCA-precipitable fractions in the mucosal and serosal reservoirs and binding and/or fixation of ^{14}C-α-La equivalent at t = 140 min after addition of 1 mg/mL ^{14}C-radiolabeled α-La in the mucosal reservoir at t = 0 min

			Ringer	Glucose	Galactose	Colchicine
TCA-Precipitable Fraction	In the mucosal reservoir (mg/mL)	at 0 min	0.95 + 0.02			
		at 140 min	0.94 ± 0.02 (23)	0.92 ± 0.03 (9)	0.92 ± 0.03 (9)	0.95 ± 0.05 (8)
	In the serosal reservoir at 140 min (μg/mL)		0.23 ± 0.10 (20)	0.39 ± 0.11* (10)	0.28 ± 0.22 (10)	0.19 ± 0.09 (6)
Binding and/or fixation of ^{14}C-α-La equivalent on tissue at 140 min (μg/cm^2)			2.20 ± 0.68 (20)	3.20 ± 1.97 (9)	2.45 ± 1.05 (9)	1.88 ± 0.86 (8)

Values are means ± SE. Number of rabbits is given in parentheses. The different media used are Ringer, Ringer + 25 mmol/L glucose, Ringer + 10 mmol/L galactose, Ringer + 0.5 mmol/L colchicine. Area of the tissues, 2.7 cm^2. Volume of the reservoirs, 6 mL.
*Significantly different from Ringer, $P < 0.05$.

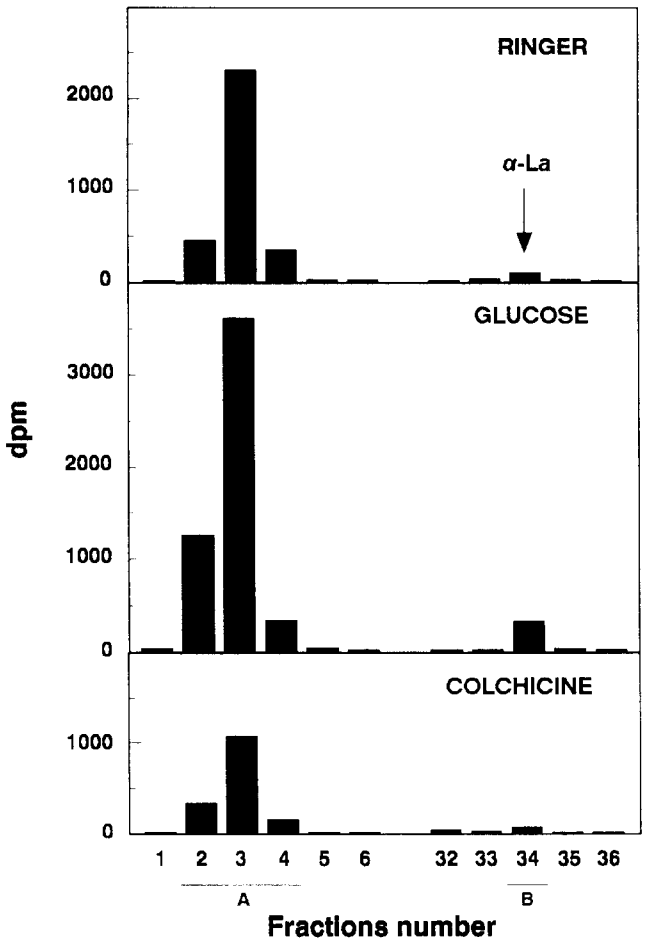

Figure 2 Typical reverse-phase high pressure liquid chromatography radioactivity pattern of the serosal reservoir content of a diffusion cell. The serosal reservoir content was analysed 140 min after the addition of 1 mg/mL radiolabeled α-La into the mucosal reservoir in Ringer for control or in the presence of 25 mmol/L glucose, 10 mmol/L galactose, or 0.5 mmol/L colchicine. Fractions of 2 mL were collected and directly used for radioactivity determination. Fraction A (short retention time) corresponded to degraded products. Fraction B was eluted at the retention time of α-La.

duced the passage of α-La but not that of PEG; (c) 25 mmol/L glucose, known to dilate tight junctions, enhanced the passage of both α-La and PEG, whereas galactose did not affect their transport. This indicates that absorption of proteins may occur through different routes, with relative importances varying according to the proteins and the experimental design considered.

The present experiment was conducted in rabbit ileum mounted in Grass diffusion cells,[19] which is a system derived from the Ussing chamber model. This in vitro model has already been used to investigate the absorption of proteins and peptides,[20,21,22] and with polypeptides it was previously shown that their transepithelial passage did not occur through a damaged edge of the tissue.[19,20] This assumption is also confirmed by the fact that the passage of α-La was modulated by colchicine and glucose and that α-La and PEG were shown to be transported in different quantities. The absence of degradation of α-La in the mucosal side of the tissue also indicates that the transport is mostly that of intact protein uptake. It appeared that more than 90% of the transported protein was composed of degraded products. In addition, a small fraction of protein escaped degradation and was found intact after crossing the tissue. It was previously observed that 6–7% of α-La was transported through the rabbit intestine in an antigenic form.[22] Moreover, intact proteins were already found in the extracellular space and in the blood or the lymph after ingestion.[1,3,12,24,25] These results agree with the idea that the major part of intact protein transport involves the following transcellular mechanism: an initial endocytotic uptake at the luminal membrane of the enterocyte, migration in endosomes along microtubules, degradation in lysosomes, and further excretion of the degraded products to the extracellular space for the major part of the protein. However, a small fraction is transcytosed and exocytosed in an intact form.[2,25] It is not yet clearly established whether the transport of protein is entirely due to a transcellular mechanism or if a paracellular pathway is also present.

The involvement of intracellular structure was assessed by colchicine, which is believed to inhibit microtubule assembly.[16] No effect on both tissue conductance and PEG's permeability was noted, thus indicating that in the present study colchicine did not affect the paracellular route. In a previous study,[8] colchicine did not significantly affect tissue conductance but slightly reduced PEG's permeability. This effect did not appear as a direct action of colchicine on tight junctions because tissue conductance remained unaffected but rather an indirect one probably in relation to a higher sensitivity of the tissue to colchicine. Moreover, colchicine probably does not affect endocytosis per se, but rather affects later steps in the endocytosis pathway.[26,27] In those conditions, colchicine would not modify luminal uptake of the protein but would act on its intracellular migration. In fact, the results indicated that colchicine mainly reduced degraded protein transport without affecting tissue-associated protein.

The existence of a paracellular pathway, currently controlled by the tight junctions or zonula occludens, is still controversial. The properties of these intercellular junctions remain partially unknown and it is now generally agreed that they are a relative, rather than an absolute, barrier.[28,29] The transepithelial resistance of intestinal epithelium decreases in the presence of insulin,[30] cytochalasin D, aminoacids, and glucose. The presence of glucose or bile acids dilates the tight junctions.[17,31] In the present experiments, glucose, unlike galactose, significantly increased both tissue conductance and permeability to PEG. In those conditions, the larger transport of α-La in the presence of 25 mmol/L glucose can be interpreted as the stimulation of an additional paracellular transport of the proteins by this sugar. Both degraded and intact protein transport were enhanced in the presence of glucose, but the higher stimulation of the intact fraction suggested that the

paracellular pathway is mainly a transport with no degradation. The concomitant stimulation of degraded fraction transport could be in relation to a recycling of degraded products at the apical membrane and further transport via the paracellular route. Though it is not possible to exclude a stimulation of the transcellular degradative pathway, to our knowledge such an effect of glucose has not been reported, whereas a glucose-induced transepithelial fluid circuit has already been reported.[32] This hypothesis of a paracellular pathway for macromolecules is not yet common, but has already been mentioned.[1,13,14] Atisook and Madara[15] showed that in the presence of glucose a paracellular pathway exists for an 11-amino acid hemepeptide of 1,900 molecular weight, whereas it does not exist for HRP of 40,000 molecular weight. In our experiments, α-La and PEG, with molecular weights of 14,100 and 4,000 respectively, are in an intermediate position.

In conclusion, our results indicate that in control conditions the transepithelial transport of α-La through the isolated rabbit ileum is mainly transcellular, with more than 90% of the transport acting through a degradative route and a small percentage by transcytosis. In the control conditions, the paracellular route is probably very minor or even absent, but it can be stimulated in some conditions, i.e., in the presence of glucose. These observations are probably of physiological significance. Even if they are mostly degraded by proteolytic enzymes, a small but significant quantity of ingested protein remains intact in the lumen and may be absorbed in an intact form. Whey proteins are incompletely degraded when they reach the intestinal epithelium,[33,34] and it has already been shown that both β-Lg and α-La were absorbed partly in an intact form.[3,25] The present results demonstrate that different routes exist for transepithelial protein transport, and that they are modulated by some mechanisms.

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