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The active transport of polypeptides in the rabbit nasal mucosa is supported by a specific vesicular transport inhibited by cytochalasin D

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Abstract

We have previously demonstrated that polypeptides (elcatonin and ACTH) can be actively absorbed across the rabbit nasal mucosa. In this paper we show that elcatonin is also transported when it is adsorbed onto microspheres (diameter: 0.5 μm), whereas the elcatonin-uncovered microspheres do not display any net transport. Cytochalasin D (0.1 μg/ml) abolishes the net absorption of elcatonin presented either alone or adsorbed. At the same concentration the inhibitor does not affect cellular active ion transports (and hence metabolism); although it increases intercellular ion and elcatonin permeability, it does not affect intercellular and paracellular permeability of the elcatonin-covered microspheres. Altogether, these results show that polypeptide transport is supported by a specific vesicular transfer inhibited by cytochalasin D by disassembly of the actin cytoskeleton, probably at the apical border of the cell.

Keywords: Actin filament; Endocytosis; Occluding junction; Microsphere

1. Introduction

We have previously shown that a specific, active transport of polypeptides (perhaps involved in antigen sampling from nasal lumen to blood) is present in the nasal respiratory mucosa of the rabbit [1]. The transcellular active transport of macromolecules is generally based on vesicular transport which in principle can result from fluid-phase nonspecific endocytosis (pinocytosis) or receptor-mediated endocytosis [2]; however, the macromolecule transport is generally specific. At the apical membrane of the epithelial cells the vesicle formation for both processes requires the action of an actin-microfilament network [3] which can be specifically disassembled by cytochalasin D [3–5]. Thus, as for the active transfer of polypeptides observed, it may be predicted that: (1) the test-molecule, presented adsorbed on microparticles, should be equally actively transported, (2) this transfer on microparticles should be specific, (3) the net transfer of the test-molecule, either presented alone or adsorbed on microparticles, should be inhibited by cytochalasin D.

The aim of this paper is to examine these points, to establish whether the active transport observed in the nasal mucosa is supported by specific vesicular transport. Cytochalasin D and polystyrene microspheres, covered with the test molecule, were used as the investigation tool. The microsphere diameter (0.5 μm) was compatible with vesicular transport, based on pinocytosis or receptor-mediated endocytosis [6].

2. Materials and methods

Male New Zealand rabbits (weighing about 3 kg) were killed by cervical dislocation; the fore-half nasal mucosae from both nostril roofs were excised, washed with Krebs-Henseleit saline at room temperature and mounted between two teflon chambers (exposed area: upper concha, 0.3 cm^2). Each chamber was filled with 1 ml Krebs-Henseleit saline, bubbled with prehumidified 95% O_2 +5% CO_2 to oxygenate the tissue, to maintain pH at 7.4 and to agitate the solution. The experiments were carried out at open electrical circuit and at 27 ± 1°C as at this temperature mucosa functionality not only does not degrade, but also improves with time for many hours [7].

As for transepithelial flux measurements the test-molecule used was elcatonin ([1,7 Asu] eel calcitonin; carbocalcitonin, CCT; Mw = 3362). It was determined by radioimmunoassay (RIA), so as to measure only the intact or

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nearly intact molecule (Ref. [1] [1]), with the aid of a γ-counter (Autogamma 800C, Packard Instr. Co., Meriden, CT, USA). Teflon chambers were used to avoid surface adsorption of the polypeptide molecules. The unidirectional mucosa-submucosa and opposite fluxes (T_{ms}, T_{sm}) were determined one on the right, the other on the left mucosa from the same animal (with random combinations), the functional symmetry of the mucosae having been demonstrated in advance [1,7]. After a 30-min preincubation to allow the tissue to recover after isolation, 1 ml saline was introduced with the test molecule (20 μg/ml) into the mucosal or submucosal chamber (donor chamber) and the appropriate transepithelial flux subsequently determined after a 2-h incubation, by complete withdrawal of the saline. Both fluxes were considered unidirectional since the maximal concentration reached in the flux chamber was 10^{-3}–10^{-5} compared to that applied in the opposite donor chamber. On the basis of 25-μl samples taken every 30 min, the elcatonin concentration in the donor chamber originating the flux was observed to decrease slightly with time. The metabolized fraction over the 2-h period was at most 17%. Although this value was relatively small, the transports actually measured were corrected by multiplying their value by the ratio between the initial elcatonin concentration and the mean concentration during the 2-h period (see also Ref. [1]). The viability of the preparation was checked at the beginning and end of the experiment by measuring the transepithelial electrical potential difference (V_{ms}): tissues with initial V_{ms} less than +1 mV (submucosa positive) were discarded [1,7].

In some experiments, after the 30-min preincubation, elcatonin was introduced (into the mucosal or submucosal chamber) adsorbed on polystyrene microspheres made fluorescent with fluoresceinisothiocyanate (FITC) (Polyscience Inc., Warrington, PA, USA); their diameter was about 0.5 μm. The final concentration in the donor chamber was about $3.5 \cdot 10^{11}$ microspheres/ml. The actual concentration of the microspheres was measured on 25-μl samples (taken at the beginning and end of the experiment) with the aid of a Luminescence spectrometer (Ls 50, Perkin-Elmer Corp., Norwalk, CT, USA) and a calibration straight-line (excitation wavelength, 440 nm; emission wavelength, 485 nm). The reference microsphere concentrations were predetermined by a Burker's chamber after adequate dilution, with microspheres observed by a fluorescence microscope (400x, Orthoplan MPV2, Leitz GMBH, D-6330 Wetzlar, Germany). During the experiment the initial and final bead concentrations proved not to be significantly different (initial concentration: $(3.5 \pm 0.1) \cdot 10^{11}$ microspheres/ml, $n = 30$; final concentration: $(3.4 \pm 0.0) \cdot 10^{11}$ microspheres/ml, $n = 30$). The microspheres transported in the 2-h experiment were measured exclusively by Burker chamber due to their very low concentration.

Elcatonin was adsorbed on microspheres by a method previously set up [8]. Briefly, a 510-μl volume of native microsphere suspension was spun down at 11 000 rpm for 10 min, the precipitate resuspended in 510 μl of Krebs-Henseleit saline containing 22 μg/ml CCT ($6.5 \cdot 10^{-6}$ M) and then incubated for 90 min at 37°C. Successively, to block still unbound bead sites, the CCT-coated microspheres were washed four times with Krebs-Henseleit saline containing 5% (w/v) bovine serum albumin (BSA). Finally, the BSA saline was removed and replaced with a Krebs-Henseleit saline.

In one set of experiments, the microspheres used were not covered by elcatonin. Since under these conditions, when placed in the Krebs-Henseleit saline, they tend to adsorb and aggregate, both donor saline and saline with transported beads were sonicated at the beginning and end of the experiment, just before carrying out the determinations. Moreover, the donor saline was completely withdrawn and renewed every 30 min, this operation being proved sufficient to maintain a constant free bead concentration.

The transepithelial electrical potential difference (V_{ms}) and short-circuit current (I_{sc}) were measured using an automatic device (S. Hénin, Milano) that allowed automatic subtraction of the bathing fluid resistance for correct measurement of I_{sc}. Bridges of 3% agar/3 M KCl and 3% agar/Krebs-Henseleit saline were used for the V_{ms} and I_{sc} determinations respectively. The transepithelial electrical resistance (R_{ep}) was calculated as the V_{ms}/I_{sc} ratio.

The Krebs-Henseleit saline had the following composition (mM): 142.9 Na^+, 5.9 K^+, 2.5 Ca^{2+}, 1.2 Mg^{2+}, 127.7 Cl^-, 24.9 HCO_3^-, 1.2 SO_4^{2-}, 5.5 glucose. The cytochalasin D was previously dissolved in ethanol and added with it to the saline (2.5 μl ethanol/ml); at this concentration, ethanol alone had no effect on V_{ms} for at least 150 min (5 experiments). Cytochalasin D was supplied by Sigma (St. Louis, MO). Elcatonin and its antibody were kindly do-

[1] Carbocalcitonin is composed of 32 amino acids and is prepared from eel calcitonin by replacing the disulphide bridge (closing the 1-7 ring) with an ethylene bridge: this makes it stable at 37°C in the absence of proteases. The polyclonal antibody used for RIA was specifically raised against CCT. It does not react with human and porcine calcitonin and displays less than 20% cross-reactivity with eel and salmon calcitonin. Its target is in the 1-15 part of the molecule. The antibody does not react with all the possible 11-32 fragments (11-32, 15-32, 20-32, 24-32, 27-32). Conversely, it displays 100% cross-reactivity with 1-11 and 1-18 fragments. If, on the action of proteases present in the nasal mucosa fragments recognizable by the antibody were formed, in particular on the luminal side, the measured net flux of CCT [1] might be the result of these metabolites diffusing faster from the mucosal to the submucosal side than the unchanged CCT. However, we have shown [1] that CCT net flux is abolished in the presence of monoiodoacetate and dinitrophenol, inhibiting cellular ATP production without any likely interference with protease activities; under these conditions J_{sm} is not modified, but J_{ms} is reduced to overlap it. Hence, in spite of the fact that slight CCT degradation is observed during the experiment (see Ref. [1] and further in this paper), J_{net} is not related to CCT fragments and their passive diffusion.

nated by ISF Laboratories for Biomedical Research (Trezzano s/N, Milan, Italy); elcatonin labeling was carried out by Calbiochem Nova Biochem (Nottingham, UK).

The results are expressed as means ± standard errors; Student's *t*-test for unpaired data or, when possible, paired data, was used for statistical analysis of the results reported in Table 1; conversely, the one-way ANOVA test was used to statistically analyze the data in Fig. 1.

3. Results

Tables 1 and 2 show the results of experiments in which elcatonin unidirectional and net transports were determined by presenting the polypeptide to the epithelium either alone or adsorbed on microspheres of 0.5 μm diameter. Since net elcatonin transport was observed to be exhausted in 2 h [1], the overall unidirectional (T_{ms}, T_{sm}) or net (T_{net}) transfers during this period were reported in the two tables. The net absorption from nasal lumen to blood was observed not only when the polypeptide was presented alone (Table 1), as expected, but also when it was adsorbed on microspheres (Table 2). In the former case the transport is specific for polypeptides [1]; similarly in the latter case the transfer of microspheres is strictly dependent on the polypeptide covering, since no significant T_{net} was measured with native CCT-uncovered microspheres (Table 2).

The effects of cytochalasin D are also shown in the two tables (experimental protocol: treatment on both sides of the tissue for the whole experiment, beginning just after V_{ms} was measured at zero min time to check the viability of the mucosa). With 0.1 μg/ml ($2 \cdot 10^{-7}$ M) inhibitor

Table 1
Effect of cytochalasin D (0.1 μg/ml) on the overall transepithelial transport of elcatonin (CCT) during the two experiment hours

	CCT transport (ng cm^{-2})	
	Control	Cytochalasin D
T_{ms}	21.6 ± 3.0°°	22.6 ± 2.0
	(18)	(11)
T_{sm}	9.4 ± 0.4	23.6 ± 2.4 **
	(18)	(11)
T_{net}	12.2 ± 3.2 *	−0.8 ± 3.0
	(18)	(11)

After 30 min preincubation 20 μg/ml CCT was added to the mucosal or submucosal chamber and the overall corresponding unidirectional transports during 2 hours (T_{ms}, T_{sm}) were determined by RIA. Based on their difference the overall net transport (T_{net}) was calculated. When present, cytochalasin D was on both sides of the tissue during the 30 min preincubation and 2 h subsequent incubation.
Results presented as means ± S.E. (number of experiments in parentheses).
°° $P < 0.01$ compared the respective T_{sm}.
** $P < 0.01$ compared to the corresponding control.
* $P < 0.05$ compared to zero.

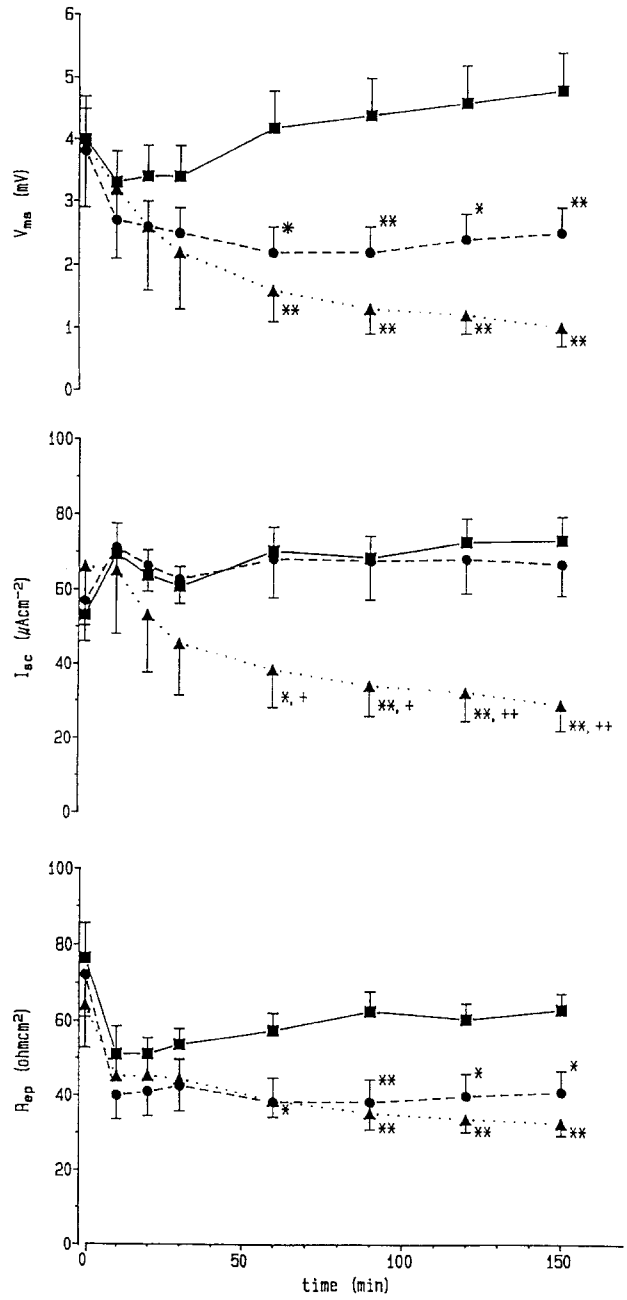

Fig. 1. Effect of cytochalasin D at different concentrations on the transepithelial electrical potential difference (V_{ms}), shortcircuit current (I_{sc}) and transepithelial electrical resistance (R_{ep}) of nasal mucosa. Results reported as means ± standard error. Control ($n = 21$): ■. Cytochalasin D: 0.1 μg/ml ($n = 13$) •; 1 μg/ml ($n = 8$) ▲. *, ** $P <$ 0.05 or 0.01 compared to the control; +, ++ $P < 0.05$ or 0.01 compared to the condition with 0.1 μg/ml inhibitor.

both net absorptions (CCT alone or on microspheres) were abolished, since T_{ms}, significantly greater than T_{sm} under control conditions, on treatment overlapped T_{sm}. However, it is worth noting that: (1) when CCT was presented alone, T_{sm} was significantly increased about 2.5 times by treatment, (2) when CCT was adsorbed on microspheres, T_{sm} was not significantly modified by the inhibitor.

Table 2
Effect of cytochalasin D (0.1 μg/ml) on the overall transepithelial transport of CCT-covered microspheres during the two experiment hours

	Microsphere transport (10^6 cm^{-2})		
	CCT-uncovered	CCT-covered	
		Control	Cytochalasin D
T_{ms}	9.0±0.8 (6)	21.9±1.6°° (9)	11.3±0.6 ·· (6)
T_{sm}	8.3±0.8 (6)	10.4±0.7 (9)	11.5±0.5 (6)
T_{net}	0.7±0.7 (6)	11.5±1.0** (9)	−0.2±0.5 (6)

After 30 min preincubation $3.5 \cdot 10^{11}$/ml CCT-uncovered or CCT-covered microspheres (polystyrene, 0.5 μm diameter) were added to the mucosal or submucosal compartment and the overall corresponding unidirectional transports during 2 hours (T_{ms}, T_{sm}) were determined by a Burker chamber. Based on their difference the overall net transport (T_{net}) was calculated. When present, cytochalasin D was on both sides of the tissue during the 30 min preincubation and 2 h subsequent incubation.
Results presented as means ± S.E. (number of experiments in parentheses).
°° $P < 0.01$ compared the respective T_{sm}.
·· $P < 0.01$ compared to the corresponding control.
** $P < 0.01$ compared to zero.

Fig. 1 reports the effects on V_{ms}, I_{sc} and R_{ep} elicited by treatments with cytochalasin D at different concentrations (0.1 and 1 μg/ml; treatment, as above, on both sides of the tissue for the whole experiment, beginning just after electrical measurements were taken at zero min time). With both inhibitors concentrations R_{ep} proved to be significantly reduced after 30 min of treatment, the decrease already being maximal with 0.1 μg/ml inhibitor. This result, which indicates an increase in intercellular junctional permeability, is in accordance with the reduction in junctional resistance observed with cytochalasin B (10 μg/ml) in other epithelia, in which it has been shown that tight-junction permeability is under cytoskeletal control [9]. The same figure also shows that 0.1 μg/ml cytochalasin D does not significantly affect I_{sc} which is an index of the active ion transports through the cellular pathway (in this epithelium: active Na^+ absorption, active Cl^- secretion [10]). It is only with a concentration of 1 μg/ml ($2 \cdot 10^{-6}$ M) that, after 30 min of treatment, this cellular parameter also decreases significantly compared to the control. V_{ms}, which is a function of both R_{ep} and I_{sc}, is obviously modified at concentrations of both 0.1 and 1 μg/ml.

4. Discussion

The results obtained show that the three predictions reported in Section 1 are all fulfilled.

Net absorption is also present with elcatonin adsorbed on microspheres. This net absorption is specific, as no net transfer is observed with CCT-uncovered microparticles (which, conversely, should also display a net transfer in the presence of pinocytosis).

As for the third prediction, of the cytochalasins, cytochalasin D is considered to be the most specific agent on actin microfilaments, mainly at the concentration of 0.1 μg/ml [4], although it is often used at higher concentrations. Unlike cytochalasin B, it does not display any inhibition on monosaccharide-facilitated transport, which supplies glucose to the cellular metabolism [4].

The results reported here show that in the nasal mucosa 0.1 μg/ml cytochalasin D does not affect cellular active ion transports, which are only modified at higher inhibitor concentrations. This fact supports the notion that metabolism is not influenced. Nevertheless, at the same inhibitor concentration, the net CCT transport is abolished. Clearly, this effect cannot be due to cellular ATP depletion. At a first sight it seems rather to be evidence in favor of the presence of vesicular transport supported by the actin cytoskeleton. On the other hand, in agreement with the findings for other epithelia [9], in the nasal mucosa intercellular junction permeability also proves to be regulated by the actin cytoskeleton: in fact, the transepithelial electrical resistance decreases and CCT T_{sm} increases on treatment with 0.1 μg/ml cytochalasin D. Hence the abolition of the net CCT transport apparently might also be due to a mere increase in T_{sm}, with an action of cytochalasin D only on the actin cytoskeleton regulating tight-junctions. However, some considerations are against this explanation.

T_{sm} is a paracellular/intercellular passive unidirectional flux; T_{ms} has two components, i.e., a paracellular/intercellular passive unidirectional flux, equal to T_{sm} at any situation, and a cellular active net flux. If the intercellular fraction of T_{sm} increases owing to the opening of tight-junctions, the passive intercellular component of T_{ms} also increases to the same extent and the overall T_{ms} must increase. Conversely, in the present case T_{ms} is unmodified, J_{net} disappears and the remaining component of T_{ms} is the passive one equal to T_{sm}. Thus, concomitantly T_{ms} has undergone an increase in the passive component (which would have increased the overall T_{ms}) and a decrease of the active component (which would have decreased the overall T_{ms}): due to these two opposite changes, T_{ms} is apparently unmodified. The point is what is the cause of the disappearing of the active component of T_{ms}. One possibility is that the cellular active transport is abolished; however, it is also possible that it remains, but simply is not measurable transepithelially, if the CCT actively transported by the cell into the intercellular channels back-diffuses to the lumen, owing to the tight-junction increased permeability. Thus, the results obtained with CCT alone cannot lead to a univocal interpretation neither for nor against the abolition of active transport at the cellular level. However, CCT, adsorbed on microspheres, displays a T_{sm} which can be only paracellular (owing to the micro-

sphere diameter [2]) and cannot be affected by changes in tight-junction permeability, even if they take place (as occurs during cytochalasin D treatment). In this way it is possible to observe whether actin cytoskeleton regulates directly the active transport, the importance of the effects on tight-junctions having been eliminated by the particular experimental condition. Actually, with this trim cytochalasin D does not modify T_{sm}, but equally abolishes T_{net}, revealing the decrease in T_{ms}.

The univocal conclusion is that CCT active transport is directly and completely inhibited at the cellular level and that the active polypeptide transport previously evidenced in the nasal mucosa is supported by specific vesicular transport driven by actin microfilaments, probably at the apical border of the cell. These conclusions are in agreement with those reported by a parallel paper in which we show that active transport of the polypeptide (presented alone to the tissue) is also inhibited by cytochalasin B (although the possibility of indirect actions in that case remains open) and by some typical endocytosis inhibitors (colchicine, aluminum fluoride, monensin) [11].

[2] Since microspheres cannot cross the epithelium intercellularly through the leaky junctions, owing to their 0.5 μm large diameter, the T_{sm}, already present under control conditions, is likely to be due to paracellular passage through a very few large pores due to points of cellular damage on which cytochalasin D obviously cannot display any effect. That the number of these pores is very small is demonstrated by the fact that bead concentration in the flux chamber, at the end of the 2-h experiment, was 10^5 times less than in the donor chamber. A similar paracellular passage through a few points of cell damage can also explain the absorption of uncovered microspheres measured 'in vivo': in this case, the absorption is actually a unidirectional flux (= T_{ms}) [12].

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