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The effect of chitosan and other polycations on tight junction permeability in the human intestinal Caco-2 cell line

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Abstract

Chitosan is a polycationic compound widely employed as dietary supplement and also present in pharmaceutical preparations. Although it has been approved for human consumption, its possible side effects have not been widely investigated and the available data in the literature are still controversial. Several polycationic substances have been shown to affect tight junction permeability in epithelial cell models *in vitro.* In this study we have compared the effects of chitosan and other polycations (polyethylenimine, poly-L-lysines of different molecular weights) on the integrity of tight junctions and of the actin cytoskeleton in the human intestinal Caco-2 cell line. We have measured trans-epithelial electrical resistance and paracellular passage of the extracellular marker inulin, and we have localized F-actin and tight junctional proteins (ZO1 and occludin) in cell monolayers treated with various concentrations of each polycation. Fluorescent poly-L-lysines were also employed to determine their association with the cell monolayer. Our results indicate that all polycations investigated are able to induce a reversible increase in tight junction permeability. This effect is concentration and energy dependent, affected by the extracellular concentration of divalent cations (calcium, magnesium and manganese) and it is associated with morphological changes in the F-actin cytoskeleton, as well as in the localization of tight junctional proteins. Chitosan, in particular, was the only cationic polymer that displayed an irreversible effect on tight junctions at the highest concentration tested (0.01%). These results indicate that oral ingestion of chitosan may have more widespread health effects by altering intestinal barrier function, thus allowing the entrance into the circulation of potentially toxic and/or allergenic substances.

Keywords: Tans-epithelial electrical resistance; Inulin passage; Poly-L-lysine; Polyethylenimine; F-actin; ZO1; Occludin

1. Introduction

Chitosan is a cationic polysaccharide obtained by partial alkaline N-deacetylation of chitin. This polymer is present in several over the counter products employed as dietary supplements for the control of overweight and hyperlipidemia. As a nutritional supplement chitosan has been reported to reduce fat absorption in the intestine by binding fatty acids, triglycerides and bile acids and increasing their excretion. It is believed that by reducing the recycling of bile acids to the liver, chitosan induces hepatic synthesis of new bile constituents from cholesterol, thus contributing to a reduction of cholesterolemia [1]. However, the molecular mechanisms of the hypocholesterolemic and lipid lowering effects of chitosan in humans are still not fully understood [2]. Human trials have shown that chitosan is effective for overweight control only when associated with a hypocaloric diet. Conversely, the possibility of adverse effects of chitosan administration has been raised, such as deprivation from the diet of trace elements and liposoluble vitamins due to the chelating properties of chitosan and to its high affinity for lipids**,** as well as alterations of the composition of the intestinal microflora due to its antimicrobial properties [3,4].

In addition to their use as food supplements, chitosan and its derivatives are under investigation as agents to be added to pharmacological preparations to improve peroral delivery of poorly absorbed drugs, such as peptides [5,6]. The pharmacological effects of chitosan may result from its mucoadhesive properties, delaying intestinal permanence of drugs in the lumen, and from a direct effect on tight junc-

Abbreviations: AP = apical; BL = basolateral; 2-DOG = 2-deoxyglucose; HBSS = Hanks balanced salt solution; FITC = fluorescein isothiocyanate; HEPES = N-2-hydroxyethyl piperazine-N-4-butanesulfonic acid; HMW = high molecular weight; LMW = low molecular weight; MES = morpholinoethane sulfonic acid; PBS^+ = phosphate buffered saline; PEI = polyethylenimine; TEER = trans-epithelial electrical resistance; TRITC = tetramethylrodamine isothiocyanate.

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tions, increasing intestinal permeability, thus allowing paracellular absorption of hydrophilic drugs. Recently, different polycationic compounds, have also been proposed as vectors for *in vivo* gene transfer [7].

Food supplements are commonly regarded as harmless compounds with beneficial effects for the health of consumers. However, potential adverse metabolic effects have been associated with prolonged ingestion of chitosan [3]. In addition, the possibility of toxic effects at the level of the intestinal mucosa should be considered.

Several reports have shown that chitosan and other cationic polymers are able to induce a reversible opening of tight junctions in different epithelial cell models, including the human intestinal Caco-2 cell line [6,8–14]. In addition, natural cationic products secreted by activated neutrophils, such as defensin and cathepsin G, increase the permeability of epithelial cell monolayers at least in part by their positive charge [15]. However, despite several lines of evidence indicating that polycations can increase the tight junction permeability of epithelial cells, the mechanisms involved have not yet been clarified.

The human intestinal Caco-2 cell line, grown and differentiated on permeable filter supports, has been extensively employed in the study of absorption enhancers [6] as well as in studies of sub-lethal toxicity of different cytotoxic agents [16,17]. An increase in tight junction permeability can be regarded as a sub-lethal toxic effect, as it allows indiscriminate flow of molecules from the intestinal lumen to the blood circulation, thus by-passing the innate barrier function of the intestinal mucosa.

The present study was therefore aimed at comparing the effects of chitosan and of other polycations on the tight junction permeability in the human intestinal Caco-2 cell line. In particular, we have carried out a comprehensive study of the functional and morphological effects of four different polycations: chitosan, polyethylenimine (PEI) and poly-L-lysine with high (300,000 Da) and low (30,000 Da) molecular weight, under different experimental conditions. Since the effects of chitosan on epithelial membranes are mediated by positive charges, we chose two other polycations (namely poly-L-lysines and PEI) as controls to study the effects of chitosan on tight junction permeability in Caco-2 cells. Unlike chitosan, which is a mixture of polymers of various sizes, PEI and poly-L-lysines are well characterized in terms of their chemical properties and molecular weights. Although neither of these latter compounds has ever been used as nutritional supplement, they have both been shown to increase paracellular permeability due to their polymeric nature and cationic properties.

2. Materials and methods

2.1. Cell culture

The human intestinal Caco-2 cell line was obtained from Prof. Alan Zweibaum (INSERM, Villejuif, Paris, France). Caco-2 cells were grown and maintained as previously described [17] in Dulbecco Modified Minimum Essential Medium containing 25 mM glucose, 3.7 g/L $NaHCO_3$ and supplemented with 4 mM L-glutamine, 1% non-essential amino acids, 1 X 10^5 U/L penicillin, 100 μg/L streptomycin and 10% heat inactivated fetal calf serum (complete culture medium). The cells were seeded on polycarbonate filter cell culture chamber inserts (Transwell, 24 mm diameter, 4.7 cm^2 area, 0.45 μm pore diameter; Costar Europe Ltd., Badhoevedorp, The Netherlands). For morphological studies transparent filters were utilized to allow for microscopy (P.E.T. track-etched membrane, 25 mm diameter, 4.71 cm^2 area, 0.4 μm pore diameter; Becton Dickinson Labware Europe, Meylan Cedex, France). Cells were seeded at a density of 4 X 10^5 cells/cm^2 and were left to differentiate for 15–17 days after confluence; the medium was regularly changed three times a week.

2.2. Preparation of polymer solutions

Chitosan (poly-D-glucosamine hydrochloride) with a degree of acetylation of 22.3%, was obtained from Pronova Biomedical (Portsmouth, NH, USA), PEI with an average molecular weight of 25,000 Da, high molecular weight (HMW) and low molecular weight (LMW) poly-L-lysines with average MW 30,000 and 300,000 Da, respectively, were all purchased from Sigma-Aldrich (Milano, Italy). All polymers were dissolved at a concentration of 1% (w/v) in Hanks balanced salt solution (HBSS : 137 mM NaCl, 5.36 mM KCl, 0.44 mM KH_2PO_4, 0.34 mM, Na_2PO_4, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 5.6 mM glucose) containing 10 mM morpholinoethane sulfonic acid (MES) pH 6.0, and were stored at -20°C. Prior to each experiment, polymer stock solutions were diluted to the appropriate working concentration in HBSS pH 6.0.

2.3. Treatment of Caco-2 cells with polycations

Experiments were performed in a water bath at 37°C using pre-warmed solutions. Before each experiment, cells were washed twice with phosphate buffered saline containing 1 mM $CaCl_2$ and 1 mM $MgCl_2$ (PBS^+) and pre-equilibrated for 10 min in the absence of the polymer with HBSS containing 10 mM MES at pH 6.0 in the apical (AP) compartment and HBSS containing 10 mM N-2-hydroxyethyl piperazine-N-4-butanesulfonic acid (HEPES) at pH 7.4 in the basolateral (BL) compartment. After removal of the medium, Caco-2 cells were treated for 2 h at 37°C with increasing concentrations of polycations in HBSS at pH 6.0 in the AP compartment, while the BL compartment contained HBSS at pH 7.4. These pH conditions were chosen in order to reproduce the physiological pH gradient existing in vivo across the small intestinal mucosa. After treatment, the polycation solution was withdrawn and the cells were washed three times with PBS^+ and transferred to complete

culture medium at 37°C for 24 h, to determine the reversibility of the polycation effect.

To investigate the energy dependence of the polymer effects, experiments were performed in the presence of 1 mM NaN_3 and 50 mM 2-deoxyglucose (2-DOG) in the AP and BL media. To achieve energy depletion of Caco-2 cells the pre-equilibration step before polymer addition was prolonged to 30 min.

When polycation effects were re-evaluated in the presence of 20 mM Ca^{2+}, Mg^{2+} or Mn^{2+} ions, the divalent cations were added to the AP compartment together with the tested polymer.

2.4. Monitoring tight junction permeability

The trans-epithelial electrical resistance (TEER) of the cell monolayers was measured at 37°C using a commercial apparatus (Millicell ERS; Millipore Co., Bedford, MA, USA) as previously described [17]. TEER was expressed as $\Omega \cdot cm^2$ after subtracting from the reading the resistance of the supporting filter and multiplying it by the surface area of the monolayer. TEER measures were started during the pre-equilibration time in HBSS and continued during the treatment, recording values every 20 min over the 2 h of the experiment. In addition, TEER values were recorded after 24 h recovery in complete culture medium. Control filters, maintained in HBSS for 2 h, displayed a stable baseline of TEER values; these values varied between single experiments in a range comprised between 800 and 1200 $\Omega \cdot cm^2$. Due to these variations in TEER values of control cells, TEER data were expressed as the percentage of control values at each time point.

The trans-epithelial passage of the radiolabeled extracellular marker (carboxyl-^{14}C-)inulin, (NEN Life Science Products, Milano, Italy), (specific activity 409 mBq/mmol) was also measured. Briefly, 18 μM radioactive inulin was added to the AP medium in the presence of the tested polycation, and the BL medium was collected every 30 min and replaced with fresh HBSS. The radioactivity in the BL medium was measured in a liquid scintillation counter (LS1801; Beckmann Instruments Inc., Irvine, CA, USA). Inulin passage to the BL chamber was expressed as the percentage of total radioactivity applied to the donor compartment.

2.5. Morphological studies

For morphological studies cells were seeded on cell culture chamber inserts fitted with transparent membranes. LMW and HMW poly-L-lysine conjugated with fluorescent dyes were employed to investigate the distribution of the polymer in treated cells. Experiments were performed essentially as described above except for the incubation time that was reduced to 1 h. Fluorescein isothiocyanate (FITC)-labeled LMW poly-L-lysine (47,000 Da) was obtained from Sigma-Aldrich. HMW poly-L-lysine (300,000 Da) was conjugated to the fluorescent Alexa 488 dye according to manufacturer instructions (Alexa TM 488, Protein Labeling Kit, Molecular Probes Inc., Eugene, OR, USA). Cells were incubated with 0.01% LMW poly-L-lysine and 0.005% HMW poly-L-lysine in HBSS at pH 6.0. After incubation with the labeled polymer, cells were washed with PBS^+ and fixed in methanol at -20°C for 2 min.

For immunofluorescent localization of tight junctional proteins, cells were fixed in methanol at -20°C for 2 min and were treated with primary antibodies and secondary tetramethylrodamine isothiocyanate (TRITC)-conjugated antibodies (Cappel-Organon Tecknika Co., Durham, NC, USA) according to conventional techniques. The antibodies used were: rabbit polyclonals anti-ZO1 and anti-occludin (Zymed Laboratories Inc., S. Francisco, CA, USA). For-F-actin localization cells were incubated for 30 min with 0.33 μg/ml FITC-phalloidin (0.25 μM; Sigma-Aldrich) in PBS^+ containing 0.2% BSA. When labeling of nuclei was required, filters were incubated for 30 min at 37°C in 20 μg/ml RNAse A (from bovine pancreas, Boehringer Mannheim Italia, Monza, Italy, previously boiled for 10 min to denature DNAse), rinsed in PBS^+ and stained with 0.001% propidium iodide (Sigma-Aldrich) in PBS^+ for 10 s. Filters were mounted cells-side up in Vectashield medium (Vector Laboratories, Burlingame, CA, USA). The cells were viewed with a fluorescent microscope (Axioskop 2, C. Zeiss; Jena, Germany).

Statistical analysis. Data were analyzed by one-way ANOVA followed by the Scheffé F-test to determine significant differences among means [18] (Statview 4.01; Abacus Concepts Inc., Berkeley, CA, USA).

3. Results

3.1. Chitosan and PEI increase tight junction permeability in a concentration-dependent fashion

The effect on the permeability of tight junctions of two cationic polymers, namely chitosan and PEI, was tested for 2 h on differentiated Caco-2 cells grown on permeable filter supports. The concentration range of the two polymers was 0.0002%–0.01% for chitosan and 0.0002–0.002% for PEI. Tight junction permeability was monitored by measuring the TEER (Fig. 1A , C) and the AP to BL trans-epithelial passage of the extracellular space marker inulin (Fig. 1B, D). Both polymers produced a concentration-dependent increase in the permeability of tight junctions in Caco-2 cells as shown by a progressive decrease in TEER with time of treatment and by an increase in the passage of inulin across the cell monolayer.

3.2. The effect of polycations on tight junction permeability is a molecular weight- and energy-dependent process

To investigate the effect of molecular weight on the polycation-induced alteration of tight junction permeability,

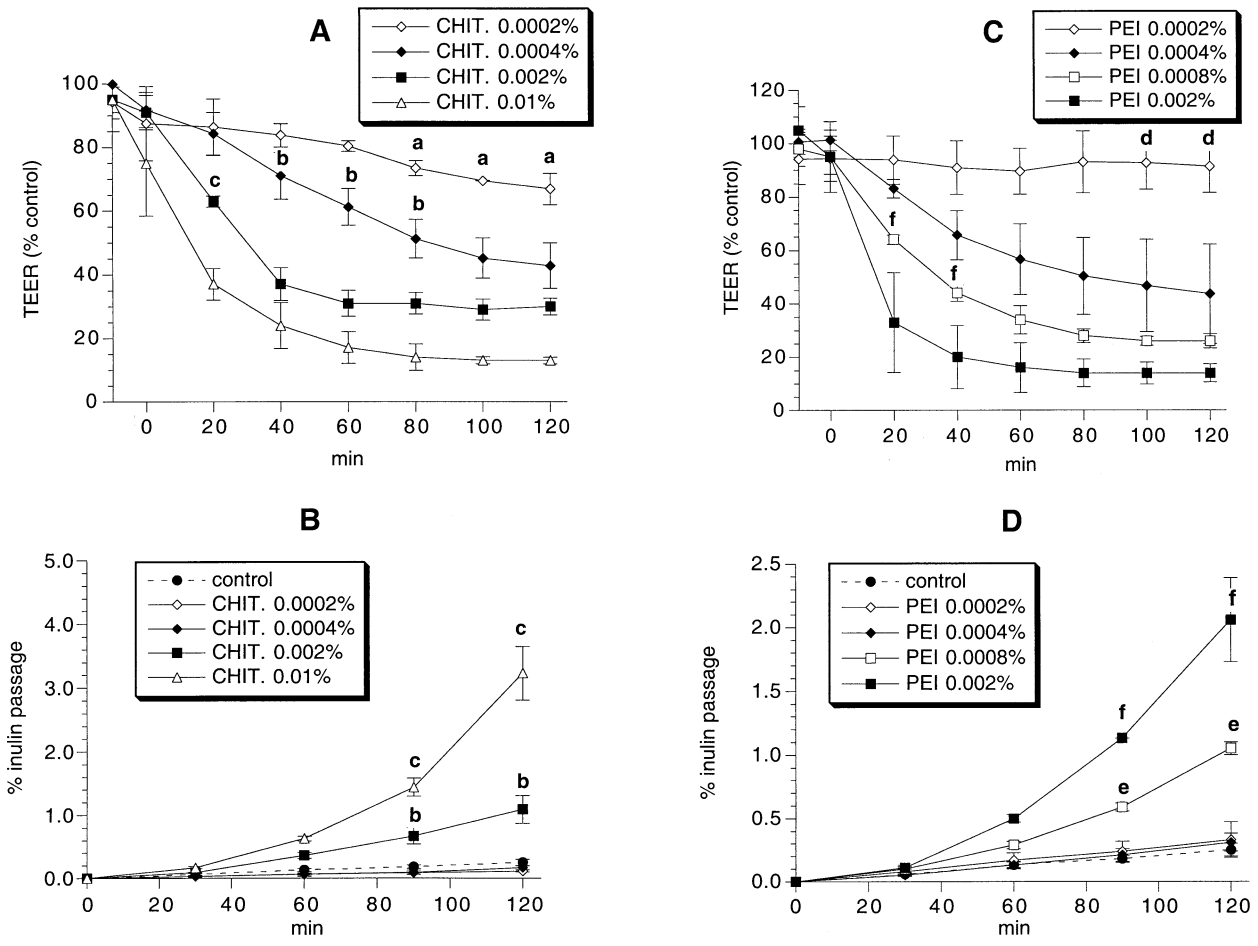

Fig. 1. ***Effect of increasing concentrations of Chitosan and PEI on tight junction permeability of Caco-2 cells grown on filter.*** Time–course of TEER variations over a 2h treatment period in the presence of chitosan (A) and PEI (C). Polymer was added at $t = 0$. TEER values were calculated as the percentage of the control TEER values at each time point. Time-course of the AP to BL passage of inulin during chitosan (B) and PEI (D) treatments. Inulin passage is expressed as the percentage of the amount of inulin added to the AP compartment at the initial time point. The plotted data are the mean ± SD of three experiments performed in duplicate. Letters above the error bars indicate significant differences (one-way ANOVA followed by Scheffé F-test) between the treatments. Chitosan: 0.0002% vs. 0.0004% (a, $P < 0.05$); 0.0004% vs. 0.002% (b, $P < 0.05$); 0.002% vs. 0.01% (c, $P < 0.05$). PEI: 0.0002% vs. 0.0004% (d, $P < 0.05$); 0.0004% vs. 0.0008% (e, $P < 0.05$); 0.0008% vs. 0.002% (f, $P < 0.05$).

poly-L-lysines of different molecular weight were assayed in Caco-2 cells. As shown in Fig. 2, HMW poly-L-lysine (300,000 Da) and LMW poly-L-lysine (30,000 Da) were tested in the concentration range between 0.002% and 0.01% for 2 h and TEER (Fig. 2A) and inulin passage (Fig. 2B) were monitored during the course of the experiment. At the same concentration, namely 0.002%, the HMW polymer exhibited a significantly higher effect on tight junctions than the LMW compound. Similar values of TEER and percentage of inulin passage were produced by 0.01% LMW poly-L-lysine and by a fivefold lower concentration of HMW poly-L-lysine (0.002%).

The different effects of HMW and LMW poly-L-lysines were also observed at the morphological level by using fluorescent dye-conjugated compounds. As shown in Fig. 3, both polycations appeared to concentrate in large fluorescent bodies. Treatment with 0.005% HMW poly-L-lysine (Fig. 3A) caused a greater number of cells to accumulate the fluorescent compound than a two fold higher concentration of LMW poly-L-lysine (Fig. 3B). Under conditions of reduced intracellular energy, obtained by treatment with 1 mM NaN_3 and 50 mM 2-DOG, (Fig. 3C), very little of the HMW polymer appeared to remain associated with the cells, while nuclear staining with propidium iodide indicated that the monolayer remained intact (Fig. 3D). The same result was obtained following energy-deprivation of LMW treated Caco-2 cells (data not shown). At the functional level, treatment of Caco-2 cells with 0.005% HMW or with 0.002% chitosan under conditions of reduced intracellular energy, significantly delayed the effect on tight junction permeability, as monitored by TEER (Fig. 4A, C). In addition, energy reduction lowered the trans-epithelial passage of inulin (Fig. 4B, D), suggesting the involvement of an energy-dependent mechanism. Treatment with 1 mM NaN_3 and 50 mM 2-DOG did not by itself alter tight junction permeability in control cells up to 2 h (data not shown).

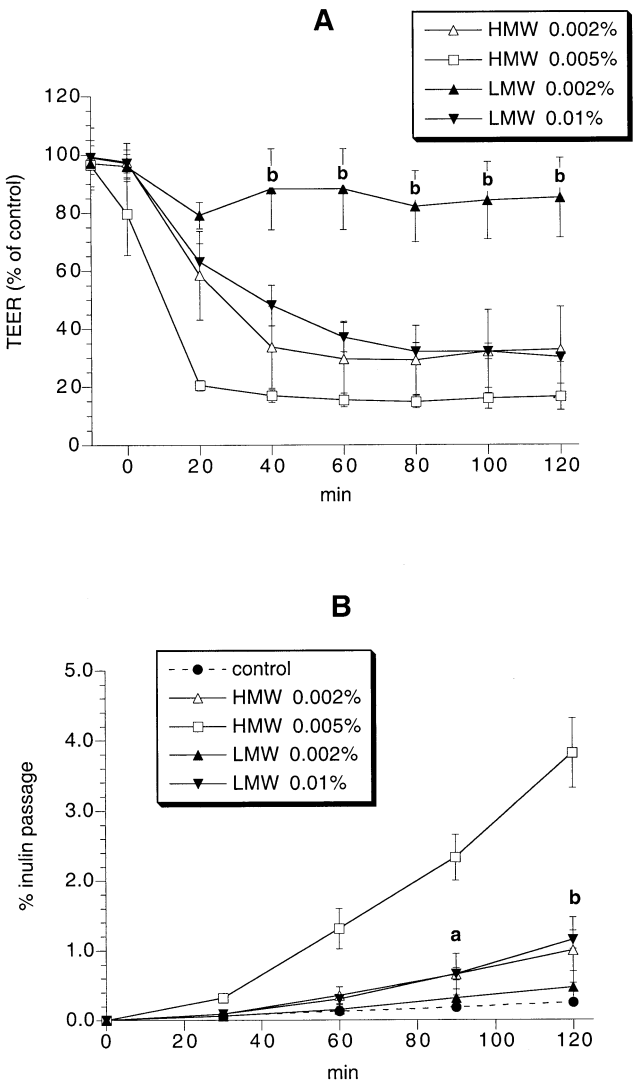

Fig. 2. ***Effect of molecular weight on polycation-induced opening of tight junctions in Caco-2 cells.*** Time course of TEER (A) and trans-epithelial inulin passage (B) following the addition of HMW (300,000 Da) and LMW (30,000 Da) poly-L-lysines. Polymer addition at $t = 0$. The plotted data are the mean ± SD of three experiments performed in duplicate. Letters above the error bars indicate significant differences (a, $P < 0.05$; b, $P < 0.01$, one-way ANOVA followed by Scheffé F-test) between 0.002% LMW and 0.002% HMW.

Table 1 shows a comparison of the effects on TEER of the three different polycations at all concentrations tested, after 2 h of treatment as well as after recovery in complete medium for 24 h following removal of the polycations. Cells treated with 0.01% chitosan showed very little recovery after 24 h, suggesting that a permanent damage had occurred. Conversely, full recovery was achieved following treatment with a fivefold lower concentration of chitosan (0.002%), despite the marked effect on tight junction permeability. For the other polymers full recovery was achieved in most cases, even when TEER values were greatly reduced by the treatments (0.01% LMW and HMW poly-L-lysine).

3.3. Divalent cations exerted different effects on the polymer-induced opening of tight junctions

The effect of divalent cations on the increase in tight junction permeability produced by treatment with different polycations is shown in Fig. 5. The presence of an excess (20 mM) $CaCl_2$, $MgCl_2$ or $MnCl_2$ in the AP medium altered in different directions the percentage of inulin passage induced by treatment with 0.002% chitosan (Fig. 5A), 0.005% HMW poly-L-lysine (Fig. 5B), and 0.0008% PEI (Fig. 5C). The presence of calcium and of manganese ions significantly reduced the passage of inulin produced by treatment with 0.002% chitosan; magnesium ions, on the other hand, had no effect (Fig. 5A). In the case of the increased inulin passage produced by treatment with 0.005% HMW poly-L-lysine, further enhancement was observed with magnesium ions, while calcium and manganese had no effect (Fig. 5B). Finally, the effect of 0.0008% PEI was significantly enhanced by magnesium and significantly reduced by manganese, while it remained unchanged in the presence of calcium (Fig. 5C).

3.4. Effect of polycations on the actin cytoskeleton and on tight junctional proteins

Since several agents that increase the tight junction permeability of intestinal epithelial cells are also known to alter the actin cytoskeleton, we compared the effects of the different polycations on the distribution of F-actin by specific staining with fluorescent falloidin. As shown in Fig. 6, control cells exhibited a regular distribution of actin filaments over the cell monolayer (panel A). Treatment with 0.005% chitosan for 1h dramatically reduced staining and altered the distribution of F-actin (panel B). In addition, single cells in the monolayer appeared to have lost staining almost completely. A similar effect was observed following treatment with 0.0008% PEI for the same amount of time (panel C), while only mild alterations in the F-actin cytoskeleton were detected following treatment of the cell monolayer with 0.005% HMW poly-L-lysine (panel D). Since treatment with 0.005% chitosan produced the most dramatic effect on the F-actin cytoskeleton, we investigated the distribution of the tight junctional proteins ZO1 and occludin, under the same experimental conditions. As shown in Fig. 7 in control cells ZO1 (panel A) and occludin (panel B) were localized at the cell periphery. Following treatment with the polycation, single cells appeared to have lost the peripheral staining for both ZO1 (panel C) and occludin (panel D). Double staining with fluorescent falloidin showed that the disappearance of the tight junctional proteins was associated with loss of staining for F-actin (panels E and F).

4. Discussion

The purpose of the present research was to determine the effect of the polycationic polymer chitosan, a widely used dietary supplement for weight control, on tight junction

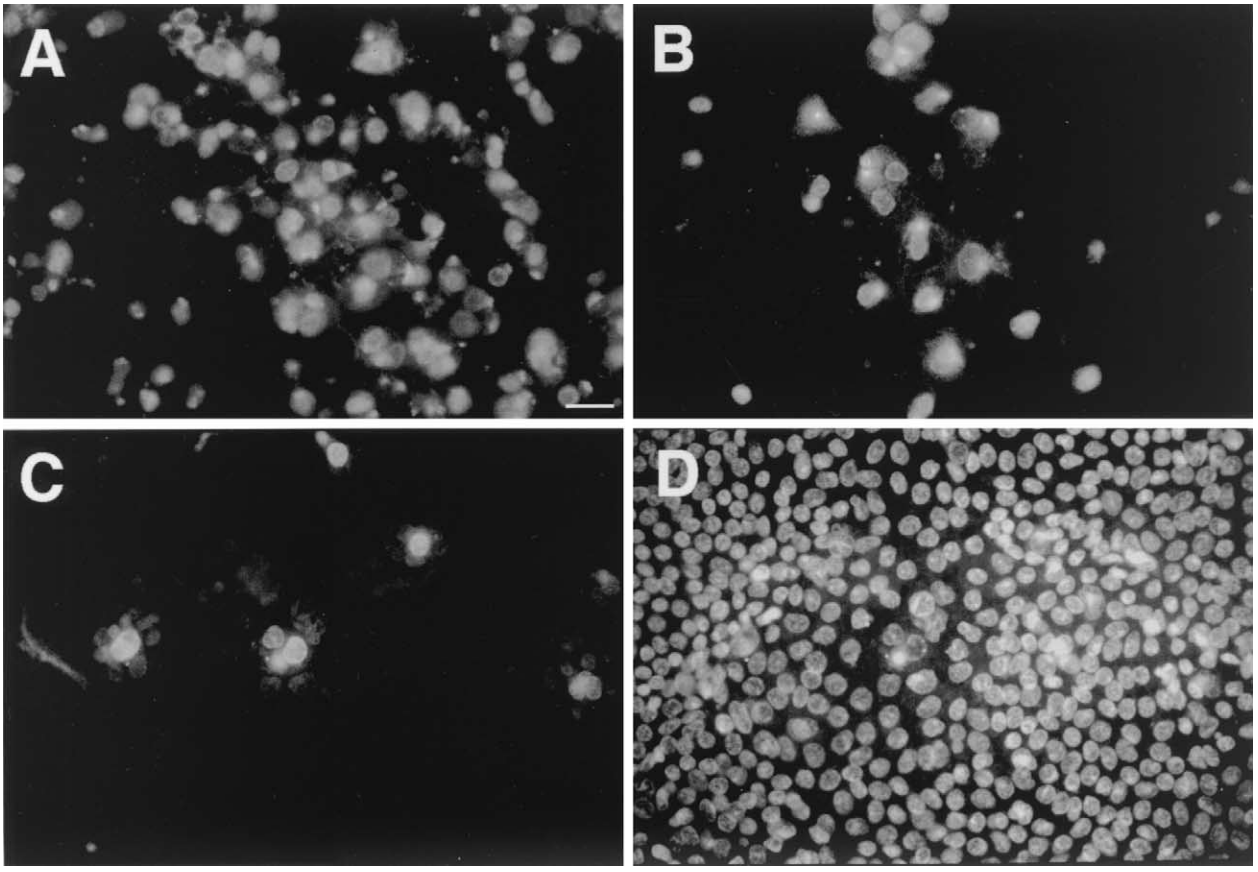

Fig. 3. ***Morphological localization of fluorescent HMW and LMW poly-L-lysines on Caco-2 cell monolayers.*** Cells were grown on transparent filter supports and treated for 1h with 0.002% Alexa 488-labeled HMW poly-L-lysine (A) and 0.01% FITC-labeled LMW poly-L-lysine (B). Cell monolayer treated with 0.002% Alexa 488-labeled HMW poly-L-lysine under conditions of reduced intracellular energy (C). Same field as in C, stained with propidium iodide to label cell nuclei (D). Magnification is the same in all panels. The bar in panel A corresponds to 20 μm.

permeability in intestinal epithelial Caco-2 cell cultures. Chitosan is an amino polysaccharide derived from the shells of marine crustaceans and composed of glucosamine and N-acetyl-glucosamine subunits. Besides its use as a nutrient supplement, chitosan is also employed in food processing and in some functional foods [19] as well as in pharmaceutical preparations [6]. The mechanisms proposed for its action in reducing the intestinal absorption of dietary lipids and cholesterol are all based on its cationic properties [2]. Emulsification of lipids and hydrophobic compounds at low pH in the stomach would lead to polar entrapment in lipid droplets, thus reducing digestibility and absorption of lipids. However, the remaining free charges on chitosan molecules could still interact with cell membranes of epithelial cells in the intestinal tract, ultimately altering the barrier function of the mucosa.

We have compared chitosan to other polycations, PEI and poly-L-lysines, to ascertain the factors that affect the permeability enhancement in the *in vitro* intestinal Caco-2 cell model. Tight junction permeability can be measured *in vitro* by different methods: TEER, as a measure of ionic permeability of the cell monolayer, and paracellular passage of extracellular markers of different sizes that are not absorbed or metabolized by the cells (e.g. mannitol, inulin). TEER does not distinguish between membrane and paracellular conductance and is a useful indicator of early perturbations of the ionic permeability of the cell monolayer. Conversely, paracellular marker passage is a more suitable parameter to describe long-term variations in the permeability of tight junctions to larger molecules [13]. For these reasons both methods were utilized in the present study to assess the effects of polycations on differentiated Caco-2 cells.

Increasing concentrations of chitosan and PEI resulted in a dose-dependent effect on tight junction permeability for both polymers. However, the time course of such effect was different depending on the method applied to measure tight junction permeability, TEER changes being observed earlier than the increase in inulin passage. A similar trend in TEER decrease as a function of chitosan concentration was previously reported in Caco-2 cells [8]. In addition, these authors also reported changes in the paracellular flux of mannitol, expressed as Papp (apparent permeability). We have not expressed the passage of inulin with Papp values as this parameter assumes a linearity of the process. Conversely, as shown by our data in Fig. 1 B, D, inulin passage exhibits a lag of approximately 30 min and increases during the following 90 min of treatment. This lag probably reflects

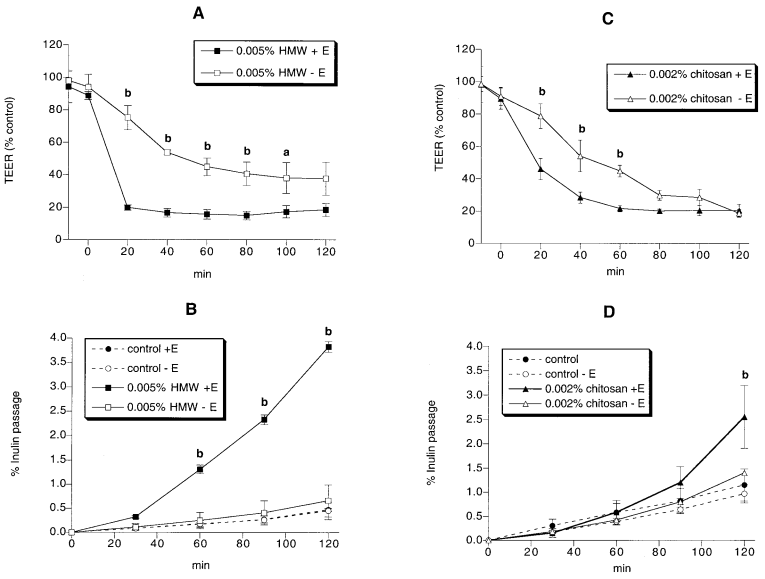

Fig. 4. ***Energy-dependence of the effect of HMW poly-L-lysine and chitosan on tight junction permeability.*** Time-course of TEER and inulin passage following treatment of cell monolayers with HMW poly-L-lysine (A, B) and chitosan (C, D) under control conditions (+E) and reduced intracellular energy conditions (-E). Polymer addition at $t = 0$. The plotted data are the mean ± SD of three experiments performed in duplicate. Letters above the error bars indicate significant differences (a, $P < 0.05$; b, $P < 0.01$; one-way ANOVA followed by Scheffé F-test) between +E and -E.

a progressive increase in the opening of tight junctions, and suggests that TEER has to decrease below a threshold level (about 50% of control values) before paracellular passage of larger molecules can occur [20].

Other polycations, such as poly-L-lysine, have previously been reported to increase paracellular permeability in epithelial cells [13,15]. Our results indicate that the effect of poly-L- lysine on tight junction permeability in Caco-2 cells is affected by molecular weight. As shown in Fig. 2, similar changes in TEER and inulin passage, were obtained using a fivefold lower concentration of HMW (0.002%) than of LMW (0.01%) poly-L-lysine. An analogous effect of molecular weight was previously reported for this polycation in alveolar epithelium *in situ* [15] and for chitosan in Caco-2 cells [21]. Thus, molecular weight appears to influence the effect of different polycations on epithelial tight junctions with no tissue-specificity. The mechanism responsible for this effect may therefore be related to the different structural conformations assumed by molecules of distinct molecular weights and the resulting variations in charge density at the cell surface.

Table 1
Comparison of the effects of polycations on TEER in Caco-2 cells

Polycation	Concentration % (w/v)	2 h Treatment % Control (±SD)	24 h Recovery % Control (±SD)
Chitosan	0.002	28.5 ± 8.7	93.5 ± 6.6
	0.01	14.1 ± 2.5	35.8 ± 4.5
HMW	0.002	23.0 ± 4.6	90.1 ± 17.6
poly-L-lysine	0.01	15.0 ± 2.2	113.0 ± 5.7
LMW	0.002	84.7 ± 13.8	97.3 ± 2.6
poly-L-lysine	0.01	21.0 ± 5.3	106.7 ± 16.6
PEI	0.0004	43.7 ± 18.6	90.5 ± 12.0
	0.001	14.2 ± 7.0	76.7 ± 17.1

The data refer to TEER measurements, expressed as the % of control after 2 h of treatment with the polymer and following 24 h of recovery in complete culture medium. Each value is the mean ± SD of duplicate filters from 3 experiments.

Coupling of HMW and LMW poly-L-lysines with a fluorescent marker allowed morphological analysis of the interaction of these polycations with Caco-2 cells. As shown in Fig. 3 A, B the polymer appeared to associate with the cells in aggregates of varying sizes. Treatment with HMW poly-L-lysine resulted in a higher number of cell-associated aggregates (Fig. 3A) than treatment with LMW poly-L-lysine (Fig. 3B), despite a twofold lower concentration of the former polymer. This result suggests that the HMW polycation presents structural features that promote autoaggregation, which may in turn be related to the stronger effect on permeability exhibited by HMW as compared to LMW poly-L-lysine. When HMW poly-L-lysine treatment was performed under conditions of lowered intracellular energy the number of fluorescent aggregates associated with Caco-2 cells was strongly reduced (Fig. 3C). This observation correlates well with the diminished effect of HMW poly-L-lysine under conditions of intracellular energy reduction, as monitored by TEER changes and by inulin passage (Fig. 4A, B). Taken together, these observations indicate that the effect of the polycation on tight junction permeability correlates with the number of aggregates associating with the cells and involves an energy dependent step**.** Also in the case of chitosan, energy depletion reduced

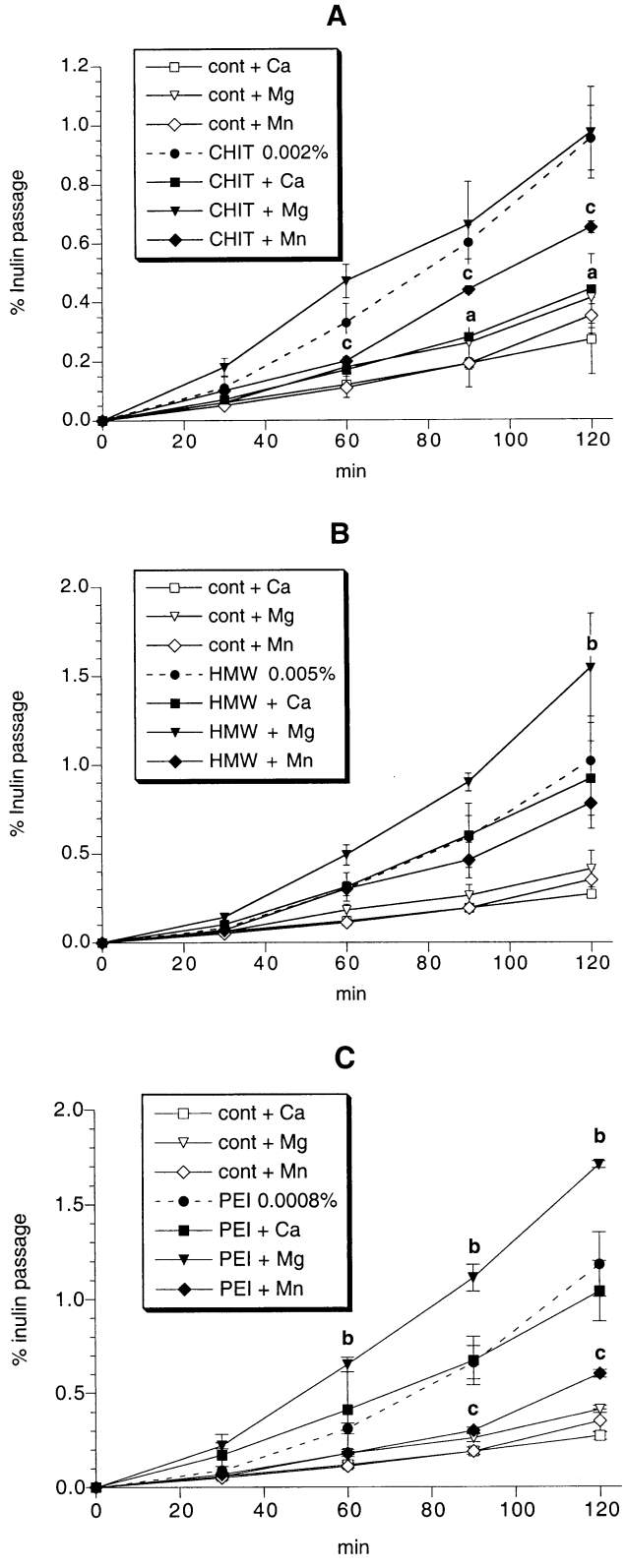

Fig. 5. ***Effect of divalent cations on polymer-induced opening of tight junctions.*** Inulin passage was measured during treatment with chitosan (A), HMW poly-L-lysine (B) and PEI (C), in the presence or absence of 20 mM calcium, magnesium or manganese salts in the AP medium. The plotted data are the mean ± SD of three experiments performed in duplicate. Letters above the error bars indicate significant differences (one-way ANOVA followed by Scheffé F-test) between the polycation alone and the polycation in the presence of Ca (a, $P < 0.05$), Mg (b, $P < 0.05$), or Mn (c, $P < 0.05$).

and delayed the opening of tight junctions in Caco-2 cells (Fig. 4C, D). Therefore, for both polycations energy appears to be required for maximal effect. It is important to note that since the paracellular "gate" function of tight junctions is an energy-dependent process [22], treatment of Caco-2 cells with inhibitors of energy metabolism can only be aimed at a partial reduction of intracellular energy levels to maintain the integrity of tight junctions in the absence of polycations.

The energy-dependence of polycation-induced opening of tight junctions may be related to the intracellular uptake of the polymer. Although there are contradictory data in the literature as to the ability of polycations to physically enter the cells [23], several lines of evidence indicate that these polymers can be internalized. Rapid uptake of polycations in glomerular epithelial cells has been associated with increased paracellular leakage of albumin [9]. Poly-L-lysine conjugated to proteins has been shown to promote their uptake in fibroblasts [24]. Moreover, polycations can act as DNA carriers by binding the negatively charged nucleic acids and delivering them inside the cells, following interaction with the negative charges on the cell membrane. These features of polycations have been widely exploited to transfect DNA in different cell culture systems [7]. In light of all these results, the morphological evidence shown in Fig. 3 suggests internalization of poly-L-lysine as the process affected by energy depletion.

The time-course and extent of recovery of the end-point under investigation indicates the severity of cytotoxic effects. In our study, full recovery of tight junction integrity was achieved with most treatments; only in the case of Caco-2 cells treated with 0.01% chitosan, we did not observe recovery of TEER to control values after 24 h in complete medium (Table 1). However, even in this case, we did not detect any increase in lactate dehydrogenase activity in the culture medium over the recovery period (data not shown), a clear indication that the plasma membrane was not permanently damaged. The extent of recovery of tight junction integrity following treatment of Caco-2 cells with chitosans has previously been shown to depend on molecular weight, concentration and length of treatment [8,25]. In addition, recovery was shown to require *de novo* protein synthesis only in the case of high concentrations of chitosan (0.5% for 30 min [8]). Our data indicate that recovery of tight junction integrity cannot be achieved even after treatment with much lower concentrations of chitosan (0.01%

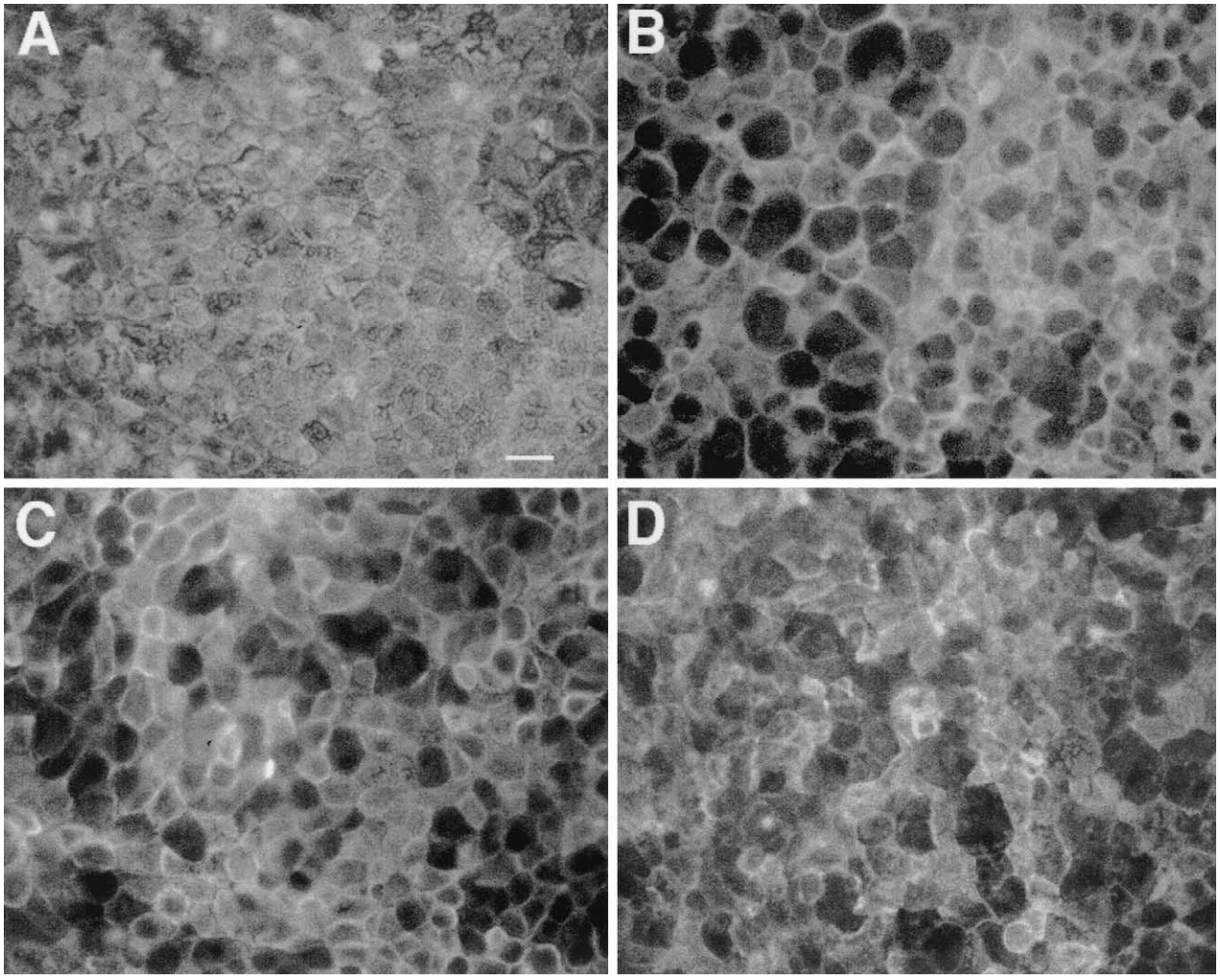

Fig. 6. ***F-actin staining of Caco-2 cell monolayers following polycation treatment.*** Caco-2 cells grown and differentiated on transparent filters (A) were treated for 1h with: 0.005% chitosan (B), 0.0008% PEI (C), 0.005% HMW poly-L-lysine (D). F-actin was visualized in all four panels by staining with FITC-labeled phalloidin. Magnification is the same in all panels. The bar in panel A corresponds to 20 μm.

for 2h). Since the chitosans employed in the two studies had a similar degree of acetylation (22% vs. 20%), these differences are likely due to the longer time of treatment used in our study. In addition, there may be differences in the molecular weight distribution of distinct batches of chitosan, since each preparation contains a mixture of different size polymers similarly to the commercial dietary supplements.

Several conditions that alter the permeability of the epithelial barrier also result in changes in the expression and distribution of the actin cytoskeleton and of tight junctional proteins [16,17,26,27]. Treatment of canine kidney MDCK cells with protamine or poly-L-lysine and of Caco-2 cells with chitosan were reported to affect the F-actin cytoskeleton and the tight junctional protein ZO1 [8,13,28]. We have extended these observations to verify whether this effect could be generalized. In this paper we report that the observed alterations in tight junction permeability following treatment of Caco-2 cells with HMW poly-L-lysine, chitosan and PEI, were accompanied by a reduction of F-actin staining. Moreover, double staining of F-actin with either of two tight junctional proteins (ZO1 and occludin), showed that cells exhibiting a reduction in F-actin staining had also lost peripheral signal for both tight junctional markers. Overall, the morphological alterations induced by polycation treatment appear to be related to the intensity of the effect of each polycation on tight junction permeability, as measured by TEER and inulin passage.

In addition to increasing tight junction permeability of epithelial cells, polycations have been reported to alter plasma membrane conductance: both effects appear to be influenced by the extracellular concentration of divalent cations [29–31]. In all cases reported in the literature divalent cations reduced the effect of polycations on cellular permeability. Although the mechanisms involved are still unclear, two possible modes of action have been proposed: divalent cations may interact with putative binding sites either on the polycation itself or on the cell membrane [29]. In the present study, an excess of Ca^{2+}, Mn^{2+} or Mg^{2+} in the experimental medium exhibited distinct effects on each

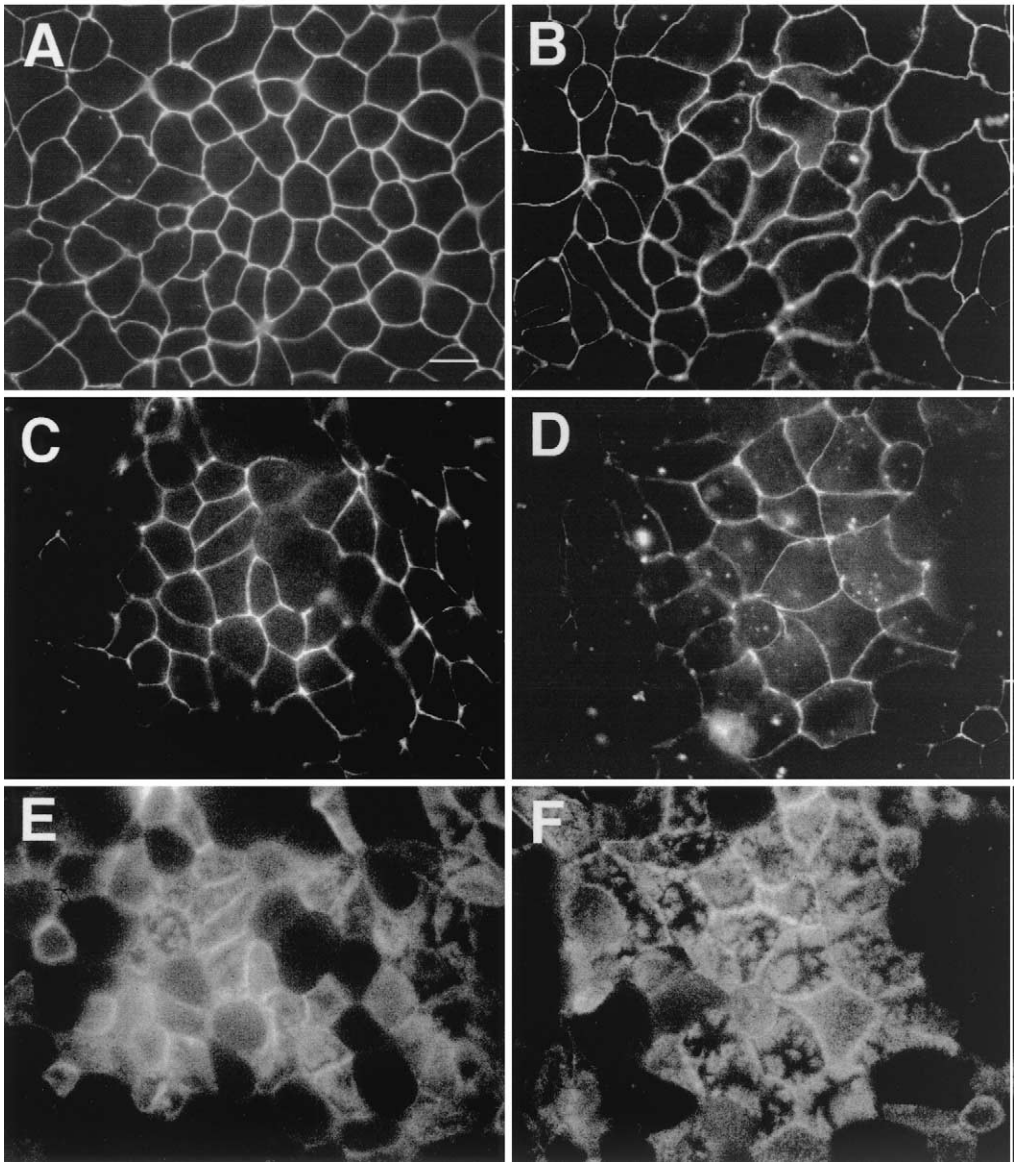

Fig. 7. ***Fluorescent staining of Caco-2 cells before and after chitosan treatment.*** Cell monolayers grown on transparent filters were stained by immunofluorescence with anti-ZO1 (A, C) or with anti-occludin (B, D) primary antibodies. Control cells are shown in panels A and B. Cells treated for 1h with 0.005% chitosan are shown in panels C and D. Double staining for F-actin of the same fields shown in C and D is shown in panels E and F. Magnification is the same in all panels. The bar in panel A corresponds to 15 μm.

polycation tested. We observed both an enhancement (Mg^{2+} with PEI and HMW poly-L-lysine) and a reduction (Ca^{2+} with chitosan and Mn^{2+} with PEI) of the effect of each polycation on the permeability of Caco-2 cells, as measured by the paracellular flow of inulin. Such diversity of effects may be ascribed to structural differences in solution among the polycations employed, which in turn may determine distinct affinities and binding capacities for the divalent cations. Conversely, it seems unlikely that the polycations employed possess distinct membrane binding sites on a relatively homogeneous cell population.

In conclusion, the results reported in the present paper indicate that the nutritional properties of the commonly used dietary supplement chitosan are not free of toxic side effects at the level of the epithelial cells of the intestinal mucosa. Our data indicate that the interaction of chitosan with intestinal cells induces a relatively rapid increase in paracellular permeability. Daily consumption of this compound as dietary supplement or food additive may lead to long-lasting changes in intestinal mucosal barrier function. Increased intestinal permeability allows the uncontrolled entry into the bloodstream of undesired molecules that could either be toxic or able to promote allergies. In order to fully establish the safety of chitosan for human consumption, further work is therefore necessary to determine the concentration levels achieved by this compound in the microenvironment of the intestinal lumen, to design preparations that would prevent it from reaching potentially toxic levels.

References

[1] R. Muzzarelli, Recent results in the oral administration of chitosan, in: R. Muzzarelli (Ed.), Chitosan *per os*: from dietary supplement to drug carrier, Atec, Grottammare, Italy, 2000, pp. 3–40.

[2] I. Furda, Reduction of absorption of dietary lipids and cholesterol by chitosan and its derivatives and special formulation, in: R. Muzzarelli (Ed.), Chitosan *per os*: from dietary supplement to drug carrier, Atec, Grottammare, Italy, 2000, pp. 41–63.

[3] S. Koide, Chitin-chitosan: properties, benefits and risks, Nutr. Res. 18 (1998) 1091–1101.

[4] R. Muzzarelli, Clinical and biochemical evaluation of chitosan for hypercholesterolemia and overweight control, in: P. Jolles and R. Muzzarelli (Eds.), Chitin and chitinase, Birkhauser Verlag, Basel, 1999, pp. 293–304.

[5] A. Bernkop-Schnurch, Chitosan and its derivatives: potential excipients for peroral peptide delivery systems, Int. J. Pharm. 194 (2000) 1–13.

[6] B. Aungst, Intestinal permeation enhancers, J. Pharm. Sci. 89 (2000) 429–442.

[7] M. Garnett, Gene-delivery systems using cationic polymers, Crit. Rev. Ther. Drug Carrier Syst. 16 (1999) 147–207.

[8] V. Dodane, M. Amin Khan, J.R. Merwin, Effect of chitosan on epithelial permeability and structure, Int. J. Pharm. 182 (1999) 21–32.

[9] M. Hammes, A. Singh, Effect of polycations on permeability of glomerular epithelial cell monolayers to albumin, J. Lab. Clin. Med. 123 (1994) 437–446.

[10] A. Kotze, M. Thanou, H. Luessen, A. de Boer, J. Verhoef, H. Junginger, Enhancement of paracellular drug transport with highly quaternized N-Trimethyl chitosan chloride in neutral environments: in vitro evaluation in intestinal epithelial cells (Caco-2), J. Pharm. Sci. 88 (1999) 253–257.

[11] A. Kotze, H. Luessen, A. de Boer, J. Verhoef, H. Junginger, Chitosan for enhanced intestinal permeability: prospects for derivatives soluble in neutral and basic environment, Eur. J. Pharm. Sci. 7 (1999) 145–151.

[12] A. Kotze, H. Luessen, B. de Leeuw, A. de Boer, J. Verhoef, H. Junginger, Comparison of the effect of different chitosan salts and N-trimethyl chitosan chloride on the permeability of intestinal epithelial cells (Caco-2), J. Control Release 51 (1998) 35–46.

[13] G. McEwan, M. Jepson, B. Hirst, N. Simmons, Polycation-induced enhancement of epithelial paracellular permeability is independent of tight junctional characteristics, Biochim. Biophys. Acta. 1148 (1993) 51–60.

[14] M. Thanou, A. Kotze, T. Scharringhausen, H. Luessen, A. de Boer, J. Verhoef, H. Junginger, Effect of degree of quaternization of N-trimethyl chitosan chloride for enhanced transport of hydrophilic compounds across intestinal Caco-2 cell monolayers, J. Control Release 64 (2000) 15–25.

[15] G. Saumon, P. Soler, G. Martet, Effect of polycations on barrier and transport properties of alveolar epithelium in situ, Am. J. Physiol. 269 (1995) L185–L194.

[16] I. De Angelis, O. Vincentini, G. Brambilla, A. Stammati, F. Zucco, Characterization of furazolidone apical-related effects to human polarized intestinal cells, Toxicol. Appl. Pharmacol. 152 (1998) 119–127.

[17] S. Ferruzza, M. Scarino, G. Rotilio, M. Ciriolo, P. Santaroni, A. Onetti Muda, Y. Sambuy, Copper treatment alters the permeability of tight junctions in cultured human intestinal Caco-2 cells, Am. J. Physiol. 277 (1999) G1138–G1148.

[18] D. Kleinbaum, L. Kupper, Applied regression analysis and other multivariable methods, Duxbury Press, Boston, MA, 1978.

[19] F. Shahidi, J. Arachchi, Y.-J. Jeon, Food applications of chitin and chitosans, Trends in Food Sciences Technology 10 (1999) 37–51.

[20] M. Briske-Anderson, J. Finlay, S. Newman, The influence of culture time and passage number on the morphological and physiological development of Caco-2 cells, Proc. Soc. Exp. Biol. Med. 214 (1997) 248–257.

[21] N. Schipper, K. Varum, P. Artursson, Chitosans as absorption enhancers for poorly absorbable drugs 1: Influence of molecular weight and degree of acylation on drug transport across human intestinal epithelial (Caco-2) cells, Pharm. Res. 13 (1996) 1686–1692.

[22] L. Mandel, R. Bacallao, G. Zampighi, Uncoupling of the molecular "fence" and paracellular "gate" functions in epithelial tight junctions, Nature 361 (1993) 552–555.

[23] N. Schipper, S. Olsson, J. Hoogstraate, A. de Boer, K. Varum, P. Artursson, Chitosans as absorption enhancers for poorly absorbable drugs 2: mechanisms of absorption enhancement, Pharm. Res. 14 (1997) 923–929.

[24] W. Shen, H. Reiser, Conjugation of poly-L-lysine to albumin and horseradish peroxidase: a novel method of enhancing the cellular uptake of proteins, Proc. Natl. Acad. Sci. U. S. A. 75 (1978) 1872–1876.

[25] H. Holme, A. Hagen, M. Dornish, Influence of chitosans with various molecular weights and degrees of deacetylation on the permeability of human intestinal epithelial cells (Caco-2), in: R. Muzzarelli (Ed.), Chitosan *per os*: from dietary supplement to drug carrier, Atec, Grottammare, Italy, 2000, pp. 127–136.

[26] T. Ma, D. Nguyen, V. Bui, H. Nguyen and N. Hoa, Ethanol modulation of intestinal epithelial tight junction barrier, Am. J. Physiol. 276 (1999) G965–G974.

[27] J. Madara, Regulation of the movement of solutes across tight junctions, Ann. Rev. Physiol. 60 (1998) 143–159.

[28] M. Peterson, D. Gruenhaupt, Protamine increases the permeability of cultured epithelial monolayers, J. Appl. Physiol. 68 (1990) 220–227.

[29] C. Bashford, G. Alder, G. Menestrina, K. Micklem, J. Murphy, C. Pasternak, Membrane damage by hemolytic viruses, toxins, complement, and other cytotoxic agents, J. Biol. Chem. 261 (1986) 9300–9308.

[30] C. Tzan, J. Berg, S. Lewis, Mammalian urinary bladder permeability is altered by cationic proteins: modulation by divalent cations, Am. J. Pysiol. 267 (1994) C1013–C1026.

[31] D. Uchida, C. Irvin, C. Ballowe, G. Larsen, G. Cott, Cationic proteins increase the permeability of cultured rabbit tracheal epithelial cells: modification by heparin and extracellular calcium, Exp. Lung Res. 22 (1996) 85–99.