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Intestinal Safety of Water-soluble β-Cyclodextrins in Paediatric Oral Solutions of Spironolactone: Effects on Human Intestinal Epithelial Caco-2 Cells

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Abstract

Effects of water-soluble β-cyclodextrins (βCDs) on intestinal epithelial integrity were investigated, to establish the safe use of these βCDs as solubilizers of spironolactone in paediatric enteral solutions. Mannitol permeability and transepithelial resistance (TER) of human intestinal epithelial Caco-2 cell monolayers during exposure to dimethyl-β-cyclodextrin (DMβCD), hydroxypropyl-β-cyclodextrin (HPβCD) and sulphobutyl ether β-cyclodextrin (SBEβCD) were followed. Staining methods were used to discern cells with damaged membranes and to study the integrity of cytoskeletal actin and tight junctions. Cytotoxicity of the βCDs was tested by effects on intracellular dehydrogenase activity.

Exposure to HPβCD and SBEβCD solutions had only minor effects on the integrity of Caco-2 cell monolayers. In contrast, DMβCD clearly increased the epithelial permeability for the hydrophilic marker [^{14}C]mannitol across Caco-2 monolayers, decreased TER and showed a dose-dependent cytotoxicity. According to staining, DMβCD increased the permeability of the apical cell membrane without discernable effects on cytoskeletal actin.

HPβCD and SBEβCD appear to be safe additives for use in enteral spironolactone preparations with respect to their acute local effects on epithelial integrity.

Spironolactone is a potassium-sparing diuretic with a solubility in water of only 0·03 mg mL^{-1}. The poor solubility of spironolactone presents a problem when formulating liquid preparations for paediatric patients who take their medication through a nasogastric tube. Liquid preparations of spironolactone described in literature contain either an abundance of organic co-solvents (Pramar et al 1992) or are made in heavy syrups (Committee on Extemporaneous Formulations 1987; Mathur & Wickman 1989). These organic excipients, as well as the high-osmolality syrups, are potentially toxic to paediatric patients (Leff & Roberts 1987). The use of water-soluble derivatives of β-cyclodextrin as solubilizers offers an alternative for the formulation of spironolactone.

Cyclodextrins are cyclic oligosaccharides that improve the solubility of lipophilic drugs by inclusion of the drug into the hydrophobic cavity of the cyclodextrin (Uekama et al 1991). Cyclodextrin-drug inclusion complexes differ from the free drug in properties such as chemical stability, solubility and bioavailability (Szejtli 1988). β-Cyclodextrin (βCD) has been used to increase the oral bioavailability of spironolactone from solid dosage forms administered to adult patients (Yusuff 1990). The low solubility of βCD and the spironolactone-βCD complex make it, however, unuseful for liquid formulations containing more than 1 mg mL^{-1} of spironolactone. Hydrophilic derivatives of βCD have been developed to overcome the low solubility of the parent cyclodextrin without loss of their complexing ability (Szejtli 1988).

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Adverse effects of cyclodextrins on human erythrocytes have been thoroughly studied (Irie et al 1982; Szejtli et al 1986; Yoshida et al 1988; Ohtani et al 1989; Leroy-Lechat et al 1994; Shiotani et al 1995). The cytotoxicity correlated with the capability of βCDs to solubilize cholesterol and other membrane components (Szejtli et al 1986; Irie et al 1992a). Water-soluble βCD derivatives have been used as additives to improve the uptake of poorly absorbable hydrophilic drugs across nasal and oral mucosa (Schipper et al 1992; Shao et al 1994; Hovgaard & Brønstedt 1995). The reduced barrier properties of the epithelia as affected by the cyclodextrins were also linked with their ability to extract lipids and proteins from mucosal membranes (Schipper et al 1992; Shao et al 1994; Hovgaard & Brønstedt 1995). In addition, aspects of oral safety of βCDs have been studied concerning possible effects on excretion of cholesterol and bile acids from the gastrointestinal tract (Gerloczy et al 1994).

In the present study, adverse effects of water-soluble βCDs on intestinal epithelium were investigated to determine their safety as solubilizers of spironolactone in paediatric enteral solutions. The effects of dimethyl-β-cyclodextrin (DMβCD), hydroxypropyl-β-cyclodextrin (HPβCD) and sulphobutyl ether β-cyclodextrin (SBEβCD) on the epithelial barrier properties were studied by determination of [^{14}C]mannitol permeability and effects on transepithelial electrical resistance in monolayers of human intestinal epithelial Caco-2 cells. Cytotoxicity of the βCDs was tested by the effects on intracellular dehydrogenase activity. Finally, propidium iodide staining and actin staining with rhodamine phalloidin were used to discern cells with damaged membranes and to study the integrity of cytoskeletal actin in tight junctions, respectively.

Materials and Methods

Materials

2-Hydroxypropyl-β-cyclodextrin (molar substitution 0·44; degree of substitution (DS) 3·1) (Encapsin HPB) (HPβCD) was a gift from Janssen Biotech, Beerse, Belgium. Sodium sulphobutyl ether β-cyclodextrin (DS 7) (SBEβCD) was kindly donated by Cydex, L. C., Kansas City, KS and spironolactone by Orion Corporation, Espoo, Finland. Heptakis 2,6-di-*O*-methyl-β-cyclodextrin (DS 14) (DMβCD) and 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide (MTT; 98% purity) were purchased from Sigma Chemicals, St. Louis, MO. [^{14}C]Mannitol was obtained from New England Nuclear, Boston, MA. Rhodamine phalloidin and propidium iodine were purchased from Molecular Probes Inc., Eugene, OR. Dulbecco's modified Eagles medium, foetal calf serum, nonessential amino acids, benzylpenicillin, streptomycin were obtained from Gibco.

Cells

Caco-2 cells originating from a human colorectal carcinoma were obtained from the American Type Culture Collection, Rockville, MD, USA. Cells were seeded at a density of $0{\cdot}4 \times 10^6$ cells cm^{-2} onto polycarbonate filters (Transwell, mean pore size 0·45 μm, Costar, Badhoevedorp, The Netherlands) and cultivated for 21–28 days. The cells were maintained in Dulbecco's modified Eagle's medium containing 10% foetal calf serum, 1% nonessential amino acids, and benzylpenicillin (100 int. units mL^{-1}) and streptomycin (100 μg mL^{-1}) as described previously (Artursson 1990). Cells of passages 95–104 were used.

Methods

Preparation of βCD solutions. The βCDs were dissolved in Hank's balanced salt solution (HBSS) containing 25 mM *N*-[2-hydroxyethyl]piperazine-*N'*-[2-ethanesulphonic acid] (HEPES). The pH of the solutions was adjusted to 7·4. Osmolalities were measured with a 5500 Vapor Pressure Osmometer (Wescor Inc., Logan, UT, USA). HBSS with reduced NaCl content was used to obtain iso-osmotic solutions of SBEβCD. The osmolalities of DMβCD and HPβCD solutions were not corrected, and were 380 and 430 mOsm kg^{-1} at the highest concentrations of 50 mM and 100 mM used in the MTT method, respectively. Osmolalities of βCD solutions used in other experiments were between 290 and 350 mOsm kg^{-1}. Experiments with HBSS with mannitol have shown that osmolalities up to 350 mOsm kg^{-1} do not affect the integrity of Caco-2 cell monolayers (Anderberg et al 1993).

Intracellular enzyme activity. The effects of the βCDs on the intracellular dehydrogenase activity were determined by the MTT method (Anderberg et al 1992). MTT is a water-soluble yellow tetrazolium salt that is converted by active mitochondrial dehydrogenases in living cells to insoluble purple formazan. Cells were seeded at a density of 50 000 cells per well in 96-well tissue culture plates and cultivated for 20–24 h before the experiment. The plates were inverted to remove culture medium and carefully washed with HBSS. Subsequently, cells were incubated with 100 μL of different concentrations of βCDs at 37°C. After 60 min the cells were washed with HBSS to remove βCDs, 100 μL of fresh HBSS and 10 μL of MTT-solution (5 mg mL^{-1} in HBSS) added and the plates were incubated for another 60 min. Formazan crystals were solubilized in a solvent containing sodium dodecyl sulphate, isobutanol and hydrochloric acid and the developed colour was measured at $\lambda = 590$ nm in a multiwell scanning spectrofotometer (Multiscan MCC/340, Labsystems Oy, Helsinki, Finland).

[^{14}C]Mannitol transport. The transport of [^{14}C]mannitol across Caco-2 cell monolayers was determined as described previously (Artursson 1990). Transport studies were performed at 37°C and 95% r.h. in HBSS at pH 7·4. The monolayers were equilibrated with HBSS at 37°C before starting the experiments. The experiments were started by addition of [^{14}C]mannitol solutions containing βCDs or both βCDs and spironolactone to the apical side of the monolayer. Samples from the basolateral side were taken at regular time intervals up to 60 min. The amounts of [^{14}C]mannitol in the basolateral solutions were measured using a liquid scintillation counter (Tricarb 1900 CA, Canberra Packard Instrument, Downers Grove, IL, USA). The apparent permeability coefficients (P_{app}) (cm s^{-1}) were determined according to the following equation:

$$P_{app} = dQ/dt \times 1/AC_0 \quad (1)$$

where dQ/dt is the permeability rate (steady-state flux mmol s^{-1}), C_0 is the initial concentration in the donor chamber (mM) and A is the surface area of the monolayer (cm^2).

Transepithelial electrical resistance. The transepithelial electrical resistance (TER) of Caco-2 monolayers was measured in HBSS at 37°C as previously described (Karlsson 1995). Caco-2 cell monolayers grown on 12 mm Snapwell polycarbonate filters were mounted in Ussing type chambers. The chambers were each connected via salt bridges to voltage-sensitive Ag/AgCl current-passing electrodes. The voltage response was measured at five different currents, and TER obtained by a linear least square fit of the current voltage pairs. Experiments were started by addition of 1·0 mL of preheated βCD solutions to the apical side of the chambers to give final concentrations of 15, 19 and 30 mM for DMβCD, SBEβCD and HPβCD, respectively. Control experiments were done in HBSS without βCDs. Resistances were measured during 60 min.

Fluorescence microscopy. Propidium iodide, an intercalating dye, was used to discern cells with damaged membranes. Propidium iodide does not permeate intact cell membranes (Anderberg et al 1993). The monolayers were incubated with βCDs in HBSS for 60 min at 37°C and rinsed twice with PBS before staining for exactly 4·5 min with propidium iodide solution (30 μg mL^{-1}). The monolayers were then rinsed twice with PBS, fixed for 10 min at room temperature in 3% formaldehyde and rinsed four times with PBS. F-actin was stained with rhodamine-labelled phalloidin according to the manufacturers specifications (Anderberg et al 1993). Following incubation with βCDs in HBSS for 60 min at 37°C, the monolayers were then rinsed twice with PBS. Subsequently, they were fixed for 10 min in 3% paraformaldehyde at room temperature, rinsed with PBS three times, and permeabilized

with 0·1% Triton X-100 in 0·1% bovine serum albumin in PBS for 1 min at room temperature. The monolayers were rinsed twice with PBS, air dried and stained with rhodamine-labelled phalloidin for 30 min in the dark on a plate shaker and rinsed three times with PBS.

Excised monolayers were mounted on glass slides in a 1 : 1 solution of PBS and glycerol. Subsequently, cells were observed by a fluorescence microscope (Zeiss Axioskop, Oberkochen, Germany). Control experiments were incubations of monolayers in HBSS solutions without βCD. All experiments were performed in duplicate at least.

Statistics. All results are presented as means ± s.d. The effects of βCDs on mannitol permeability were analysed using the unpaired Students *t*-test, comparing each treatment with respective control. Effects on propidium iodide permeability were analysed using the one-factor analysis of variance, followed by the Tukey honestly significant difference test for pairwise comparisons.

Results

Intracellular enzyme activity

Intracellular enzyme activity of Caco-2 cells was decreased after exposure to DMβCD at concentrations above 12 mM, with an IC50 value of 14 ± 2 mM (Fig. 1). At concentrations below 12 mM, DMβCD increased enzyme activity. SBEβCD solutions decreased enzyme activity slightly at the highest concentration studied of 50 mM, whereas HPβCD only increased enzyme activity in the concentration range studied of 3 to 100 mM (Fig. 1).

[^{14}C]Mannitol transport

The integrity of the intestinal epithelial barrier function following exposure to βCDs was studied by determination of the permeability of the poorly permeable substance [^{14}C]mannitol across Caco-2 cell monolayers. The baseline permeability of mannitol across the monolayers was low, the P_{app} being 0·98 ± 0·11 cm s^{-1} ($\times 10^{-7}$) (n = 33). Addition of HPβCD and SBEβCD up to concentrations of 30 and 28 mM, with or without spironolactone, did not change mannitol transport as compared with control (Table 1). DMβCD increased mannitol permeability in a time- and concentration-dependent manner (Fig. 2, Table 1). At 30 mM DMβCD the mannitol permeability was 5·8 ± 0·8 cm s^{-1} ($\times 10^{-7}$) (n = 4) over the first 40 min of exposure, increasing to 23 ± 2 cm s^{-1} ($\times 10^{-7}$) (n = 4) over the last 20 min (Fig. 2). Effects of DMβCD on mannitol permeability were reduced when it was added as a complex with spironolactone (Table 1).

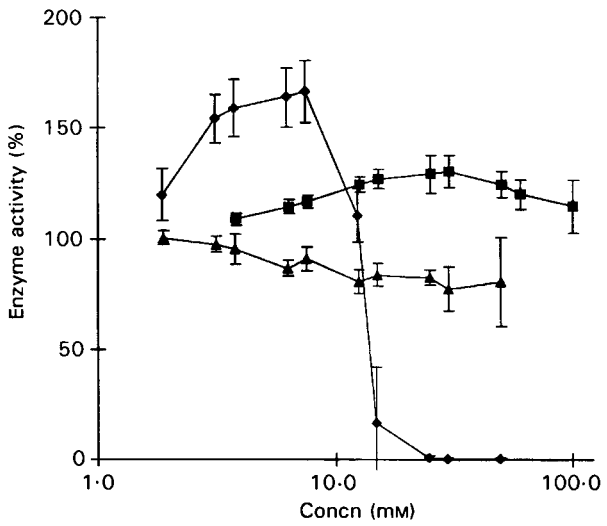

FIG. 1. Effects of DMβCD, HPβCD and SBEβCD on intracellular dehydrogenase activity of Caco-2 cells (mean ± s.d., n = 8). ▲, SBEβCD; ◆, DMβCD; ■, HPβCD.

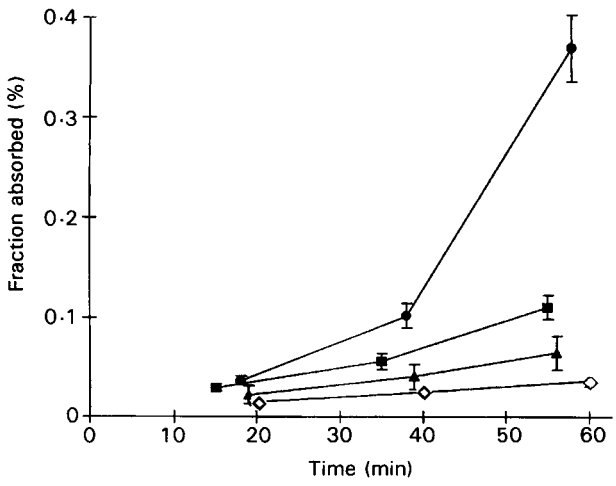

FIG. 2. Effects of DMβCD on [^{14}C]mannitol permeability across monolayers of Caco-2 cells (mean ± s.d., n = 4, control n = 14). ▲, 15 mM DMβCD; ■, 23 mM DMβCD; ●, 30 mM DMβCD; ◇, control.

Transepithelial electrical resistance

The transepithelial electrical resistance of the control Caco-2 cell monolayers was 320 ± 38 Ω cm^2, and decreased slightly during the experiment (Fig. 3). Addition of SBEβCD hardly affected the TER of the monolayers as compared to control. Addition of HPβCD and DMβCD solutions initially increased TER values (Fig. 3). For HPβCD the TER remained above the initial resistance during the 60 min of exposure, whereas exposure to DMβCD reduced TER values to 46·8 ± 17·5% of the initial resistance at the end of the experiment.

Fluorescence microscopy

DMβCD increased the permeability of the apical cell membrane, as detected with the fluorescent probe propidium iodide. Untreated control monolayers had intact membranes and were unaccessible for propidium iodide (Table 2). After treatment with 23 mM DMβCD for 60 min the amount of stained cells increased from 0·17 ± 0·25% (control) to 1·43 ± 0·19% (Table 2). According to results from staining with rhodamine-labelled phalloidin, DMβCD did not affect the distribution of cytoskeletal actin (not shown). HPβCD and SBEβCD did not damage the apical membrane producing percentages of stained cells similar to control treatment (Table 2).

Table 1. Permeability of [^{14}C]mannitol across Caco-2 monolayers in βCD solutions with and without spironolactone.

Treatment	P_{app} (× 10^7) (cm s^{-1})[a]	Treatment	P_{app} (× 10^7) (cm s^{-1})[a]	Treatment	P_{app} (× 10^7) (cm s^{-1})[a]
Control[b]	0·97 ± 0·13 (n = 14)	Control[b]	0·99 ± 0·11 (n = 15)	Control[b]	1·02 ± 0·09 (n = 12)
15 mM DMβCD	2·06 ± 0·41 (n = 4)*	15 mM HPβCD	0·82 ± 0·08 (n = 4)	14 mM SBEβCD	0·94 ± 0·06 (n = 4)
23 mM DMβCD	3·59 ± 0·40 (n = 4)***	23 mM HPβCD	0·82 ± 0·19 (n = 4)	21 mM SBEβCD	1·03 ± 0·03 (n = 4)
30 mM DMβCD	14·8 ± 1·4 (n = 4)***	30 mM HPβCD	0·87 ± 0·05 (n = 4)	28 mM SBEβCD	1·07 ± 0·22 (n = 4)
18 mM DMβCD with SP (1 : 2·5)	1·49 ± 0·28 (n = 3)				
22 mM DMβCD with SP (1 : 3)	1·41 ± 0·19 (n = 3)*	22 mM HPβCD with SP (1 : 3)	1·01 ± 0·14 (n = 4)	22 mM SBEβCD with SP (1 : 3)	0·96 ± 0·06 (n = 4)

[a]P_{app} values were calculated up to 60 min of exposure; mean values ± s.d. [b]Controls combined from all experiments with respective βCD. *Significant difference, $P < 0.05$; comparison with respective control (Student's t-test). ***Significant difference, $P < 0.001$; comparison with respective control (Student's t-test).

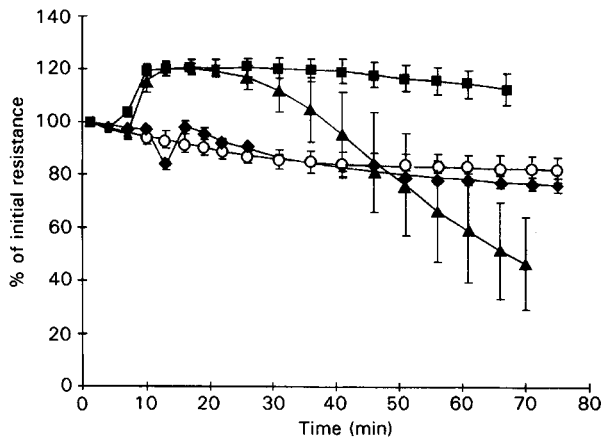

FIG. 3. Effects of DMβCD, HPβCD and SBEβCD on transepithelial resistance (TER) of Caco-2 monolayers (mean ± s.d., n = 3, control n = 8). ▲, 15 mM DMβCD; ◆, 19 mM SBEβCD; ■, 30 mM HPβCD; ○, control.

Table 2. Nuclei stained by propidium iodide as a percent of the total number of cells.

Treatment[a]	Number of stained nuclei[c] (%)[b]
Control	0·17 ± 0·25
23 mM DMβCD	1·43 ± 0·19***
23 mM HPβCD	0·12 ± 0·14
21 mM SBEβCD	0·20 ± 0·23

[a]Caco-2 monolayers were incubated for 60 min with βCD solutions before staining with propidium iodide. [b]Number of stained nuclei are presented as % of total number of cells; mean ± s.d. Four areas of 0.45 cm^2 on 2 filters were calculated. [c]Significantly different from other treatments, one-way analysis of variance ($P < 0.001$). ***Significantly different from control, $P < 0.001$, pairwise comparison (Tukey HSD).

Discussion

Spironolactone is one of the diuretics used to reduce lung congestion and thereby to improve lung function in low birthweight infants (Atkinson et al 1988). Present daily dosage recommendations for infants are 3–5 mg kg^{-1} of spironolactone administered in 1–2 doses. A solution should preferably contain 3–5 mg mL^{-1} of spironolactone to minimize the extra water-load to the kidneys. A clear liquid formulation containing 2 mg mL^{-1} spironolactone has been described in the literature (Pramar et al 1992). Here solubilization of spironolactone is aquired by the use of a co-solvent blend of PEG 400, propylene glycol, glycerol and ethanol comprising 60% of the liquid formulation. According to a preliminary phase-solubility study a 15 mM solution of HPβCD in water could maximally solubilize 3 mg mL^{-1} of spironolactone and the solubilizing capacity of DMβCD is reported to be 3·6 mg mL^{-1} spironolactone in an 11 mM solution (Uekama 1985). As co-solvents are undesirable excipients for premature infants (Leff & Roberts 1987), water-soluble βCDs could offer an alternative way for the solubilization of spironolactone.

The ability of cyclodextrins to solubilize molecules in their lipophilic cavity is the basis for their use in drug delivery, as well as their physiological effects. The cellular effects of βCDs are based on their capacity to extract cholesterol and other membrane components (Szejtli et al 1986; Ohtani et al 1989; Irie et al 1992a,b; Shiotani et al 1995). Of the parent cyclodextrins, α-cyclodextrin (αCD) has the highest potency for solubilizing phospholipids and β-cyclodextrin for solubilizing cholesterol from erythrocytes (Ohtani et al 1989). In nasal membranes DMβCD has shown nearly equal ability to extract phospholipids as DMαCD, whereas the extraction of cholesterol followed the order DMβCD > > DMαCD > HPβCD (Irie et al 1992b). The haemolytic effects of βCDs are of the same order as their capacity to solubilize cholesterol and decrease in the order DMβCD > trimethyl-βCD > βCD > HPβCD > hydroxyethyl-βCD > SBEβCD (Yoshida et al 1988; Leroy-Lechat 1994; Shiotani et al 1995). Since cholesterol is a rigidifier of lipid bilayers (Yeagle 1985), extraction of cholesterol will influence the stability of the membrane, and eventually result in membrane damage. This is corroborated by the fact

that prehaemolytic concentrations of βCDs caused shape changes in erythrocytes (Irie et al 1982; Ohtani et al 1989; Shiotani et al 1995). Cyclodextrins have also been shown to release proteins from erythrocytes (Ohtani et al 1989) as well as nasal mucosa (Shao et al 1994). This has, however, been regarded as a secondary process in which the extraction of lipid components from the membrane causes the extrusion of proteins (Ohtani et al 1989; Shao et al 1994).

Caco-2 cell monolayers have been successfully used to study the toxicity and mechanisms of action of pharmaceutical additives and absorption enhancers on the intestinal epithelium, showing good correlations with animal studies (Anderberg et al 1992). The intracellular dehydrogenase activity was found to correlate linearly to the number of viable Caco-2 cells (Anderberg et al 1992), making the MTT method a tool for screening cytotoxic effects of substances. Of the βCDs presently studied, only DMβCD had clear toxic effects on the intracellular enzyme activity of Caco-2 cells. HPβCD, as well as DMβCD at lower concentrations, increased intracellular enzyme activity possibly through subtoxic interactions of the cyclodextrins with the epithelial membrane. Similar increases in enzyme activity have been demonstrated with a bile acid derivative at subtoxic concentrations (Anderberg et al 1992). DMβCD also increased enzyme activity in Caco-2 cells in a study by Hovgaard & Brønstedt (1995), where the absorption enhancing effects of α-, β-, γ-, HPβ- and DMβCD were screened.

DMβCD was the only βCD in the present study to affect the permeability of the Caco-2 monolayers to [^{14}C]mannitol. Increased mannitol transport across the monolayer correlated with an increased membrane permeability as evidenced by propidium iodide staining. No changes in actin staining could be discerned after exposure to DMβCD compared with control monolayers. Changes in cytoskeletal actin staining may be used as an indirect measure of effects on tight junctions and therewith the integrity of the paracellular barrier to solute transport, since strong evidence excists for association of the cytoskeleton with the tight junction (Madara 1987). DMβCD, therefore, seems to affect cell permeability through membrane fluidization only, at this concentration.

The decrease in TER as caused by 15 mM DMβCD, however, suggests a dilatation of tight junctions in the monolayers. In epithelia such as the small intestine the majority of passive ion flow is presumed to occur through the paracellular space (Madara 1983). Therefore, as TER is a measure of the ion permeability of the epithelial layer, it is also a measure of the function of tight junctions (Madara 1987). The fact that DMβCD did not change tight junction integrity according to RP staining but decreased TER may be explained by the different total amounts of DMβCD per cm^2 used in the two experiments. Although the concentrations used were similar, due to differences in volumes and monolayer areas, the amount of DMβCD was 0·080 mmol cm^{-2} in TER experiments and only 0·010 mmol cm^{-2} in the RP staining experiments. The total amount of free cyclodextrins, rather than the cyclodextrin concentration, has earlier been found to determine the cytotoxicity (Leroy-Lechat et al 1994). Similarly, in the present study, decreasing the amount of free cyclodextrin molecules by addition of spironolactone to DMβCD solutions showed reduced effects on mannitol permeability. Disruptions of tight junction integrity at large amounts of DMβCD per cm^2 monolayer as evidenced by TER decreases are, therefore, a likely development to membrane interruptions already seen at much smaller amounts of DMβCD.

Exposure to HPβCD and SBEβCD solutions had very little effects on the integrity of Caco-2 cell monolayers as shown by mannitol and propidium iodide permeability and the TER of the monolayers. This agrees with the study by Hovgaard & Brønstedt (1995), and Shao et al (1994), where DMβCD, but not HPβCD, affected the permeability of Caco-2 monolayers to PEG-4000 and increased the intestinal absorption of insulin, respectively.

In summary, we conclude that DMβCD showed clear cytotoxic effects on human intestinal epithelial Caco-2 cells. At the concentrations studied, DMβCD increased epithelial permeability probably due to membrane fluidization as evidenced by increased propidium iodide uptake. HPβCD and SBEβCD did not affect the integrity of intestinal epithelial Caco-2 cells and did not display toxicity in the intracellular enzyme activity assay, and seem in these respects to be safe additives for use in enteral preparations.

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