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Cell-polarity dependent effect of chelation on the paracellular permeability of confluent Caco-2 cell monolayers

Arthur B.J. Noach, Yuji Kurosaki, Margret C.M. Blom-Roosemalen, Albertus G. de Boer and Douwe D. Breimer

Center for Bio-Pharmaceutical Sciences, Division of Pharmacology, Leiden University, P.O. Box 9503, NL-2300 RA Leiden (The Netherlands)

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Summary

To investigate the effect of extracellular chelation at the apical, basolateral or both sides on the resistance and permeability of epithelial cell layers, we used 15 days cultures of a human intestinal adenocarcinoma cell line (Caco-2) and hydrophilic FITC-labeled dextran model compounds of various molecular weights. Transport of these hydrophilic compounds is restricted to the paracellular pathway in which the tight junctions form a barrier. Tight junctions are dependent on extracellular calcium and magnesium for their integrity and function. Calcium and magnesium chelation with 2.5 mM EDTA at the apical and basolateral side of the monolayer resulted in a drastic drop, up to 80% of the initial value, in trans-epithelial electrical resistance after 60 min. Application at the basolateral side resulted in a drop of 40% in resistance, while application on the apical side almost did not give any effect. The same pattern was also found in transepithelial clearance studies with fluorescein-Na and FITC-labeled dextran model compounds with molecular weights ranging from 4000 to 500 000. After 2.5 mM EDTA treatment on both sides a maximal (1400-fold) enhancement in transport clearance occurred for the dextran molecule with molecular weight 20 000 (Stokes-Einstein molecular radius 30 Å). For basolateral calcium and magnesium chelation similar results were found, however, with lesser maximal effects. For apical application no transport enhancement could be found with 2.5 mM EDTA. These results have shown that transport of hydrophilic compounds through epithelial monolayers is enhanced more effectively by basolateral application of EDTA than by apical application.

Introduction

A major barrier for absorption (transport) of hydrophilic compounds is the fact that these compounds are unable to cross the membranes of the cells lining the lumen of the gastro-intestinal tract.

For this reason they will have a preference for transport via the intercellular spaces, i.e., the paracellular route. A significant impediment to this route is that the surface area is very small compared to the cell membrane area and that the intercellular spaces are sealed by the tight junctions between adjacent cells. These junctions in general have a very limited pore diameter, which

Correspondence to: A.B.J. Noach, Center for Bio-Pharmaceutical Sciences, Division of Pharmacology, Leiden University, P.O. Box 9503, NL-2300 RA Leiden, The Netherlands.

greatly inhibits the transport of larger molecules via this route.

There is growing evidence that tight junctions are dynamic structures and can be influenced in many ways (Cereijido et al., 1981, 1988; Gumbiner, 1987; Madara et al., 1987; Madara, 1989) and that thereby paracellular transport is regulated (Madara, 1988). Factors which influence the tight junctions have been extensively discussed elsewhere and consist, among others, of cAMP, protein kinase C, cytochalasin D and hormones (Duffey et al., 1981; Madara et al., 1986; Coleman and Kan, 1990). It is also known that the tight junctions are connected to the cytoskeleton of the cell and that changes in the cytoskeleton may have an influence on the shape of the tight junctions and on their permeability (Madara, 1987; Madara et al., 1987; Schnittler et al., 1990). In addition, calcium and magnesium are indispensable ions for the integrity and functioning of tight junctions (Meza et al., 1980; Pitelka et al., 1983; Gonzalez-Mariscal et al., 1990).

Our main interest in influencing tight junctions is to investigate if it will be possible to enhance the transport of large and/or polar molecules over epithelial gastro-intestinal barriers, possibly by interfering with extracellular calcium and magnesium levels.

In previous studies on the effect of calcium removal on the integrity of the tight junctions calcium depletion was performed simultaneously at the apical as well as at the basolateral side of monolayer cultures (Artursson and Magnusson, 1990).

This is an unrealistic situation compared to the in vivo situation where an exogenous treatment can only be carried out from the luminal side of the epithelium. For this reason we were interested to see if the effect of calcium and magnesium removal on the tight junctions by EDTA is dependent on the side of administration to a cell layer and so to evaluate the applicability of this compound as an absorption enhancing agent. As a model for gastro-intestinal epithelium we used Caco-2 cell monolayers, a human adenocarcinoma cell line (Fogh et al., 1977) which has been characterised as being a good model for gastro-intestinal epithelium (Pinto et al., 1983; Hidalgo et al., 1989; Artursson, 1990; Hilgers et al., 1990; Wilson et al., 1990). As a model for hydrophilic compounds we used fluorescein-Na and FITC-labeled dextran molecules of various molecular weights.

Materials and Methods

Cells

Caco-2 cells, originating from a human adenocarcinoma, were a kind gift from Dr G. van Meer (Department of Cell Biology, Utrecht University Hospital, Utrecht, The Netherlands) and were used between passages 70 and 85.

The cells were maintained at 37°C in 25 cm^2 cell culture flasks (Greiner, Nürtingen, Germany) in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 1% non-essential amino acid solution (100 ×), 10% heat-inactivated fetal bovine serum, benzylpenicillin G (160 U/ml) and streptomycin sulphate (100 μg/ml) in an atmosphere of 95% air and 5% CO_2. The pH of the medium was 7.4.

Medium, amino acid solution and antibiotics were obtained from Sigma Chemical Co., St Louis, MO, U.S.A.; fetal bovine serum was from HyClone® Laboratories Inc., Logan, UT, U.S.A., through Greiner BV, Alphen a/d Rijn, The Netherlands. The medium was changed every other day.

The cells for experiments were grown on Transwell-COL™ collagen treated cell culture filter inserts (Costar, Cambridge, MA, U.S.A.; cat. no. 3425) with a surface area of 4.71 cm^2.

The seeding density was 1×10^4 cells/cm^2. Culture medium (2.5 ml) was added on each side of the filter. The growth of the cells and degree of confluency was checked daily microscopically. Confluent monolayers were used on day 15–20 after seeding, at which time the transepithelial electrical resistance of the monolayers was at a stable value of approx. 1200 Ω cm^2.

Model compounds

Fluorescein-sodium (Flu) and FITC labeled dextran molecules with molecular weights 4000 (FD-4), 10000 (FD-10), 20000 (FD-20), 40000

(FD-40), 70000 (FD-70), 150000 (FD-150) and 500000 (FD-500) were purchased from Sigma Chemical Co., St. Louis, MO, U.S.A. The range in molecular weights was the stated average ±1%, according to the manufacturer's statement.

In all FITC-labeled dextrans no free fluorescein could be detected by the HPLC method as described under *Transport studies*.

Resistance experiments

The transepithelial electrical resistance (TEER) of the monolayers was recorded with a four-electrode system in a custom made measurement cup (Department of Physiology, Leiden University Medical School, Leiden, The Netherlands) filled with DMEM and kept at 37°C.

The filter cup was placed between two sets of Ag/AgCl electrodes. Via a round electrode-set an a.c. current of 10 μA was passed through the monolayer. Via the other set of electrodes the resulting potential difference over the monolayer was measured. The 10 μA current was supplied by a 10 V, 10 Hz block pulse, generated by a PM 5131 function generator (Philips Nederland BV, Eindhoven, The Netherlands) via a 1 MΩ series resistance. The current remained constant throughout the experiments. The resulting potential difference was amplified by a Model 113 Differential Amplifier (Princeton Applied Research Corp., Princeton, NJ, U.S.A.). Signal recording was carried out by a PM 3350 Digital Storage Oscilloscope (Philips Nederland BV, Eindhoven, The Netherlands).

The mean of 10 subsequent potential difference measurements was corrected for the intrinsic value of the empty filter and expressed as TEER in Ω cm^2.

30 min prior to the start of the experiment the culture medium was changed with fresh DMEM with the same composition as stated above. At $t = 0$ the resistance of the monolayer was measured and directly after the measurement the culture medium was changed to DMEM containing 1.75, 2.5 or 5 mM EDTA at the apical and/or basolateral side. For experiments with EDTA treatment at the basolateral side, the medium in the resistance measurement cup was also changed to EDTA containing medium in the same concentration, in order to avoid changes in the environment in the cluster plate where the incubation was performed and during the resistance measurement.

The resistance was measured every 15 min up to 120 min and at various time points the medium was changed back to DMEM with serum and without EDTA and the recovery of the resistance after EDTA treatment was recorded. During the experiment the cells were kept in the 37°C incubator with 95% air/5% CO_2 atmosphere and only removed from there for the measurement.

The resistance was calculated as percentage of the initial ($t = 0$) value of the same filter.

Microscopic examination of the monolayer was performed before and directly after the experiment.

Transport studies

The compounds were dissolved in DMEM with serum (pH 7.4) in a final concentration of 1 mg/ml (Flu) or 10 mg/ml (for all FDs). For filter and monolayer experiments at $t = 0$, 250 μl DMEM was removed from the apical chamber of the filter and replaced with 250 μl compound containing solution. In this way the initial drug concentrations were 100 μg/ml (Flu) or 1 mg/ml (for all FDs). 200 μl samples were taken every 15 min up to 90 min from the basolateral chamber. This volume was replaced with 200 μl non-drug-containing DMEM.

At $t = 0$ min and $t = 90$ min, 50 μl samples were taken from the apical chamber.

For experiments with EDTA the culture medium was changed to medium with 2.5 mM EDTA (pH 7.4) at the apical and/or the basolateral side 30 min prior to the start of the transport experiment to achieve an initial opening of the tight junctions. Na_2 EDTA was obtained from J.T. Baker Chemicals BV, Deventer, The Netherlands.

Samples were analyzed, after dilution with water (Milli-Q, Millipore SA, Molsheim, France), on a reversed-phase HPLC system. This system consisted of a WISP™-710B autoinjector (Waters Associates, Milford, MA, U.S.A.) an Ultrasphere™ 3 μm ODS column, 4.6 mm i.d. × 7.5 cm (Altex, Berkeley, CA, U.S.A.) or a Spherisorb

ODS-2 column, 4.6 mm i.d. × 10 cm (Alltech, Deerfield, IL, U.S.A.). For protection of the analytical column a guard column (1.4 inch × 2.1 mm i.d.) packed with C_{18} Pellicular Column Refill (Alltech, Deerfield, IL, U.S.A.) was placed between the injector and the analytical column.

The mobile phase consisted of 90% 5 mM phosphate buffer pH 7.4 and 10% acetonitrile (Westburg, Leusden, the Netherlands), flow 1.0 ml/min.

To the autoinjector a RF-530 fluorescence HPLC monitor (Shimadzu Corporation, Kyoto, Japan) was connected. Excitation wavelength was 488 nm, and emission wavelength 512 nm.

Detector output was processed and recorded on a C-R3A reporting integrator (Shimadzu Corp., Kyoto, Japan).

Transport-clearance values of the compounds over monolayers or filters were calculated according to Van Bree et al. (1988) with a custom made APL*PLUS v8.0 program (STSC, Inc, Rockville, MD, U.S.A.) and expressed in μl/min.

Results

Effect of EDTA on the monolayer resistance

In Fig. 1 the results of EDTA treatment on the TEER are visualized. When monolayers were exposed to medium containing 1.75 mM EDTA apically, basolaterally or at both sides, there were only minor effects on the TEER after 60 min compared to control experiments without EDTA, where a drop in resistance of maximally 10% was found after 2 h (data not shown). After exposure to an EDTA concentration of 2.5 mM apically a small effect could be seen, similar to that of the 1.75 mM concentration on apical and basolateral side (Fig. 1). 2.5 mM EDTA on basolateral application resulted in a more pronounced effect on the resistance (Fig. 1). Apical plus basolateral application was even more effective and gave rise to a drop in resistance of up to about 80% (Fig. 1).

At a very high concentration of EDTA (5 mM) apical application was more effective than at 2.5

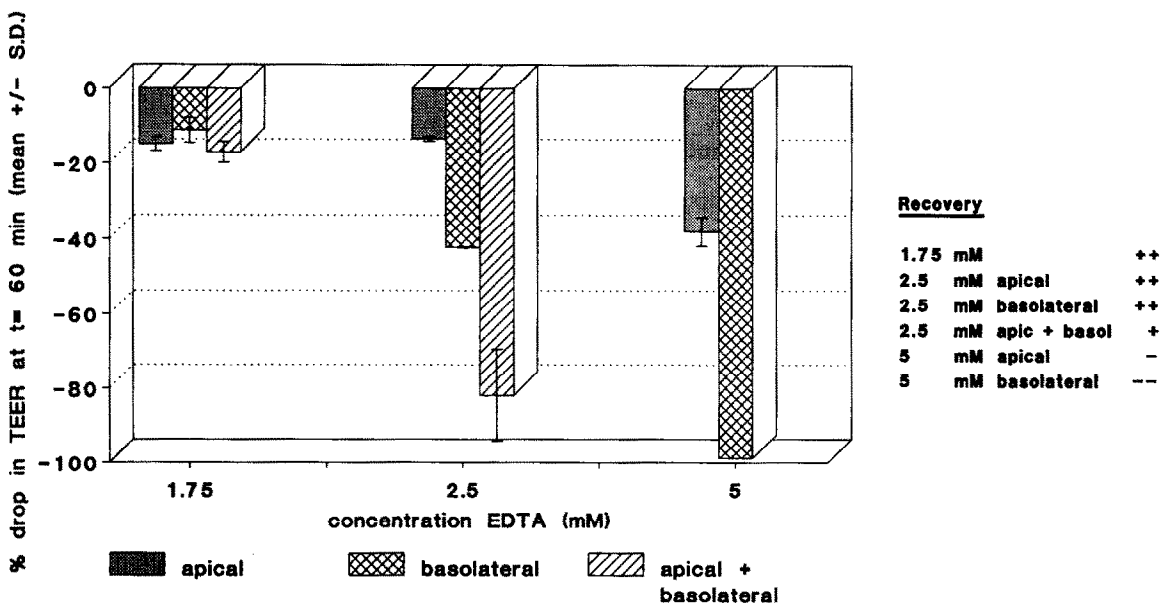

Fig. 1. Effect of different concentrations of EDTA on the resistance of Caco-2 monolayers after 60 min treatment and recovery after treatment (ranging from + + (quick and full recovery) to – – (very poor recovery)) ($N = 3$–4).

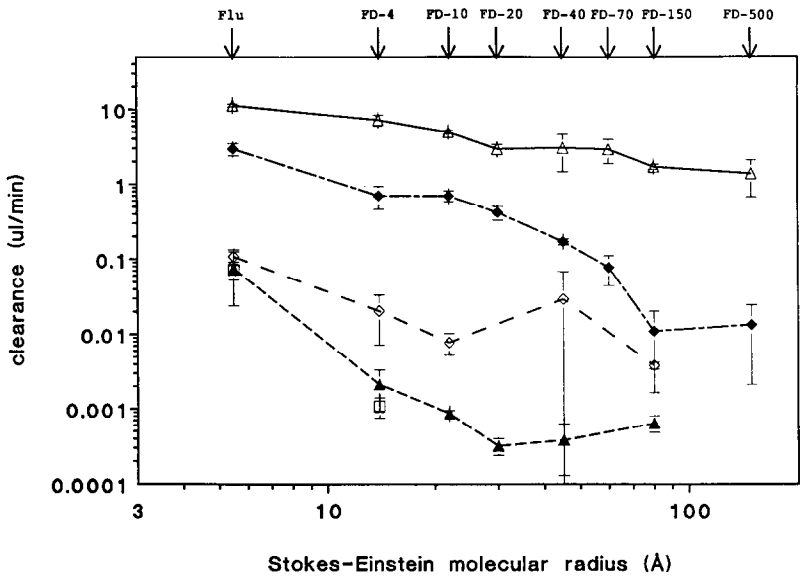

Fig. 2. Clearance of fluorescein-Na and FITC-dextran molecules with various molecular weights over empty control filters, untreated Caco-2 monolayers and Caco-2 monolayers after treatment with 2.5 mM EDTA. (△) Filter without cell monolayer, (▲) untreated monolayer, (□) apical, (◇) basolateral, (◆) apical and basolateral EDTA treatment ($N = 2$–6).

mM, but less effective than 2.5 mM applied on both sides. 5 mM EDTA at the basolateral side reduced the TEER almost to values of the empty Transwell™ filter (Fig. 1). When checked microscopically following this last treatment the cells no longer appeared to be a confluent monolayer.

Since 5 mM EDTA at the basolateral side damaged the integrity of the cell layer we did not carry out experiments where this concentration was applied at both sides at the same time.

After removal of EDTA the resistance increased again. The greater the drop in TEER with EDTA, the less rapid was the return to control values in TEER. However, within 4 h after removal of EDTA in the 1.75 and 2.5 mM groups, resistance values approached those values

TABLE 1

Transport-clearance (in μl/min) of fluorescein-Na and FITC-dextran molecules for control monolayers and after various EDTA treatments

	Untreated monolayer	EDTA (2.5 mM) apical and basolateral	EDTA (2.5 mM) apical	EDTA (2.5 mM) basolateral
Flu-Na	0.075 ± 0.051	2.9642 ± 0.5611 [a]	0.0724 ± 0.0188	0.1087 ± 0.0257
FD-4	0.0021 ± 0.001	0.6968 ± 0.2373 [a]	0.0011 ± 0.0003	0.0203 ± 0.0131 [c]
FD-10	0.0009 ± 0.0001	0.6931 ± 0.1131 [c]	N.D.	0.0077 ± 0.0025
FD-20	0.0003 ± 0.0001	0.4129 ± 0.0842 [b]	N.D.	N.D.
FD-40	0.0004 ± 0.0003	0.1726 ± 0.0159	N.D.	0.0294 ± 0.0392
FD-70	N.D.	0.0779 ± 0.0332	N.D.	N.D.
FD-150	0.0006 ± 0.0002	0.0110 ± 0.0093 [c]	N.D.	0.0038 ± 0.0004
FD-500	N.D.	0.0132 ± 0.0111	N.D.	N.D.

N.D. not determined ($N = 2$–6).

[a] $P \leq 0.01$; [b] $P \leq 0.05$; [c] $P \leq 0.1$; all compared to untreated monolayer (Mann-Whitney U-test).

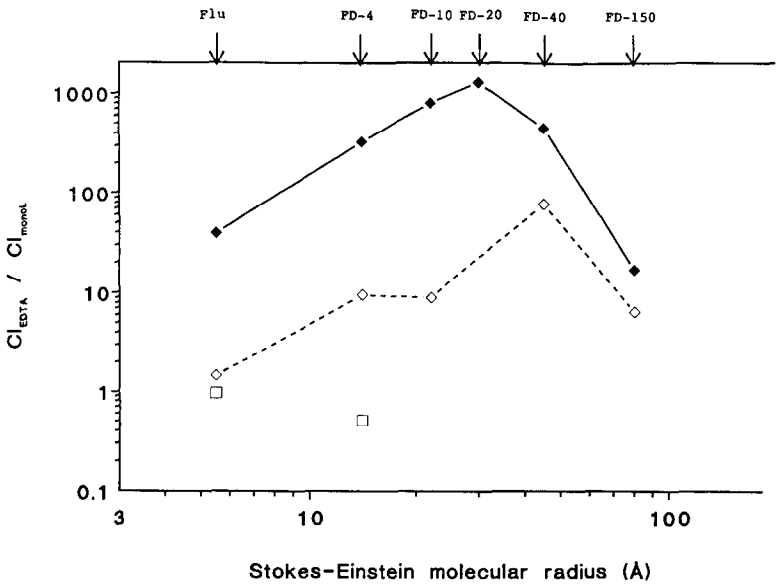

Fig. 3. Ratio of clearance values of EDTA treatment (2.5 mM) over untreated monolayers. (□) Apical, (◇) basolateral, (◇) apical and basolateral EDTA treatment.

of the control experiments. In the 5 mM group hardly any recovery of TEER occurred. This is depicted in the 'recovery chart' in Fig 1.

Effect of EDTA on the permeability for hydrophilic model compounds

Transport-clearance values of the fluorescent model compounds across the Transwell™ filters with and without a confluent monolayer of Caco-2 cells are shown in Fig. 2

In the absence of EDTA the clearance values were 150–9000-times lower when a monolayer was present vs empty control filters, depending on the compound used (Fig. 2), indicating that the monolayer is the main barrier for transport, and also that there is a relation between the molecular radius of the compound and its transport clearance through the monolayer.

Since, according to the results of the resistance experiments, 2.5 mM EDTA seemed to be the optimal level to work with and this concentration just chelates all Ca^{2+} and Mg^{2+} in the culture medium, this concentration was applied either apically, basolaterally or on both sides of the monolayer. Following EDTA treatment at the basolateral side or at both sides transport-clearance values increased when compared to untreated monolayers. The most pronounced effect was observed after application on both sides. Application at the basolateral side gave lesser effects and application only at the apical side did not provide any enhancement (Fig. 2 and Table 1).

When the relative increase in transport-clearance ($Cl_{EDTA}/Cl_{monolayer(control)}$) was calculated for both sides treatment, a bell-shaped curve was obtained (Fig. 3) with a maximum effect for the FITC-dextran with a molecular weight of 20 000 (Stokes-Einstein radius 30 Å), while for basolateral treatment a similar but less pronounced curve resulted.

Discussion

In the present investigations we have shown that complexation of extracellular calcium and magnesium on the apical, the basolateral or on both sides of confluent monolayers of Caco-2 cells has differential effects on the trans-epithelial electrical resistance and the transport of hydrophilic model compounds from the apical to the basolateral side.

In previous experiments (Artursson and Magnusson, 1990) it has been shown that chelating extracellular calcium on both sides of filter-grown Caco-2 cells with 2.5 mM EGTA results in a rapid drop in TEER, with a relatively fast recovery of the resistance after removal of the chelating agent. This could be confirmed in the present experiments with EDTA.

Until now, however, nothing has been published with respect to the differential effects of calcium and magnesium chelation at the apical, the basolateral or at both sides of intestinal cells in monolayer culture. An effect of calcium removal from the tight junctions on the resistance could be anticipated since it is well known that tight junctions require Ca^{2+} and Mg^{2+} for their integrity and thus for sealing of the paracellular transport-route (Martínez-Palomo et al., 1980; Meza et al., 1980; Palant et al., 1983; Pitelka et al., 1983; Gonzalez-Mariscal et al., 1990). Since it is known that tight junctions are dependent on Ca^{2+} as well as on Mg^{2+} we used EDTA to investigate the influence of chelation on the paracellular permeability. Interestingly, we found that the effect of EDTA on the TEER and on paracellular transport was side dependent. Application of EDTA to the basolateral side of the cell layers resulted in much more pronounced effects on TEER than at the apical side (Fig. 1). Application on both sides had an even larger effect, whereas application to the apical side only was effective in the highest EDTA concentration.

Since a lower resistance points to a more leaky barrier and thus is an indication for opening of tight junctions, a differential increase in transport of hydrophilic compounds via the paracellular route could be anticipated (Madara et al., 1986, 1988; Artursson and Magnusson, 1990) and was indeed found.

With the intermediate (2.5 mM) EDTA concentration, where all Ca^{2+} and Mg^{2+} in the culture medium is chelated, a substantial increase in paracellular transport of the model compounds (Fig. 2 and Table 1) could be achieved after basolateral or bilateral EDTA while there were no severe undesirable effects in terms of cell death (trypan blue exclusion) or detachment of the cells from the support. Apical application had no effect. Morphological data on transport route are in accordance with these results (to be published). Higher concentrations of EDTA gave rise to cellular detachment while the percentage of trypan blue positive stained (dead) cells remained the same (data not shown).

Enhancement of transport clearances by EDTA (Fig. 3) may be explained by its effect on pore diameters in (opened) tight junctions. Transport facilitation is particularly effective for the dextran molecule with a molecular weight of 20000 (Stokes-Einstein radius 30 Å) where a 1400-fold increase in transport-clearance was found. For larger molecules the pores are not sufficiently widened to bring about a similar clearance enhancement. A firm explanation for the smaller effect with fluorescein-sodium and FITC dextrans with molecular weights 4000 and 10000, compared to that for the dextran with a molecular weight of 20000, as can be seen in Fig. 3, is more difficult to give. A reason could be that these compounds (especially fluorescein-Na) are already transported quite well, so that the size of the tight junction pores is not the major limiting factor for transport. It is also possible that these components have a different molecular shape in solution (e.g., linear vs globular) than the larger compounds. Alternatively, the components may have a different charge distribution, so that differential effects occur with respect to adherence to the tight-junctional channel.

An explanation for the differential effect of calcium and magnesium chelation at the apical and basolateral side of the cell monolayer might be that there is a difference in composition of the tight junctions at the apical vs the basolateral side so that there is a difference in susceptibilty towards the effect of EDTA. Gumbiner (1987) and Gumbiner et al. (1988) have shown that the adhesion molecule uvomorulin at the zonula adherens on the basolateral side of the tight junctions is of importance for maintaining the integrity of tight junctions and calcium dependent and that splitting the zonula adherens has drastic effects on the tight junctions. Probably, apical calcium chelation has lesser effects because it does not directly affect the more calcium-susceptible zonula adherens.

We found in our experiments that the action of EDTA is most effective when this compound is applied at the basolateral side of the cell layer. In vivo it will be rather difficult to reach this side of the cells. This may explain why in previously reported in vivo experiments from our laboratory (Van Hoogdalem et al., 1989) it was found that EDTA (6.7 mM) applied by rectal infusion did not affect absorption of the peptide des-en-kephalin-γ-endorphin (DEγE). Furthermore, our data on cell-polarity dependence of chelating agents on tight junctions are fully supported by recently published similar findings in thyroid follicular epithelium (Nilsson, 1991).

In conclusion, we found that in the human intestinal adenocarcinoma cell line Caco-2, which is a good model for human intestinal epithelium, chelation of extracellular calcium and magnesium results in a polarity-dependent drop in trans-epithelial electrical resistance and an increase in transport clearance for hydrophilic model compounds. Therefore, we may conclude that opening of tight junctions between the cells by chelation is polarity dependent.

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