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Bisphosphonates increase tight junction permeability in the human intestinal epithelial (Caco-2) model

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Abstract

The human Caco-2 monolayer model was used to elucidate the potential role of bisphosphonates in the regulation of the junctional complex opening. Experiments were performed in parallel with EGTA, a potent free Ca^{2+} chelator, which was already demonstrated to increase intestinal epithelium permeability both in vitro and in vivo. Following treatment of Caco-2 cells with increasing non-toxic concentrations of tiludronate, a new bisphosphonate, different phenomena could be observed such as (i) free Ca^{2+} complex formation at tiludronate concentrations above 0.3 mM and a Ca^{2+} IC_{50} around 10–20 mM, (ii) increase in monolayer permeability, i.e., decrease in the transmembrane epithelial electrical resistance, at tiludronate concentrations above 10 mM and a TEER IC_{50} around 40 mM, associated with (iii) an opening of the junctional complex (desmosomes, intermediate and tight junctions) as demonstrated by scanning electron microscopy of the cell monolayer and electron microscopy of the apical pole of cells. (iv) The direct consequence of these observations was an increase in monolayer permeability at the level of the paracellular route. Hence, the paracellular transport not only of the PEG_{400} probe was increased following treatment of Caco-2 monolayers with tiludronate concentrations above 20 mM but also that of tiludronate itself at concentrations above 15 mM. These results are in agreement with a previous study (Boulenc et al., *Biochem. Pharmacol.*, 46 (1993) 1591–1600) which showed that tiludronate was specifically transported across Caco-2 cells via the paracellular route. Therefore, by its specific effect on free Ca^{2+} concentration, tiludronate could regulate the junctional complex opening of the intestinal epithelium monolayer and enhance its own transport across the intestinal wall.

Keywords: Paracellular transport; Caco-2 intestinal model; Tight junction; Bisphosphonate; Calcium chelator

1. Introduction

It is now well established that despite its colonic origin, the human intestinal epithelial cell line Caco-2 spontaneously differentiates into po-

Abbreviations: TEER, transepithelial electrical resistance; PEG, polyethylene glycol; FCS, fetal calf serum

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larised columnar cells which are more representative of the small intestine(Pinto et al., 1983; Rousset et al., 1985). These cells, when grown on collagen-coated filters, exhibit well-characterised microvilli and a polarised distribution of brush border enzymes (Zweibaum et al., 1983, 1984) including alkaline phosphatase and sucrase-isomaltase.

During the last decade, this cell line has been extensively used as a model (Artursson, 1991; Wilson, 1991) for the characterisation of transport properties of numerous compounds (Hidalgo et al., 1989; Artursson and Magnusson, 1990; Hidalgo and Borchardt, 1990) including ions, drugs, sugars, peptides, bile acids and vitamins, for the mechanistic evaluation of recovery and repair of surfactant injuries to the intestinal epithelium and for the study of the effect of pharmaceutical additives on epithelial integrity and drug absorption (Anderberg and Artursson, 1993).

When cultured on permeable filters, polarised Caco-2 cells are joined by a junctional complex which is comprised of desmosomes, intermediate junctions and tight junctions and which is governed by the extracellular Ca^{2+} concentration (Artursson and Magnusson, 1990). The paracellular barrier and junctional protein distribution depend on extracellular calcium (Collares-Buzato et al., 1994). This intercellular space is utilised by hydrophilic drugs (that do not partition significantly into the cell membrane) to cross the intestinal epithelium and penetrate the circulation. Hence, the paracellular route of hydrophilic drugs, i.e., the hydrophilic β-blockers (Artursson and Magnusson, 1990), vasopressin analogue (Lundin and Artursson, 1990) and a bisphosphonate (Boulenc et al., 1993) has been investigated. It is now well recognised that not only calcium chelators (Boulenc et al., 1993) but also commonly used pharmaceutical wetting agents, such as sodium dodecyl sulphate, perturb the junctional complex and increase the absorption of hydrophilic marker molecules across the intestinal epithelium (Anderberg et al., 1992). Exposure of the cell line to such compounds was associated with decreases in transepithelial electrical resistance and increases in the permeability to low and high molecular weight marker molecules as well as to peptides (Thwaites et al., 1993). Among various pharmaceutical xenobiotics reported to improve the absorption of hydrophilic drugs through the paracellular route, a bisphosphonate, i.e., 3-amino-1-hydroxypropylidene-1,1-diphosphonate, was shown to potentiate the rectal absorption of cefoxitin in rat (Van Hoogdalem et al., 1989). Since bisphosphonates have also been reported to bind Ca^{2+} (Bonnery et al., 1988), we investigated their roles in tight junction opening and membrane permeability. The results were compared to those observed following treatment of Caco-2 monolayers with a potent calcium chelator, i.e., EGTA. In addition to transport studies, electron microscopy was used to examine the surface morphology of treated cells as well as the integrity of the junctional complexes. Since previous studies from our laboratory (Boulenc et al., 1993) demonstrated that the new bisphosphonate, i.e., tiludronate, was transported via the paracellular route in the Caco-2 monolayer model and also binds calcium and magnesium at high drug concentration, tiludronate could affect its own paracellular transport. This hypothesis was further investigated.

2. Materials and methods

2.1. Chemicals

^{14}C-labelled (spec. act. 33 mCi/mmol) and unlabelled tiludronate were obtained from SANOFI Recherche, Montpellier, France. EGTA was obtained from Sigma, St Louis, USA. [^{14}C]Polyethylene $glycol_{400}$ (spec. act. 15.3 μCi/mg) was purchased from Amersham. [^{3}H]Mannitol (spec. act. 30 Ci/mmol) was purchased from New England Nuclear Products (Boston USA).

2.2. Cell culture

Caco-2 cells, originating from a human colorectal carcinoma (Fogh et al., 1977), were obtained from Dr A. Zweibaum (INSERM U-178, Villejuif, France). Caco-2 cells were grown in 75 cm^2 flasks at 37° C in an atmosphere of 10% CO_2 using Dulbecco's modified Eagle medium

(DMEM) supplemented with 15% heat-inactivated foetal calf serum (FCS), 1% nonessential amino acids, 10 mM L-glutamine, 50 IU/ml penicillin, and 50 μg/ml streptomycin. D-Glucose concentration in the culture medium was 4.5 g/l. The medium was changed every other day until the flasks reached 90% confluence and daily thereafter. Under these culture conditions, cells became confluent 5–6 days after seeding. For cell production, cells were detached from the flasks before they attained the stationary growth phase, with trypsin (0.25% in phosphate-buffered saline at pH 7.4) containing 0.2% EDTA for 10 min at 37° C. All tissue culture media were obtained from Eurobio Laboratories (Paris, France). Cells used in this study were between passages 70 and 90.

For transport studies Caco-2 cells were seeded onto collagen type I-coated inserts (Millicell-CM; pore size, 0.4 μm; diameter, 30 mm; Millipore, Bedford, MA) at 63 000 cells/cm^2. The monolayers used in this study were 12–20 days post-seeding or 7–16 days post-confluence.

2.3. Integrity of the monolayers

The integrity of the monolayers was determined by measurement of the potential difference (transepithelial electrical resistance; TEER). The potential difference was expressed as transmembrane resistance (Ω/cm^2) after subtraction of the intrinsic resistance of the model (i.e., the resistance obtained over the cell-free inserts). A monolayer with low TEER was assumed to exhibit extensive leakage through imperfect occluding junctions or holes in the monolayer. Under our incubation conditions, TEER values remained constant from day 12 to day 20.

[^{3}H]Mannitol was added to the apical side of the monolayers. The radiolabelled marker transported through Caco-2 cells was evaluated after 3 h at 37° C on a 0.5 ml aliquot part, withdrawn from the basolateral chamber. Sample radioactivity was determined by liquid scintillation counting (Tricarb; Packard Instruments). The results were expressed as the transported percentage of the dose as a function of incubation duration. The rate of [^{3}H]mannitol transported was 2.0 ± 05% per h. These results are in agreement with those previously reported by others (Artursson and Magnusson, 1990) who showed that undamaged differentiated monolayers are slightly permeable to macromolecules and to drugs transported via the paracellular route, i.e., mannitol.

2.4. Intracellular enzyme activity

Intracellular dehydrogenase activity was determined as follows; Caco-2 cells were seeded in 96-well tissue culture plates and were incubated for 10 days in order for cells to achieve a continuous differentiated monolayer. Monolayers were then treated for 1 h with increasing EGTA or tiludronate concentrations. The incubation medium containing the drug was then removed and the monolayers washed twice. Monolayers were then incubated for an additional 1 h period with 5 mg/ml (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT). In living cells, MTT is cleaved by the mitochondrial dehydrogenase activity to a dark-blue product. The intensity of the coloration, which was proportional to the overall activity of the cells, was measured in a multiwell scanning spectrophotometer (Multiscan MCC/340, Labsystem).

2.5. Preparation for transmission electron microscopic examination

The inserts were washed in PBS buffer (pH 7.2) and fixed in a solution containing 2% glutaraldehyde and 0.1 M sucrose, in 0.1 M cacodylate buffer (pH 7.3), for 1 h at 25° C. The cells were rinsed in 0.1 M Hanks buffer and fixed with 1% osmium tetroxide in 0.1 M sodium cacodylate. HCl buffer for 1 h at 25° C. After desiccation, the preparation was embedded in Epon resin, sliced with a Reichert ultramicrotome, stained with uranyl acetate and lead acetate, and examined and photographed on a transmission electron microscope (Jeol 100S).

2.6. Scanning electron microscopy

The cell monolayers were treated with either EGTA or tiludronate, fixed with a 1.5% glu-

taraldehyde solution and then coated with gold and palladium by a partly simplified tannic acid-osmium method. The specimens were studied in a JEOL JSM-820 scanning electron microscope.

2.7. Measurement of drug transport

Solutions of drugs and their radiolabelled isotopes were prepared from the radiolabelled isotopes and the corresponding unlabelled compounds in Hanks buffer to give final concentrations up to 10^{-3} M. All transport experiments were performed over 2 h, in a 10% CO_2 incubator at 95% relative humidity and 37° C in serum-free Hanks buffer (pH 7.4) containing 1 g D-glucose per l.

The monolayers were agitated on a mixer (Red Rocker, Hoefer Scientific Instruments, San Francisco, USA) at 16 rpm and a 10° angle. Preliminary experiments already demonstrated that agitation did not affect the rate of transport of hydrophilic drugs such as mannitol or tiludronate (data not shown). The drug solutions were added to the apical side of the monolayer and aliquot parts, usually 0.2 ml, were withdrawn from the basolateral compartment at previously determined intervals. Usually, drug solutions were added to the apical compartment, except where specified, and the rate of appearance of the label in the basal compartment was monitored. After withdrawal, the same volume of buffer was added in the basal compartment in order to maintain the receiver fluid volume constant. A minimum of three and a maximum of four samples were taken from each chamber at regular time intervals. All inserts were checked for monolayer integrity by evaluating the transepithelial electrical resistance before each experiment, and the transport of a paracellular probe after the end of the experiment.

2.8. Radioactive scintillation counting

Radioactivity was determined by liquid scintillation counting of 0.05–0.2 ml aliquot parts of the incubation medium in a Tricarb liquid scintillation spectrometer (Packard Instruments). Results were corrected to dpm by comparison with standard quench curves.

2.9. Calculations

The apparent permeability coefficient (P_{app}) was expressed in cm/s and was determined as previously reported (Boulenc et al., 1993):

$$P_{app} = dQ/[dt \times A \times C_0]$$

where dQ/dt is the transport rate ($\mu g/s$) and corresponds to the slope of the regression line determined on at least four different time points, C_0 denotes the initial concentration in the donor chamber ($\mu g/ml$; $\mu g/cm^3$), and A is the area of the membrane (5.7 cm^2)

2.10. Treatment with a specific calcium chelator

The selective calcium chelator, EGTA, was used to bind free extracellular Ca^{2+}. All EGTA experiments were performed under the same conditions as the drug transport studies. Serum-free Hanks medium (containing 1.2 mM Ca^{2+}) was supplemented with increasing EGTA concentrations. The medium containing EGTA was added to the two compartments, apical and basal, for periods of time up to 1 h. When the effect of tiludronate was investigated, drug was also added in both compartments. The integrity of the monolayers was checked a few hours (less than 6 h) after the end of each experiment by determination of [^{3}H]mannitol transport and by measurement of the transmembrane epithelial electrical resistance.

2.11. Determination of extracellular Ca^{2+} concentration

The concentration of Ca^{2+} in the incubation medium, i.e., Hanks buffer = 1.2 mM, was determined in the absence or presence of increasing concentrations of either EGTA or tiludronate, using an Ingold-type electrode. Calculations were determined relative to the control value.

3. Results

3.1. Comparative effects on intracellular enzyme activity

The intracellular dehydrogenase activity determined by the MTT method was evaluated following a 1 h exposure of 12-day-old Caco-2 monolayers to increasing EGTA or tiludronate concentrations ranging between 0.05 and 20 mM and between 1.5 and 80 mM, respectively (Fig. 1). Dehydrogenase activity remained unaffected by the presence of either tiludronate or EGTA up to 80 and 20 mM, respectively. Higher drug concentrations could not be evaluated, due to the lack of solubility of these compounds at this specific neutral pH.

Another way to investigate drug cytotoxicity, was to evaluate [^{3}H]mannitol transport across the monolayer several hours following treatment of monolayers with high drug concentrations. Caco-2 monolayers were incubated for 1 h with increasing EGTA (0.6, 1.25, 2.5 and 5 mM) or tiludronate (12.5, 25, 50 and 100 mM) concentrations, extracellular medium was removed and replaced by free-drug incubation medium. After a subsequent 12 h period, the transport of mannitol across the monolayers was determined. Caco-2 monolayers treated with concentrations up to 2.5 mM for EGTA and 50 mM for tiludronate remained almost impermeable to mannitol transport as observed for untreated cells. However, at higher drug concentrations, Caco-2 monolayers remained leaky to mannitol suggesting an irreversible opening effect at the level of the junctional complex.

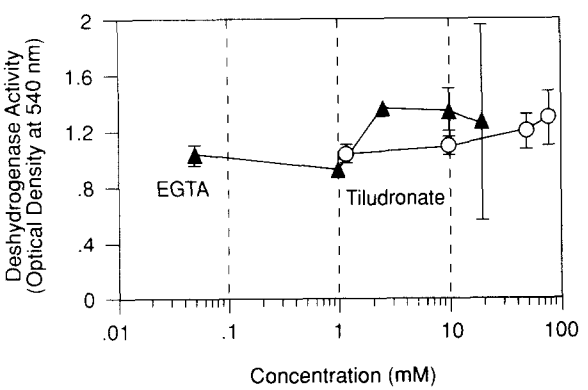

Fig. 1. Comparative effect of EGTA and tiludronate on intracellular dehydrogenase activity. Caco-2 monolayers were incubated for 1 h with increasing EGTA or tiludronate concentrations and the intracellular dehydrogenase activity was determined according to the MTT method as described in section 2. Values are expressed as mean ± S.D. of eight different determinations.

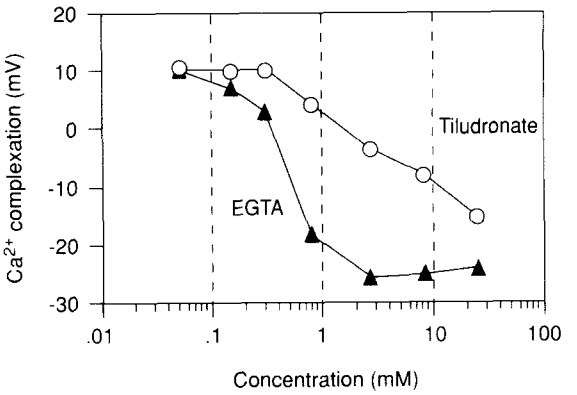

Fig. 2. Comparative effects of EGTA and tiludronate on Ca^{2+} complex formation. Culture medium was incubated with increasing EGTA or tiludronate concentrations for about 30 min and Ca^{2+} complexation was determined. Reported results were obtained in a single experiment, each value being the mean of two different determinations.

3.2. Comparative effects on Ca^{2+} complex formation

EGTA has already been reported to be a potent and selective calcium chelator. Similarly, Bonnery et al. (1988) for tiludronate, as well as other authors (Lamson et al., 1984) for different analogues, demonstrated that bisphosphonates formed polynuclear complexes with calcium and could be precipitated as calcium bisphosphonate. The effect of a 30 min exposure of culture medium to increasing EGTA or tiludronate concentrations on free Ca^{2+} chelation was evaluated. The results are illustrated in Fig. 2. Most of the Ca^{2+} was complexed following incubation with 2.5 mM EGTA. The Ca^{2+} IC_{50} (concentration which complexed 50% of the free Ca^{2+}) was about 0.5 mM for EGTA and 10–20 mM for tiludronate.

3.3. Comparative effects on transepithelial electrical resistance (TEER)

Preliminary experiments demonstrated that a 1 h exposure of Caco-2 monolayers to 10 mM EGTA was associated with a dramatic decrease in the TEER value which could not be restored following a further 24 h incubation of monolayers with EGTA-free medium. This was also confirmed when PEG_{400} paracellular transport was investigated just after or 24 h after a 1 h exposure to 10 mM EGTA. Under the latter conditions, monolayers remained leaky to PEG_{400}, in contrast to functional monolayers (data not shown). However, a low monolayer permeability to PEG_{400} was fully restored 24 h after a 1 h exposure to 2.5 mM EGTA.

The effect of the presence of EGTA in the apical compartment or at both sides of the Caco-2 monolayer was also investigated. When EGTA (2.5 mM final concentration) was added in the apical compartment, the tiludronate permeability coefficient was increased 3.7-fold ($n = 3$), while a 31.7-fold increase ($n = 3$) was observed when EGTA was added in both compartments.

Fig. 3 illustrates the effects of 2.5 mM EGTA and 50 mM tiludronate on the transepithelial electrical resistance of Caco-2 monolayers. At these specific concentrations, both compounds

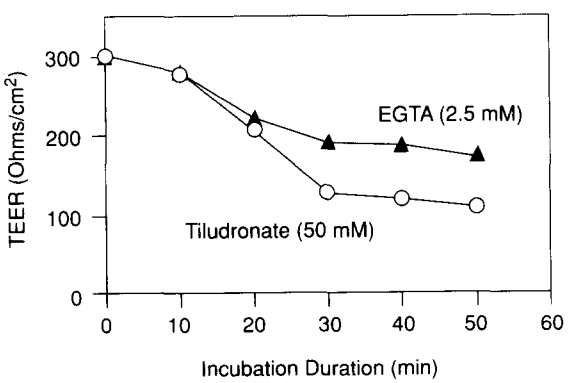

Fig. 3. Comparative effect of EGTA and tiludronate on transepithelial electrical resistance. 14-day-old Caco-2 monolayers were exposed to either 2.5 mM EGTA or 50 mM tiludronate and the transepithelial electrical resistance was monitored every 10 min for 50 min (representative experiment).

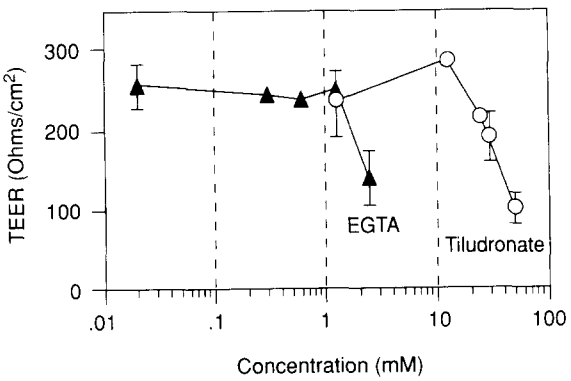

Fig. 4. Comparative effects of EGTA and tiludronate on transepithelial electrical resistance. 14-day Caco-2 monolayers were exposed to increasing EGTA or tiludronate concentrations for about 40–50 min and transepithelial electrical resistance was determined. When S.D. were reported, values were mean ± S.D. of data obtained in at least three different experiments performed on triplicate inserts.

affected the permeability of the cell monolayer in a similar manner. The maximal effect was observed following a 30 min exposure to the drug. This effect remained constant over the remaining 30 min of observation. The effect of increasing concentrations on monolayer permeability was further evaluated following a 40–50 min exposure to increasing EGTA or tiludronate concentrations. EGTA and tiludronate concentrations up to 1.5 and 10 mM, respectively, did not affect the transmembrane epithelial electrical resistance of the monolayer (Fig. 4). The TEER IC_{50} (concentration resulting in a 50% decrease of the transepithelial electrical resistance of the monolayer) was about 40 mM for tiludronate. A 50% decrease was also achieved at 2.5 mM EGTA concentration. However, due to the effect of EGTA on membrane permeation and cell viability, higher EGTA concentrations could not be investigated. At these drug concentrations, the transepithelial electrical resistance was fully restored following an additional 4 h incubation in EGTA- or tiludronate-free fresh culture medium.

3.4. Comparative effects on monolayer morphology

Using scanning electron microscopy, Caco-2 cells treated with 2.5 mM EGTA or 50 mM

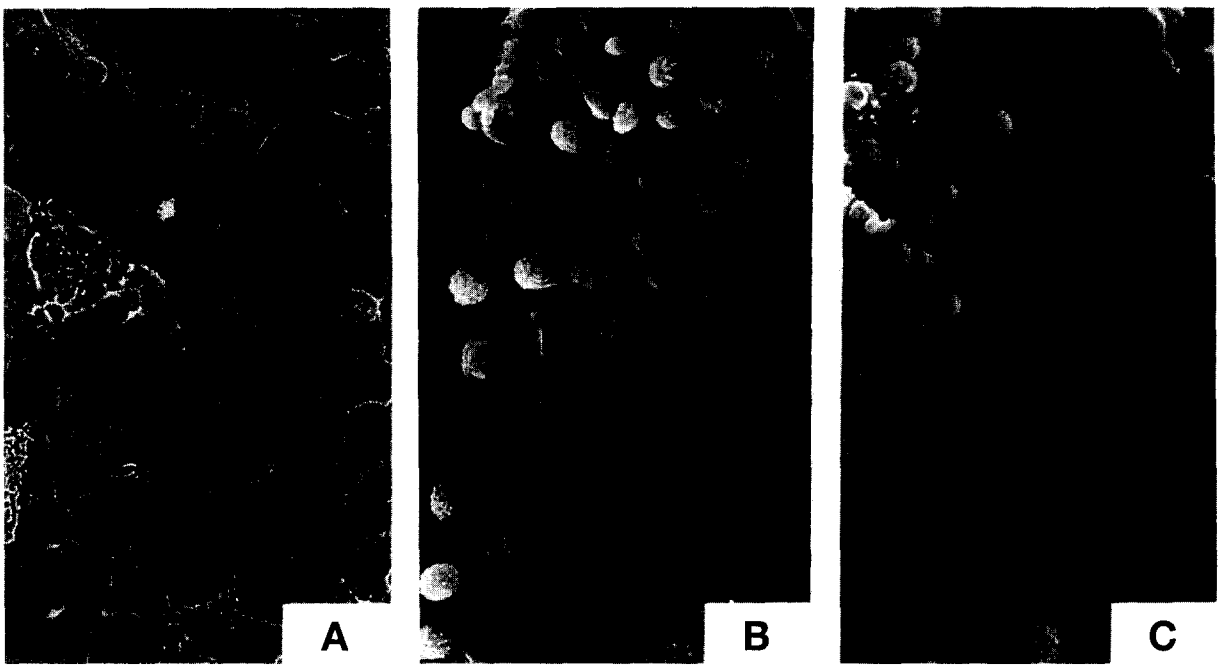

Fig. 5. Scanning electron micrographs showing the surface of the Caco-2 cell monolayers. Post-confluence 12-day Caco-2 cells were incubated for 40 min in the absence (A) or presence of either 2.5 mM EGTA (B) or 50 mM tiludronate (C) and scanning electron micrographs were recorded. The cracks are artefacts from the preparation of the specimens. Reproduced from Boulenc et al. (1993).

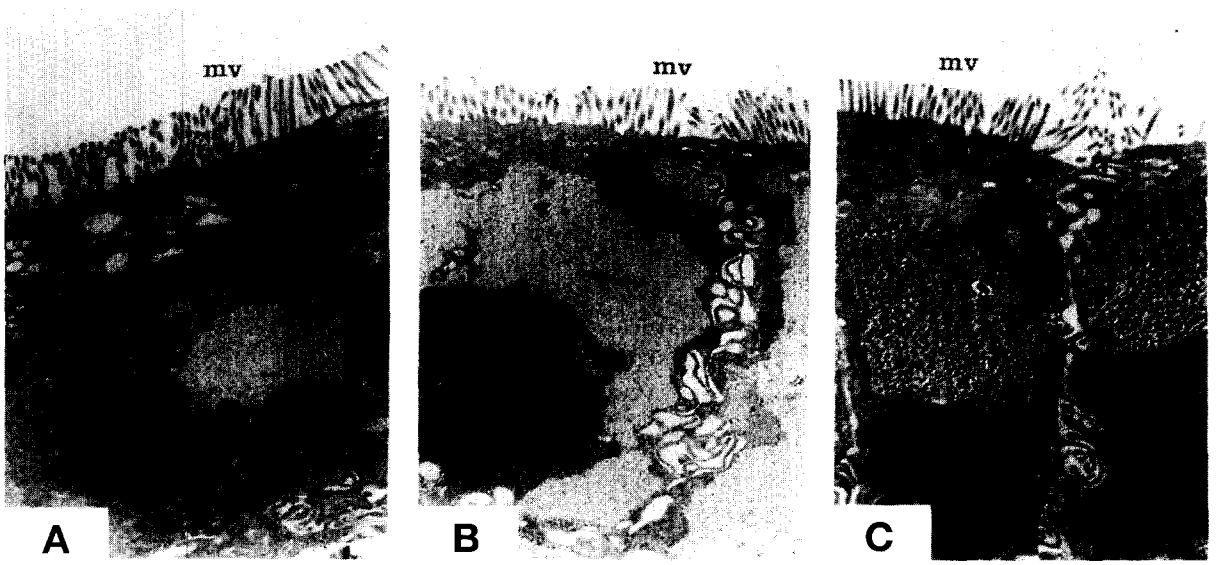

Fig. 6. Electron micrographs of Caco-2 monolayers. Electron micrographs of 14-day-old Caco-2 monolayers cultured for 40 min in the absence (A) or presence of either 2.5 mM EGTA (B) and 50 mM tiludronate (C). mv, microvilli

tiludronate appeared to be contracted and rounded as compared with the untreated cells (Fig. 5). To investigate better the effects of both compounds on the intercellular junctional complex, transmission electron microscopy was also performed. As previously reported by numerous laboratories (Pinto et al., 1983; Zweibaum et al., 1984; Grasset et al., 1985; Hilgers et al., 1990), the cells have a morphological appearance similar to that of well-differentiated absorptive cells. Caco-2 cells are polarized with the nuclei generally located in the basal part of the cells and they form a homogeneous monolayer with well-developed apical brush borders (Fig. 6). Different components of the intercellular junctional complex (desmosomes, intermediate and tight junctions) are observed. Following treatment of cells with either 2.5 mM EGTA or 50 mM tiludronate, pronounced differences were observed particularly at the level of the paracellular space (Fig. 7). Components of the junctional complex were often separated. At these drug concentrations, the junctional structures of adjacent cells appeared in parallel which suggests that the cells were still in contact. Moreover, the apical brush border appeared to be only slightly affected, if at all, by drug treatment.

3.5. Comparative effects on PEG_{400} paracellular transport

The transepithelial transport of PEG_{400}, a marker for the paracellular route, was evaluated on 12-day Caco-2 monolayers pretreated for 30 min with increasing EGTA or tiludronate concentrations. Under control conditions, the permeability coefficient (P_{app}) for PEG_{400} was around 0.8 ± 0.1 cm/s.10^6 ($n = 5$). No effect on P_{app} of PEG_{400} was demonstrated for EGTA and tiludronate concentrations below 0.1 and 15 mM, respectively. As illustrated in Fig. 8, the permeability of Caco-2 monolayers to PEG_{400} was increased following a 30 min incubation with higher EGTA or tiludronate concentrations. Similar effects were achieved following a 30 min incubation period of Caco-2 monolayers with either 2.5 mM EGTA or 40–50 mM tiludronate.

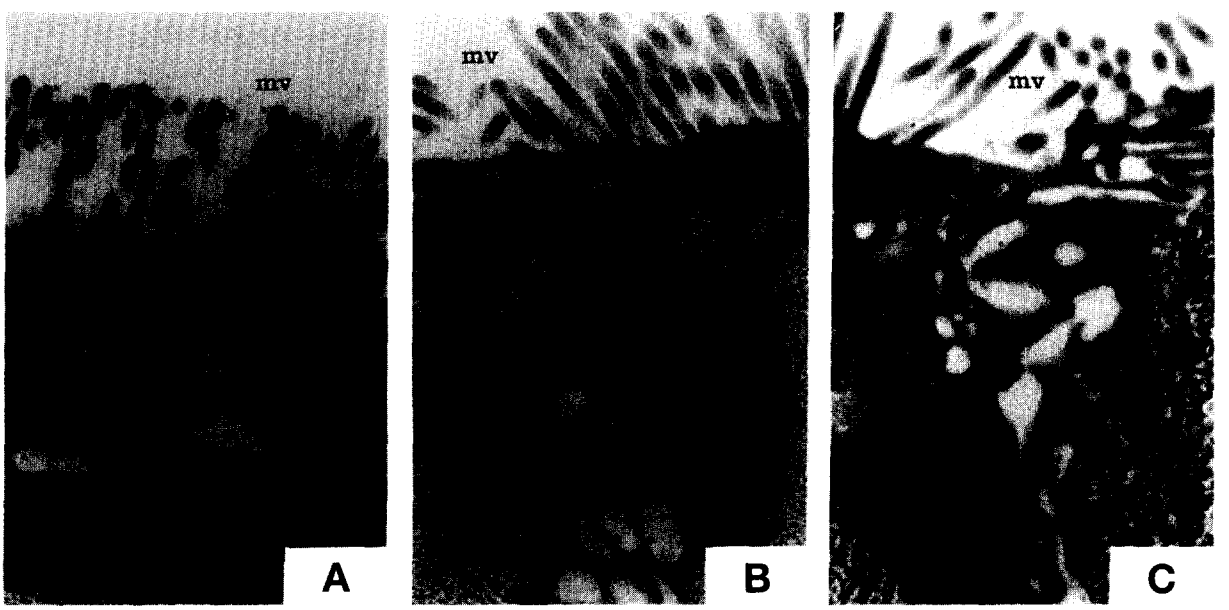

Fig. 7. Electron micrographs of the junctional complex. Junctional complexes (desmosomes, intermediate junctions and tight junctions) obtained in either untreated 14-day Caco-2 monolayers (A) or cells treated with either 2.5 mM EGTA (B) or 50 mM tiludronate. mv, microvilli; arrowheads indicate elements of the junctional complex, i.e., tight junction and desmosomes.

3.6. Comparative effects on tiludronate paracellular transport

Previous studies performed on the Caco-2 monolayer epithelial model demonstrated that tiludronate was mainly transported by the paracellular route (Boulenc et al., 1993). The permeability coefficient for tiludronate transport across the Caco-2 monolayer was around $0.4 \pm 0.1 \times 10^6$ cm/s ($n = 7$). P_{app} for tiludronate was increased following treatment of Caco-2 monolayers with EGTA concentrations as low as 0.05 mM. The P_{app} of tiludronate was then increased in a concentration-dependent manner following treatment of Caco-2 monolayers with increasing EGTA concentrations. The results are illustrated in Fig. 9. Tiludronate pretreatment of Caco-2 monolayers also affected its own transport. Hence, the P_{app} of tiludronate was increased up to $13.4 \pm 1.0 \times 10^6$ cm/s when Caco-2 cells were pretreated for 40–50 min with 30 mM tiludronate.

Of particular interest was the observation that the P_{app} of tiludronate was increased at EGTA and tiludronate concentrations of 0.05 and 15 mM, respectively, conditions under which the P_{app} of PEG_{400} was not affected. This difference could be linked at least in part to the difference in the molecular weights of PEG_{400} and tiludronate (Mol. Wt 302 g/mol). These results are in agreement with recent data (Artursson et al., 1993). These authors demonstrated that the paracellular permeability of different metabolically inert molecules of increasing molecular weights, ranging between 60 and 502 g/mol, was dependent on both molecular weight and structure flexibility.

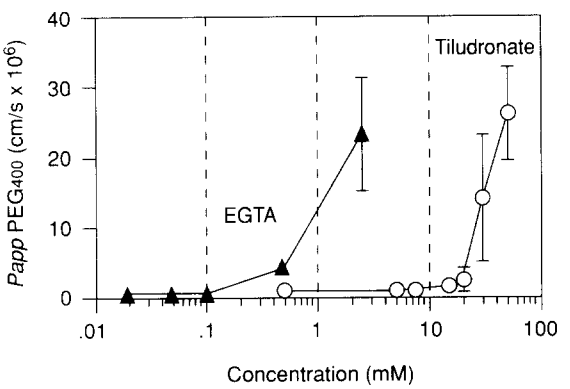

Fig. 8. Comparative effects of EGTA and tiludronate on the permeability coefficient for PEG_{400}. 14-day Caco-2 monolayers were exposed to increasing EGTA or tiludronate concentrations for about 30 min and P_{app} for PEG_{400} was determined. When S.D. were reported, values were mean ± S.D. of three experiments performed on duplicate inserts and on different days.

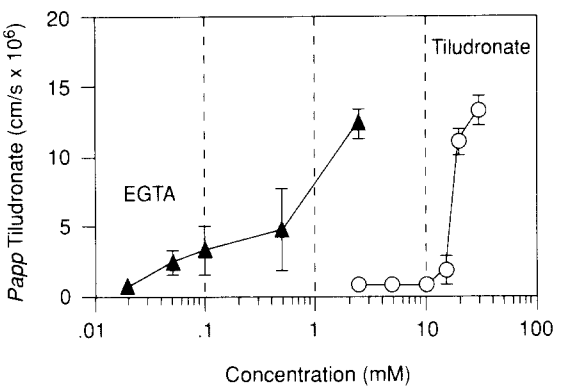

Fig. 9. Comparative effects of EGTA and tiludronate on the permeability coefficient for tiludronate. 14-day Caco-2 monolayers were exposed to increasing EGTA or tiludronate concentrations for about 30 min and P_{app} for tiludronate was determined. When S.D. were reported, values were mean ± S.D. of three different experiments performed on duplicate inserts and at different days.

4. Discussion

The human intestinal epithelial cell line Caco-2 provides a cellular model to study the differentiated functions of intestinal enterocytes. Caco-2 cells spontaneously differentiate in culture into polarised cells possessing microvilli and enterocytic properties. Confluent monolayers form tight junctions between cells. The region of the normal human GI tract represented by the Caco-2 model is still under investigation. While the presence of brush border hydrolases and transport pathways for bile acids and cobalamin are properties of the distal ileum, the electrical properties are more indicative of a colonic epithelium (Hidalgo and Borchardt, 1990; Artursson, 1991; Artursson and Karlsson, 1991). When compared to the normal

rat intestine in vivo, Caco-2 monolayers exhibited similar permeability profiles (Artursson et al., 1993).

The epithelial monolayer is comprised of the lipophilic cell membranes and the intercellular junctions, i.e., tight junctions, desmosomes and occluding junctions, between the cells. The intercellular spaces are sealed by tight junctions which reduce their pore radius to a few ångströms (Madara and Dharmsathaphorn, 1985). The contribution of this paracellular pathway to the total permeability of the epithelial monolayer is only significant for drugs that are transported slowly across the cell membrane, e.g., hydrophilic compounds with a low molecular weight and very low octanol/water partition coefficients (Artursson and Magnusson, 1990; Artursson and Karlsson, 1991).

In a previous study (Boulenc et al., 1993), we demonstrated that tiludronate was transported across the Caco-2 monolayers by the paracellular route. Indeed, tiludronate transport was relatively slow, independent of the temperature, of the polarity of the cell membrane and of the presence of an energetic inhibitor. On the other hand, it was highly dependent on EGTA, a calcium chelator which reduces the extracellular free calcium ion concentration, i.e., conditions under which the intercellular spaces were widened and the tight junctions were resolved at the ultrastructural level. This increase in tiludronate transport following EGTA treatment was preserved only 10–30 min after EGTA removal, consistent with its reversal effect on tight junction widening.

Consistent with the use of the paracellular route, the intestinal absorption of various bisphosphonates, i.e., 1-hydroxyethylidene-1,1-bisphosphonate (HEBP) and dichloromethylene bisphosphonate (Cl_2MBP), has already been described (Fogelman et al., 1986) to be low in humans. Based on the observations that bisphosphonates can bind free Ca^{2+} and form polynuclear complexes with calcium (Lamson et al., 1984; Janner et al., 1991) and were precipitated as calcium bisphosphonates, Janner et al. investigated whether the calcium chelator EDTA could improve intestinal absorption of different bisphosphonates, including Cl_2MBP, in the rat. Indeed, EDTA did increase the absorption of these bisphosphonates but only at certain doses of these compounds and at high EDTA concentrations, making this chelator unsuitable for clinical use. Among various explanations for this increase in bisphosphonate availability, they suggested that EDTA could directly enhance intestinal permeability, a consequence of the reversible decrease in the calcium and magnesium content of the intestinal epithelium associated with ultrastructural alterations at the level of tight junctions. EDTA as well as other promoters such as sodium taurocholate, sodium caprylate and sodium laurate have been demonstrated (Tomita et al., 1988) to increase the paracellular route of inulin, a consequence of the increase of the colonic pore size in an everted sac procedure. A number of non-steroidal anti-inflammatory drugs such as indomethacin, phenylbutazone and diclofenac enhance drug absorption from the gastro-intestinal tract by both transcellular and paracellular routes (Yamashita et al., 1987). Apart from the permeability enhancing effects, the functional alterations of the intestinal membranes, however, may limit their use for clinical applications as promoter agents. One of the major drawbacks of the use of EDTA is that its promoting effect on intestinal drug absorption appears to be accompanied by a damaging effect on mucosal integrity. Concentrations of 0.8–1% induced severe damage of the small intestinal epithelium in rats (Nakanishi et al., 1983) as well as jejunal blood loss in dogs (Tidball and Lipman, 1962).

It was then suggested that strong chelating agents, such as EDTA, could cause the solubilization and the release of proteins from the mucosal membrane, resulting in significant enhancement of the absorption of cefoxitin from the rectum. EDTA, with its high chelating activity and by the formation of calcium-EDTA complexes, could alter the structure of the epithelial cell layer by removing calcium from the cell junction. This action was expected to cause permeability enhancement of the intestinal membrane to drugs. Yamashita et al. (1987) demonstrated that 1 mM EDTA treatment of the rat jejunum induced gradual reduction in the membrane resistance in

relation to the permeability enhancing effects. When the concentration of EDTA became higher, irreversible changes in membrane function occurred (Nishihata et al., 1985).

In the present study, we demonstrated that tiludronate could mimic the effect of a potent chelating agent on the regulation of tight junction opening. Hence, when Caco-2 cells were exposed to tiludronate concentrations above 10 mM, we observed (i) the complexation of free Ca^{2+}, (ii) a decrease in the transepithelial electrical resistance, (iii) a widening of the intercellular space, (iv) an increase in the paracellular transport of PEG_{400} and (v) an increase in the permeability coefficient of tiludronate. These concentration-dependent effects can be compared to those observed following incubation of Caco-2 monolayers with increasing EGTA concentrations. Similar drug effects were achieved with either 2.5 mM EGTA or 30–50 mM tiludronate. As demonstrated by monitoring the intracellular dehydrogenase activity, the increase in monolayer permeability was not linked to drug cytotoxicity. These observations are in agreement with those of Van Hoogdalen et al. (1989) who reported that, due to its calcium binding properties, 3-amino-1-hydroxypropylidene-1,1-diphosphonate could be a potential paracellular absorption promoting agent. Similarly, pharmacokinetic studies performed in human healthy volunteers demonstrated the non-linear pharmacokinetics of tiludronate (Necciari et al., 1989; Thiercelin et al., 1992) following oral administration. These studies suggest that following treatment of patients with tiludronate, i.e., either single high dose or chronic administration, the drug could increase its own transport across the intestine wall and hence increase its bioavailability.

Although studies on various drugs and comparison with their percentage of absorption in vivo will be required to determine how far one can extrapolate from the in vitro system to the whole animal or to man, the current results indicate that the Caco-2 monolayer cell culture system provides a convenient model not only for characterizing transport systems but also for investigating the effect of different pharmaceutical drug additives on monolayer permeability.

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