Biochemical and Biophysical Research Communications 418 (2012) 128–133

Contents lists available at SciVerse ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

Evaluation of calcium depletion as a strategy for enhancement of mucosal absorption of macromolecules

Driton Vllasaliu [a,*], Saif Shubber [a], Martin Garnett [a], Cameron Alexander [a], Mike Eaton [b], Snow Stolnik [a]

[a] Division of Drug Delivery and Tissue Engineering, Boots Science Building, University of Nottingham, University Park, Nottingham NG7 2RD, UK
[b] UCB Pharma, 208 Bath Road, Slough, Berkshire SL1 3WE, UK

ARTICLE INFO

Article history:
Received 28 December 2011
Available online 8 January 2012

Keywords:
Calu-3
Caco-2
Tight junctions
TEER
Calcium depletion
Mucosal protein delivery

ABSTRACT

Extracellular calcium is crucial for functioning of the epithelial barrier. Compounds that bind calcium, reducing its extracellular levels, have therefore been investigated as mucosal absorption enhancers. However, the conditions under which calcium reduction sufficiently modulates the epithelial barrier to result in meaningful improvements in mucosal drug absorption are unclear. Present work investigated the settings in which calcium depletion leads to optimal epithelial barrier-modulating effects. Using Calu-3 and Caco-2 cell layers and inducing calcium depletion site-specifically (apically, basolaterally or on both sides) we demonstrate that apical calcium removal produces a modest effect on the tight junctions (the extent of the effect being dependent on the duration of apical calcium unavailability), whilst basolateral calcium exhaustion leads to a prominent effect on the epithelial barrier. However, using polyacrylic acid as an example, we show that polymeric calcium-binding agents proposed as mucosal absorption-enhancing excipients alter calcium levels exclusively on the apical side of the epithelium, which explains their modest effect on epithelial barrier modulation (also demonstrated in our work). Therefore the use of calcium-depleting agents, especially those based on macromolecular polymers, is a relatively inefficacious strategy to promote mucosal absorption of macromolecules.

1. Introduction

Mucosal surfaces offer potential for non-invasive delivery of biotherapeutics. However, these surfaces are naturally designed to provide a barrier to the movement of material from the external environment (mucosal lumen) into the systemic circulation. This barrier is especially prominent for hydrophilic macromolecules, a category within which most biotherapeutics fall. For any given drug, absorption in its active form across the mucosal surfaces requires the drug to overcome a series of barriers, including mucus, mucociliary clearance and degradative enzymes. The main barrier restricting mucosal absorption of macromolecules is the epithelial cell layer. The diffusion of hydrophilic macromolecules through epithelial cell membranes is not feasible, while the presence of tight junctions that interconnect adjacent epithelial cells limits transepithelial (paracellular) movement of hydrophilic molecules larger than 1000 Da [1].

Attempts to deliver biologicals across different mucosal surfaces *in vivo* have often resulted in poor bioavailability, leading to investigation of different strategies to improve their absorption. These include the use of agents to improve drug access, stability and contact time with the mucosal surfaces, for instance mucolytics [2], enzyme inhibitors [3], mucoadhesive polymers [4], and compounds that temporarily disrupt the epithelial barrier. The latter approach aims to achieve transient opening of the epithelial tight junctions, facilitating macromolecular translocation via the paracellular corridor.

The presence of calcium in the extracellular environment of epithelial cells is essential for the maintenance and regulation of the epithelial barrier [5,6]. Since the late seventies the effect calcium on epithelium has been extensively studied, particularly using the Madin–Darby Canine Kidney (MDCK) epithelial cell line as a model where a calcium-free medium was demonstrated to prevent the formation of tight junctions [7] and increase tight junction permeability [8]. These studies often employed calcium-chelating compounds, such as ethylenediaminetetra-acetic acid (EDTA) and ethylene glycol tetra-acetic acid (EGTA), to deplete calcium, opening the tight junctions [9,10] and increasing the paracellular permeability [11,12].

Compounds that bind calcium therefore have, and continue to be, investigated for their absorption-enhancing potential. The application of 'classical' calcium binding agents, such as EDTA

* Corresponding author. Address: Division of Drug Delivery and Tissue Engineering, School of Pharmacy, University of Nottingham, Nottingham NG7 2RD, UK. Fax: +44 115 9515102.

E-mail addresses: Driton.Vllasaliu@nottingham.ac.uk (D. Vllasaliu), paxss@exmail.nottingham.ac.uk (S. Shubber), martin.garnett@nottingham.ac.uk (M. Garnett), cameron.alexander@nottingham.ac.uk (C. Alexander), nanomedicine@btinternet.com (M. Eaton), snow.stolnik-trenkic@nottingham.ac.uk (S. Stolnik).

0006-291X/$ - see front matter © 2012 Elsevier Inc. All rights reserved.
doi:10.1016/j.bbrc.2011.12.146

and EGTA, has generally been limited to *in vitro* investigational situations due to their local intolerability. However, a considerable number of studies have also investigated various polymeric compounds with calcium-binding properties and attributed absorption-enhancing effects to extracellular calcium depletion. These compounds typically include, but are not limited to, carboxylic acid-containing polymers such as poly(acrylic acid) and its derivatives [13,14] and poly(methacrylic acid) [15–17]. These polymers do not penetrate cell membranes.

Despite the accepted importance of extracellular calcium in maintaining the epithelial barrier, it still remains unclear whether administration of calcium-binding absorption enhancers is a viable option to disrupt the epithelium and achieve therapeutically adequate mucosal absorption of macromolecules. This is due to conflicting reports with regards to conditions under which extracellular calcium depletion produces sufficient effects on the epithelial barrier and/or attributing the absorption-enhancing effect of some polymers to calcium depletion without indisputable experimental confirmation of the effect. Yet novel calcium-binding polymers [18] and chemical modification of existing polymers to include calcium-chelating moieties [19] continue to be proposed as drug delivery options for improving mucosal absorption of macromolecules.

The present work attempts to clarify whether luminal calcium depletion augments mucosal absorption of macromolecules to an extent that may present a realistic drug delivery strategy. In this respect, the settings where extracellular calcium depletion leads to modulation of the epithelial barrier were systematically investigated using relevant epithelial models – cell layers of a bronchial cell line, Calu-3, and those of a commonly used colorectal cell line, Caco-2. Calcium depletion was achieved site-specifically using calcium-free medium and calcium-chelating compounds and effects on the epithelial barrier investigated. To gain a mechanistic insight into the process of calcium-regulated modulation of tight junctions and confirm the calcium-depleting action of compounds investigated, relative changes in calcium levels in the extracellular solution were assessed using a fluorescent calcium-sensing probe.

2. Materials and methods

2.1. Materials

See Supplementary Information.

2.2. Cell culture

Calu-3 and Caco-2 cells were seeded on Transwell® filters at 10^5–2×10^5 cells/cm^2 and cultured using EMEM and DMEM for 9–14 days and 21–23 days, respectively.

2.3. Effect of calcium-free HBSS on tight junction modulation

2.3.1. Effect on TEER

Baseline TEER was measured following replacement of culture medium with HBSS and equilibration for 45 min. Thereafter:

(1) Calu-3 layers were incubated with calcium-free HBSS (CFH BSS) on both *apical and basolateral* sides for 30, 60 or 90 min, following which the solution was replaced with HBSS.
(2) Calu-3 and Caco-2 layers were incubated with CFHBSS on the *apical* side and HBSS on the basolateral side.

TEER was measured periodically in all cases. CFHBSS used in this work was also magnesium-free, therefore control experiments were conducted using CFHBSS, supplemented with 140 mg/L $CaCl_2$ (found in standard HBSS) and determining its effect on TEER.

2.3.2. Effect on permeability

FD4 was applied apically in CFHBSS (500 μg/ml) to Calu-3 layers and basolateral solution (HBSS) sampled every 30 min for 3 h, with replacement of sampled solutions with fresh HBSS. FD4 permeability was expressed as the apparent permeability coefficient (P_{app}) (see Supplementary Information).

In a different experimental set-up, FD4 was dissolved in CFHBSS (500 μg/ml) and applied apically in Calu-3 layers. This solution was replaced with fresh FD4/CFHBSS every 30 min. FD4 permeability was measured by sampling the basolateral solution (HBSS) periodically as previously. The control experiment was conducted by measuring FD4 permeability following its application and re-application apically in HBSS every 30 min. In this instance, permeability was expressed as cumulative FD4 amount traversing the cell layers.

2.4. Effect of EDTA on tight junction modulation

2.4.1. Effect on TEER

EDTA was applied to the apical side of Calu-3 and Caco-2 layers at 0.125% w/v. TEER was measured periodically (every 30 min for 3 h), following which EDTA was removed, cells washed and incubated with culture media overnight and TEER measured to determine cell recovery. In a separate experiment, EDTA was applied either apically or basolaterally in Caco-2 monolayers with subsequent regular measurement of TEER.

2.4.2. Effect on permeability

EDTA (0.125% w/v) was applied in Calu-3 layer apically in conjunction with FD4 (500 μg/ml) or basolaterally, with apical addition of FD4. Basolateral solution was sampled periodically for 3 h, with replacement of the sampled solution with either fresh HBSS or 0.125% w/v EDTA/HBSS (to maintain basolateral EDTA concentration constant).

2.4.3. Effect of CFHBSS and EDTA on calcium levels

Calu-3 layers were washed and equilibrated in HBSS. CFHBSS or EDTA (0.125% w/v in HBSS or CFHBSS) were added apically (and HBSS basolaterally). Apical solution was subsequently withdrawn (50 μl) regularly for 3 h. Calcium-sensing fluorescent probe, Fluo-4, was applied (10 μM) to the sampled solutions and fluorescence measured (485/535 nm excitation/emission) using an MFX microtiter plate fluorometer (Dynex Technologies, USA). Cell layers incubated with HBSS (on both apical and basolateral sides) served as the control, with the apical solution sampled at 2 and 180 min and treated with Fluo-4.

The relative change in *basolateral* calcium level following apical application of EDTA was measured by applying EDTA (0.125% w/v in HBSS) apically and regular sampling of the basolateral medium (HBSS). Fluo-4 was added to the sampled solutions and fluorescence determined. Control experiments were performed by measuring Fluo-4 fluorescence of basolateral solutions consisting of HBSS or EDTA in HBSS (0.125% w/v) (with apical HBSS in both cases).

2.5. Effect of polyacrylic acids on tight junction modulation and calcium levels

PAA of ~100 and ~250 kDa were applied (0.25% w/v in HBSS, pH 7.4) to the apical side of Calu-3 layers and TEER measured regularly over 80 min. PAA solutions were then removed, cells washed with PBS and culture medium applied. TEER was also measured at 110, 140 and 170 min (in culture medium) to determine TEER recovery. Control experiments were conducted by applying HBSS to the cells and monitoring TEER.

The permeability experiment was conducted as described earlier by adding FD4 (500 μg/ml) apically in conjunction with PAA (0.25% w/v) at pH 7.4.

The effect of PAA on relative changes in calcium levels was determined following the addition of PAA (0.25% w/v; in HBSS, pH 7.4) to Calu-3 layers apically and periodic sampling of the apical and basolateral solution (50 μl). Fluo-4 (10 μM) was then applied to the sampled solutions and fluorescence measured. Cells incubated with HBSS (both apically and basolaterally) served as a control, whereby the solution was sampled from the apical side 5 and 180 min after its application.

2.6. Statistical analysis

See Supplementary Information.

3. Results and discussion

3.1. Effect of CFHBSS on tight junction modulation

To confirm the role of extracellular calcium on the tight junctions, Calu-3 layers were initially exposed to CFHBSS on both *apical* and *basolateral* sides. This resulted in a sharp decrease in TEER, the extent being dependent upon the incubation period with calcium-free medium (Fig. 1A). The TEER-decreasing effect lasted up to the point when calcium-containing solution was re-introduced, after which TEER reversed, assuming levels similar to control. In the control experiment there was a slight decrease in TEER initially, but reversal to near-baseline values was apparent within 1 h.

The heavy dependence of the epithelial barrier on extracellular calcium has been demonstrated by work showing that extracellular calcium exhaustion induces global changes in epithelial cells, including disruption of actin filaments and adherent junctions, reduction of cell adhesion and activation of protein kinases [6]. Interestingly, our data shows that when calcium removal was conducted selectively on the apical side of the cell layers, the change in TEER followed a different pattern. In this instance, the drop in TEER was transient, with recovery observed whilst calcium-free medium was present on the apical side of the cell layers (Fig. 1B). The extent of this recovery was especially apparent in Calu-3 layers (up to 97% of the baseline value), whilst a lower level reversal was evident in Caco-2 monolayers (to 63%). The effect in TEER was reflected in the permeability study, whereby apical addition of FD4 in CFHBSS in Calu-3 and Caco-2 layers resulted in a modestly higher permeability (1.8-fold) compared to control (Fig. 1C) in both Calu-3 and Caco-2 layers ($p = 0.001$ and 0.007, respectively).

A further experiment assessing the role of apical calcium on the epithelial barrier employed a design whereby FD4 permeability was determined in conditions of continually low levels of apical calcium. In this scenario, FD4-containing CFHBSS was repeatedly removed and re-applied apically in Calu-3 layers (to counteract recovering apical calcium levels, shown later). FD4 permeability in this instance amounted to 0.52 μg/h, 3.3-fold higher compared to 0.16 μg/h in the control experiment (application of FD4 in the same manner, but in calcium-*containing* medium) (Fig. 1D).

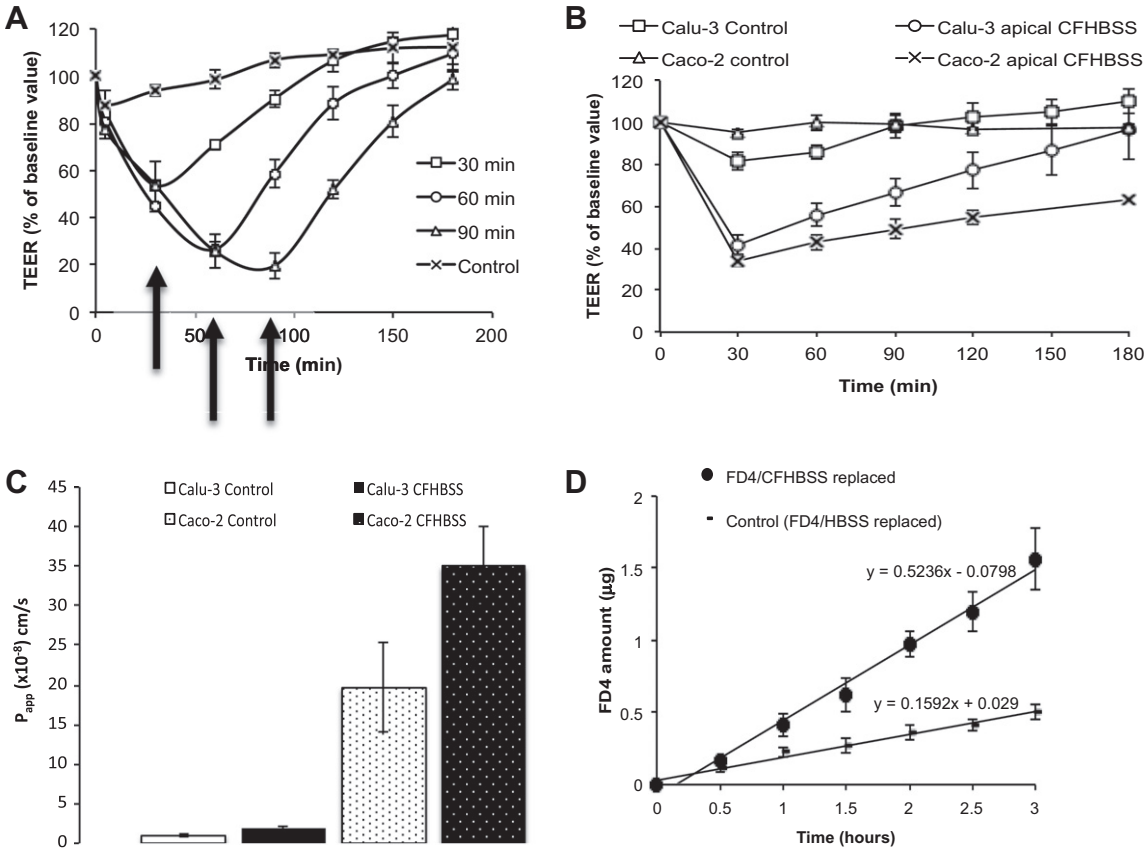

Fig. 1. Effect of calcium-free HBSS (CFHBSS) on tight junction modulation. (A) Effect on TEER. CFHBSS was applied apically and basolaterally in Calu-3 layers. Arrows indicate replacement of CFHBSS with calcium-containing HBSS. (B) CFHBSS was applied exclusively apically in Caco-2 and Calu-3 layers (calcium-containing HBSS was present basolaterally). Control: cells incubated with $CaCl_2$-supplemented CFHBSS. TEER expressed as % relative to the baseline value. (C) FITC-dextran (~4 kDa, FD4) permeability following its apical application in CFHBSS at 500 μg/ml (HBSS was present basolaterally). Control: FD4 permeability in HBSS. (D) FD4 permeability across Calu-3 layers subjected to regular replacement of apical solution consisting of FD4 dissolved in CFHBSS or HBSS (control) at 500 μg/ml. Results presented as the mean ± SD ($n = 3$) in all cases.

Although this is still a modest improvement in paracellular permeability, our data suggests that prolonged apical calcium depletion produces a larger epithelial barrier-modulating effect – a previously unreported finding of significance when considering the design of an absorption-enhancing mucosal delivery system.

The lack of significant barrier-modulating effects following the apical exposure of cell layers with calcium-free medium indicates that the presence of calcium on the apical surface of polarized epithelial cells is relatively unimportant for the maintenance of the tight junction integrity. Existing publications reporting similar findings are surprisingly rare and include a few studies conducted solely on intestinal Caco-2 cell monolayers [20–22]. Unlike the previous publications in this area, our studies were conducted on the airway epithelial cell line, Calu-3, as a mucus-producing (hence more relevant) epithelial model, in addition to Caco-2 cells.

3.2. Effect of EDTA on tight junction modulation

In contrast to CFHBSS, apical EDTA produced a significantly larger reduction in TEER (to 22% and 28% of the baseline value in Calu-3 and Caco-2 layers, respectively), which was sustainable for the duration of incubation (Fig. 2A). Comparing the site of application in Caco-2 monolayers (Fig. 2B), both apical and basolateral application of EDTA led to a similar initial drop in TEER, although at later intervals this decrease was more prominent with basolateral compared to apical application (to 11% and 23% of baseline TEER, respectively). Apical EDTA also produced a notably higher permeability enhancement (Fig. 2C) compared to apical CFHBSS in Calu-3 layers. Mirroring the TEER data, the extent of permeability enhancement was markedly larger with basolateral compared to apical EDTA (45-fold increase relative to the control versus 15-fold).

EDTA chelates an equivalent molar amount of calcium, so the concentration used (4.3 mM) would be expected to produce total calcium depletion on the apical side of the cells. However, the permeability-enhancing effect of EDTA cannot be explained wholly by apical calcium depletion since the level of FD4 permeability increase in the conditions of repeated apical addition of calcium-free medium was notably lower compared to apical EDTA (3.3- versus 15-fold). It should be considered that the pronounced effect of apical EDTA may be, at least in part, a result of its cell toxicity [23]. Another possible factor could be apical-to-basolateral translocation of EDTA and the resulting reduction of calcium in the basolateral milieu.

3.3. Effect of CFHBSS and EDTA on calcium levels

Fig. 3A shows the levels of Fluo-4 fluorescence when the probe was applied to the cell medium bathing the cell layers apically. Incubation of Calu-3 layers with CFHBSS on the apical side only resulted in an initial marked reduction of calcium level relative to control (Fluo-4 fluorescence decreased from 2725 to 891) at 5 min time point. Thereafter, fluorescence increased gradually with time, indicating increasing apical calcium levels, reaching a plateau close to control values (2337–2358) at 90–180 min. In contrast, for apical media containing EDTA (in HBSS or CFHBSS), Fluo-4 fluorescence immediately assumed low values of 50–160, which remained constant throughout the course of the experiment. To the best of our knowledge, these findings have not been reported previously and could explain the modest impact of apical calcium on the tight junctions and the consequent effect on the epithelial barrier. The manner in which the reversibility in apical calcium levels arises is, at the present moment, unclear, though it is possible that calcium traverses the epithelium in the basolateral-to-apical direction via the paracellular route [24].

Fluo-4 fluorescence of basolateral solution following apical addition of EDTA demonstrated an interesting pattern. There was a decline in the fluorescence intensity towards the later measurement intervals, reaching a reduction by approximately 25% (from 2100 to 1560), 180 min post-application (Fig. 3B). This suggests some level of apical-to-basolateral movement of EDTA and reduction of

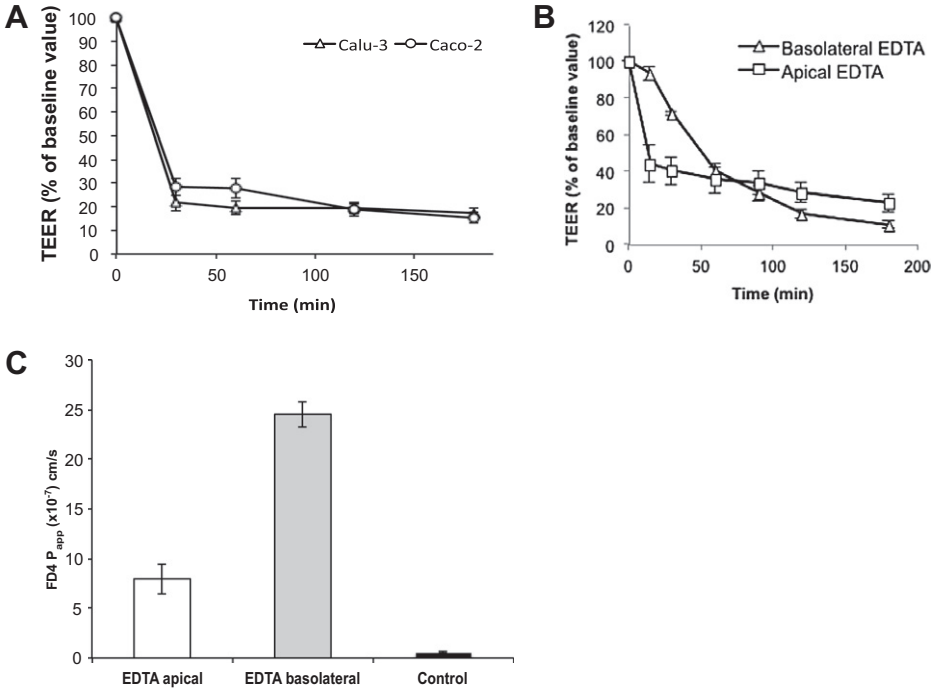

Fig. 2. Effect of EDTA (0.125% w/v in HBSS) on tight junction modulation. (A) Effect on TEER following apical EDTA application in Calu-3 and Caco-2 layers. (B) Effect on TEER following apical or basolateral EDTA application in Caco-2 monolayers. TEER shown as % of the baseline value. (C) Effect of apical or basolateral EDTA application on (apical-to-basolateral) FD4 permeability across Calu-3 layers. Control: FD4 permeability in HBSS. Results are presented as the mean ± SD ($n = 3$) in all cases.

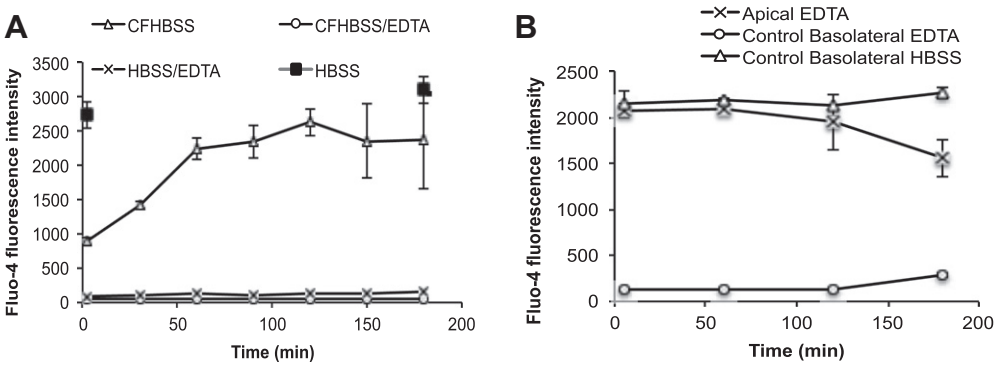

Fig. 3. Fluo-4 fluorescence intensity, indicating relative calcium levels, of cell-conditioned solutions consisting of CFHBSS or EDTA (0.125% w/v). (A) Solutions consisting of CFHBSS, EDTA in CFHBSS, EDTA in HBSS and HBSS were applied to, and sampled from, the *apical* side of Calu-3 layers. (B) EDTA in HBSS was applied on the apical side, with HBSS present on the basolateral side. Samples were taken from the *basolateral* side of the cell layers. Controls represent basolaterally-applied EDTA (in HBSS, 0.125% w/v) or HBSS and apically-present HBSS. Results presented as the mean ± SD (n = 3).

basolateral calcium. This is perhaps not surprising given the molecular size of EDTA (M_W 292 Da), which may enable paracellular transport across the cell layers. A reduction in basolateral calcium levels, even if not dramatic, could produce a prominent effect on the epithelial barrier, as demonstrated by the study where application of EDTA basolaterally resulted in 45-fold increase in FD4 permeability.

3.4. Effect of polyacrylic acid on tight junction modulation

The last part of the work assessed the potential of polyacrylic acid (PAA), as an example of a previously investigated calcium-binding absorption enhancer, in modulating the epithelial barrier. Several studies have demonstrated the ability of this material to facilitate mucosal absorption of macromolecules [25,26]. In terms of its absorption-enhancing mechanism, Kriwet et al. [27] postulated that the depletion of extracellular calcium and other metal ions by PAA could explain its biological effects, including drug absorption enhancement. Similarly, the PAA derivative, carbomer (Carbopol® 934P), was shown to significantly decrease TEER and markedly increase fluxes of [^{14}C] mannitol and FD4 following its application apically in Caco-2 monolayers, which the authors attributed to extracellular calcium depletion [28].

In this work, the application of PAA of ~100 and ~250kDa to the apical side of Calu-3 layers (at pH 7.4) led to TEER reduction to approximately 77% and 59% of the baseline value, respectively (Fig. 4A) – a decrease that was sustainable for the duration of cell incubation and reversed to near-baseline values following their removal. However, the tested PAA produced an insignificant (p = 0.21) to modest (1.88-fold; p = 0.045) increase in FD4 permeability (~100

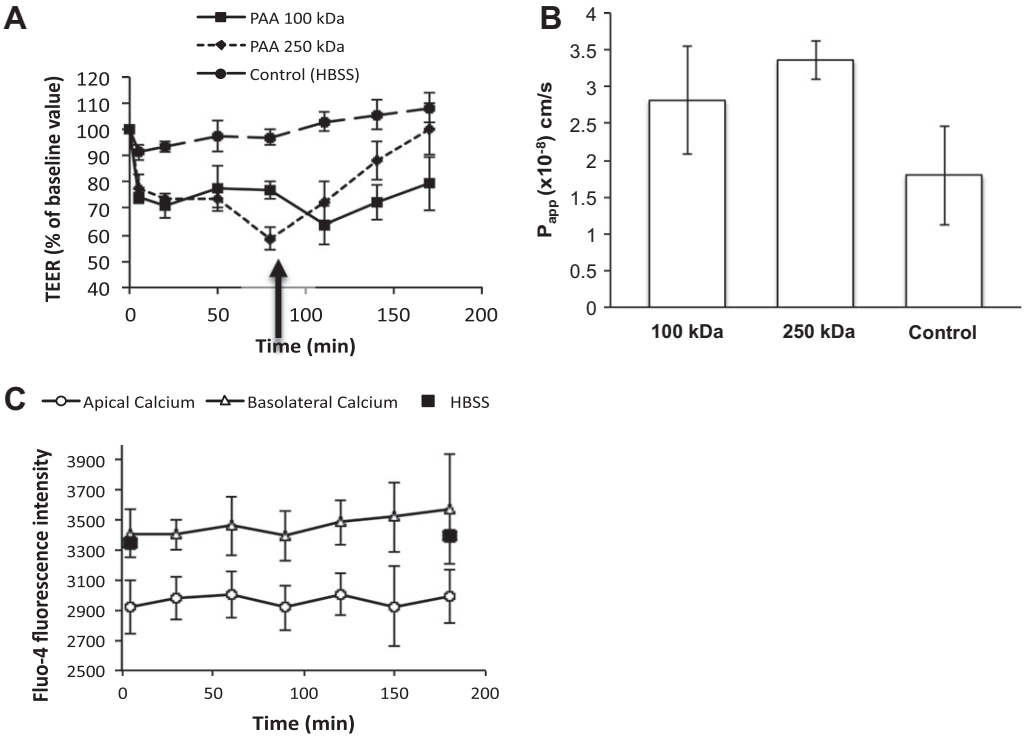

Fig. 4. Effect of polyacrylic acids (PAA, 0.25% w/v, pH 7.4) of ~100 and ~250 kDa on Calu-3 barrier. (A) Effect on TEER. Arrow indicates removal of the samples and application of HBSS. Results expressed as % relative to the baseline value. (B) Effect on FD4 permeability. Control: FD4 permeability in HBSS. (C) Effect of PAA (~100 kDa) on relative calcium levels in the apical and basolateral solutions, as indicated by Fluo-4 fluorescence. Control: cell layers incubated with HBSS (apically and basolaterally) and sampled at 5 and 180 min. Results presented as the mean ± SD (n = 3).

and ~250 kDa, respectively) (Fig. 4B). Taken together, the data shows that PAA had a moderate effect on TEER, which was insufficient to affect the barrier capacity of the cell layers in a significant way. Examining the impact on extracellular calcium levels, we noted some level of apical calcium depletion following apical addition of PAA (~100 kDa) in Calu-3 layers, as indicated by Fluo-4 fluorescence that was lower than that of calcium-containing control throughout the time-course of the experiment (Fig. 4C). However, unlike EDTA, apical PAA had no effect on basolateral calcium levels as Fluo-4 fluorescence of basolateral samples was similar to that of calcium-containing HBSS during the course of the experiment.

Several studies have proposed calcium-binding polymers as agents with the potential to improve mucosal absorption of macromolecules by the paracellular route [27,28]. The capacity of PAA to bind and deplete calcium from the extracellular medium, was suggested as an explanation for increased paracellular permeability of epithelial cell monolayers [29,30]. Similarly, the absorption-enhancing effect of several polymeric powder formulations following nasal application in rats has also been attributed to tight junction opening through a local decrease in calcium concentration [31]. However, it is important to highlight that absorption enhancers based on hydrophilic polymers are expected to predominantly locate at the luminal side of the epithelium following their mucosal administration *in vivo*. Consequently, the reduction in extracellular calcium is likely to occur exclusively on the luminal side. Our study systematically provides evidence that meaningful modulation of the tight junctions requires calcium depletion on the serosal side of epithelial cell layers and adds to existing literature (which is somewhat limited) [20–22] supporting this line of reasoning. We therefore argue and demonstrate that because polymeric calcium-chelating agents such as PAA do not penetrate the epithelial surfaces (their luminal-to-serosal translocation is limited by the same barriers that prevent the absorption of macromolecular biotherapeutics), they can only influence apical calcium levels. Given the limited role of apical calcium on the tight junctions, calcium-sequestering polymers are ineffective as absorption-enhancing excipients.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2011.12.146.

References

[1] L. Illum, Transport of drugs from the nasal cavity to the central nervous system, Eur. J. Pharm. Sci. 11 (2000) 1–18.
[2] S. Takatsuka, T. Morita, Y. Horikiri, H. Yamahara, H. Saji, Absorption enhancement of poorly absorbed hydrophilic compounds from various mucosal sites by combination of mucolytic agent and non-ionic surfactant, Int. J. Pharm. 338 (2007) 87–93.
[3] T. Goto, M. Morishita, K. Nishimura, M. Nakanishi, A. Kato, J. Ehara, K. Takayama, Novel mucosal insulin delivery systems based on fusogenic liposomes, Pharm. Res. 23 (2006) 384–391.
[4] A. Bernkop-Schnurch, A.H. Krauland, V.M. Leitner, T. Palmberger, Thiomers: potential excipients for non-invasive peptide delivery systems, Eur. J. Pharm. Biopharm. 58 (2004) 253–263.
[5] C.A. Rabito, C.A. Rotunno, M. Cereijido, Amiloride and calcium effect on the outer barrier of the frog skin, J. Membr. Biol. 42 (1978) 169–187.
[6] S. Citi, Protein kinase inhibitors prevent junction dissociation induced by low extracellular calcium in MDCK epithelial cells, J. Cell Biol. 117 (1992) 169–178.
[7] L. Gonzalez-Mariscal, B. Chavez de Ramirez, M. Cereijido, Tight junction formation in cultured epithelial cells (MDCK), J. Membr. Biol. 86 (1985) 113–125.
[8] A. Martinez-Palomo, I. Meza, G. Beaty, M. Cereijido, Experimental modulation of occluding junctions in a cultured transporting epithelium, J. Cell Biol. 87 (1980) 736–745.
[9] M. Thanou, J.C. Verhoef, H.E. Junginger, Chitosan and its derivatives as intestinal absorption enhancers, Adv. Drug Deliv. Rev. 50 (Suppl. 1) (2001) S91–101.
[10] J.D. Schulzke, A.H. Gitter, J. Mankertz, S. Spiegel, U. Seidler, S. Amasheh, M. Saitou, S. Tsukita, M. Fromm, Epithelial transport and barrier function in occludin-deficient mice, Biochim. Biophys. Acta 1669 (2005) 34–42.
[11] D. Ameye, J. Voorspoels, P. Foreman, J. Tsai, P. Richardson, S. Geresh, J.P. Remon, Trypsin inhibition, calcium and zinc ion binding of starch-g-poly(acrylic acid) copolymers and starch/poly(acrylic acid) mixtures for peroral peptide drug delivery, J. Control Release 75 (2001) 357–364.
[12] M. Roumi, E. Kwong, R. Deghenghi, V. Locatelli, S. Marleau, P. Du Souich, R. Beliveau, H. Ong, Permeability of the peptidic GH secretagogues hexarelin EP 51389, across rat jejunum, Peptides 22 (2001) 1129–1138.
[13] L. Li, N.R. Mathias, C.L. Heran, P. Moench, D.A. Wall, R.L. Smith, Carbopol-mediated paracellular transport enhancement in Calu-3 cell layers, J. Pharm. Sci. 95 (2006) 326–335.
[14] S. Sajeesh, K. Bouchemal, C.P. Sharma, C. Vauthier, Surface-functionalized polymethacrylic acid based hydrogel microparticles for oral drug delivery, Eur. J. Pharm. Biopharm. 74 (2010) 209–218.
[15] A.C. Foss, N.A. Peppas, Investigation of the cytotoxicity and insulin transport of acrylic-based copolymer protein delivery systems in contact with Caco-2 cultures, Eur. J. Pharm. Biopharm. 57 (2004) 447–455.
[16] S. Sajeesh, C. Vauthier, C. Gueutin, G. Ponchel, C.P. Sharma, Thiol functionalized polymethacrylic acid-based hydrogel microparticles for oral insulin delivery, Acta Biomater. 6 (2010) 3072–3080.
[17] S. Sajeesh, K. Bouchemal, V. Marsaud, C. Vauthier, C.P. Sharma, Cyclodextrin complexed insulin encapsulated hydrogel microparticles: an oral delivery system for insulin, J. Control Release 147 (2010) 377–384.
[18] T. Ooya, M. Eguchi, A. Ozaki, N. Yui, Carboxyethylester-polyrotaxanes as a new calcium chelating polymer: synthesis calcium binding and mechanism of trypsin inhibition, Int. J. Pharm. 242 (2002) 47–54.
[19] M.C. Bonferoni, G. Sandri, S. Rossi, F. Ferrari, S. Gibin, C. Caramella, Chitosan citrate as multifunctional polymer for vaginal delivery – evaluation of penetration enhancement and peptidase inhibition properties, Eur. J. Pharm. Sci. 33 (2008) 166–176.
[20] P. Artursson, C. Magnusson, Epithelial transport of drugs in cell culture. II: Effect of extracellular calcium concentration on the paracellular transport of drugs of different lipophilicities across monolayers of intestinal epithelial (Caco-2) cells, J. Pharm. Sci. 79 (1990) 595–600.
[21] M. Tomita, M. Hayashi, S. Awazu, Comparison of absorption-enhancing effect between sodium caprate and disodium ethylenediaminetetraacetate in Caco-2 cells, Biol. Pharm. Bull. 17 (1994) 753–755.
[22] A.V. Kamath, R.A. Morrison, N.R. Mathias, S.A. Dando, A.M. Marino, S. Chong, Modulation of tight junctions does not predict oral absorption of hydrophilic compounds: use of Caco-2 and Calu-3 cells, Arch. Pharm. Res. 30 (2007) 1002–1007.
[23] S. Zakelj, L. Vadnjal, A. Kristl, The effect of clodronate on the integrity and viability of rat small intestine in vitro-a comparison with EDTA, Biol. Pharm. Bull. 28 (2005) 1249–1253.
[24] S.L. Davies, C.E. Gibbons, M.C. Steward, D.T. Ward, Extracellular calcium- and magnesium-mediated regulation of passive calcium transport across Caco-2 monolayers, Biochim. Biophys. Acta 1778 (2008) 2318–2324.
[25] K. Morimoto, K. Morisaka, A. Kamada, Enhancement of nasal absorption of insulin and calcitonin using polyacrylic acid gel, J. Pharm. Pharmacol. 37 (1985) 134–136.
[26] A. Ryden, P. Edman, Effect of polymers and microspheres on the nasal absorption of insulin in rats, Int. J. Pharm. 83 (1992) 1–10.
[27] B. Kriwet, T. Kissel, Interactions between bioadhesive poly(acrylic acid) and calcium ions, Int. J. Pharm. 127 (1996) 135–145.
[28] G. Borchard, H.L. Lueßen, A.G. de Boer, J.C. Verhoef, C.M. Lehr, H.E. Junginger, The potential of mucoadhesive polymers in enhancing intestinal peptide drug absorption. III: Effects of chitosan-glutamate carbomer on epithelial tight junctions in vitro, J. Controlled Release 39 (1996) 131–138.
[29] H.L. Lueßen, C.M. Lehr, C.O. Rentel, A.B. Noach, A.G. de Boer, J.C. Verhoef, H.E. Junginger, Bioadhesive polymers for the peroral delivery of peptide drugs, J. Controlled Release 29 (1994) 329–338.
[30] H.L. Lueßen, C.O. Rentel, A.F. Kotze, C.M. Lehr, A.G. de Boer, J.C. Verhoef, H.E. Junginger, Mucoadhesive polymers in peroral peptide drug delivery. IV. Polycarbophil chitosan are potent enhancers of peptide transport across intestinal mucosae in vitro, J. Controlled Release 45 (1997) 15–23.
[31] C.R. Oechslein, G. Fricker, T. Kissel, Nasal delivery of octreotide: absorption enhancement by particulate carrier systems, Int. J. Pharm. 139 (1996) 25–32.