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Research paper

The role of citric acid in oral peptide and protein formulations: Relationship between calcium chelation and proteolysis inhibition

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ABSTRACT

The excipient citric acid (CA) has been reported to improve oral absorption of peptides by different mechanisms. The balance between its related properties of calcium chelation and permeation enhancement compared to a proteolysis inhibition was examined. A predictive model of CA's calcium chelation activity was developed and verified experimentally using an ion-selective electrode. The effects of CA, its salt (citrate, Cit) and the established permeation enhancer, lauroyl carnitine chloride (LCC) were compared by measuring transepithelial electrical resistance (TEER) and permeability of insulin and FD4 across Caco-2 monolayers and rat small intestinal mucosae mounted in Ussing chambers. Proteolytic degradation of insulin was determined in rat luminal extracts across a range of pH values in the presence of CA. CA's capacity to chelate calcium decreased ~10-fold for each pH unit moving from pH 6 to pH 3. CA was an inferior weak permeation enhancer compared to LCC in both *in vitro* models using physiological buffers. At pH 4.5 however, degradation of insulin in rat luminal extracts was significantly inhibited in the presence of 10 mM CA. The capacity of CA to chelate luminal calcium does not occur significantly at the acidic pH values where it effectively inhibits proteolysis, which is its dominant action in oral peptide formulations. On account of insulin's low basal permeability, inclusion of alternative permeation enhancers is likely to be necessary to achieve sufficient oral bioavailability since this is a weak property of CA.

1. Introduction

Development of oral delivery systems for proteins and peptides offers the promise of improved patient compliance compared to conventional parenteral administration. Moreover, in the case of certain protein therapeutics (e.g., insulin), the physiological response elicited may exhibit a pharmacodynamic profile which more closely resembles the natural physiological response. However, delivery of protein therapeutics is severely hindered by poor absorption across the intestinal barrier and extensive degradation by proteolytic enzymes. Thus, to effectively overcome these impediments, a formulation strategy which can modulate both of these processes is necessary to achieve acceptable oral bioavailability with low intra-subject variation.

Although degradation of proteins by gastric enzymes and low pH may be overcome via inclusion of an enteric coating, the approach to minimise proteolytic activity in the small intestine, while simultaneously ensuring efficient release and permeation represents a more significant challenge. In this regard, one such concept extensively explored is that of acidic inhibition of proteolysis. Luminal proteases, such as trypsin and chymotrypsin, exhibit maximum activity at pH $\geqslant$ 6.5 [1,2] i.e., that typically observed in the pH microenvironment of the jejunum and ileum. Via adjustment of the local pH to values corresponding to pH < 6.5, proteolytic activity of enzymes such as chymotrypsin [1], the primary luminal degrading enzyme for insulin [2], can be significantly diminished.

Indeed, acidic inhibition of proteolysis as a strategy for the oral delivery of therapeutic peptides recently gained attention following Tarsa Therapeutics (Philadelphia, PA) successful completion of a phase III trial ('ORACAL') for orally delivered salmon calcitonin (sCT) [3]. Such technology typically comprises of an enteric coated capsule or tablet, which bypasses the stomach unchanged, along

Abbreviations: CA, citric acid; Cit, citrate; DOC, sodium deoxycholate; EDTA, ethylenediaminetetraacetic acid; ISE, ion selective electrode; KH, Krebs–Henseleit; LCC, lauroyl carnitine chloride; sCT, salmon calcitonin; TDC, taurodeoxycholate; TEER, transepithelial electrical resistance.

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with a pH-lowering excipient contained in vesicles (e.g., an organic acid such as citric acid). Upon entry into the duodenum with its luminal pH range of between 5 and 6, pH-dependent disintegration of the polymer coating of the dosage form commences, followed by release from the vesicle of both co-localised API and citric acid (CA). Concomitant association of CA maintains a decrease in local pH, thus stabilising the co-released peptide. In this way, it facilitates a reduction in the luminal enzymatic activity, providing a higher concentration gradient of the API over time, which in turn promotes improved absorption and bioavailability [4,5].

Alongside pH-lowering agents, co-administration of an absorption enhancer(s) has generally been regarded as indispensable due to the inherently poor epithelial permeability properties of proteins and peptides [5,6]. Indeed, previous publications exploring this technology have employed LCC, an amphiphilic surfactant [5–7]. However, based upon the recent ORACAL sCT study, where an absorption enhancer was omitted, one may speculate that either the need for co-administration is diminished on account of the proposed permeation enhancing properties of citric acid or citrate (Cit) [3], or that enhancers might not be required for oral sCT where bioavailability of 1–3% is typical for marketed nasal versions of this particular potent molecule [8]. CA and Cit are GRAS excipients and have been widely employed in oral formulations of small molecules. Thus, despite this formulation strategy being comparatively new, a body of literature exists examining the multiple mechanisms by which CA, in its salt form (i.e., tri-sodium citrate) may promote oral absorption. Cit exhibits calcium chelating properties and evidence exists to suggest that it may increase paracellular absorption, by triggering disruption of tight junction complexes via depletion of intracellular calcium [9–11].

In this report, the potential mechanism of action of CA as both an acidic proteolysis inhibitor and calcium chelator/permeation enhancer was addressed and conclusions made as to which might be its dominant action at relevant pH values in the upper small intestine. *In silico* and *in vitro* determination of CA's calcium chelation activity and its capacity to prevent insulin degradation by peptidases across a broad range of pH values were obtained. From this data we assessed whether or not a common pH range existed over which *both* proteolysis inhibition and calcium chelation occurred. Finally, the capacity of CA/Cit to enhance permeability was investigated in Caco-2 monolayers and rat intestinal tissue and compared to that of lauroyl carnitine chloride (LCC), an established amphiphilic permeation enhancer [5–7] previously employed as an additional agent in pH-lowering oral peptide formulations.

2. Materials and methods

2.1. Materials

Caco-2 cells (ATCC-HTB-37) were obtained from American Type Culture Collection (ManassasVA). Cell culture media (Dulbecco's modified essential media (DMEM)) and penicillin/streptomycin were purchased from Lonza (Verviers, Belgium). All other supplements i.e., foetal bovine serum (FBS), HEPES buffer and non-essential amino acids (NEAA) as well as Hanks' balanced salt solution (HBSS) and trypsin were purchased from Gibco (Naerum, Denmark). Corning Transwell® filter inserts (1.12 cm^2 surface area, 0.4 μm pore diameter) were purchased from Fisher Scientific (Slangerup, Denmark). FITC-dextran 4 kDa (FD4) and D-glucose were purchased from Sigma Aldrich (Dublin, Ireland). Bovine serum albumin (BSA) was purchased from Sigma Aldrich (Copenhagen, Denmark). All other reagents were of the highest analytical grade.

The Iso-Insulin ELISA assay kit was purchased from Mercodia (Uppsala, Sweden). Lauroyl-DL-carnitine (LCC) was purchased from Chemos (Regenstauf, Germany). [^{3}H]-mannitol, [^{14}C]-mannitol and Ultima Gold® scintillation fluid were purchased from Perkin Elmer (Waltham, MA). Liquid scintillation counting was carried out using a TopCount C990201 or a TriCarb 2900TR liquid scintillation counter (both Perkin Elmer). Luminescent measurements were performed using a Spectramax® 250 or Gemini® microplate reader (both Molecular Devices, Sunnyvale, CA). Fluorescent measurements were performed on a Tecan® GENios fluorescent microplate reader (Tecan, Durham, NC).

2.2. Cell culture

Caco-2 cells (passage numbers 40–60) were seeded at a density of 2.5×10^5 cells/flask and grown to 70–90% confluence in DMEM (supplemented with 10% FBS, 100 U/ml penicillin and 100 μg/ml streptomycin and 1% (v/v) NEAA). For transport studies, Caco-2 monolayers were cultured on permeable Transwell® 12 mm diameter inserts with pore sizes of 0.4 μm at a density of 10^5 cells/cm^2 and used after 14–17 days in culture. Cells were cultured at 37 °C and 5% CO_2 atmosphere and the medium was changed every other day.

2.3. Modelling chelation activity of citric acid (CA)

A model to predict free calcium fraction was constructed as described in Supplementary materials. The conditional pKa and citrate (Cit) calcium chelation constant, K, corresponded to previously published values in which similar ionic strengths were applied [12–14]. The model was not corrected retrospectively to take account of the experimentally determined calcium electrode measurements.

2.4. Calcium electrode measurements

A pH-meter (744; Metrohm, Herisau, Switzerland) was fitted with a micro pH-electrode (6.0224.100; Metrohm), a calcium selective electrode (6.0508.110; Metrohm) and an AgCl reference electrode (Dri-Ref-L; World Precision Instruments, Sarasota, FL). All titrations were performed in calcium-free transport media at room temperature (RT). To 20 ml of calcium-free HBSS 300–500 μl CA or Cit (1.5–2 M) was added to yield a final solution of CA/Cit (30 mM) and pH values of 4, 5, 6, and 7.4. The solution was titrated with 40 mM $CaCl_2$ from 0.5 μl to 1310 μl [5×10^{-3}–2.5×10^0] mM $CaCl_2$. Electrical motive force (EMF, mV) and pH were concomitantly monitored during titration. Double standard curves of calcium added to transport media (without CA/Cit), assuming that free calcium concentration was equivalent to total calcium. Titrations were performed at room temperature to improve reproducibility. All solutions were maintained at room temperature for 4 h prior to titration, as the ISE was sensitive to temperature changes. Activity of the ISE was assessed within the pH range of 3–7.4.

2.5. In vitro inhibition of proteolysis

Cit and CA were added to zinc-free transport medium (see "Transepithelial transport studies in Caco-2″; zinc-free) to give Ca/Cit stock solutions a total concentration of 12.5 mM of CA species and a range of pH values (3.5–7.4). Subsequently, enzyme-rich washes were extracted from fasted rat duodenal lumens by rinsing 10 cm fresh duodenum with 10 ml water and instantly freezing the eluate at −80 °C until use. At time point zero, 100 mM recombinant human insulin (Novo Nordisk A/S, Copenhagen, Denmark) was mixed with duodenal extracts and CA stock solutions in a ratio of 1:1:8 respectively, yielding 10 mM insulin and 10 mM CA species. The kinetic study was performed using an autosampler robot

(Gilson 215 liquid handler; Middleton, WI) running 16 separate samples simultaneously. The reaction was sampled at six time points over a period of 120 min. Upon collection of each 20 μl sample, 50 μl trifluoroacetic acid (5% v/v) was immediately added and the samples refrigerated (4 °C) in order to stop the proteolytic degradation. Insulin content was quantified by Acquity UPLC consisting of an autosampler (Model Acq-SM), pump (Model Acq-BSM), column oven (Model Acq-SM) and detector (Model Acq-TUV; Waters, Milford, MA). RP-UPLC separation was achieved by Acquity BEH 1.7 μM C18 1 × 50 mm column (Waters), using a linear gradient of acetonitrile in 0.2 M sodium sulfate, 0.04 M sodium phosphate, pH = 7.2. Peaks were detected by UV absorption at 220 nm and quantified using a human insulin standard. Reaction rates were calculated as the slope of the linear least squares fit to the semi log-plot of concentration versus time, see Eq. (1). All reactions rates were derived by the natural logarithm, *e*.

$$C_t = C_0 \cdot e^{-kt} \Rightarrow \ln(C_t) = -k \cdot \mathbf{t} + \ln(C_0) \quad (1)$$

where t (s) is a given time point, C_t (μM) the remaining concentration of insulin at time point, t; k (min^{-1}) is the reaction constant and C_0 (μM) is the initial concentration of insulin.

2.6. Transepithelial transport studies across Caco-2 monolayers

Filter-grown monolayers were washed with warm HBSS buffer (138 mM NaCl, 5.3 mM KCl, 1.3 mM $CaCl_2$, 0.40 mM $MgSO_4$, 0.44 mM KH_2PO_4, 4.2 mM $NaHCO_3$ and 5.6 mM glucose; pH 7.4) supplemented with 0.1% BSA and 10 mM HEPES and allowed to equilibrate. Transepithelial electrical resistance (TEER) was measured with a chop-stick electrode (Millicell-ERS®, Millipore, Billerica, MA) prior to testing and monolayers with TEER values <600 Ω cm^2 were discarded. The buffer in the respective apical side was then replaced with a solution containing insulin (10 μM) and [^{3}H]-mannitol (0.8 μCi/ml) alone or in combination with CA (5 mM; pH 4.5) or LCC (1 mM), and the monolayers were incubated at 37 °C for 60 min. In some studies, Cit (20 mM; pH 7.4) was added to the basolateral side. Samples containing fluxed insulin from the donor apical side were collected from the basolateral compartments every 15 min for 1 h and human insulin content, was diluted to 100–10,000 ppm (~14–1400 mU/l), and was assayed using ELISA. Flux (J [mol/s]) was determined from steady-state appearance rates of insulin in the receiver fluid. The apparent permeability coefficient, P_{app} [cm/s], was calculated according to Eq. (2)

$$P_{app} = J/(A \cdot C_i) \quad (2)$$

where C_i (mol/cm^3) is the initial concentration of insulin in the donor fluid and A is the nominal surface area:1.12 cm^2 for Caco-2 monolayers and 0.63 cm^2 for intestinal mucosae.

2.7. Preparation of rat intestinal tissue for Ussing chamber studies

Studies were carried out in accordance with the UCD Animal Research Ethics Committee policy, on the use of post mortem animal tissue in research, as well as in adherence to the "Guide for the care and use of laboratory animals", (8th Edition, National Academy of Sciences, 2011. http://www.aaalac.org/resources/theguide.cfm). Male Wistar rats (250–300 g) (Charles River, Margate, UK) were euthanised by stunning and cervical dislocation. The lower jejunum and ileum (lower small intestine) was removed, opened along the mesenteric border and rinsed in warm oxygenated Krebs–Henseleit solution (KH; 118 mM NaCl, 4.7 mM KCl, 2.5 mM $CaCl_2$, 1.2 mM $MgSO_4$, 1.2 mM KH_2PO_4, 25 mM $NaHCO_3$ and 10 mM glucose) according to previous methods [15]. Tissue was pinned with the mucosal side down on a dissection board to expose the external muscularis, which was carefully removed with a size 5 fine forceps. The tissue was then mounted in Ussing chambers with a circular window area of 0.63 cm^2, bathed bilaterally with 5 ml KH and continuously gassed with 95% CO_2/5% O_2 at pH 7.4 and maintained at 37 °C. The transepithelial potential difference (PD, mV) and short circuit current (I_{sc}, μA) were measured across the lower small intestine. The tissue was voltage clamped to zero for 30 s and switched to open circuit configuration for 3 s by an automatic voltage clamp (EVC-4000 amplifier) and Pro-4 timer (both WPI, Hertfordshire, UK). Analogue data were digitised with a Mac Powerlab® data acquisition unit and analysed with Chart® software (AD Instruments, Oxford, UK). Following an equilibration period of 15 min, PD and I_{sc} were measured and TEER was calculated at regular time points from 0 to 120 min using Ohm's law.

2.8. Transepithelial transport studies in rat lower small intestinal tissue

Transport of [^{14}C]-mannitol and FITC-Dextran 4000 (FD4), non-degradable hydrophilic flux marker, was examined across lower small intestinal mucosae mounted in Ussing chambers. Briefly, [^{14}C]-mannitol (0.2 μCi/ml) and FD4 (1 mg/ml) were added to the apical chamber and flux was monitored periodically over 2 h by sampling the serosal chamber (200 μl) every 20 min for 2 h, and apically (200 μl) at time zero, while replenishing with fresh KH buffer at each sampling point. In some studies, CA (30 mM), Cit (30 mM) or LCC (3 mM) were simultaneously added to the apical chamber. Samples containing [^{14}C]-mannitol were mixed with scintillation fluid and read in a scintillation counter (TriCarb 2900TR, Perkin Elmer). Where samples contained FD4, fluorescence was measured in a fluorescence microplate reader (Spectramax Gemini, Molecular Devices) with $\lambda_{ex}/\lambda_{em}$ of 480/520 nm. The P_{app} was calculated according to Eq. (2).

2.9. Statistical data analysis

Statistical analysis was carried out using Prism-6® software (GraphPad, San Diego, USA) using two-tailed unpaired Student's t-tests unless otherwise stated. Results are presented as the mean ± standard deviation (SD, unbiased). The level of significance set was $P > 0.05$. Normal distribution of data was in general assumed except for apparent permeability data which elicited a relatively consistent standard deviation (%RSD) and was therefore log transformed prior to statistical analysis.

3. Results

3.1. Prediction of the chelation activity of citric acid (CA)

To estimate the chelation activity of CA, a mathematical model was employed (see Supplementary material). Chelation activity was defined as the average of the apparent formation constants of the individual species of CA, weighted according to their presence and expressed relative to the formation constant of Cit^{3-}, in theory the strongest chelator. The predicted relationship between each form of CA (H_2Cit^-, $HCit^{2-}$, Cit^{3-}) and their calcium chelation activity is depicted in Fig. 1. As illustrated, CA chelation capacity is especially high in the presence of high levels of Cit^{3-} and that is pH-dependent. Above pH 5, total chelation activity corresponds directly to the proportion of the Cit^{3-} species present. At values below pH 5 the fraction of Cit^{3-} is less than 0.1. In this range (i.e., pH < 5), $HCit^{2+}$ becomes relatively more dominant compared to Cit^{3-}. At pH > 5, while $HCit^{2+}$ exhibits some degree of chelation capacity, it is thought to be significantly less than that of Cit^{3-} at the higher pH values. Importantly, calcium chelation activity was

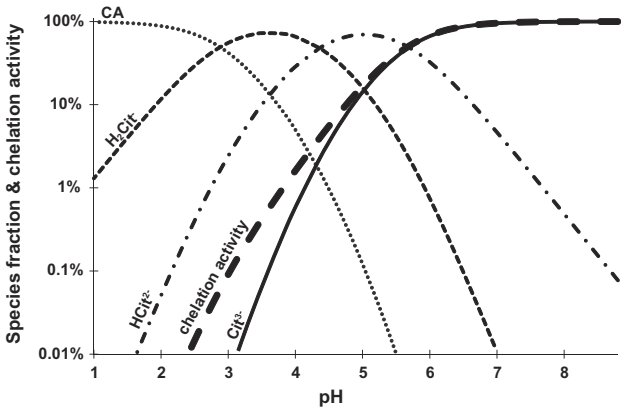

Fig. 1. Calculated CA chelation activity related to the theoretical concentration of the individual formats (ion and salt) at various pH values. The bold dashed line represents the summarised chelation activity of all formats. The model depicted applies to solutions of ionic strength between $I = 0.1–0.6$ M, but does not apply to diluted solutions.

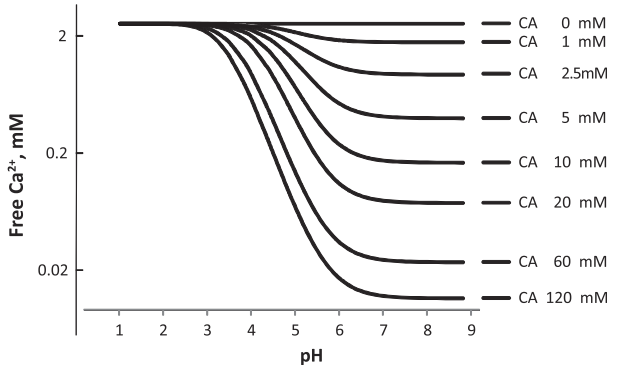

Fig. 2. Calculated free calcium concentration in media of 2.5 mM total calcium (KH buffer) at different concentrations of CA and pH values. The model depicted applies to solutions of ionic strength between $I = 0.1–0.6$ M, but does not apply to diluted solutions.

reduced ~10-fold for each unit of pH within the pH range of 3–6. As shown in Fig. 2, in order to chelate 99% of the total calcium in KH buffer at a pH of 7.4, very high concentrations of 120 mM CA (pH 5.5) or 60 mM CA (pH 7) would be required. For 90% chelation, 120 mM CA (pH 4.2) or 10 mM CA (pH 5.8) would be necessary. The prediction therefore is that at highly acidic local pH values, non-physiological concentrations of CA would be required to substantially chelate calcium, the most likely mechanism to open epithelial tight junctions for permeation enhancement.

3.2. Correlation of 'predicted' versus 'measured' free calcium (Ca^{2+})

CA, H_2Cit^-, $HCit^{2-}$ and Cit^{3-} form a buffer system in which the distribution of the species is a function of pH. Herein, all mentioned concentrations of CA or Cit are absolute and accompanied by a pH value from which the actual distribution of CA-species can be found (Fig. 1).

In order to validate the predictive model, total and free calcium were determined with a calcium-selective electrode following titration of 30 mM CA (at pH values of 4, 5, 6, and 7.4) with 10 μM–3 mM Ca^{2+}. Fig. 3 shows predicted free Ca^{2+} levels versus corresponding experimentally determined free Ca^{2+} levels. Within the range of 0.01–2 mM total Ca^{2+}, very accurate correlations were achieved ($R^2 > 0.98$). According to the conditions observed in Fig. 3, CA/Cit is at least 25-fold in excess compared to calcium. Consequently,

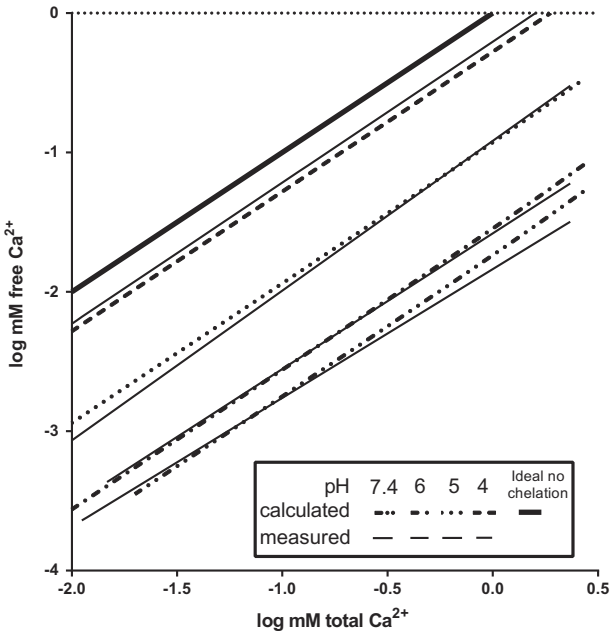

Fig. 3. Free calcium versus total calcium in solutions measured with an ion-selective electrode compared to the calculated free calcium. Solution: HBSS + HEPES (10 mM) + CA (30 mM). Dashed lines are calculated relationships of the total and free calcium. The thin black line — represents the experimentally determined calcium chelation. The thick black line ▬ represents the ideal i.e., no chelation, whereby total calcium = free calcium.

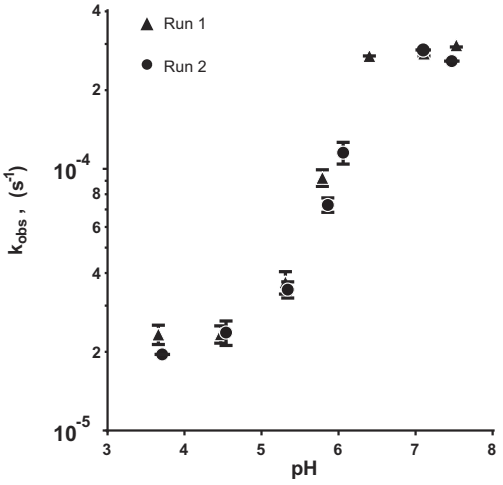

Fig. 4. Apparent reaction constants (k_{obs} natural log) of the proteolysis of insulin by rat duodenal extracts at various pH values in HBSS + HEPES (10 mM) with CA/Cit (10 mM). The proteolysis rate shows an exponential pattern of increase with pH. Insulin proteolysis was fitted as 1st order decay. Data points represent an average of two measurements ± SD from two separate runs. $n = 28$.

the titration slopes of free calcium versus total calcium achieved are highly linear and when plotted on a double-log scale display slopes close to 1. A minor departure from linearity was observed in the lower part of the standard slope (free calcium/mV) which fitted well with 2nd or 3rd order polynomial and was attributed to be a loss of sensitivity of the electrode in its lower detection range.

3.3. pH-dependent degradation of insulin

In the absence of proteolysis inhibition, insulin is readily degraded in the small intestine. However, the activity of most key

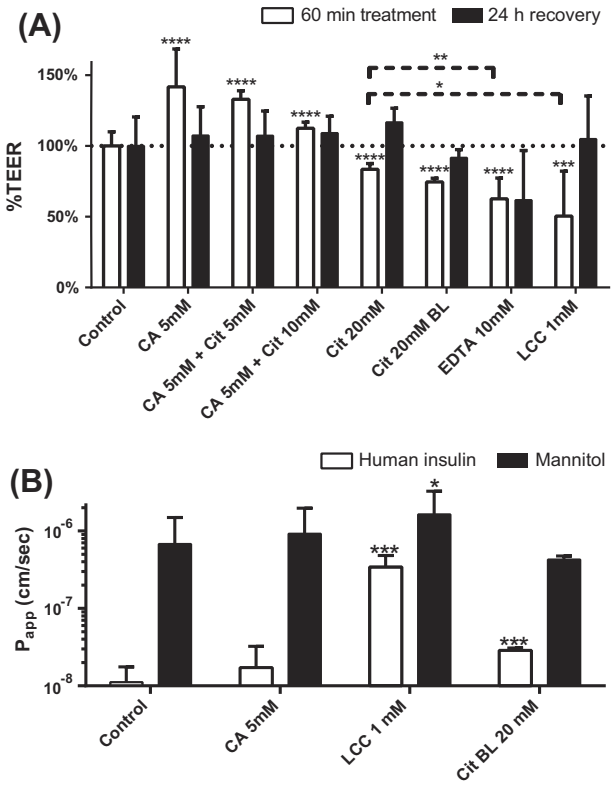

Fig. 5. (A) Effect of CA, Cit and LCC on TEER in Caco-2 monolayers. Normalised change of TEER (%) in monolayers after 60 min treatment with CA 5 mM (pH 4.5), CA 5 mM + Cit 5 mM (pH 5.2), CA 5 mM + Cit 10 mM (pH 5.5), Cit (basolateral) (20 mM, pH 7.4), EDTA (10 mM) or LCC (1 mM). Data are represented as means ± SD, n = 7–50. Separate Student's t-tests were carried out to evaluate if treatments significantly differed from the control. (B) P_{app} of insulin and [^{3}H]-mannitol across Caco-2 monolayers incubated with CA (5 mM, pH 4.5), Cit (20 mM, pH 4.5), LCC (1 mM, pH 7.4) for 60 min. Separate Student's t-tests were carried out to evaluate if treatments significantly differed from the control. Data are represented as means ± SD, n = 3–12.

proteolytic enzymes including trypsin, chymotrypsin and elastase is strongly influenced by pH. To investigate the precise relationship between pH and proteolysis rate, an *in vitro* proteolysis assay using native extracts of rat duodenum was performed in the presence of CA/Cit (10 mM). Throughout, the degradation of insulin was characterised by first order kinetics. A plot of insulin degradation versus time is provided in the Supplementary material. At pH 6.5–7.4, the native pH of the small intestine, the reaction rate proceeded much faster than at acidic pH values (Fig. 4). By decreasing the pH from 7 to 4.5 however, the reaction rate was reduced markedly (10-fold) ($p < 0.0001$). Hereafter from pH 4.5 to 4 the reaction rate remained unchanged. At a pH of 3.5, the reaction rate was an order of magnitude lower than that seen at pH 7.4. In order to exclude other potential confounding factors, the influence of both total Cit concentration and the quantity of NaCl added was evaluated. Although Cit (120 mM, pH 7.4) lowered the reaction rate, k_{obs}, twofold ($p < 0.001$), NaCl (75–250 mM) elicited no significant effect and both the independent factor of NaCl and Cit appeared to have no physiological significance. These data emphasise the dramatic protection against pancreatic peptidases afforded by simply maintaining the pH at 3–4 with a 10 fold lower concentration of CA than that required to chelate calcium.

3.4. Effects of CA, Cit and LCC on Caco-2 monolayers: TEER and P_{app} values of [^{3}H]-mannitol and insulin

Treating Caco-2 monolayers with apical addition of LCC (1 mM) elicited a decrease in TEER of 50% in 60 min compared to that of untreated monolayers (Fig. 5A) ($p < 0.001$). Exposure to various concentrations of Cit (5–20 mM pH 7.4) apically or basolaterally however, resulted in smaller albeit concentration-dependent reductions in TEER and Cit was less potent and efficacious than the well-known chelator and permeation enhancer, EDTA and LCC (Fig. 5A). Surprisingly, CA (5 mM) at pH 4.5 elicited a significant increase in TEER of 50% relative to untreated monolayers ($p < 0.001$). Comparable responses were observed with 5 mM CA in combination with 5 mM and 10 mM Cit (Fig. 5A). Following incubations, transport medium was removed and fresh DMEM introduced; monolayers were then incubated for 24 h to assess TEER recovery. All treated cultures showed a full recovery in TEER, exhibiting comparable values to that of control monolayers after 24 h. Of note, addition of excessive amounts of CA, resulted in a lowering of pH < 4 where TEER recovery was not observed due to irreversible deterioration of the monolayer barrier, as earlier reported [16]. In summary, the data reveal that CA and/or Cit only have marginal effects on TEER, but nothing like the level of decrease which would be expected from an effective permeation enhancer (i.e., >50% TEER decrease).

The P_{app} values of insulin across Caco-2 monolayers showed a significant increase in the presence of LCC (1 mM), generating a 40-fold increase in transport (8×10^{-7} cm/s) compared to basal insulin P_{app} across untreated monolayers ($p < 0.01$) while the P_{app} of [^{3}H]-mannitol was 50% greater than in untreated monolayers (1×10^{-6} cm/s) ($p < 0.05$) (Fig. 5B). In contrast, CA (5 mM) had no effect on the P_{app} of either insulin or [^{3}H]-mannitol. While high concentrations of Cit (20 mM) produced a 2-fold increase of insulin permeability ($p < 0.001$), this was still significantly less than that of LCC and observed only when applied basolaterally. The effect apically was even smaller. Overall, the data confirm that, compared to the dramatic permeation enhancement induced by LCC, CA/Cit are not effective enhancers in Caco-2 monolayers and this is consistent with their nominal effect on TEER compared to positive controls.

3.5. Effect of CA, Cit and LCC on rat lower small intestinal mucosae: TEER and P_{app} of [^{14}C]-mannitol and FD4

The effects of CA, Cit and LCC on the electrophysiological responses of rat lower small intestinal tissue were examined in Ussing chambers. Addition of very high concentrations of Cit (30 mM, pH 7.4) elicited a sustained decrease in TEER over 120 min, which was significantly lower (~20%) than that observed in control tissue (Fig. 6A). In contrast, incubation with CA (30 mM, pH 3) gave rise to a significant, albeit transient increase in TEER (~20%) relative to control during the initial 20 min exposure. Thereafter, TEER values aligned with those of untreated controls. Although LCC did not provoke any significant decrease in TEER, a 2-fold increase in the permeability of FD4 was observed (Fig. 6B). In contrast, neither the TEER reduction elicited by Cit nor the TEER increase observed with CA was associated with changes in mannitol permeability. Permeability of [^{14}C]-mannitol remained statistically unaffected by the various treatments. Overall, these data do not indicate that CA/Cit is an effective permeation enhancer in rat lower small intestinal mucosae, whereas LCC was effective, at least for FD4.

4. Discussion

This work addressed the functional role of CA in formulation-based approaches for the delivery of peptides and proteins via the oral route. Specifically, we examined the interplay between its primary use as a pH-lowering agent and its additional function as a calcium chelator that might cause both tight junction openings and contribute to further inhibition of serine proteases. Modulation of intestinal pH via formulation is an attractive means to

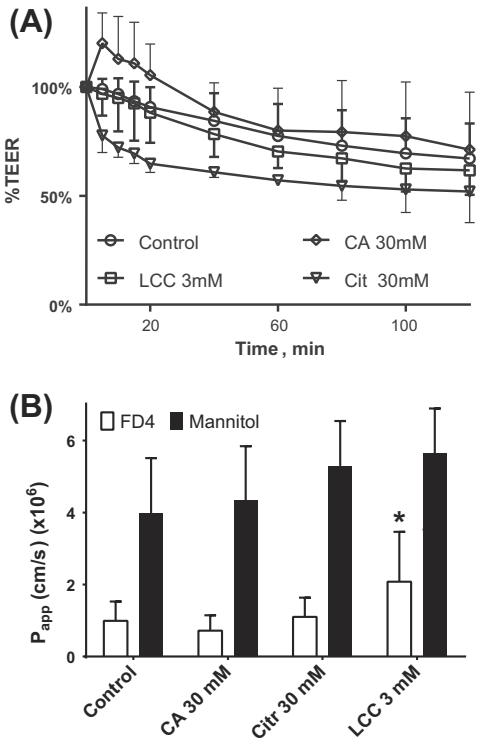

Fig. 6. (A) Effect of CA, Cit and LCC on TEER (A) and P_{app} (B) in rat lower small intestine in Ussing chambers upon mucosal-side incubation with CA (5 mM, pH 4.5), Cit (20 mM, pH 7.4) or LCC (3 mM) for 120 min. (B) P_{app} of FD4 and [^{14}C]-mannitol across rat lower small intestine incubated with CA (5 mM, pH 4.5), Cit (20 mM, pH 7.4) or LCC (3 mM) for 120 min. Data are represented as means ± SD, n = 3–4.

stabilise the protein against enzymatic degradation. Indeed, it has previously been used as a strategy for small intestinal delivery of sCT. Using CA (a prototype organic acid) and LCC (an amphiphilic surfactant), there was significant enhancement in oral bioavailability of sCT in dogs [4,5,17]. However, precise elucidation of the combined effects of CA on protein stability and permeability remains undetermined.

Chelation of calcium is an efficient means by which to modulate tight junction structures. Participation of Ca^{2+} in the establishment of epithelial cell junction networks has been widely demonstrated [18,19]. CA, a hydroxy tricarboxylic acid, is an efficient chelator in its salt form (i.e., citrate), capable of sequestering multivalent cations. Conditional chelation constants for Ca^{2+}–Cit^{3-} and Ca^{2+}–$HCit^{2-}$ are 1880 M^{-1} and 67 M^{-1}, respectively [14,12], while conditional CA $pKa_{1,2,3}$ values applied were [2.80; 4.08; 5.33] [13,14]. Such values are dependent on ionic strength and temperature. Thus, use of the applied model to predict chelation capacity potential is suggested to be restricted to conditions whereby I ranges from 0.1 M to 0.6 M and temperature ranges from 18 °C to 45 °C. KH and HBSS have ionic strengths of $I \sim 0.16$ M. In this context, our proposed model predicts that at pH values of 6–7, and above, optimal chelation activity is observed. Importantly, below pH 6 the apparent chelation constant is reduced ~10-fold for each pH unit. Therefore, at highly acidic pH values the concentration of CA/Cit necessary to chelate vast quantities of calcium dramatically increases. The pH microenvironment generated via release of CA from a pH-lowering formulation likely corresponds to a value of 4.5 or lower [17]. Under these circumstances, CA will primarily dissociate into $HCit^{2+}$ and not Cit^{3-} the predominant chelating species. In this chemical arrangement, its potency as a chelator of calcium ions is considerably reduced. Accordingly, this formulation approach is unlikely to engender significant chelation activity, but will be dominated by a capacity for pH-mediated peptidase inhibition.

Enzymatic degradation of proteins by luminal proteases represents a significant barrier to achieving a therapeutically relevant bioavailability. The ability of pH-lowering formulations to curtail this undesirable aspect of intestinal physiology is its primary attraction for oral peptide and protein delivery. Our *in vitro* investigations revealed that an EC_{50} of proteolysis is achieved at pH 6. However, given that extensive proteolytic degradation arises *in vivo*, in order to effectively protect sufficient quantities of intact protein, substantial serine protease inhibition is necessary. The kinetics studies pertaining to insulin degradation indicate that proteolysis activity exhibits an apparent linear (log scale) decrease down to a value of pH 4.5 where >90% inhibition was achieved. Given that proteins are extensively and rapidly degraded especially in the duodenal and jejunal regions of the GIT, formulations employing acidic inhibition of proteolysis should therefore aim to achieve a local pH of at least 4.5. In this regard, an *in vivo* study in dog found that capsules loaded with the maximal practicable quantity of CA, corresponding to 570 mg in a 680 mg tablet, yielded the highest bioavailability, by reducing the pH to as low as 3 [18]. It should be noted that degradation studies performed *in vitro* represent a system of optimal mixing conditions, which may not be present *in vivo*. Under such circumstances, lowering the pH beyond 4.5 likely ensures a greater acidic expanse within the small intestine, thus representing a local region in which protein degradation is limited for a period of time.

Chymotrypsin is the primary enzyme responsible for the degradation of insulin [2]. Corresponding studies examining chymotrypsin-mediated degradation of casein or denatured lysozyme exhibit a slope (k_{obs} versus pH) which closely resembles that generated in our investigations examining the degradation of insulin in rat duodenal enzyme extracts [1]. Studies suggest that the activity of chymotrypsin can be lowered 2-fold when free calcium is less than 0.05 mM [20]. Similarly, the activity of chymotrypsin has been shown to be lowered when Ca^{2+} is omitted [20] or when available Ca^{2+} is chelated by EDTA [21]. Our investigations however, indicate that the function of CA/Cit as a chelator is most pronounced at high pH values (i.e., >7). Indeed, we have observed that Cit 120 mM lowers the rate constant of chymotrypsin-mediated degradation of insulin by up to 35% at pH 7.4 due to its prevalent chelating action at that pH value (see Supplementary material). However, for pH-lowering formulations, where the local pH at the site of release is <5, its function as a chelator does not contribute to its peptidase inhibitory capacity, which is simply due to moving the pH optimum for serine proteases away from pH 7.4. Therefore, in practice the impact of CA/Cit on reduction of enzyme activity via calcium chelation is significantly less than that due to acidity *per se*. Trisodium citrate (120 mM) will provide a considerable increase to ionic strength possibly further reducing the activity of luminal proteolysis. However, on the contrary, addition of NaCl (75, 120 and 250 mM) did not significantly reduce the degradation rate of insulin by chymotrypsin (unpublished data).

On account of its large molecular weight size, transport of insulin (5800 MW, 8 Å) across the small intestinal epithelium is severely restricted. Indeed, basal permeability results in absorption of doses far below that required to achieve a therapeutic effect [22]. Amphiphilic permeability enhancers, a class of absorption enhancers including LCC and sodium taurodeoxycholate (TDC) promote trans- and paracellular absorption via mild recoverable membrane perturbation and disruption of tight junction complexes [23–25]. For example, permeation of sCT across rat ileal tissue in Ussing chambers can be increased 5-fold and 14-fold for LCC and TDC, respectively [6]. However, their successful incorporation in a pH-lowering formulation requires that they exhibit sufficient

solubility at low pH values. In this regard, LCC satisfies this requirement. As a consequence, it has been widely co-entrapped in formulations designed for acidic inhibition of proteolysis [5,7].

Nevertheless, in the ORACAL study for orally delivered sCT, no amphiphilic enhancer was required. One interpretation was that CA/Cit might be enhancing paracellular transport presumably via modulation of tight junctions [3], in addition to their effect on inhibiting peptidases by acidifying the pH. Supporting this, work by Okada and colleagues [26] revealed that addition of organic acids facilitated vaginal absorption enhancement of the luteinising hormone releasing hormone (LH–RH)-analogue, leuprolide. Furthermore, they observed a weak correlation between this effect and the chelating properties of the acids. However, the influence of pH on such effects was not addressed in this study. Collectively, our investigations indicate that even if intestinal luminal calcium chelation is associated with permeability enhancement, the pH conditions of acidic inhibition of proteolysis (i.e., pH $\leqslant$ 4.5) will extensively reduce chelation activity. Conceivably, the positive results yielded in the Phase III trial with sCT in the absence of a recognised permeability enhancer could be ascribed to the fact that sCT is half the molecular weight of insulin and exhibits a higher baseline permeability [27–31]. Moreover, since sCT is highly potent and marketed nasal versions are associated with no more than 1–3% bioavailability for required efficacy, the hurdles for an oral sCT are therefore much lower and would not require a recognised permeation enhancer once the excipient organic acid performs the pH-lowering role. This would not be the case for insulin where a much higher oral bioavailability would be required for a commercially-viable oral formulation. Consistent with this theory, ileal instillation of insulin with soy-bean proteolysis inhibitor alone did not lower blood glucose in rats, whereas glucose levels were significantly reduced when insulin was co-administered with the bile salt, sodium deoxycholate (DOC) [32], an efficient permeation enhancing agent.

There are numerous reports which demonstrate the effect of CA as a permeability enhancer. CA (1%, pH 7) enhanced nasal absorption of oil/water (O/W) emulsions containing indomethacin by 6.5-fold [33]. However, it should be noted that the nasal clearance/dilution is smaller than that observed in the GIT [34]. Moreover, the CA partitions in water yielding a concentration of 100 mM; thus, the concentration of CA reaching the nasal mucosa is likely much higher than in the intestine.

Insulin formulated with CA (10%, pH 1.72) for vaginal administration effectively lowered blood glucose to the same level as a 10 times lower intramuscularly injection. However, although such high concentrations of CA and low pH can increase permeability, they have also been shown to lead to extensive damage of mucosal tissue [26]. In the context of chronic administration, such adverse effects are undesirable.

Our *in vitro* (Caco-2 monolayers) and *ex vivo* (rat small intestinal tissue) transport studies indicate that the ability of CA/Cit (5–30 mM) to increase permeability of insulin or FD4 is negligible, exhibiting effects which are lower than that of LCC (1 or 3 mM), regardless of pH. Examination of absorption processes *ex vivo* can be confounded by enzymatic degradation. Thus, FD4 being a non-degradable hydrophilic macromolecule-sized compound represents a useful surrogate marker compound for insulin in Ussing chamber-based transport studies, facilitating exclusive examination of the absorptive process alone. The concentrations of LCC employed correspond to those which lie below that which can adversely impact tissue viability over the course of permeation study [25]. In the case of CA, the concentrations used represent those which are anticipated to be found locally in the lumen at the site of release of such oral formulation(s). Compared to excised intestinal tissue, Caco-2 monolayers represent a more fragile model system. Thus, pH should not be reduced beyond 4 [16] and LCC and CA concentrations can likewise be reduced to appropriate functional concentrations which ensure the cell monolayers recover following treatment.

In line with earlier findings for EDTA [19], basolateral application of Cit elicited a more marked decrease in TEER. This differential susceptibility could be attributed to compositional differences in tight junction structures at the apical and basolateral interfaces as previously shown for EDTA [18]. Application to the basolateral side ensures direct exposure of the highly calcium-dependent zonula adherens proteins – structures which are implicated in preservation of monolayer integrity. In this regard, apical addition of Cit will have little impact. However, translation of these basolateral specific effects *in vivo* is not particularly relevant; given the fact that exposure is restricted to the apical membrane of the intestinal epithelia upon release from the dosage form. Collectively, these results strongly suggest that apically applied CA, by means of significant calcium chelation at pH 7.4, is not sufficient to elicit significant augmentation of insulin permeability, notwithstanding its ability to trigger acidic inhibition of proteolysis. Nevertheless, this observation does not preclude the possibility that CA/Cit could potentially chelate calcium in a pH neutral micro-environment below the mucus layer covering the intestinal epithelium.

5. Conclusions

It is evident that the pH range over which CA effectively inhibits proteolysis and that whereby Cit exerts calcium chelating properties does not coincide. At pH 3–4, the capacity of CA to inhibit small intestinal serine proteases is high, and this is due to sub-optimal pH values for those enzymes rather than to calcium chelation. Moreover, *in vitro* and *ex vivo* investigations indicate that the capacity of Cit/CA to exert significant permeation enhancement on human intestinal monolayers and isolated rat small intestinal mucosae is extremely low. While oral delivery of a few potent small peptides including sCT may be successfully achieved in the presence of formulated peptidase inhibitors (e.g., CA) in the absence of permeation enhancers, larger and more impermeable peptides and proteins will require an absorption enhancing agent in the formulation.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.ejpb.2013.12.017.

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