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Research Paper

Melittin as an Epithelial Permeability Enhancer I: Investigation of Its Mechanism of Action in Caco-2 Monolayers

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Purpose. Melittin is an amphipathic antimicrobial peptide which has been shown to enhance the permeability of mannitol and reduce transepithelial electrical resistance (TER) across Caco-2 monolayers. The aim of this work was to further examine the potential of melittin as a paracellular permeability enhancer and to investigate the mechanism of interaction with tight junction proteins in Caco-2.

Materials and Methods. The permeability of a range of fluorescent markers of differing molecular weights across monolayers was examined and immunofluorescence and western blotting analysis of tight junction proteins were also carried out. The mechanism of TER reduction was also examined using cell signalling inhibitors.

Results. Apical but not basolateral addition of melittin increased the permeability of a range FITC-dextrans (4–70 kDa) across monolayers. Melittin effects were reversible and no cytotoxicity was evident in polarized Caco-2 epithelia at the concentrations used. Altered expression of ZO-1, E-cadherin and F-actin was also detected. The phospholipase A2 inhibitors, aristolochic acid and indomethacin and the cyclooxygenase inhibitor, piroxicam, partially attenuated melittin-induced TER reduction, suggesting that part of the mechanism by which melittin opens tight junctions involves prostaglandin signalling.

Conclusions. Apically-added melittin opens tight junctions, causing dramatic TER reductions with significant increases in flux of dextrans. These effects appear mediated in part via PLA2 and involve alterations in specific tight junction proteins.

KEY WORDS: Caco-2 monolayers; epithelial tight junctions; melittin; oral peptide delivery; paracellular permeability enhancer; polarized gastrointestinal epithelial cells.

INTRODUCTION

The delivery of biotech drugs via the oral route is often limited due to poor oral bioavailability. These drugs are often delivered by invasive parenteral routes which can affect patient compliance particularly in relation to chronic therapies. The intestine maintains a low permeability to large hydrophilic drugs by means of a paracellular resistance controlled by tight junctions (1). While many approaches have been undertaken to increase the absorption of drugs across the GI tract, one of the most widely researched is co-administration with a paracellular permeability enhancer (PPE) (2). Controlled, reversible opening of intestinal epithelial tight junctions with minimal local or systemic toxicity by PPEs has improved the oral bioavailability of low molecular weight heparin (3). However, an intrinsic property of many PPEs is their low safety margin which is a limiting factor in their application in oral drug delivery (4) where repeated dosing is likely to be required. Furthermore, although tight junction opening can be demonstrated *in vitro* with agents including sodium caprate (C10), higher concentrations required *in vivo* suggest multiple mechanisms are involved (5,6).

Regulation of permeability in polarized epithelia is a complex process which has not been fully elucidated to date. The tight junctions form a barrier to the diffusion of molecules from the gut lumen and also restrict the diffusion of lipids and proteins between apical and basolateral membranes (7). The list of tight junction associated proteins is extensive (7). Tight junctions are composed of transmembrane and scaffold proteins and are regulated by numerous signalling proteins.

A limited number of proteins, including, human immunodeficiency virus Tat protein (HIV Tat) (8) and herpes simplex virus VP22 protein (9) and Zonula occludens toxin (ZOt) (10) have been shown to have potential for the delivery of therapeutic proteins and peptides. In particular, both HIV Tat and ZOt have both demonstrated tight junction opening effects (10,11). Melittin is a cationic amphipathic, antimicrobial peptide that disrupts the plasma membrane of bacterial cells through a torroidal pore mechanism (12,13). The potential use of melittin as an

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absorption enhancer was first reported by Liu *et al.*, (14), who demonstrated enhanced transport of mannitol across Caco-2 monolayers. More recently, we have shown that melittin rapidly reduced the transepithelial resistance (TER) of Caco-2 cell monolayers and visualised tight junction opening by transmission electron microscopy (15). However, melittin demonstrates both haemolytic activity and cytotoxicity in cell lines (15,16). This cytotoxicity has been attributed to the hydrophobicity of melittin and to its affects on cellular metabolism (17). A number of studies on the interactions of melittin with both prokaryotic and eukaryotic cells have shown that the peptide can interact with a range of cellular proteins which are linked with tight junction regulation and permeability. These include binding to calmodulin (18), inhibition of the sodium–potassium, hydrogen–potassium, and calcium ATPase pumps (19,20), as well as protein kinase C (PKC) (21), phosphorylase kinase (22), troponin C (18), and adenylate cyclase (23) among others.

The transient nature of the effect of melittin on TER (recovered within eight hours) is an ideal property for an enhancer of paracellular permeability. Investigation of the extent by which melittin enhances the flux of molecules of different molecular weights in the absence of cytotoxicity target cells is essential in evaluating the peptide's further potential as an absorption enhancer across the intestine. Furthermore, the assessment of the mechanism(s) by which melittin induces tight junction opening needs to be further examined to see how it compares to other agents. In this study, using fluorescence microscopy and western blotting, we examined the effect of melittin on the expression and distribution of a number of tight junction associated proteins in Caco-2 monolayers, namely ZO-1, E-cadherin, claudin-1 and F-actin. In addition, we explored the potential mechanism of melittin effects on TER. Since melittin is an activator of phospholipase A2 (PLA2) and melittin stimulates electrogenic chloride secretion through a PLA2-dependent pathway in rat colon (24), this led us to examine the role of PLA2 activation in melittin's effects on TER in Caco-2 monolayers. A series of inhibitors of the lipid signalling pathway, including PLA2 inhibitors, cyclooxygenase inhibitors, PKC inhibitors and phosphoinositol hydrolysis inhibitors were assessed to examine this potential mechanism. In addition, the role of melittin antagonism of PKC was also explored. The aim of this investigation was thus to further examine the potential of melittin as a paracellular permeability enhancer and evaluate the potential interaction with tight junction proteins/associated proteins in Caco-2 monolayers.

MATERIALS AND METHODS

Reagents

Melittin (Serva Inc, Germany) was purchased as a lyophilised powder and exhibited purity in excess of 98% by micro-reverse phase chromatography and SDS PAGE. The peptide was reconstituted in sterile ultra pure water and frozen in single use aliquots. Piroxicam and aristolochic acid were purchased from Sigma (Ireland). Sphingosine, neomycin sulphate, COX-2 inhibitor peptide, as well as calmodulin inhibitor, W7 and myosin light chain kinase (MLCK) inhibitor peptide 18 were purchased from Calbiochem (UK). All other reagents were purchased from Sigma (Ireland) unless otherwise stated.

Tissue Culture

Caco-2 cells were purchased from ECACC, UK and were grown in DMEM with 2 mM L-Glutamine, 1% v/v non-essential amino acids, and 10% v/v fetal bovine serum (Sigma-Aldrich, Ireland). Caco-2 cells were used between passages 35–60 and cultivated in 75 cm^2 tissue culture flasks at 37°C in a humidified atmosphere with 5% CO_2. The viability of the test cells prior to use in experimentation exceeded 99%, as determined by exclusion of the vital dye trypan blue.

Measurement of Transepithelial Electrical Resistance (TER)

The effect of melittin on the integrity across absorptive mucosa was evaluated in polarized Caco-2 monolayers prepared by 21 day continuous culture on 12 mm Transwell® polycarbonate inserts (Corning Costar Corp. USA). Cells were seeded at a density of 5×10^5 cells/insert with culture medium changed bilaterally on alternate days and TER monitored over the 21 day period. Prior to use all monolayers exhibited resistance of greater than 1,200 $\Omega.cm^2$. Melittin was added to either the apical or basolateral side of the monolayers and TER measurements taken periodically over a 24 h period using an EVOM® voltohmmeter with a chopstick-type electrode (World Precision Instruments, USA). The data were displayed as percentage change in TER over untreated monolayers.

Immunofluorescence Microscopy

Differentiated monolayers, 21-days old, were treated with melittin concentrations close to IC_{50} value previously obtained for non-polarized Caco-2 cells (15). The monolayers were rinsed with prewarmed HBSS for 5 min and subsequently permeabilised, stained and fixed. For ZO-1 and E-cadherin labelling, monolayers were permeabilised with cold methanol (−20°C) for 30 min. Non-specific binding sites were blocked with PBS containing 1% w/v BSA for 10 min. The cells were then immunoprobed with 2.5 μg/ml mouse anti ZO-1 antibody (Invitrogen, USA) or mouse anti-E-cadherin antibody (BD Biosciences, UK) for one hour. Each well was then washed three times with 1% BSA-PBS for 5 min, before incubating with 5 μg/ml FITC conjugated goat anti mouse antibody (Dako Cytomation, Denmark) for one hour protected from light. The monolayers were then washed five times with 1% BSA-PBS for 5 min and post-fixed with 4% w/v paraformaldehyde prepared in PBS for 10 min. The filters were then removed from the plastic supports, mounted on slides with Vectashield™ containing DAPI (Vector Laboratories, USA) and examined on a Nikon Eclipse 80i fluorescence microscopy. F-actin labelling was carried out using phalloidin, conjugated to rhodamine (Invitrogen, USA), as described by the manufacturer. Briefly, melittin-treated monolayers were rinsed with pre-warmed HBSS, fixed with 3.7% paraformaldehyde in PBS, permeabilised with cold acetone (−20°C), blocked with 1% BSA-PBS, and treated with 1 U (33 nM) rhodamine-phalloidin in 1% BSA-PBS. The monolayers where then rinsed five times with 1% BSA-PBS and examined by fluorescence microscopy.

Transport Studies

The permeability-enhancing effects of melittin were evaluated across monolayers by transepithelial flux of sodium fluorescein and the dextrans, FITC Dextran 3,000 (FD3), FITC Dextran 10,000 (FD10) and FITC Dextran 70,000 (FD70) (Molecular Probes, Netherlands). Monolayers were rinsed with pre-warmed HBSS supplemented with 11 mM glucose and 25 mM HEPES (15 mM free acid, 10 mM sodium HEPES, pH 7.4)). Monolayers were equilibrated in HBSS until resistance stabilised. Monolayers were then treated with 0.5 mg/ml FITC-dextran followed by addition of melittin, both to the apical side. Apical sampling (10 μl) was carried out at time 0 and 180. Basolateral samples of 0.5 ml were taken every 20 min over 3 h, replenishing with fresh pre-warmed HBSS at each sample timepoint. The excitation and emission maxima of each FITC dextran were determined in HBSS supplemented with glucose (11 mM) and HEPES (25 mM) and were 495/520 nm. Excitation and emission slit widths were set at 2.5 nm. The apical-to-basolateral flux of fluorescent dextrans were quantitatively measured with a HS750 spectrofluorimeter (Perkin Elmer, UK) using an external standard curve. The apparent permeability coefficients (Papp) were calculated according to $Papp = dQ/dt (1/AC_0)$, where dQ/dt is the permeability rate derived from the slope of the line, A is the diffusion area of the monolayer, and C_0 is the initial donor solution concentration (25).

Cytotoxicity Testing

The cytotoxicity of melittin on 21 day-old monolayers was evaluated using the methylthiazolydiphenyl-tetrazolium bromide (MTT) conversion assay. Following apical application with melittin at a range of concentrations and incubation for 3 or 24 h, each monolayer was rinsed and treated with apically applied MTT (0.5 $mg.ml^{-1}$ in DMEM) at 37°C in a humidified atmosphere for two hours. MTT-formazan was solubilised with DMSO without shaking for 120 min. Aliquots were transferred to a 96-well plate and absorbance read in a multiwell plate reader at 550 nm. The cytotoxic effects of melittin were determined relative to media-treated control cells.

Western Blotting Analysis

The effect of melittin on the expression of tight junction and adherens junction proteins was semi-quantitatively evaluated by western blotting with chemiluminescent immunodetection. Polarized Caco-2 cells grown on Transwell® supports were lysed by gently scraping the cells into buffer containing 50 mM Tris [pH 7.4], 0.1% SDS, 150 mM NaCl, 5 mM iodoacetamide, 1% v/v Igepal, and 1% w/v sodium deoxycholate with complete protease inhibition cocktail (Roche Applied Sciences, UK). The protein content of each monolayer was quantified (BCA protein quantification, Pierce, UK) and diluted to ensure 15 μg of protein was loaded in each lane of a 7.5%/10% SDS PAGE gel. Proteins were separated and transferred to nitrocellulose (AGB, Ireland) and successful separation and transfer confirmed with Coomassie blue and Ponceau S staining of gels and nitrocellulose, respectively. Membranes were subsequently blocked with either 5% w/v dried milk powder (Marvel™) or 4% w/v BSA (for E-cadherin) over night at 2–8°C. Each blot was probed with anti-ZO-1, anti-E-cadherin, anti-claudin, anti β-actin and anti-F-actin (Serotec) antibodies (5 μg/ml) and equivalent loading confirmed with anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (Abcam, Cambridge UK). An alternative anti-F-actin antibody was also used (Abcam). The membranes were stringently washed, probed with HRP conjugated polyclonal anti-murine IgG antibody (ZO-1, E-cadherin, β-actin, and GAPDH), goat anti-rabbit IgG (claudin), or HRP-polyclonal murine IgA (F-actin) and detected with enhanced chemiluminescence.

Melittin Changes in TER: Effects of Selected Cell Signalling Inhibitors

Effects of cell signalling inhibitors on melittin-induced TER changes were examined by pre-incubation of monolayers with bilateral additions of each inhibitor for 60 min prior to application of melittin at the apical side (either 1.6 or 3.2 μM). Monolayers were treated with inhibitor alone, melittin alone or melittin in combination with an inhibitor. The cyclooxygenase inhibitors, piroxicam (50 μM), COX-II inhibitor peptide (100 nM) and indomethacin (200 μM); phospholipase A2 (PLA2) inhibitor, aristolochic acid (75 μM); phospholipase C (PLC) inhibitor, neomycin (150 μM); PKC inhibitor, sphingosine (20 μM); calmodulin inhibitor W7 (100 μM) and MLCK inhibitor peptide 18 (10 μM) were used at their EC_{50} concentrations that inhibit respective enzymes. The effect of PKC activation was examined with phorbol myristate acetate (PMA, 1.7 μM). TER was monitored at 15, 30, 45 min, and every hour from 1 to 9 h.

Statistical Analysis

All experiments were carried out on three independent occasions unless otherwise stated. All values are expressed as the means ± standard error mean (SEM). Statistical comparisons were carried out by unpaired Student *t* test or by analysis of variance (ANOVA) with Prism 4.0® and Sigma Stat® software packages, respectively.

RESULTS

Effects of Melittin on TER in Caco-2 Cell Monolayers

The exposure of polarized Caco-2 cell monolayers to apical additions of melittin resulted in a concentration-dependent decrease in TER over 12 h with a threshold concentration of 0.7 μM, up to a maximum of 3.5 μM (Fig. 1). For 0.7 μM melittin, the rapid drop in TER was optimal 45 min after melittin addition and was statistically different from untreated control ($P < 0.05$). At higher concentrations of melittin the effect was quicker, and a significant and maximal TER reduction was observed within 15 min of addition of 1.8 μM ($P < 0.01$), 3.5 μM ($P < 0.001$), and 5.3 μM ($P < 0.001$). The TER recovered to control levels within 24 h of melittin removal when treated with concentrations up to 1.7 μM, indicating that the effects of melittin were reversible at lower concentrations. The lowest concentration examined (0.2 μM)

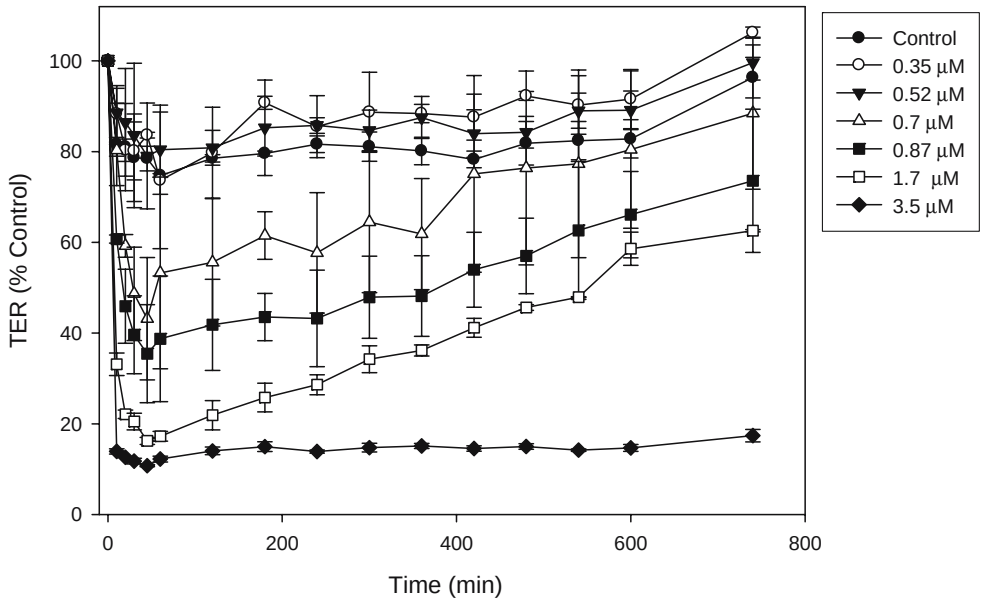

Fig. 1. Effect of melittin concentration on TER of Caco-2 monolayers following 12 h exposure. Results represent mean ± SD, three independent experiments carried out in triplicate.

had no effect and its profile was superimposable with control (data not shown).

To evaluate whether the effects of melittin on Caco-2 monolayers were polarized, the antimicrobial peptide was added at equal concentrations to either the apical or basolateral compartments and TER was measured over 24 h (Fig. 2). The apical administration of 3.2 μM melittin resulted in an irreversible decrease in TER to ~ 15% of media control, however the equivalent basolateral application did not significantly reduce TER relative to media treated control indicating the effect of melittin is polarized under these conditions (Fig. 2). The difference in TER response between apical and basolateral application of 3.2 μM melittin was statistically significant ($P < 0.001$) at each time point. The basolateral administration of 5.3 μM melittin was different to apical administration at shorter timepoints. The TER was reduced to only 80% of control within 30 min of basolateral application, as compared with 14% following apical application; ($P < 0.001$) and to 40% of control one hour after basolateral application, compared with 11% following apical application ($P < 0.05$). At longer time points of 7 and 24 h, the TER values were comparable (data not shown) following both apical and basolateral application of melittin.

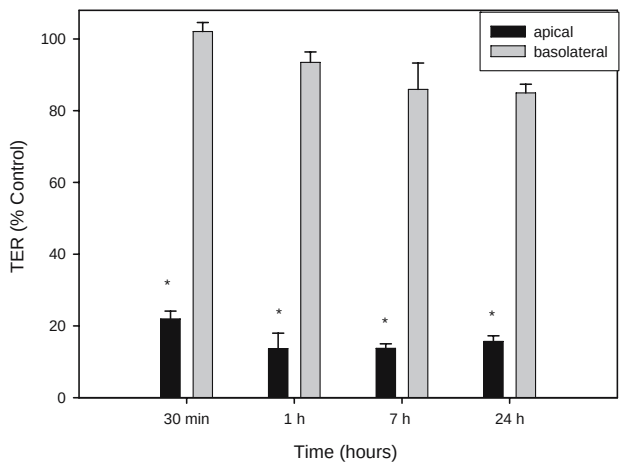

Fig. 2. Decrease in TER induced by apical or basolateral treatment of Caco-2 monolayers with melittin over 24 h. Results represent the mean of three independent experiments carried out in triplicate. *$p < 0.001$ compared with zero time-point.

Effect of Melittin on the Permeability of Fluorescent Markers

The potent effects of melittin on TER are an indirect indication of an alteration in paracellular permeability. Permeability enhancing effects of melittin have been demonstrated for mannitol fluxes in Caco-2 cells (14). To further evaluate melittin's direct ability to increase permeability, it was important to study the effect on a range of hydrophilic sugars of different molecular weight. Apically-applied melittin had a concentration-dependent effect on apical-to-basolateral permeability of four fluorescent markers ranging in molecular weight from 400 Da to 70 kDa, at all four concentrations tested (Fig. 3). In addition, an inverse relationship was found between the permeability enhancing effect and molecular weight of the marker molecule. The four-fold increase in transport of sodium fluorescein was statistically significant at the lowest concentration of melittin tested (0.8 μM, $P < 0.05$). At melittin concentrations of 1.6, 3.2 and 5.3 μM the flux of sodium fluorescein was 7, 11 and 13 times greater than control respectively. Melittin also resulted in significantly enhanced flux of the three FITC-dextrans (3, 10, and 70 kDa) across Caco-2 monolayers.

The polarized effect of melittin on transport was again demonstrated using fluorescein permeability as a marker. The Papp of sodium fluorescein was comparable to control Papp when concentrations of below 3.2 μM were applied basolaterally (Papp values were 2.6 and 3.11×10^{-6} (cm/s) at 0.8 and 1.7 μM melittin basolaterally). However, when

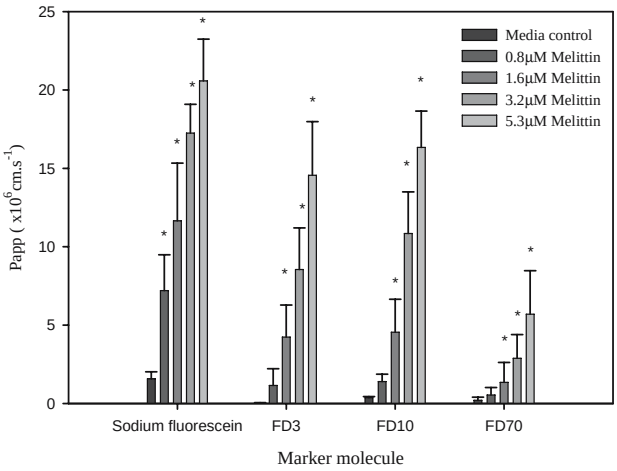

Fig. 3. Permeability coefficients of sodium fluorescein, FD3, FD10 and FD70 across Caco-2 monolayers exposed to melittin for 3 h. Each Papp represents the mean of three independent experiments carried out in triplicate ± SEM, $*p < 0.05$ compared to medium treated controls.

3.2 μM or higher concentrations of melittin were applied basolaterally, the Papp values for sodium fluorescein were comparable with those achieved with the same concentration of melittin applied apically (Papp of $15.6 \pm 4.3 \times 10^{-6}$ (cm/s) basolaterally versus $17.2 \pm 1.8 \times 10^{-6}$ (cm/s) following apical application). This suggests that a threshold value of 3.2 μM melittin was required basolaterally in order to increase permeability of markers.

Cytotoxicity Studies

We have previously shown that melittin was cytotoxic to Caco-2 cells grown on plastic in log-phase of growth (15), however, its effect on polarized filter-grown cells is more relevant to investigations as a permeability enhancer. MTT assays on polarized Caco-2 cells showed that melittin was not cytotoxic following 24 h incubations with concentrations up to 5.3 μM of peptide (Fig. 4).

Effect of Melittin on Tight Junction Proteins

In order to investigate how melittin resulted in opening of tight junctions, the expression and distribution of three tight junction associated proteins were examined by immunofluorescence. When Caco-2 cells were exposed to 1.6 μM melittin, a concentration that resulted in a reversible drop in TER, no apparent change in the distribution of ZO-1 was observed by immunofluorescence relative to control (Fig. 5b). However, at twice that concentration (3.2 μM), there was a dramatic reduction in detectable ZO-1 by immunofluorescence microscopy (Fig. 5c). A comparable pattern was also evident for the two other proteins examined, F-actin and E-cadherin. Phalloidin staining was not greatly altered following exposure of Caco-2 cells to 1.6 μM melittin relative to medium treated control, however, at 3.2 μM melittin, complete actin depolymerisation was observed (Fig. 5f). Consistent with this, E-cadherin expression was unaltered at 1.6 μM melittin, but virtually undetectable at double that concentration (Fig. 5g–i).

Confirmation of the alterations in expression of these proteins was demonstrated by Western blotting (Fig. 6). ZO-1 expression was significantly reduced relative to control at concentrations of 3.2 μM and greater. However, at the lowest concentration examined, 0.8 μM, there was a 1.5-fold increase in ZO-1 expression, when data was normalised for expression of the house-keeping protein, GAPDH. E-cadherin expression also reduced following treatment with 3.2 and 5.3 μM melittin, however, no apparent increase in expression at 0.8 μM melittin was observed. An increase in the level of actin monomer at 43 kDa and a concomitant reduction in high-molecular weight F-actin was observed with increasing concentrations of melittin of 1.6 μM and above. These changes in F-actin were also observed using an alternative F-actin antibody (data not shown). There was no alteration in the β−actin expression, indicating the immunofluorescence change was due to depolymerisation of filamentous actin, rather than reduced expression of actin. No change in occludin-1 expression was observed at any of the concentrations examined (data not shown).

Investigation of Mechanism of Action of Melittin Using Cell Signalling Inhibitors

Regulation of tight junction function is multifactorial, therefore we examined a series of cell signalling inhibitors in attempt to investigate the mechanism by which melittin opened tight junctions, increasing epithelial permeability. The effects of melittin on both TER and tight junction proteins were different at concentrations of 1.6 μM compared with 3.2 μM, thus we explored the effects of cell signalling inhibitors on cells treated at both of these melittin concentrations. The PLA2 inhibitors, aristolochic acid (75 μM) and indomethacin (200 μM) partially attenuated the effect of 3.2 μM melittin (Fig. 7a, b). The effect was significant (<0.05) at each timepoint after 5 h. When piroxicam, neomycin, sphingosine, Cox-II inhibitor peptide, W7 and

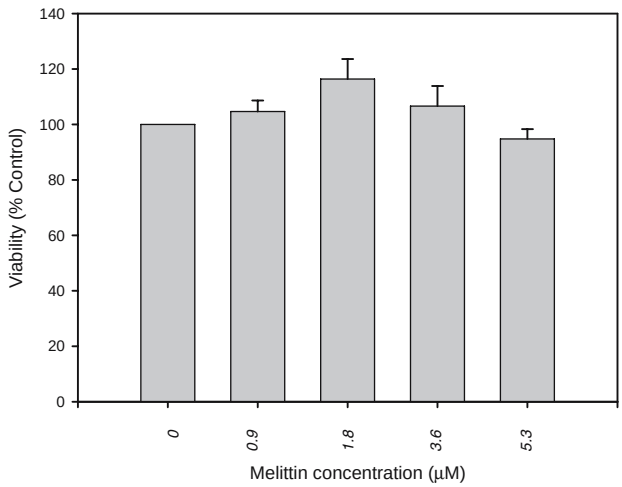

Fig. 4. Evaluation of the cytotoxic potential of melittin on Caco-2 cell monolayers following 24 h treatment using the MTT assay. Each value is displayed as the mean of three independent experiments carried out in triplicate ± SEM.

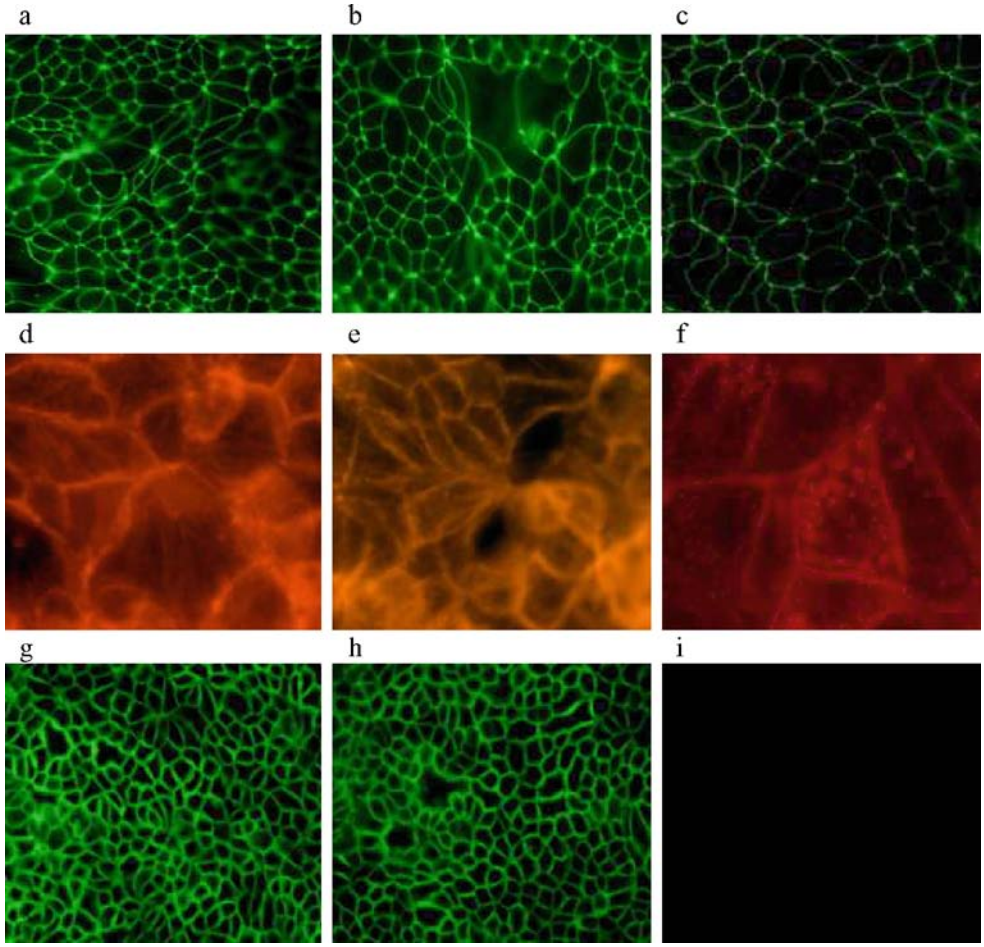

Fig. 5. Fluorescent micrographs of Caco-2 cell monolayers treated with melittin for two hours and stained for ZO-1, F-actin and E-cadherin. Panels (**a**–**c**) are as follows: (**a**) media control, (**b**) 1.6 μM melittin treated, (**c**) 3.2 μM melittin treated and all stained for ZO-1. Panels (**d**–**f**): (**d**) media control, (**e**) 1.6 μM melittin, and (**f**) 3.2 μM melittin treated monolayers stained for E-cadherin. Panels (**g**–**i**): (**g**) media control, (**h**) 1.6 μM melittin, and (**i**) 3.2 μM melittin treated monolayers stained for F-actin. Each image is representative of monolayers treated and stained on three independent occasions.

MLCK peptide 18 were applied the % control TER profiles were superimposable with that of melittin (3.2 μM) alone (data not shown), indicating that these inhibitors did not have any effect on the melittin induced tight-junction opening at irreversible concentrations of the peptide. When the lower concentration of melittin (1.6 μM) was examined, neomycin significantly modulated the drop in TER at 15–30 min ($P < 0.05$) (Fig. 7e). Piroxicam also attenuated the TER-reducing effects of melittin alone at this lower concentration, but only at the longer time points (after 5 hours, $P < 0.05$ for each time point), comparable with aristolochic acid (Fig. 7d, e). Neither Cox-II inhibitor peptide nor MLCK inhibitor had an inhibitory effect when a lower concentration of melittin (1.6 μM) was used. (data not shown).

The role of the PKC activator, PMA, was also examined. Pre-treatment of Caco-2 cells with PMA alone resulted in a dramatic increase in TER relative to untreated controls, prior to addition of melittin (1.6 μM) (Fig. 7f). Four hours after the addition of PMA, the TER began to decrease due to time-dependent down regulation of PKC. When melittin was applied to PMA-pretreated monolayers the drop in TER was slower and less dramatic than cells treated with melittin alone, i.e. TER was maintained at 40% of untreated controls relative to 20% of untreated controls) for a 2 h period (Fig. 7f), ($p < 0.05$).

DISCUSSION

Liu *et al.* (14) previously evaluated the permeability increasing effects of melittin across Caco-2 monolayers. Mannitol absorption was enhanced in their study; however, no further analysis of the mechanism of permeability has been carried out since. Due to the many effects of melittin, there may be safety considerations in its use as a drug delivery agent, therefore, in order to assess the scope of melittin as a PPE and to enable the development of potentially safer analogues, the mechanism of melittin action on the intestinal epithelial barrier needs to be further elucidated.

In this study we have shown that melittin induced a concentration-dependent reduction on TER of polarized Caco-2 cells *in vitro*, which suggested that the enhanced absorption of mannitol seen by Liu *et al.* (14) was due to an effect on tight junction opening. The effect on TER is reversible at lower concentrations of melittin (0.7 μM), with

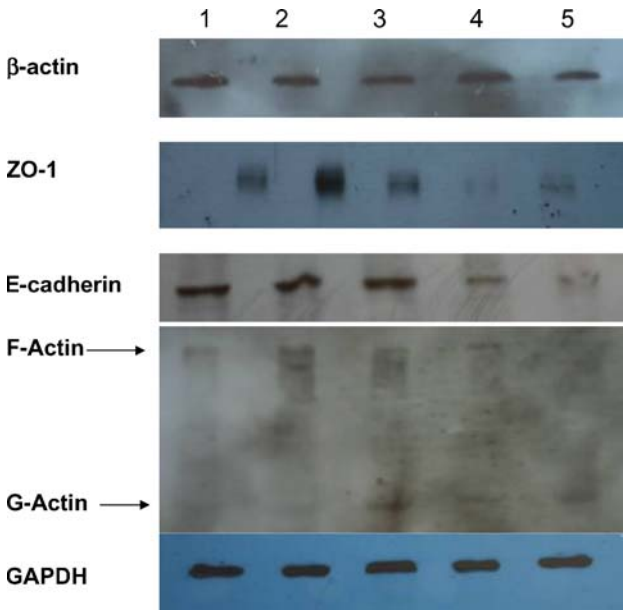

Fig. 6. Effect of melittin (2 h; 0–5.3 μM) on the expression of tight junction associated proteins ZO-1, E-cadherin, F-actin, G-actin, β-actin on Caco-2 cell monolayers. *Lanes* refer to the following melittin concentrations: Lane 1: control; Lane 2: 0.8 μM; lane 3: 1.8 μM; lane 4: 3.6 μM; Lane 5 5.3 μM. To ensure equal loading of protein to each well the protein concentration was quantitatively measured in triplicate on three independent occasions ($n = 3$) with a BSA protein assay kit (Pierce, USA). The loading control protein concentrations were verified with anti-GAPDH antibody.

a maximal reduction in TER observed 45 min after application, which was virtually fully reversible after 8 h.

This drop in TER was consistent with increased permeability of FITC dextrans and mannitol (14). The effect of melittin on the transport of FITC dextrans was inversely proportional to dextran molecular weight, in contrast to the effect of C10, a medium chain fatty acid which has been shown to increase paracellular transport of a range of molecules (26), and which was shown to transport larger FITC dextrans to a comparable extent to lower molecular weight dextrans or a combination of both (27). Paracellular transport can be enhanced by increasing both the radius of the paracellular pore and the number of pores constituted by tight junctions (28). This suggests that the tight junction pore size induced by C10 is more substantial than that of melittin. The concentration of melittin that reversibly altered TER effect (0.8 μM) resulted in a 4.5 fold increase in sodium fluorescein, and between 3.1 and 3.7 fold increase in FITC dextrans up to the FD10 size. There was only a two-fold increase in Papp for FD70 at this reversible concentration of melittin. This suggests that melittin has limitations in terms of delivery of larger molecular weight therapeutic proteins. This may be of significance in terms of the safety of melittin, as limited effects on tight junction diameter will not enable potential entry of pathogens.

The effects of melittin were considerably reduced when it was applied basolaterally which suggests that melittin has a specific interaction with the apical side of the Caco-2 cell membrane. This is in contrast to C10, which has comparable effects irrespective of which surface it is applied (29) and suggests that melittin has a direct interaction with a receptor at the apical surface, whereas C10 may dissolve in the lipid bilayer. Furthermore, the sideness of the melittin effect indicates that systemic delivery of melittin would have limited toxicity. The polarised effect of melittin on both TER and drug permeability was also observed in rat colon (30).

Although melittin resulted in an irreversible effect on TER at concentrations of 3.2 μM and greater, no toxicity was observed in monolayers treated with either 3.2 or 5.3 μM melittin. This lack of cytotoxicity is in contrast to our previous findings where melittin was quite toxic to logarithmically growing Caco-2 cells grown in 96 well plates (15). This indicates that the more differentiated polarized cells are, the more resistant they become to the cytotoxic effects of melittin. Cytotoxicity may therefore not be as great a problem as anticipated, if more advanced cell culture and tissue culture models are used. In any event, Caco-2 are considerably more prone to cytotoxic effects than those seen in less reductionist intestinal models (31). This resistance of polarized cells to protein toxins has previously been reported, *Pseudomonas aeruginosa* Exotoxin T induced cell rounding and cell death in non-polarized MDCK cells, while polarized MDCK cells were found to be resistant to its cytotoxic effects (32). This resistance was proposed to be related to the regulation of cortical actin in the polarized epithelium.

The immunofluorescence studies revealed that ZO-1 expression or distribution was only slightly altered at a concentration of 1.6 μM melittin, but was dramatically reduced at twice that concentration. This observation was in agreement with changes in TER, i.e. the drop in TER was reversible up to and including 1.6 μM, but did not recover at twice that concentration. The dramatic loss of ZO-1 at the higher concentration helps explain why the TER is not recoverable following 3.2 μM melittin treatment. The reduction in ZO-1 expression at higher melittin concentrations was confirmed by western blotting. Interestingly, the loss of ZO-1 is preceded by an increase in ZO-1 signal at the lowest concentration of melittin examined. This increase in ZO-1 may be due to a compensatory response to transient disruptions of expression of tight junction proteins. It has previously been reported that disruption of tight junctions in cultured Caco-2 cells caused mRNA levels for ZO-1 to increase by 10-fold relative to Caco-2 cells with intact tight junctions (33).

Redistribution of actin was more dramatic following treatment of Caco-2 monolayers with 3.2 μM melittin compared with 1.6 μM. A clear depolymerisation of F-actin was observed with an overall decrease in expression along intercellular junctions. There is strong evidence to support the relationship between actin filaments and the epithelial barrier effects of other absorption enhancers. Chitosan has been shown to form an ionic interaction with the tight junction, opening the junction in a process involving a decrease in ZO-1 proteins and a change in F-actin from a filamentous structure to a globular structure (34,35). C10 is considered to act, in part, via activation of PLC and upregulation of intracellular Ca^{2+} resulting in contraction of calmodulin-dependent actin–myosin filaments mediated by myosin light chain kinase (36). Zonula occludens toxin (ZOt), on the other hand, induces PKC-α related polymerisation of soluble G-actin to F-actin. This causes the rearrangement of the actin filaments and displacement of proteins including ZO-1

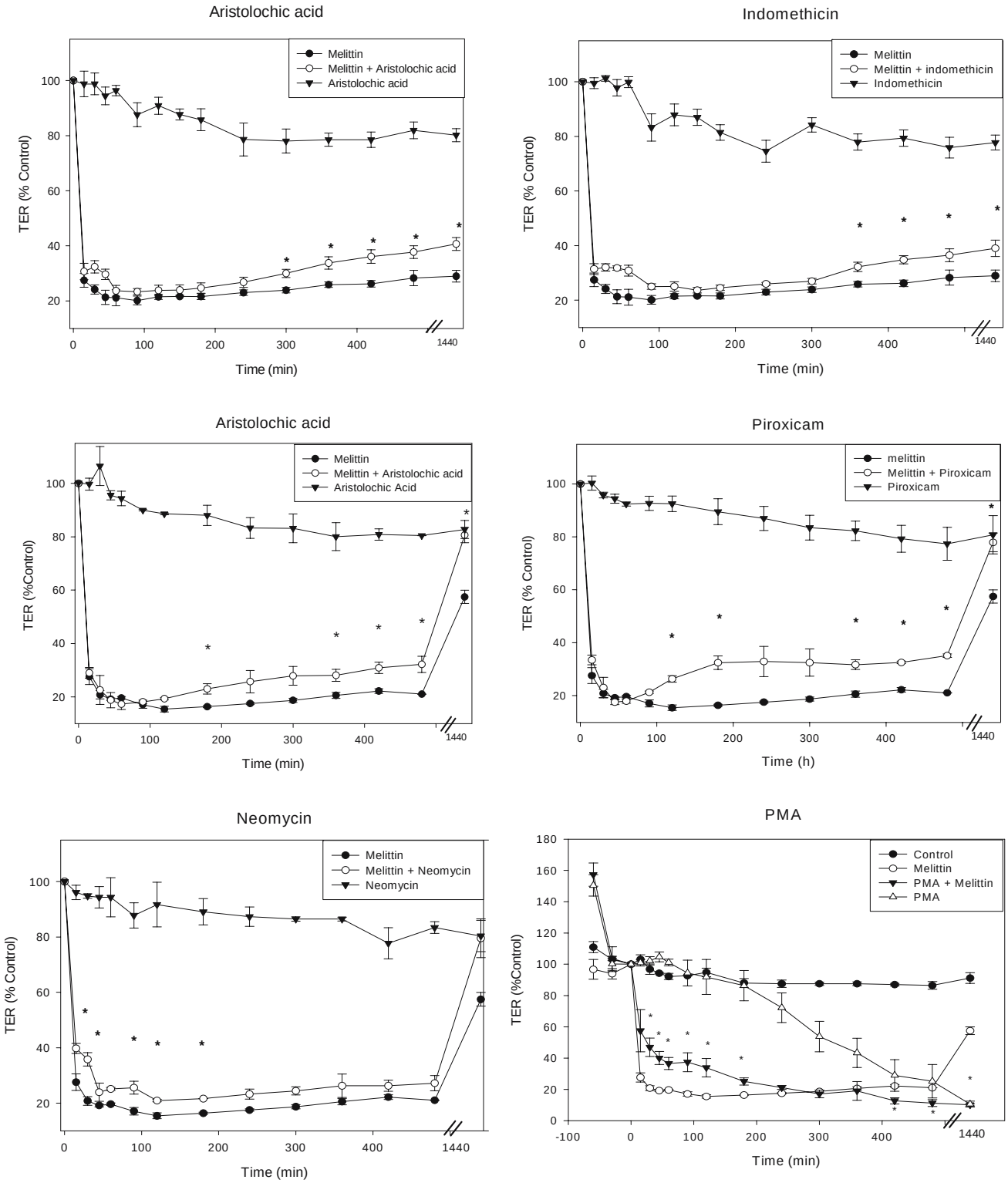

Fig. 7. Effect of pre-treatment of monolayers with cell signalling inhibitors on TER change induced by melittin. Panels refer to the following inhibitors: (**a**) aristolochic acid; (**b**) indomethacin; (**c**) aristolochic acid; (**d**) piroxicam; (**e**) neomycin and (**f**) PMA. Monolayers were treated with 3.2 μM melittin (**a**) and (**b**) or 1.6 μM melittin (**c**–**f**). Data shown represent mean TER expressed as a percentage of the zero time control +/− SEM, from three independent experiments. $^{*}P < 0.05$ relative to melittin treated monolayers at respective time point.

from the junctional complex (37). While the current study demonstrates that melittin is likely to act through modification of cellular actin in junctional complexes, the mechanism of action needs to be elucidated further.

The study on the mechanism by which melittin acts on epithelial barrier function is complex due to the number of reported activities of the peptide. Melittin is known to have calmodulin binding activity (18,19) suggesting a possible role

for calmodulin in melittin's absorption enhancing effects, or indeed one of the many proteins that calmodulin regulates. Myosin movements along actin are regulated by phosphorylation of calmodulin activated MLCK (38) causing contraction of the perijunctional actinomysin ring resulting in tight junction opening. Furthermore, reported PKC inhibition by melittin (21) and the documented role of this protein in ZOt permeation enhancing effects (10,39) suggests a role of PKC in melittin's activity. The fact that the TER effects of melittin and ZOt were both reversible, time and dose-dependant suggests melittin's TER reducing effects may be PKC-dependent. It has been previously been shown that melittin is an activator of PLA2, (40) which resulted in an increase in electrogenic chloride secretion across rat colonic epithelium (24). Activation of PLA2 has been shown to decrease TER of Caco-2 cells via hydrolysis of phosphatidylcholine (41). A number of inhibitors of reported melittin activities were screened to assess the mechanism of action of the peptide on tight junctions. We have shown that the effect melittin on TER is partly modulated by the presence of indomethacin and aristolochic acid and piroxicam, all of which exert their inhibitory effects on the arachidonic acid metabolic pathway. Aristolochic acid inhibits PLA2. Indomethacin is a more potent inhibitor of Cox-2 as are Cox-2 inhibitor peptide and piroxicam. Given that PLA2 activation results in activation of cyclooxygenase-2, these data suggest that the TER reduction is partially mediated via prostaglandin signalling pathway. In particular, the enhanced effect at longer time points suggests that the maintenance of the open tight junctions is mediated via this pathway. Studies on the role of arachidonic metabolism on tight junction regulation are limited. It was recently shown that exposure of differentiated Caco-2 cells to prostaglandin E2 resulted in a disruption of epithelial barrier function (42). In addition, cyclooxygenase inhibitors modulated the effects of certain short chain fatty acids on tight junction permeability (43) and restored E-cadherin expression in rat uroepithelium (44). Furthermore, it has been reported that arachidonic acid reduced the transendothelial resistance of endothelial cells (45).

The concentration of melittin which resulted in reversible tight junction opening, was inhibited by neomycin and PMA. This suggests that a different pathway involving PKC and PLC is stimulated by reversible melittin concentrations. At the higher melittin concentrations, complete breakdown of actin filaments and complete loss of ZO-1 is mediated by a prostaglandin signalling pathway. The involvement of PKC is not surprising. There are 11 isoforms of PKC, which are not all PMA-responsive and which have can have opposing effects on cellular processes, including tight junction assembly and disassembly (7). PKC activation has previously been shown to increase TER in Caco-2 cells via MLCK phosphorylation (46). This effect was observed within 15 min and was sustained for over 2 h in agreement with the observed melittin kinetics. MLC phosphorylation has been associated with condensation of the actinomyosin ring (47) however, PKC was shown to phosphorylate MLCK resulting in decreased MLC phosphorylation and activity (46). If this is the mechanism triggered following melittin treatment, the effect would be independent of MLCK inhibition, consistent with the lack of effect with W7.

Further studies will need to be carried out to explore these mechanisms. All of these attenuating effects are small, suggesting that there are additional mechanisms also involved. However, a possible mechanism may relate to melittin affinity for the bradykinin receptor (24). Bradykinin can mediate biphasic prostaglandin synthesis via at least two pathways, an immediate response involving PLC and PLA2 and a delayed response involving PKCε and cyclooxygenase-2 (48). PLC and PKC have both been implicated in the reversible melittin response, with PLA2 and cyclooxygenase-2 inhibitors only having effects after extended periods of time (>3 h). PLA2 activation has been shown to indirectly decrease TER of Caco-2 monolayers (41). While further studies are needed to elucidate a mechanism, it is likely that these prostaglandins play some role in the melittin effects on tight junctions.

Overall, melittin has been shown to reversibly open tight junctions in a mechanism involving ZO-1 and F-actin. The process may involve prostaglandin signalling and/or PKC. Further studies will be carried out to elucidate this further. However, the lack of cytotoxicity of melittin in polarized cells suggests that melittin itself may have potential applications as a drug delivery agent. Furthermore, the relatively simple chemistries involved in modification of the melittin structure may allow derivatives with greater selectivity to be developed without compromising the absorption enhancing properties of melittin.

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