Pharmaceutical Research, Vol. 24, No. 7, July 2007 (© 2007)
DOI: 10.1007/s11095-007-9246-z

Research Paper

Melittin as a Permeability Enhancer II: *In Vitro* Investigations in Human Mucus Secreting Intestinal Monolayers and Rat Colonic Mucosae

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Received November 22, 2006; accepted January 19, 2007; published online March 23, 2007

Purpose. Melittin has shown potential as a non-cytotoxic absorption enhancer in Caco-2 monolayers. Our objectives were to assess *in vitro* efficacy and cytotoxicity of melittin in two intestinal permeability models and investigate the potential mechanism by which melittin might enhance gastrointestinal absorption.

Materials and methods. The effects of melittin were examined in the mucus-secreting intestinal cell monolayers, HT29-MTX-E12 (E12), using transepithelial electrical resistance (TER), transmission electron microscopy (TEM) and the MTT viability assay. The effects of melittin on TER, permeability and short circuit current (Isc) were also investigated in rat colon mucosae mounted in Ussing chambers. Ion transporting capacity of tissue was measured in response to secretagogues as surrogate markers of cytotoxicity. Melittin stability was examined by a means of a hemolytic assay. The mechanism by which melittin decreases TER across the rat mucosa was examined with a range of enzymatic inhibitors.

Results. Apical addition of melittin resulted in a reversible non-cytotoxic concentration-dependent decrease in TER across E12 monolayers, which was independent of the presence of mucus. Apical addition of melittin reduced TER and increased the permeability of [^{14}C]-mannitol across rat colonic mucosae. The melittin-induced drop in TER in rat colon was significantly attenuated by W7 suggesting partial mediation by calmodulin.

Conclusions. The rapid and reversible nature of melittin's permeation enhancing properties and its limited cytotoxicity in polarized intestinal epithelia, suggests a potential drug delivery role for the peptide in oral formulations of poorly absorbed drugs.

KEY WORDS: absorption enhancer; gastrointestinal permeability models; melittin; oral peptide delivery; ussing chambers.

INTRODUCTION

The oral route of administration is the most common and desirable mode of delivering drugs to systemic targets. The use of the oral route can be unavailable for drugs that have increased hydrophilicity and/or high molecular weight (1). This is primarily as a result of the intestinal epithelial barrier lining the GI tract which maintains polarity across the GI tract, permits access to nutrients, and restricts absorption of xenobiotics (2). Tight junctions of the GI tract limit oral absorption of more water miscible peptides (3). Hence injected delivery routes must be used for most biotech drugs. Parenteral routes can often result in decreased patient compliance, and therefore advanced formulations which enhance oral bioavailability of class III type molecules are desired (4). The use of paracellular permeability enhancers (PPEs) such as sodium caprate (C10) (5) and chitosans (6) has been shown to increase oral bioavailability of selected drugs to therapeutic levels. Absorption enhancers of greater efficacy and potency are required to represent a realistic option in the routine delivery of biotech therapeutics (7).

Melittin is a 26-amino acid hemolytic (8) antimicrobial peptide which interacts with a broad range of metabolic functions in mammalian cells *in vitro* (9–14). The pore forming ability of melittin is responsible for the antimicrobial effects in bacteria. The mechanism of action of melittin is believed to follow a toroidal pore mechanism were membrane disruption results in rapid cell death (15). The selective toxicity of antimicrobial peptides is based on eukaryotic cell membranes possessing a more neutral charge opposing initial surface interaction (16). The increased lytic effects of melittin over conventional cationic antimicrobial peptides results from a higher hydrophobicity (17). Although the use of this antimicrobial peptide in the treatment of systemic infections is not feasible due to interaction with eukaryotic plasma membranes (9,15) at concentrations equivalent to antimicrobial activity, melittin has demonstrated potent ability to enhance the absorption of a range of molecules, including macromolecules in Caco-2 monolayers with minimal cytotox-

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icity in the polarized target cells (18–20). Melittin resulted in a rapid concentration-dependant decrease and subsequent recovery in TER in Caco-2 monolayers (20) with 1000-fold greater potency than that of C10 (5,21). This decrease in TER was coupled with an increased flux of a range of paracellular flux markers. Furthermore, melittin decreased expression of the tight junction associated proteins, ZO-1, and E-cadherin as well as inducing depolymerisation of filamentous actin (19).

The human intestine has eight sub-populations of cells of which ~24% are goblet cells (22), whereas Caco-2 monolayers comprise only enterocytes (23,24). The production of mucus in the GI tract may potentially reduce the effects of melittin on permeation enhancement. Hence the effects of melittin on a mucus-secreting intestinal monolayer HT29-MTX-E12 (E12) (25) and also on isolated rat colonic mucosae were assessed. The muscle stripped rat colon is a more complex model of the GI tract, which is also composed of normal (non-transformed) cell membrane compositions which may potentially exhibit reduced cytotoxicity in relation to melittin's SAR with eukaryotic cells (16,17).

Our hypothesis is that melittin's interaction with the more robust epithelia of these two gastrointestinal models may be more informative of effects *in vivo* than permeability and cytotoxicity data arising from Caco-2.

MATERIALS AND METHODS

Reagents

Melittin (Serva Inc, Germany) was purchased as a lyophilised powder and exhibited purity in excess of 98% by μRPC and SDS PAGE. The peptide was reconstituted in sterile ultra pure water and frozen in single-use aliquots. Molecular weight determination for final verification of peptide preparation was carried out using MALDI-TOF. The inhibitors: piroxicam (non-specific COX inhibitor), bumetanide (inhibitor of $Na^+K^+Cl^-$ co-transporter), and quinacrine (phospholipase A_2 inhibitor) were purchased from Sigma (Ireland). The calmodulin and myosin light chain kinase (MLCK) inhibitor, N-6-(6-aminohexyl)-5-chloro-1-naphthalenesulfonamide (W7), was purchased from Calbiochem (UK). The concentrations were chosen according to their EC_{50} values for their respective enzyme inhibition. All other reagents were purchased from Sigma (Ireland) unless otherwise stated.

Tissue Culture

Caco-2 (European Collection of Animal cell Cultures, UK) and HT29-MTX-E12 (E12) cells (gift from Professor Per Artursson, Uppsala University, Sweden) are both human intestinal epithelial cell lines that become polarized in continuous culture and display differentiated cell structure and function in a monolayer format. Both Caco-2 and E12 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) with 2 mM L-Glutamine, 1% v/v non-essential amino acids, and 10% v/v fetal bovine serum (All from Sigma-Aldrich, Ireland) at 37°C in a humidified atmosphere with 5% CO_2.

Evaluation of Polarized Epithelial Cell Monolayers

E12 (P40–50) and Caco-2 (P35–50) epithelial monolayers were grown for 21 day continuous culture on 0.4 μm polycarbonate Transwell® inserts (Corning, UK). E12 were seeded at a density of 2×10^4 cells per 12 mm insert and Caco-2 cells were seeded at 5×10^5 cells per insert fed bilaterally every other day (20,26). TER was measured periodically over 24 h in monolayers treated with melittin. The final TER value was obtained by subtracting the intrinsic resistance of the insert from the total resistance of insert and cell monolayer while compensating for filter area. The data was displayed as percentage TER relative to untreated control at time zero.

The apparent permeability coefficient (Papp) was calculated for E12 monolayers with sodium fluorescein as previously described (19). Briefly, flux of sodium fluorescein (500 μg/ml) from apical to basolateral bathing solution (HBSS, supplemented with 11 mM glucose and 25 mM HEPES (pH 7.4) was monitored over 3 h with spectrofluorimetry (495/520 nm).

The effect of melittin on E12 cells in the presence and absence of mucus was evaluated. E12 monolayers were pretreated with 20 mM N-acetyl-L-cysteine (NAC) for 15 min at 37°C to remove the apical mucin secretions (27). The presence and absence of mucins was verified by light microscopy staining with mucicarmine (28). Briefly, monolayers rinsed with pre-warmed HBSS and mounted on slides were treated with mucicarmine staining solution (Fluka, Ireland) rinsed and examined. The uncalibrated optical density of each image was calculated with ACT-1® software package.

The polarity and differentiated cell ultrastructure was visualised by transmission electron microscopy (TEM). E12 and Caco-2 monolayers were rinsed three times with HBSS and fixed with 2.5% (w/v) glutaraldehyde. Sections were embedded in epoxy resin, mounted on 300 mesh copper grid and stained with uranyl acetate and lead citrate and examined (29).

Cytotoxicity Testing

The cytotoxicity of melittin on polarized E12 cells with and without NAC pre-treatment was evaluated using the MTT (methylthiazolydiphenyl-tetrazolium bromide) conversion assay as previously described (20). Melittin was removed from monolayers and MTT (0.5 $mg.ml^{-1}$) added for 2 h. Production of MTT-formazan by metabolically active cells was measured spectrophotometrically (550 nm).

Rat Colonic Tissue Preparations

All animals were treated according to good animal welfare protocols which comply with EU legislation governing the use of animals in research. Animals were maintained on a 12–12 h light-dark cycle with free access to normal food and water. Male Wistar rats (200–250 g) were sacrificed by stunning and cervical dislocation. The colon was removed and placed in Krebs-Heinsleit solution containing 118 mM NaCl, 4.7 mM KCl, 2.5 mM $CaCl_2$, 1.2 mM $MgSO_4$, 1.2 mM KH_2PO_4, 25mM $NaHCO_3$, and 11.1 mM D-glucose continuously bubbled with 95% O_2/5% CO_2 maintaining the pH at 7.4. The mucosa-submucosa preparations were prepared by opening the colon longitudinally

along the mesenteric border followed by blunt dissection removing underlying smooth muscle.

Electrophysiology

Stripped tissue preparations were mounted in Ussing chambers avoiding segments containing lymphoid tissue in distal and proximal regions with a surface area of 0.63 cm^2, bathed bilaterally with 5 ml of Krebs-Henseleit buffer at 37C and bubbled with 95% O_2/5% CO_2 (30). The potential difference (PD, mV) of the mounted tissue was measured in the open circuit configuration. Tissue was then voltage clamped to zero potential difference by insertion of a required short circuit current (Isc, $\mu A.cm^{-2}$) by means of an automatic voltage clamp (EVC-4000 amplifier, WPI, UK). Isc and PD were alternatively monitored by periodically switching to open circuit conditions for 3 s every 30 s using a Pro-4 timing device (WPI, UK). Analogue data signals were digitised with a Powerlab® data acquisition unit and analysed with Chart® software package (AD instruments, UK). The TER ($\Omega.cm^2$) across the tissue was determined using Ohm's law ($R = V/I$). Both Isc and TER were monitored and allowed to equilibrate over a 30 min period until a basal steady state condition was achieved. The tissue was pharmacologically stimulated by either apical or basolateral additions and Isc, PD, and TER monitored for up to 2 h. At the end of each experiment, tissue Isc was stimulated by the cholinergic agonist carbachol (10^{-8}–10^{-4} M), thereby assessing ion transporting capacity, an indirect marker of tissue viability (31).

Transport Studies

The effect of melittin on paracellular flux of mannitol (MW 182) was performed across colonic mucosae mounted in Ussing chambers by luminal addition of 1 μCi/ml D-[^{14}C] mannitol (Amersham Biosciences, UK). Transport of the mannitol was monitored from apical to basolateral chambers over 2 h (32). Apical sampling (100 μl) was carried out at 0 and 120 min, and basolaterally (0.5 mls) every 20 min replenishing with Krebs-Henseleit buffer. Samples were immediately mixed with liquid scintillation fluid and analysed in a scintillation counter (Tricarb, Hewlett

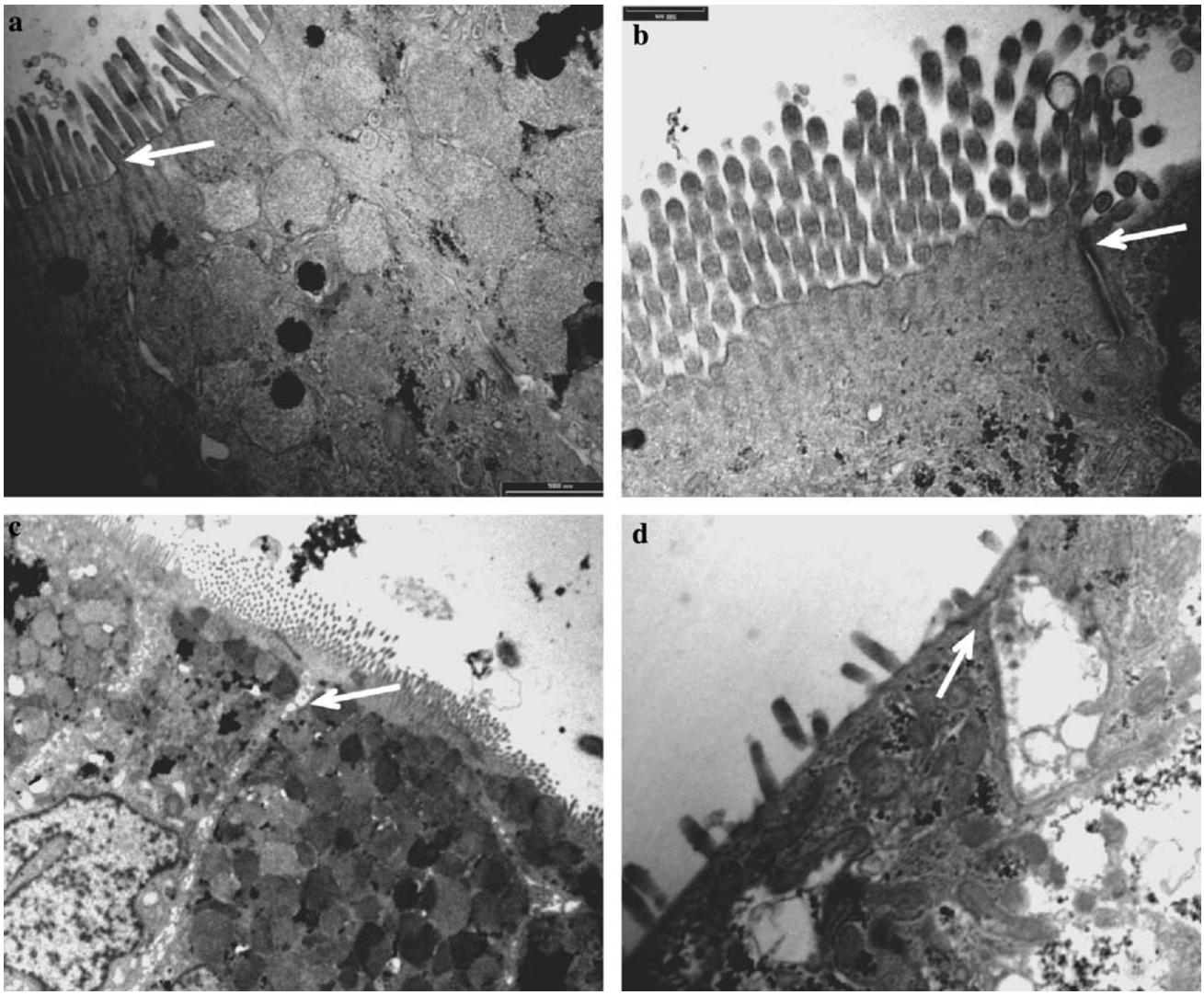

Fig. 1. TEMs showing the effects of apical addition of 3.2 μM melittin on Caco-2, and E12 cultured epithelial monolayers compared with untreated controls. **a** E12 control, **b** Caco-2 control, **c** melittin (3.2 μM) treated E12 monolayers, **d** melittin (3.2 μM) treated Caco-2. Bar in all cases 500 nm. *Arrows* show tight junction in Figure 1a and 1b and disrupted junction in 1c and 1d.

Packard). The amount of transepithelial flux resulting from melittin treatment was determined using the apparent permeability coefficient (Papp). The Papp (cm/s) values were calculated according to Papp = dQ/dt ($1/AC_0$), where dQ/dt is the permeability rate, A is the diffusion area of the monolayer, and C_0 is the initial donor solution concentration (33).

Hemolysis Assay

The hemolytic activity of melittin was used as a marker of melittin stability in apical and basolateral bathing solutions from rat colonic tissue mounted in Ussing chambers. The hemolytic potential was measured using a modified spectrophotometric hemoglobin release assay. An aliquot (100 μl) of fresh sheep erythrocytes (4% v/v) suspended in Krebs-Henseleit solution were treated with 100 μl samples taken periodically from apical and basolateral bathing solutions over 2 h and hemolysis measured as previously described (20).

Statistical Analysis

All experiments were carried out with a minimum of three independent studies unless otherwise stated. All values are expressed as the means ± standard error of the mean. Statistical deductions were carried out by unpaired Student *t*-tests or one way analysis of variance (ANOVA) with Prism® and Minitab® software packages, respectively.

RESULTS

Effect of Melittin on the E12 Monolayers

The TER of untreated E12 and Caco-2 monolayers was ~80 ± 8 Ω.cm^2 ($n = 60$) and 1565 ± 252 Ω.cm^2 ($n = 12$), respectively, on day 21 in culture. The E12 monolayers demonstrated continuous rings of the tight junction protein ZO-1 at junctional sites (data not shown), as well as differentiated morphological features under TEM (Fig. 1a). E12 monolayers exhibited bundles of mucin-like secretory granules at the apical-most surface with exocytosis at various points across the section (Fig. 1a). The columnar E12 monolayers displayed microvilli at the apical surface, and nuclei were localised nearer the basolateral surface (Fig. 1a). The junctional complexes were more densely packed in Caco-2 compared with E12 monolayers (Fig. 1a–b). In comparison, Caco-2 monolayers exhibited brush border like dense microvilli at the apical surface with strong cell-to-cell adhesion, tight junction formation and the noticeable absence of mucin like secretory granules (Fig. 1b). The E12 monolayers exhibited visibly increased mucus production after day 17 in culture.

The effect of melittin on the polarity, differentiation and ultrastructural morphology of E12 monolayers was evaluated after a 2 h exposure using TEM and compared with Caco-2 (Fig. 1c and d, respectively). Melittin (3.2 μM) had significant effects on the Caco-2 cell model compared to control with a decrease in cell-to-cell adhesion and formation of large intercellular spaces, loss of tight junctions, and a decrease in the density of cytoplasmic material. Melittin also had a profound effect on microvilli after 2 h exposure. In the case of E12 monolayers, melittin did not effect the expression of microvilli on the apical surface or result in an increase in cellular debris, however the peptide visually affected cell-to-cell adhesion and tight junction formation.

The effect of apically added melittin on TER was assessed across E12 monolayers by monitoring TER relative to untreated control. Melittin-induced a decrease in resistance across E12 monolayers, which was concentration- and time-dependent with the greatest decrease in TER observed at 180 min and maximal drop observed with 3.2 μM melittin resulting in a 40% decrease in TER (Fig. 2a). The TER then stabilised for approximately 5 h. Apically applied melittin increased permeability to sodium fluorescein in a concentration dependant manner with the greatest effect seen at 3.2 μM ($P < 0.05$) (Fig. 2e). The characteristic layer of translucent mucus at the apical surface of E12 monolayers was removed by pre-treatment with NAC. When stained with the empirical epithelial mucin stain, mucicarmine, for one hour, a five-fold decrease in uncalibrated optical density (OD) (ACT-1 Image Software) of stained light micrographs was observed resulting in E12 monolayers with comparable OD with non-mucus secreting Caco-2 monolayers (Fig. 2c–d). The removal of the mucus layer from the apical surface of E12 monolayers did not impact on the TER reducing effects of the peptide (Fig. 2b). The effects of melittin on TER were also recoverable to within control TER levels after 24 h in a concentration-dependent manner. The removal of mucus however did significantly increased transport of fluorescein in E12 monolayers treated with 3.2 μM melittin compared E12's with mucus ($P < 0.05$) (Fig. 2e).

Effect of Melittin on the Viability of E12 Monolayers

The cytotoxicity of melittin on E12 monolayers was evaluated using the MTT assay after 24 h exposure (Table I). Apical addition of melittin did not affect the viability of E12 monolayers up to concentrations of 5.3 μM. The pre-treatment of E12 monolayers with NAC and subsequent melittin treatment also had no effect on the viability of E12 monolayers.

Effect of Melittin Across Rat Colonic Epithelium

The absorption enhancing effects of melittin were also evaluated across rat colonic epithelial segments mounted in Ussing chambers. While the effects of melittin on TER and Isc were examined over 2 h, the most significant effects were seen over the first 40 min. The basal TER across the rat colonic epithelial sheets ranged from 125–220 Ω.cm^2 and tissues were paired according to comparable resistance following equilibration. Apical treatment with melittin ranging from 5–100 μM resulted in a rapid concentration-dependant decrease in TER with the maximal effects at 10 min with both 5 and 10 μM treatments (Fig. 3a). This was followed by an intermediate recovery over 120 min (data not shown). The lowest concentration of melittin tested (1 μM) did not result in a significant reduction in TER. Melittin resulted in a greater decrease in TER on apical application compared with basolateral application (Fig. 3a). The basolateral addition of melittin (10 μM) had little effect on TER across isolated rat segments (Fig. 3a). To conclusively demonstrate melittin's potential in reducing TER, the

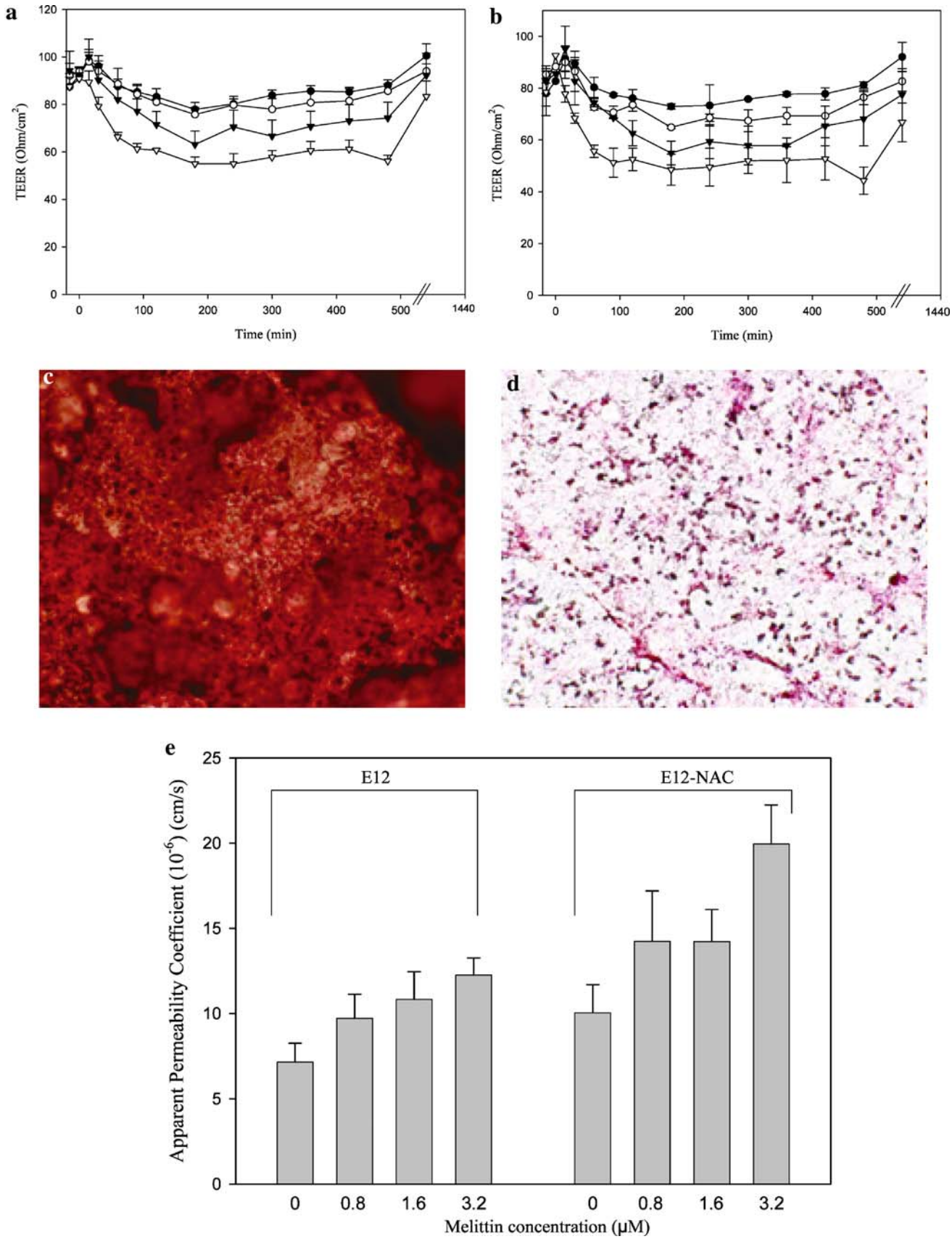

Fig. 2. Effect of melittin on TER and permeability E12 monolayers over 24 h before **a** and after **b** pre-treatment with NAC. Results represent the mean ± SEM of three independent experiments carried out in triplicate. Media control (*open circle*), 0.8 μM melittin (*filled circle*), 1.6 μM (*filled inverted triangle*), and 3.2 μM melittin (*open inverted triangle*). Removal of mucus was confirmed by light microscopy of E12 monolayers stained with mucicarmine (magnification ×10) **c** untreated E12 monolayers, **d** NAC-treated E12 monolayers. **e** Permeability of fluorescein before and after NAC treatment; * $p < 0.05$ relative to media control.

Table I. Viability of E12 Monolayers After Melittin Addition

Melittin (μM)	Viability	
	E12 Monolayers	E12 Monolayers Pretreated with NAC (20 mM)
0	100 ± 0.0	100 ± 0.0
0.8	89.9 ± 4.5	100.4 ± 5.3
1.6	82.9 ± 1.6	135 ± 20.5
3.2	98.7 ± 10.7	98.9 ± 4.7

Viability of E12 monolayers following 24 h treatment with melittin using the MTT assay. Results represent the mean ± SEM of three independent experiments carried out in triplicate.

peptide was tested at 100 μM which was found to irreversibly reduce TER across the rat colonic epithelium by 93% (Fig. 3).

Melittin increased the Papp of [^{14}C]-mannitol across rat colonic mucosa in a concentration-dependent manner (Fig. 3b). Under steady state conditions, the Papp of control mucosae was 2.2×10^{-7} cm/s, which increased almost two- and five-fold following apical addition of 5 and 10 μM melittin treatments, respectively. The direct application of 10 μM melittin to the basolateral bathing solution had no effect on mannitol flux. Addition of 100 μM melittin to the apical bathing solution resulted in a 60-fold increase in Papp to 76.9×10^{-7} cm/s.

Channel forming antimicrobial peptides have been shown to elicit a physiological secretory response across the mammalian GI tract (34). The potential of melittin to interact with secretory homeostasis by altering ion movements was evaluated across the rat colonic mucosa by continuous monitoring of the Isc (μA/cm^2) over 2 h (Fig. 4a). The apical application of melittin resulted in a transient, concentration-dependent increase in Isc which rapidly reached a plateau in less than 5 min, gradually returned to steady state control Isc over 120 min. The basolateral addition of 10 μM melittin increased currents to a plateau of ~180 μA/cm^2 at 15 min ($P < 0.01$) (Fig. 4a). Tissue treated with basolaterally added melittin showed a more gradual decline over 120 min and did not return to steady state following 120 min

The electrogenic ion transporting capacity of colonic tissue, used as an index of viability, was evaluated at the end

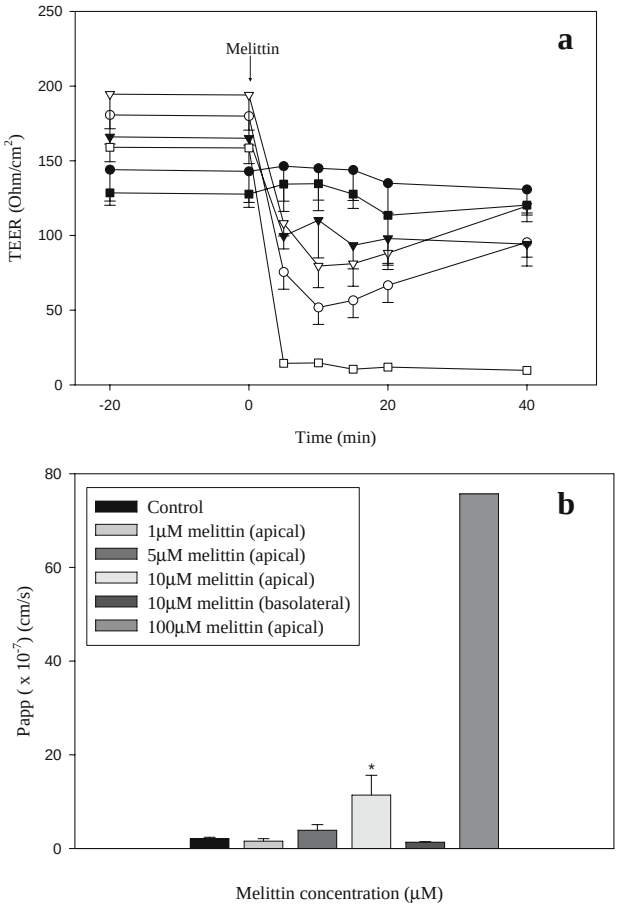

Fig. 3. TER (**a**), and Papp of [14C]-mannitol (**b**) across rat colonic mucosa treated with a range of melittin concentrations applied apically unless otherwise stated. Control (*filled circle*), 10 μM melittin (*open circle*), 10 μM melittin applied basolaterally (*filled inverted triangle*), 5 μM melittin (*open inverted triangle*), 5 μM melittin, 1 μM melittin (*filled square*), 100 μM melittin (*open square*). (Panel **b** (*) significant increase in Papp of [^{14}C]-mannitol on apical application of 10 μM melittin $P < 0.05$) ($n = 4$–8).

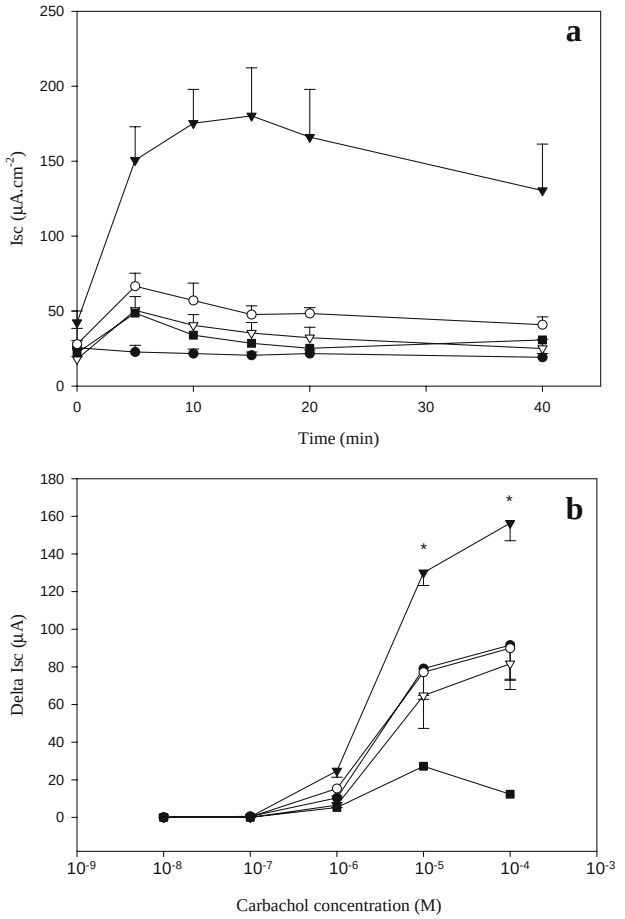

Fig. 4. Effect of melittin on **a** basal (μA.cm^{-2}) and **b** carbachol stimulated (μA) Isc across rat colonic epithelium ($n = 4$–8) were Control (*filled circle*), Melittin 10 μM, apically applied (*open circle*), Melittin 10 μM, basolaterally applied (*filled inverted triangle*), Melittin 5 μM, apically applied (*open inverted triangle*), Melittin 100 μM, apically applied (*filled square*). Panel **a** (*) significant increase in carbachol stimulated Isc when melittin is applied basolaterally ($P < 0.05$). Panel **b** (*) significant increase in basal Isc stimulated by basolaterally applied melittin relative to apical application ($P < 0.05$).

Table II. Effect of Inhibitors on Absorption Enhancement and Ion Transport of Melittin

Inhibitor (conc)	TEER ($\Omega.cm^{-2}$)	Papp ($cm.sec^{-1}$)	Isc(μA)
Control	145.0	2.03	0
Melittin (10 μM)	44.0	10.7	118.0
Melittin + W7 (100 μM)	80.9	3.7	51.74
Melittin + Bumetanide (100 μM)	66.2	4.77	79.0
Melittin + Piroxicam (10 μM)	39.4	12.2	109.1
Melittin + Quinacrine (10 μM)	41.5	12.3	118.0

Effect of quinacrine (10 μM), W7 (100 μM), bumetanide (10 μM), and piroxicam (10 μM) on TER (after 10 min of melittin treatment), Papp of Papp of [14C]-mannitol (over 40 min), maximal change in Isc (μA), cumulative carbachol ion transport (10^{-8}–10^{-4} M) and the Isc after melittin treatment for 10 min across rat colonic mucosa ($n = 3$–6).

of each 2 h melittin treatment period using the cholinomimetic, carbachol (10^{-8}–10^{-4} M). The short circuit current responses of tissue mucosae after 2 h melittin exposure are shown (Fig. 4b). The apical application of 5 and 10 μM melittin did not significantly affect the responsiveness of tissue to the agonist carbachol, however, when applied basolaterally resulted in a significant increase in carbachol-stimulated chloride secretion ($P < 0.05$) (Fig. 4b). The apical addition of 100 μM melittin resulted in a decrease in carbachol-stimulated electrogenic chloride secretion indicating melittin may impede secretion at higher concentrations.

Analysis of Modulators of Reported Melittin Activities

To elucidate the mechanisms of melittin's permeation enhancing effects, and stimulation of Isc, mucosae were pretreated with a selection of inhibitors at concentrations equivalent to their EC_{50} values, and the effect on TER and Isc was evaluated (Table II, Fig. 5). Bilateral addition of the calmodulin/MLCK inhibitor, W7 (100 μM), significantly attenuated the subsequent effect of melittin on TER ($P < 0.05$) (Fig. 5a). W7 also resulted in a 2.3-fold decrease in mannitol permeability relative to melittin alone ($P < 0.05$) (Fig. 5b). Furthermore W7 was found to decrease the maximal ΔIsc induced by apical addition of 10 μM melittin from 118 μA to 51.7 μA ($P < 0.05$) (Table II).

Non-specific downstream blockage of cyclooxygenase I and II by piroxicam significantly reduced ion transport induced by 10 μM melittin (Table II). In addition the loop diuretic, bumetanide significantly reduced the extent of melittin's apical induced secretory response (Table II). Melittin has been reported as an agonist of phospholipase A2 (35). However, the phospholipase A2 (PLA_2) inhibitor, quinacrine (10 μM) had no significant effect on melittin-induced decreases in TER or on the induced increase in ion secretion/absorption (Table II). With the exception of bumetanide (36) each inhibitor/melittin combination assayed did not effect the cumulative carbachol response.

Stability of Melittin During Incubation with Rat Colonic Mucosae

The stability of melittin in Ussing chambers was assessed using a hemolysis assay with a limit of sensitivity of 0.35 μM melittin ($n = 5$). Solutions taken from apical or basolateral tissue incubated for set periods in Ussing chambers and bathed in Krebs-Henseleit solution did not result in an increase in hemolysis of sheep erythrocytes. The addition of 10 μM melittin to the apical bathing solution at time zero resulted in 100% hemolysis which was abolished after 20 min incubation and not detected following 40 min indicating a rapid loss of structural integrity (Fig. 6). The addition of complete protease inhibitor cocktail® to the apical bathing solution significantly slowed the deterioration of melittin to at 20 min ($P < 0.01$). Hemolytic activity was still detectable (3.5%) after 40 min incubation of mucosae with melittin in the presence of the cocktail®. Basolateral addition of melittin

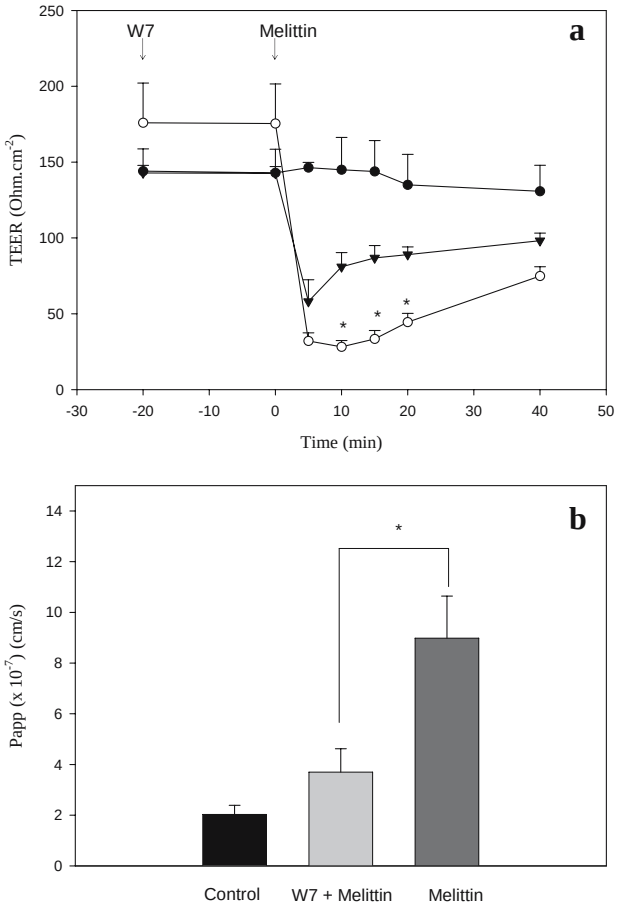

Fig. 5. Effect of the calmodulin antagonist, W7 (100 μM), on **a** TER and **b** Papp of [14C]-mannitol across rat colonic mucosa treated with apically-added melittin (10 μM) ($n = 3$–6). Control (*filled circle*), Melittin 10 μM, apically applied (*open circle*), bilateral W7 (100 μM) and apical melittin (10 μM) (*filled inverted triangle*). Panel **a** (*) significant difference in TER between 10 μM melittin in the presence of W7 ($P < 0.05$). Panel **b** (*) significant decrease in Papp between 10 μM melittin in the presence of W7 ($P < 0.05$).

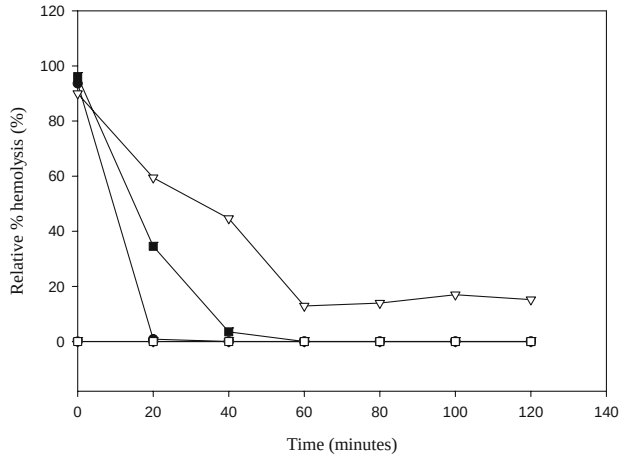

Fig. 6. Bioactivity of melittin apical and basolateral bathing solutions of mounted rat colon using hemolytic activity. Apically applied melittin sampling the apical chamber (*filled circle*). Apically applied melittin sampling the basolateral chamber (*open circle*). Basolaterally applied melittin sampling the apical chamber (*filled inverted triangle*). Basolaterally applied melittin sampling the basolateral chamber (*open triangle*). Apically applied melittin pretreated with 1x complete protease inhibitor cocktail sampling the apical chamber (*filled square*). Apically applied melittin pretreated with complete protease inhibitor cocktail sampling the apical chamber (*open square*). All data represents the mean of two independent events ± SEM.

exhibited hemolytic activity in the basolateral chamber of 58 and 44% after 20 and 40 min, respectively, suggesting greater stability than apically applied melittin. Melittin was not detected in basolateral or apical chambers when the peptide was applied to apical or basolateral chambers suggesting the peptide does not travel across via its own permeation enhancing effects.

DISCUSSION

There are several reports on the interaction of melittin with mammalian cell structure and function including pathways which are linked with paracellular permeability. These include signalling molecules such as protein kinase C (PKC) (37), phospholipase A2 (PLA2) (35) and calcium dependant calmodulin (38). These aspects of melittin's activity and its ability to disrupt the integrity of the cell membrane may be involved in the peptide's cytotoxicity which is considered a limiting factor in the use of melittin in infectious disease (20). Cytotoxicity analysis showed melittin was less toxic towards polarized Caco-2 monolayers relative to non-polarized Caco-2 or hematopoietic cells (19,20). The fact that melittin had no effect on the viability of E12 monolayers in the current study and Caco-2 monolayers (19) suggests that the peptide is less cytotoxic than we previously thought (20). This phenomenon was not unique to melittin, reports show *Pseudomonas* Exotoxin T and poly(N,N-dimethylaminoethyl methacrylate) (pDMAEMA) were found to exhibit greater cytotoxicity in cells that were not polarized (26,39). Should melittin be restricted to the gastrointestinal epithelium, the potential site of action, the cytotoxicity in the gut and the efficacy of the peptide as a permeation enhancer become key factors.

Melittin decreased TER and increased the Papp of fluorescein across E12 monolayers in a concentration-dependent fashion, however it did not exhibit the same magnitude of effect as in Caco-2 monolayers (19). At all concentrations tested in E12 monolayers, melittin treatment was at least partially recoverable to within control TER, which was not the case in the equivalent melittin treated Caco-2 monolayers. The reduced effects of melittin in E12 monolayers, albeit significant, may be related to the relative tightness of the Caco-2 monolayer (TER > 1,000 $\Omega.cm^{-2}$) compared with E12 monolayers (~80 $\Omega.cm^{-2}$). The effect on E12 monolayers suggest that melittin may have efficacy in human GI which has a TER much closer to that of E12 monolayers than to Caco-2 monolayers (40,41). In addition, the fact that the peptide's effects on TER were at least partially recoverable at all concentrations, along with reduced extent of transport of sodium fluorescein, lack of visual damage and lack of cytotoxicity suggests melittin has effects on E12 monolayers which are quantitatively different to Caco-2 monolayers. This was not only due to the mucus layer at the apical surface of E12 cells preventing the melittin from diffusing to the site of action, as treatment with mucolytic, NAC, did not alter melittin's effects on TER, although it did affect the extent of fluorescein transport at melittin concentrations above 3.2 μM ($P < 0.05$). This data implies that mucus has a role in the reduced absorption enhancing effects of melittin in E12 monolayers and that melittin has reduced permeability enhancing properties in E12 monolayers relative to Caco-2 monolayers.

Comparisons in Caco-2 monolayers shown melittin (μM) has greater effect with respect to TER reduction and transport of FITC dextrans over C10 (10–15 mM) (5,21), which is in advanced clinical trials as an absorption promoter in oral preparations (7). The effects of melittin were also found to be more potent than cytochalasin B, which results in a more irreversible and gradual reduction in TER (42). The effect of melittin on TER was more comparable with that of Zonula occludens toxin (Zot) (43,44) and chitosans (45,46), i.e. modulation was reversible, time- and concentration-dependant. Unlike melittin, Zot does not affect the permeability of the colon, due to lack of appropriate receptors. The profile by which melittin effects TER in Caco-2 was also similar to chitosan (46) and N-trimethyl chitosan (47), which cause a rapid and reversible decrease in TER. However melittin was more effective at reducing TER with a more rapid recovery (46).

The morphology of Caco-2 cell monolayers was altered by melittin treatment (20), although, the lack of cytotoxicity suggests the apparent damage did not effect viability. The visual effects of melittin were not as dramatic in E12 cell monolayers compared with Caco-2 monolayers, which coincided with TER data. However in sections analysed, melittin reduced cell-to-cell adhesion, which in conjunction with TER and fluorescein studies is indicative of an increase in paracellular permeability. Melittin did not appear to affect cellular functions such as mucus secretion, nor expression of microvilli in E12 cells, in contrast with Caco-2 cells (Fig. 1 and (20)).

Caco-2 monolayers have been shown to be more susceptible to the effects of permeation enhancers compared with whole tissue (48), however, the absorption enhancing effects of melittin seen in cultured epithelial cells were also shown across rat colonic mucosae. The effects of apical melittin on TER across rat mucosa were rapid, although, a higher concentration of melittin was required to bring about

an increase in paracellular permeability across the tissue sections. In culture models, the peptide's permeation enhancing effects were seen at concentrations around 0.7–3.2 μM while comparable effects required 5–10 μM in rat mucosae (19). This may be due to a number of reasons. It has been suggested that antimicrobial peptides have a greater surface interaction with more negative charged phospholipids which is responsible for the selective toxicity of the peptides (17). Since transformed cells can have a greater percentage of anionic phospholipids such as phosphatidylcholine (3–9%) (16), melittin may have a reduced interaction with the normal rat mucosa. Furthermore, this may be related to varied expression of target receptors, the multilayer nature of the rat mucosa with epithelium and associated plexus, or the presence of a mucus layer in tissue. The application of 100 μM melittin demonstrated the maximum capacity of the peptide's absorption enhancing properties in rat colon by irreversibly decreasing TER and increasing mannitol flux by 35-fold relative to untreated control. Melittin (5–10 μM) had no effect on the cholinergically induced secretory response stimulated by carbachol indicating the peptide does not affect viability of the tissue sections over a 2 h treatment period. This data confirms melittin's reduced cytotoxicity in polarized cells and in isolated tissue sections. The fact that C10 abolished a carbachol secretory response in isolated colonic tissue (49) and has been given to patients as a formulated absorption enhancer in clinical trials without compromise in safety (7) suggests melittin may be adminisered safely in oral formulations.

The basolateral application of 10 μM melittin did not significantly affect the TER or the apical to basolateral flux of [^{14}C]-mannitol suggesting that the peptide interacts with an apical receptor in the rat mucosa. This data compared well with our previous observations in Caco-2 models (19). Since melittin did not reduce TER when applied basolaterally in Caco-2 monolayers, it is likely melittin absorption enhancing effects are limited to apical application, a desirable property for an orally administered delivery agent.

Stimulation of Isc following basolateral addition of melittin shows the peptide not only acts on the paracellular permeability and to a lesser extent ion secretion on the apical surface, but also possesses site specific electrolyte secretion on the basolateral surface. The basolateral effects of melittin appear to be independent of apical effects. The increase in ion transport when melittin is applied apically, while rapid, transient and significantly lower than basolateral addition, is linked with the peptides absorption enhancing effects across rat mucosae as the inhibitors W7, and bumetanide significantly attenuate both paracellular and transcellular secretion pathways concurrently. The increase in Isc following basolateral addition was more gradual, plateaued at a significantly higher Isc, and furthermore was independent of permeation enhancement further suggesting melittin effects are polarised, similar to the absorption enhancement in Caco-2 monolayers (19). The effect of the basolateral application of melittin was partially attributable to electrogenic chloride secretion which has previously been shown across rat colonic mucosae (50). The increase in ion secretion may be an undesirable property of the peptide leading to diarrhoea and dehydration.

The rapid concentration-dependent increase in paracellular permeability and apical specific effects of the peptide in Caco-2 and rat colonic mucosa suggests that melittin acts via an apical signalling mechanism. In our previous reports melittin resulted in a concentration-dependent decrease in the expression of the ZO-1 with reorganisation of filamentous actin (19). The absorption enhancing effects of melittin involve actin comparable with other potent absorption enhancers; cytochalasin B, Zot (*V. cholera*), and C10. Melittin is a potent competitive inhibitor of calmodulin (51), an essential mediator of cytoskeletal assembly through MLCK phosphorylation (52), suggesting a role for calmodulin in the absorption enhancing effects of melittin. Furthermore, melittin has both PLA_2 activating and PKC inhibitory effects which have been reported to play a central role in paracellular permeability. Our previous reports show both melittin's PKC and PLA2 activation play a role in the preliminary decrease in TER or the rate of recovery (19).

Within the current report W7 reduced the extent by which melittin increased paracellular permeability, similar to C10 (53), indicating that the activity of melittin in rat tissue is partially calmodulin-dependant. Melittin is a calmodulin inhibitor (9,51,54) therefore the W7-mediated attenuation in melittin's effects may be due to a W7-induced conformational change in the calmodulin structure, resulting in reduced melittin binding. Melittin has previously demonstrated reduced binding to calmodulin in the absence of calcium (55). Calcium binding increased the exposure of hydrophobic moieties on the proteins surface resulting in greater hydrophobic interactions with the hydrophobic moieties of melittin. The effects of W7 across the rat colonic mucosa did not correlate with Caco-2 monolayers as pre-treatment of Caco-2 cells prevented a significant recovery of TER (19). The role of calcium in the melittin response in epithelial is not clear-cut and will be the subject of future studies.

Ion transport stimulation on basolateral melittin treatment has been previously examined in rat colon mucosae (50). Low concentrations of melittin (0.17–0.7 μM) increased Isc due to the peptide's PLA2 activation, with resultant prostaglandin E2 production. PLA2 is also linked with absorption enhancement, its activation mediates the hydrolysis of phosphatidylcholine to lysophosphatidylcholine which decreased TER in Caco-2 monolayers (35), suggesting that the concurrent permeation enhancing and ion secretory effects of melittin may involve PLA2. Quinacrine, an inhibitor of PLA2, had little effect on apical melittin-induced alterations in barrier function or Isc, suggesting a limited role for PLA2. However, the inhibition of COX by piroxicam which prevents the production of prostaglandin E2 partially inhibited melittin-induced increase in Isc. Other activities of melittin include pore formation in prokaryotic, eukaryotic and artificial membranes. This ability is based on the hydrophobicity of melittin. It has previously been shown that channel forming peptides, cryptdins 2 and 3 form anion conductive pores in the plasma membrane of T84 monolayers which led to a reversible and dose dependant increase in chloride secretion (34). The physiological chloride secretory response induced by these secretagogues describes a novel mechanism of paracrine secretion which may to some extent apply to melittin.

The stability of melittin across the rat colonic mucosa was examined by evaluating the peptide's hemolytic activity at time intervals during tissue incubations. Since pore formation requires the intact peptide, this assay was an indirect surrogate

bioassay of melittin, however does not demonstrate the complete deterioration of all bioactive elements. This is emphasised as tryptic fragments of melittin have also been shown to bind to calmodulin (51,54). The hemolytic activity of melittin was rapidly reduced in solutions bathing colonic tissue segments mounted Ussing chambers. Following apical addition of melittin, no hemolytic activity was detected in the basolateral bathing solution at any time point indicating the peptide had not transported across the monolayer, at least to the level of sensitivity (a 10% flux). The decrease in melittin's hemolytic activity was, at least in part, related to actions of apical proteases. Pre-treatment with protease inhibitor cocktail significantly attenuated melittin deterioration suggesting it is digested by peptidases. Basolateral addition resulted in a more gradual deterioration, with hemolytic activity remaining after 120 min. The significant increase in melittin in free solution is like to be due to reduced prevalence of digestive enzymes on the basolateral surface. The proteolysis of melittin may be responsible for the recovery of TER across the rat mucosa over 2 h and may reduce its oral bioavailability, minimising the potential for systemic toxicity. Alternatively, it should be possible to use peptidase inhibitors to maintain melittin intact for longer periods in different intestinal regions.

In conclusion, low concentrations of melittin added to the apical side induces a rapid, reversible increase in paracellular permeability in intestinal monolayers and isolated rat colonic epithelium. Potent permeation enhancing effects of melittin were not cytotoxic in both cell and tissue models, although at higher concentration reduced ion transport capacity of rat colonic epithelium. This along with rapid clearance of the peptide, poor absorption across the isolated tissue, and development of delivery methods for site specific release of peptides (e.g. colonic (56)) suggests melittin or stable derivatives may be used in therapeutic delivery of oral preparations. The mechanism by which melittin reduces TER in rat colonic epithelium is partially calmodulin dependent, while PLA2 has no apparent role in melittin absorption enhancing effects.

ACKNOWLEDGEMENTS

This work was supported by the Programme for Research in Third Level Institutions (PRTLI) administered by HEA (Ireland) and the Health Research Board (Ireland). The authors would also like to express their gratitude to Professor Alan Baird, University College Dublin, for helpful discussions during these studies, Mr Pat Kearns for help with handling of rats and James Reilly (ITT Dublin) for advice with statistical analysis. HT29-MTX-E12 cells were a generous gift from Professor Per Artursson, Uppsala University, Sweden.

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