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Site of fluorescent label modifies interaction of melittin with live cells and model membranes

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ABSTRACT

The mechanism of membrane disruption by melittin (MLT) of giant unilamellar vesicles (GUVs) and live cells was studied using fluorescence microscopy and two fluorescent synthetic analogues of MLT. The N-terminus of one of these was acylated with thiopropionic acid to enable labeling with maleimido-AlexaFluor 430 to study the interaction of MLT with live cells. It was compared with a second analogue labeled at P14C. The results indicated that the fluorescent peptides adhered to the membrane bilayer of phosphatidylcholine GUVs and inserted into the plasma membrane of HeLa cells. Fluorescence and light microscopy revealed changes in cell morphology after exposure to MLT peptides and showed bleb formation in the plasma membrane of HeLa cells. However, the membrane disruptive effect was dependent upon the location of the fluorescent label on the peptide and was greater when MLT was labeled at the N-terminus. Proline at position 14 appeared to be important for antimicrobial activity, hemolysis and cytotoxicity, but not essential for cell membrane disruption.

1. Introduction

Melittin (MLT) is a small membrane-active peptide [1] which consists of 26 amino acid residues and is the major toxic component of bee venom [2–4]. Some of its properties include being antimicrobial [5], cytolytic [6,7], cationic (+5) and hemolytic [8,9]. The MLT peptide has an amphipathic structure [10,11], a strong binding affinity to lipid membranes [12] and can exist in two orientations, parallel or perpendicular to the membrane bilayer [13]. Furthermore, MLT is known to induce changes in the integrity of plasma membranes and lipid bilayers resulting in pore and ion channel formation as well as vesicle deformation [4,14]. Pore formation is likely to be the result of perpendicular insertion into the membrane while parallel orientation with the membrane may be its inactive state [13,15]. However, the underlying mechanism of membrane insertion and disruption upon MLT exposure is unclear because its interaction with the membrane is dependent on lipid bilayer composition [16] and peptide concentration [16,17].

To investigate the interaction between peptides and cell membranes, which is a fundamental biological process involved in ion channel formation, cytolysis and signaling [18], membrane fusion and disruption [19], and endocytosis and exocytosis [20], various techniques have been used including solid-state NMR spectroscopy [3,21], isothermal titration calorimetry [22], fluorescence [23,24] and circular dichroism (CD) spectroscopy [25]. Previous investigations of the action of MLT on cell membranes typically used synthetic model membranes rather than biological membranes due to the complexity and heterogeneity of biological membranes [26] as well as the deleterious hemolytic activity of MLT in vivo [27].

Melittin also exhibits anticancer properties [28,29] that may be mediated by the activation of the ubiquitous enzyme, phospholipase A2 (PLA2) [30,31], which catalyzes hydrolysis of sn-2 acyl bond of phospholipids resulting in the release of fatty acids [32]. This lipid–peptide interaction leads to changes in structure of cell plasma membranes causing vesiculation or blebbing [33]. These spherical outgrowths from the plasma membrane are due to the detachment of plasma membranes that are devoid of cytoskeleton material [34,35].

Here, we used light and fluorescence microscopy to visualize the interaction of MLT with model membranes and HeLa cells. Previous fluorescence-based studies on the interaction of melittin with membranes used fluorescence fluorescent labels attached to the N-terminal amine or lysine-7 [36]. In our study, two fluorescent analogues of MLT were prepared, the first being a wild type MLT containing a thiopropionic acid (TPA) residue at the N-terminus. Since proline at

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position 14 has been identified as an important amino acid for the antimicrobial activity of MLT [37], the second MLT analogue had the Pro replaced with a cysteine (C) residue. AlexaFluor 430 was attached to TPA and cysteine using thiol-maleimide chemistry. Using these two fluorescent MLT analogues, we identified an important role for both Pro14 and the N-terminal region for membrane disruptive activity and cytotoxicity.

2. Materials and methods

2.1. Peptide synthesis

The two analogues of MLT were prepared using Fmoc/tBu chemistry via microwave irradiation-enhanced solid phase synthesis on a CEM Liberty synthesizer [38,39]. Fmoc-protected amino acids were purchased from GL Biochem (Shanghai, PRC). Fmoc removal was via 20% piperidine solution in dimethylformamide (DMF; Merck, Sydney, Australia) at 75 °C for 3 min. Coupling of each HCTU/DIEA-activated Fmoc-amino acid was performed at 75 °C for 5 min, except for cysteine (50 °C for 5 min) and arginine (twice 50 °C for 5 min). In one MLT analogue, 3-thiopropionic acid (TPA) (Sigma-Aldrich, Sydney, Australia) was attached to the N-terminus of MLT leading to TPA-MLT. In the second, Pro-14 was replaced by a cysteine leading to MLT(P14C). After cleavage and simultaneous side-chain deprotection with trifluoracetic acid (TFA) in the presence of scavengers as previously described [38, 39], the crude peptides were isolated by precipitation with ice-cold ether and then purified by RP-HPLC using a Phenomenex C4 column (particle size 5 μm, 4.6 × 150 mm), with gradient of 10% A to 90% B 30 min (A: 0.1% TFA (Auspep, Melbourne, Australia) in H_2O; B: of 0.1% TFA in acetonitrile). The TPA-MLT and MLT(P14C) were obtained in overall yields of 15% and 18%, respectively, and 22% for native MLT calculated from 0.1 mmol starting resin.

2.2. Maleimide functionalization of AlexaFluor 430

AlexaFluor 430 is commercially available only in NHS ester form (Invitrogen, Sydney, Australia) which is amine-reactive. A major limitation of using this reagent in solution is that the peptide will be labeled at multiple sites with free NH_2 groups. To avoid multiple labeling, the NHS ester of AlexaFluor 430 can be selectively attached to the side chain of Lys by solid phase methods following on-resin N^{ε}-deprotection. However, this approach can consume a lot of dye which is very expensive [40]. Therefore, thiol-maleimide conjugation chemistry was used to attach the AlexaFluor 430 dye to the MLT peptide in solution. Subsequently, a coupling reaction was performed between the NHS-AlexaFluor 430 (1.4 μmol, 1 mg) and *N*-(2-aminoethyl)maleimide (Sigma-Aldrich, Sydney, Australia) (1.68 μmol, 0.43 mg) in the presence of DIEA (2.68 μmol, 0.05 μl) in DMF. The reaction was monitored by HPLC and purified with a gradient of 10% A to 90% B leading to 53% yield of Mal-AlexaFluor 430.

2.3. Conjugation of Mal-AlexaFluor 430 to MLT peptides

The presence of a TPA residue at the N-terminus of MLT (TPA-MLT) enabled the attachment of Mal-AlexaFluor 430 to the thiol of TPA. For the mutant analogue, MLT(P14C), the AlexaFlour 430 was attached to the side-chain of the replaced cysteine residue. MLT(P14C) or TPA-MLT and Mal-AlexaFluor 430 were dissolved separately in minimum amount of MilliQ water. Then, the TPA-MLT or MLT(P14C) solution (0.702 μmol, 2 eq) was added to Mal-AlexaFluor 430 (1.6 μmol, 1 eq) solution drop by drop. The pH was adjusted to 7.4 using 0.2 M phosphate buffer. The reaction was completed in 30 min at room temperature. The peptides were then purified using a preparative RP-HPLC C4 column with a gradient of 35% A to 65% B for 30 min. The residual TFA was removed by freeze-drying the peptide from dilute aqueous HCl. Fig. 1 shows the MALDI TOF mass spectra and RP-HPLC profiles for the purified peptides. 9.77 mg (40% yield) of Fluor-TPA-MLT (F-MLT) and 11 mg (45% yield) of MLT(P14C)-Fluor (MLT(P14C)-F) were obtained from 3 mg of dye (Mal-AlexaFluor 430).

2.4. Circular dichroism (CD) spectroscopy

CD spectroscopy was used to probe the secondary structure and folding of MLT peptides and to determine conformational changes upon addition of lipid vesicles. Phospholipids, palmitoyloleoylphosphatidylcholine (POPC), palmitoyloleoylphosphatidylethanolamine (POPE) and palmitoyloleoylphosphatidylglycerol (POPG) were purchased from Avanti Polar Lipids (Alabaster, USA). Cholesterol (Chol) was purchased from Sigma-Aldrich (St Louis, USA). Multilamellar vesicles (MLVs) were prepared by dissolving desired amounts of phospholipid ± Chol in chloroform: methanol (3:1), after which the organic solvent was evaporated under vacuum to obtain a lipid film. The lipid film was placed under vacuum overnight to evaporate residual organic solvents. The dried film was resuspended in MilliQ water, homogenized, divided into aliquots and then lyophilized for each experiment. Large unilamellar vesicles (LUVs) were prepared by extruding MLVs through 100 nm polycarbonate membranes (Whatman Nuclepore, GE Healthcare, Sydney, Australia) in 10 mM Tris buffer. The CD spectra were recorded on a Chirascan-plus instrument (Applied Photophysics, Leatherhead, UK) between 190 and 260 nm. The cell path length was 0.1 cm and all measurements recorded at 25 °C [25]. Peptides were prepared in 10 mM Tris buffer (pH 7.0) plus 10 mM NaCl, and the final concentration was 10 μM. Helix content of peptide was calculated using the formula $^{max}[\theta]_{222} = -40{,}000 \times [(1-2.5/n)] + (100 \times T)$, where n is number of amino acid residues and T is temperature in °C [41]. Percentage helicity was calculated as $100 \times [\theta]_{222} / {}^{max}[\theta]_{222}$. Q6 Q7

2.5. Giant unilamellar vesicles (GUVs)

GUVs were prepared by adding a desired amount of 0.1 M phospholipid solution of dipalmitoylphosphatidylcholine (DPPC) or dimyristoylphosphatidylcholine (DMPC) dissolved in chloroform to a mixture of chloroform:methanol in a ratio of 9.8:1 [42]. To detect the membrane bilayer of the GUVs, the red fluorescent dye, BODIPY® TR ceramide (Life Technologies, Australia) was added to the lipid solution mixture at a ratio of 1:200 of dye:lipid. Filtered HEPES buffer solution was carefully added to the organic phase of the mixture to obtain 285 μM of vesicle sample solution. Vacuum rotary evaporation was used to remove the organic phase (at 40 rpm), 40 °C and 100 mBar minimum pressure. The remaining aqueous GUV suspension was stored at room temperature for less than 1 week before analysis. Specific volume of GUVs and MLT peptides (dissolved in HEPES buffer) was transferred to a microscopy chamber (ibidi GmbH, Germany) at a molar ratio of 1:27. The fluorescent GUVs were visualized using DeltaVision DV Elite™ Restorative Wide and a 60× objective. The Alexaflour 430 was detected using the green channel filter with excitation/emission wavelength of 490/510 nm and the BODIPY® TR ceramide was detected using the red channel filter (587/610 nm). Experiments were repeated 3 times.

2.6. Hemolytic activity of test peptides

The hemolytic activity of the test compounds was measured by incubating (1–2) × 10^8 human red blood cells (RBC) with peptides in phosphate buffered saline (PBS) in a final volume of 70 μl for 6 min at room temperature. After centrifugation at 210 ×*g* for 4 min, 2 μl of supernatant was transferred into a 96-well plate and diluted with PBS to a final volume of 100 μl, after which the absorbance at 412 nm was determined. Data were normalized against RBC lysed with sodium dodecyl sulfate. Distilled water was used as the positive control and PBS (pH 7.4) was used as the negative control, respectively. One HU (hemolytic unit) was defined as the minimum amount of dissolved

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