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Imaging the membrane lytic activity of bioactive peptide latarcin 2a

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ABSTRACT

Latarcin 2a (ltc2a, GLFGKLIKKF**G**RKAISYAVKKARGKH-COOH) is a short linear antimicrobial and cytolytic peptide extracted from the venom of the Central Asian spider, *Lachesana tarabaevi*, with lytic activity against Gram-positive and Gram-negative bacteria, erythrocytes, and yeast at micromolar concentrations. Ltc2a adopts a helix–hinge–helix structure in membrane mimicking environment, whereas its derivative latarcin 2aG11A (ltc2aG11A, GLFGKLIKKF**A**RKAISYAVKKARGKH-COOH), likely adopts a more rigid structure, demonstrates stronger nonspecific interaction with the zwitterionic membrane, and is potentially more toxic against eukaryotic cells. In this work, interactions of these two ltc2a derivatives with supported "raft" lipid bilayer (1,2-dioleoyl-*sn*-glycero-3-phosphocholin/egg sphingomyelin/cholesterol 40/40/20 mol%) were studied by *in situ* atomic force microscopy in order to investigate the potential anticancer activity of the peptides since some breast and prostate cancer cell lines contain higher levels of cholesterol-rich lipid rafts than non-cancer cells. Both peptides induced reorganization of the raft model membrane by reducing line tension of the liquid ordered phase. Ltc2aG11A induced membrane thinning likely due to membrane interdigitation. Formation of large pores by the peptides in the bilayer was observed. Cholesterol was found to attenuate membrane disruption by the peptides. Finally, leakage assay showed that both peptides have similar membrane permeability toward various model membrane vesicles.

1. Introduction

Antimicrobial peptides (AMPs) are innate host defense components present in various organisms to defeat a wide spectrum of pathogens, including bacteria, viruses, and fungi. AMPs are typically short amphipathic peptides containing both cationic and hydrophobic amino acid residues [1–6]. Over 1200 AMPs have been discovered but the mechanisms of actions remain uncertain [4]. Various models—such as barrel-stave, toroidal, and carpet—have been proposed, with the Shai–Matuszaki–Huang model being the most comprehensive [3–6]. In summary, the Shai–Matuszaki–Huang model explains that the peptides carpet the membrane and the insertion causes area expansion of the outer leaflet and membrane thinning. The strain between outer and inner leaflets generates tension within the bilayers resulting in toroidal pores, which allow transportation of peptides and lipids into the inner leaflet. The peptides can then diffuse onto intercellular targets or cause physical disruption and collapse of membrane, both of which result in cell death [3].

Various studies have shown that AMPs share similar properties with cationic amphiphilic anticancer agents that can be categorized into two classes: agents toxic to both bacterial and cancer cells but not mammalian non-cancer cells, and agents toxic to all cell types, including mammalian non-cancer cells [7,8]. Cancer cell membranes contain more anionic molecules, such as phosphatidylserine lipids and O-glycosylated mucins [7,8]. Thus cationic anticancer agents preferentially bind to such anionic membranes through nonspecific electrostatic interaction. In addition, cancer cells contain higher numbers of microvilli- minute projections of the cell membrane—providing a larger surface [7,8] and were also shown to be more fluid than membranes of normal cells, making them more susceptible for disruption by anticancer agents [9]. Thus differences in lipid composition, fluidity, and surface area between normal and cancer cell membranes contribute to the activity and efficiency of anticancer agents.

Recently, a new family of antimicrobial peptides called latarcins was purified from the venom gland of the Central Asian spider *Lachesana tarabaevi* [10]. Seven structurally unrelated groups of short linear cysteine-free membrane-active peptides that are lytic to Gram-positive and Gram-negative bacteria, erythrocytes, and yeast at micromolar concentrations were identified [10]. A 26 amino acid residue peptide latarcin 2a (ltc2a, GLFGKLIKKFGRKAISYAVKKARGKH-COOH) has broad-spectrum antibacterial activity against Gram-positive and Gram-negative bacteria with MIC values in the micromolar range [10], while having moderate hemolytic activity toward human erythrocytes with an EC_{50} in the 3 to 6 μM range [11,12]. Both molecular dynamics simulation and NMR data indicate that ltc2a adopts a helix–hinge–helix structure in a small micelle with N-terminus helix embedded in the micelle and C-terminus laying on the membrane [13,14]. However, in a larger micelle, molecular dynamics simulations showed that two helices of ltc2a are angled 150º apart and lie peripherally on the membrane [14]. To investigate membrane binding

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and rupturing by ltc2a, in a previous study we compared ltc2a to its mutant ltc2aG11A, GLFGKLIKKF**A**RKAISYAVKKARGKH-COOH and found that by replacing glycine 11 with alanine at the hinge region, a more rigid peptide with reduced conformational flexibility is obtained. The mutant ltc2aG11A has stronger nonspecific interaction with zwitterionic cell membranes, thus potentially leading to higher toxicity against eukaryotic cells [15].

It has been suggested that cholesterol in eukaryotic cell membranes prevents the cytolytic effect of antimicrobial agents [16]. Membranes with higher cholesterol levels had decreased rate of peptide insertion [17]. At the same time, Li et al. suggested that some breast and prostate cancer cell lines contain higher levels of cholesterol-rich lipid rafts than non-cancer cells [18], pointing at the relevance of raft supported lipid bilayers (SLBs) for modeling cancer cells. Enriched in cholesterol and sphingolipids, rafts have been characterized as detergent-resistant domains due to their insolubility in detergents such as Triton X-100 [19,20]. These microdomains are involved in regulating various cellular functions [18,19]. Therefore in this study we investigated the interactions of ltc2a derivatives with supported raft lipid bilayers and lipid vesicles in order to gain further understanding of the mechanism of action of ltc2a, and its cytotoxic and potential anticancer properties.

2. Materials and methods

1,2-Dioleoyl-*sn*-glycero-3-phosphoethanolamine (DOPE), 1,2-dioleoyl-*sn*-glycero-3-phospho-(1′-*rac*-glycerol) (sodium salt) (DOPG), 1,1′,2,2′-tetramyristoyl cardiolipin (sodium salt) (CL), 1,2-dioleoyl-*sn*-glycero-3-phosphocholin (DOPC), egg sphingomyelin (SM), and cholesterol (Chol, ovine wool, >98%) were purchased from Avanti Polar Lipids Inc. (Alabaster, AL). Latarcin 2a (>95.1% purity) and latarcin 2a G11A (>95.4% purity) were synthesized by Gen Script Corporation (Piscataway, NJ). Stock solutions of the peptides were prepared in the required concentration in 18.2 MΩ cm^{-1} Milli-Q water. 6-Carboxyfluorescein (CF) was from ACROS Organic (NJ). Calcein and ascorbic acid were from Sigma-Aldrich (Oakville, ON). Tris, EDTA, Triton X-100, and ammonium molybdate were purchase from Bioshop (Burlington, ON).

2.1. Liposome preparation

Five different model cell membranes were used: mammalian model (DOPE/DOPC/Chol 22.2/44.4/33.4 mol%), raft model (SM/DOPC/Chol 40/40/20 mol%), *Escherichia coli* model (*E. coli*, DOPE/DOPG 80/20 mol%), *Staphylococcus aureus* model (*S. aureus*, DOPG/CL 55/45 mol%) and *Bacillus subtilis* model (*B. subtilis*, DOPE/DOPG/CL 12/84/4 mol%) [21,22]. The liposomes were prepared by dissolving appropriate amounts of lipids in chloroform followed by solvent evaporation under a stream of nitrogen. The samples were kept in vacuum for at least 24 h to ensure complete removal of the solvent. The lipid films were hydrated for 30 min in Milli-Q water or leakage buffer (10 mM Tris–HCl, 150 mM NaCl, 1 mM EDTA pH 7.45) containing 70 mM CF/calcein, for AFM imaging and leakage assay, respectively, to obtain the final lipid concentration 1 mg/mL. The hydration temperature was 50 °C for raft model and 40 °C for other model membranes. For AFM imaging, lipid suspension was vigorously stirred followed by sonication with Elma S10H Elmasonic at 50 °C. For leakage assay, the lipid suspension was sonicated at 40 °C for 5 min followed by extrusion through 200 nm polycarbonate membrane (Nuclepore Track-Etch membrane, Whatman) 31 times at 40 °C. Five freeze/thaw cycles were then performed to maximize CF/calcein encapsulation. Free CF/calcein was separated with a Sephadex G-50 size exclusion column using leakage buffer for equilibrium and elution.

2.2. Atomic force microscopy (AFM) imaging

Topography images of supported lipid bilayers were obtained in contact mode with an Ntegra (NTMDT, Russia) atomic force microscope at room temperature with 512×512 points per image and 0.7 Hz scanning rate. A 100×100 μm^2 scanner and rotated monolithic silicon cantilevers (450 μm-long, force constant 0.2 N/m ContAl, resonance frequency 13 kHz; Budget Sensor) or silicon nitride V-shaped cantilevers (100 μm-long, force constant 0.27 N/m, 30 kHz; Budget Sensor). The supported lipid bilayers were formed on freshly cleaved mica substrates sealed with a silicon O-ring in a fluid cell by adding 1 mL PBS, 40 μL of 1 M $CaCl_2$ and 1 mL of 1 mg/mL lipid vesicles. After incubating at room temperature for 50 min, the fluid cell was rinsed with Milli-Q deionized water or with 150 mM NaCl solution to remove excess vesicles. To investigate the role of cholesterol model cell membranes composed of DOPC/SM 50/50 mol% was used. Peptide was injected directly into the fluid cell by incrementally adding 15 μL of 0.29 mg/mL solution (this corresponds to increasing the total peptide concentration in the 2 mL cell by 0.3 μM) up to 0.9 μM.

AFM images (10×10 μm^2) were threaded into sequential video (1 s per frame with 0.9 s cross dissolve transition between each image) using an iMovie application. The Z scale in the images was ~6 nm; for some final images in the videos ~20 nm. Scale bar in each video is 2 μm.

2.3. Leakage assay

Leakage experiments were performed as described previously [23] by using 2 mL of CF or calcein-containing vesicles diluted 20 times with the leakage buffer on a Varian Cary Eclipse spectrofluorometer. Excitation and emission wavelengths were between 475 to 490 nm, and 510 to 525 nm, respectively, slits were 2.5 nm, and integration time was 1.5 s. The baseline fluorescence (F_0) was monitored until steady signal intensity was reached before the addition of peptide. After the peptide was added, the fluorescence signal intensity was monitored for 10 min or until no further changes were observed. The final fluorescence signal intensity (F) was then measured. The maximum fluorescence signal that corresponds to complete the disruption of the vesicles (F_M) was measured by adding 20 μL of 10% triton X-100 to the mixture at the end of each experiment. Fluorescence intensity was monitored for 5 min. The following formula was used to measure the leakage fraction: % leakage = $[(F-F_0)\times 100\%]/(F_M-F_0)$. Lipid phosphorus concentration was determined using a phosphate assay [24].

3. Results and discussion

3.1. Raft domains in supported lipid bilayers visualized by atomic force microscopy

The formation of microscale domains in the raft mixture has been extensively studied over the past decade. It is now well established that sphingomyelin can form both inter- and intra-molecular hydrogen bonds with other lipids and cholesterol whereas phosphocholine (PC) lipid could only be the acceptor for hydrogen bonding [25,26]. In addition, intramolecular hydrogen bonding between SM molecules also limits the lateral diffusion and rotational motion of SM and indirectly strengthens the interaction with Chol [25]. Moreover, Chol preferentially interacts with SM and other sphingolipids due to their structure and the saturation of the hydrocarbon chain [27]. As a result when supported by a freshly cleaved mica SM/DOPC/Chol forms a phase separated SLBs with SM and Chol in liquid ordered (L_o) phase that is 0.5–1 nm higher than DOPC in the liquid disordered (L_d) phase (Fig. 1) [20,25,27–29]. The location and size of domains remain relatively constant over the 60 min period after the addition of 30 μL of deionized water or 150 mM NaCl solution. However, the detergent resistant 5 nm domains were visualized after treatment with 0.28 mM Triton X-100 (Fig. 1), consistent with previous studies [20,30]. These supported lipid bilayers therefore represent a convenient model to investigate the activity of various bioactive peptides, such as latarcins.