Magainin-Mediated Disruption of Stratum Corneum Lipid Vesicles

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INTRODUCTION

Drug delivery across the skin has had great success for drugs such as nicotine, estradiol, and a few others (1,2). However, the vast majority of drugs cannot cross skin at therapeutic rates, due primarily to the formidable barrier presented by skin's outer layer, the stratum corneum. This barrier to transdermal transport is formed primarily by a series of multilamellar lipid bilayers found in stratum corneum's extracellular spaces.

Strategies to enhance drug delivery across the skin have focused to a large extent on chemical and physical methods to disrupt lipid structure (1,2). However, most enhancers have side effects, including irritation or safety concerns. The ideal enhancer for transdermal drug delivery would be one that is specifically targeted to disrupt stratum corneum lipids without damaging cells found deeper within the skin. The use of magainin peptides may present an opportunity to do this.

Magainins are a family of peptides originally isolated from the skin of the African clawed frog, *Xenopus laevis* (3), which show a broad spectrum of antimicrobial activity. They exhibit potent antibacterial behavior at low concentrations (4,5) and belong to a class of antimicrobial peptides that interact directly with the lipid bilayer as opposed to specific membrane proteins (6,7). This antimicrobial activity appears to be derived from the peptides' ability to increase the porosity of the membrane. Magainins specifically target bacteria because of the favorable interaction between the positively-charged magainins and the typically negatively-charged bacterial membranes. Bacterial membranes generally contain large amounts of lipid with negatively-charged head groups such as phosphatidylserine and phosphatidylglycerol. In contrast, magainins are generally not as effective against most eukaryotic cells because of unfavorable interaction with the positive charges residing on their numerous zwitterionic lipid head groups such as phosphatidylethanolamine and phosphatidylcholine.

Given this charge-dependent mechanism of magainins' effect, we sought to determine if magainins could disrupt bilayers made of lipids found in human stratum corneum. Although analysis of stratum corneum lipids is complicated by spatial and inter-individual variation, they are reported to contain fewer zwitterionic phospholipids (~5 wt. %) than typical eukaryotic cells, while containing ~16 wt. % negatively-charged fatty acids (8). Given the significant negative charge and limited zwitterion content of stratum corneum, we propose the hypothesis that magainins can disrupt stratum corneum lipid bilayers.

MATERIALS AND METHODS

Materials

Cholesterol, cholesterol sulfate, palmitic acid, calcein, and ceramides (non-hydroxy fatty acid, prepared by treating bovine brain sphingomyelin with phospholipase C) were purchased from Sigma (St. Louis, MO), methanol from Fisher Scientific (Fair Lawn, NJ) and chloroform from J. T. Baker (Phillipsburg, NJ). The Tris / EDTA / NaCl buffer (pH 7.4) contained 10 mM Tris, 150 mM NaCl, and 0.1 mM EDTA (Sigma). The phosphate-buffered saline (PBS; pH 7.4) contained 10 mM phosphate buffer, 2.7 mM potassium chloride, and 137 mM sodium chloride (Sigma).

Magainin peptides were synthesized using a PE-Biosystems (Foster City, CA) model 433A peptide synthesizer at the Microchemical Facility at Emory University School of Medicine (Atlanta, GA). Two peptides were produced: the magainin 2 peptide (NH_2-Gly-Ile-Gly-Lys-Phe^5-Leu-His-Ser-Ala-Lys^{10}-Lys-Trp-Gly-Lys-Ala^{15}-Phe-Val-Gly-Glu-Ile^{20}-Met-Asn-Ser-COOH) and its leucine-lysine analogue (NH_2-Lys-Leu-Leu-Leu-Lys^5-Leu-Leu-Leu-Lys-Leu^{10}-Leu-Lys-Leu-Trp-Leu^{15}-Lys-Leu-Leu-Lys-Leu^{20}-Leu-Leu-Lys-COOH) (9). The derivative is a simple synthetic peptide designed to mimic the behavior of magainin 2 by using leucine residues to form a hydrophobic helix and lysine residues to provide the positive charge required to achieve a favorable interaction with the negative membrane (9,10).

Preparation and Characterization of Vesicles

Vesicles were made from lipid bilayer components representative of those found in human stratum corneum. The weight percentages of compounds used were: 40% ceramides, 25% cholesterol, 10% cholesterol sulfate, and 25% palmitic acid (8,11). A total of 40.2 mg (93.5 μmol) of these 4 constituents were dissolved in 1–1.5 ml of a 2:1 volume mixture of chloroform and methanol and then lyophilized (Speed Vac SC110, Savant Instruments, Farmingdale, NY) until only a thin lipid film remained. To form calcein-loaded vesicles, the lipid film was resuspended in 10 ml of a 70 mM calcein solution in PBS and subjected to five freeze-thaw cycles in liquid nitrogen and 37 °C water. To make the vesicles uniform in size, they were subjected to repeated extrusion through Nucleopore membranes (Whatman Membrane Filtration, Clifton, NJ) of 400 nm (11 passes) and 100 nm (19 passes) pore size (12). The extruded vesicle suspensions were dialyzed (Slide-A-Lyzer, 10 kDa cut-off, Pierce Chemical Company, Rockford, IL) in Tris / NaCl / EDTA buffer to remove calcein not trapped within vesicles.

The size of vesicles was determined by dynamic light scattering (DynaPro-LSR-TC, Protein Solutions, Charlottesville, VA) to be approximately 100 nm. Based on an initial solution of 40.2 mg (93.5 μmol) lipids in 10 ml of phosphate

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buffer (see above), we estimated the vesicle stock solution to contain approximately 4.02 mg/ml (9.35 mM) lipid, assuming no loss of lipid during vesicle preparation. After dilution into Tris / NaCl / EDTA buffer for leakage experiments (see below), vesicle concentration should be approximately 21 μg/ml (49 μM) lipid.

Leakage Measurement

Measurements of calcein leakage from vesicles were made by placing 1.9 ml of 10, 40, or 100 μM magainin 2 or magainin derivative in Tris / NaCl / EDTA buffer in a 4 ml cuvette and then adding 10 μl of calcein-loaded vesicle suspension. Using a spectrofluorimeter (490 nm excitation, 520 nm emission; Photon Technologies International, S. Brunswick, NJ), sample fluorescence was measured every 10 s for 5 min. Only calcein that leaked from vesicles emitted measurable fluorescence because calcein at high concentration within vesicles (i.e., 70 mM) self quenches (13). In this way, fluorescence intensity is a measure of calcein that has leaked from vesicles. Fractional calcein leakage was calculated using background fluorescence measured from intact calcein-loaded vesicles as zero-leakage and maximum observed fluorescence as 100% leakage.

RESULTS AND DISCUSSION

Magainin-mediated Disruption of Vesicles

To address whether magainins can disrupt stratum corneum vesicles, Fig. 1 shows the result of exposing vesicles to different concentrations of two magainin peptides: magainin 2 and a magainin derivative. Both peptides at the 3 concentrations studied caused significant leakage of calcein from vesicles, indicating that they disrupted vesicle membranes. Magainin 2 disrupted vesicles very rapidly. Leakage occurred before the first measurement (i.e., within 10 s) and did not increase further over the 5 min of measurements that followed (Fig. 1A). The effects of this magainin also showed a strong concentration dependence: 10 μM had only a small effect, while 100 μM achieved extensive leakage. There was approximately a 30-fold change in leakage over the range of magainin concentrations studied (Fig. 1B).

In contrast, the magainin derivative acted somewhat less rapidly. Although most effects were seen within 10 s, leakage continued to increase for more than 2 min at 10 μM and for about 30 s at 40 μM (Fig. 1A). There was also a weaker dependence on concentration of the magainin derivative. Over the concentration range studied, there was less than a 3-fold change in leakage (Fig. 1B). Previous studies with vesicles representative of bacterial membranes similarly showed calcein leakage dependent on magainin concentration (12). However, in contrast to this study, initial rates of leakage from bacterial vesicles were faster for the magainin derivative than for magainin 2. Also, overall rates for both magainins were slower for bacterial membranes than stratum corneum vesicles.

Interpretation of Data

The different levels of calcein leakage seen in Fig. 1 presumably exist because leakage occurs over a relatively short period of time and then terminates. There are two likely

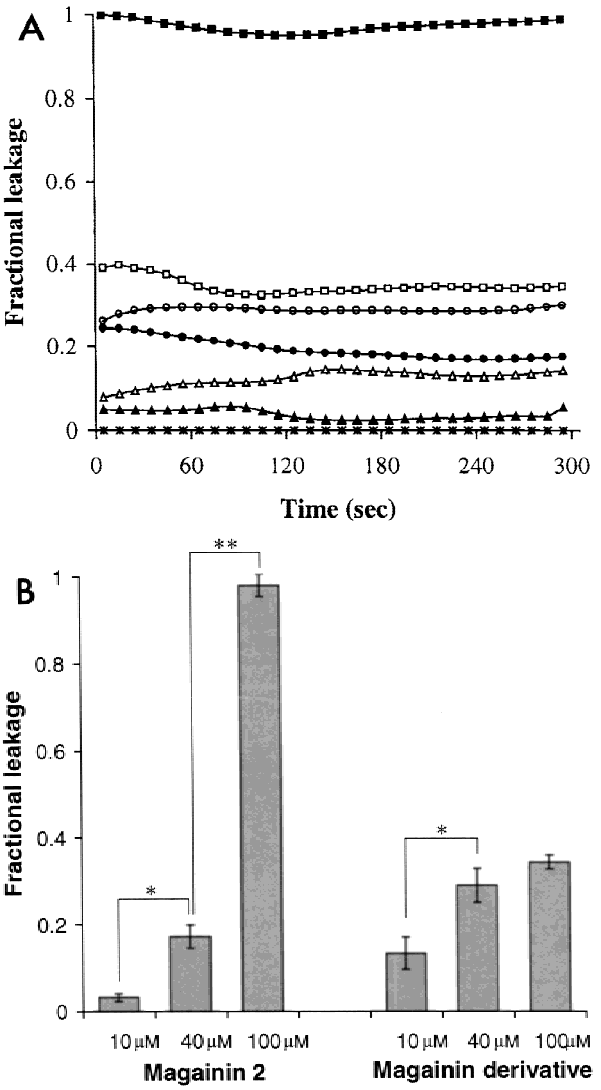

Fig. 1. Effect of magainin concentration on the leakage of entrapped calcein out of stratum corneum lipid vesicles. (A) The concentrations of magainin 2 were (*) 0, (▲) 10 ,(●) 40, and (■) 100 μM , and the magainin derivative were (*) 0, (△) 10, (○) 40, and (□) 100 μM. Each data point represents the average of 3 to 6 replicates. Average values for standard error of the mean for each data set are between 0.012 and 0.050. (B) Average magainin-mediated leakage achieved during the last 100 s of each experiment. Error bars indicate standard error of the mean. Fractional leakage is expressed relative to magainin-free controls, which have, by definition, no leakage. Statistical significance indicated by one-tailed Student's *t* test: (*) $P < 0.01$, (**) $P < 0.001$. Vesicle disruption showed dependence on magainin concentration and type, as well as a weak dependence on time.

mechanisms that could explain this termination. First, if vesicle lysis is responsible for the leakage, then lower concentrations of magainin provide an insufficient amount of peptide to lyse all of the vesicles. Once a vesicle is lysed, the peptide is likely to be agglomerated with the components of the former vesicle, which may prevent its incorporation into and lysis of another vesicle. The second possibility is that magainins form transmembrane pores that deactivate after a limited time. Previous studies on bacterial vesicles containing negatively-charged phosphatidyl glycerol lipids indicate that this latter mechanism is more likely because large fluorescent

polysaccharides were unable to leak from magainin-disrupted vesicles, presumably because they were too large to fit through the pores (12). If lysis were involved, these large molecules would be expected to leave the vesicles. In addition, high-resolution transmission electron micrographs of magainin-treated vesicles suggest that, over time, peptides may phase separate to form a nonporous region of the membrane in intact vesicles, which provides a possible explanation for the transient nature of magainin-mediated pores (12).

The time frame over which leakage occurred was much faster in the present study with stratum corneum lipids than the previous work with bacterial lipids (12). This is most likely due to the significantly higher concentrations used (i.e., 10–100 μM or ~0.20–2.0 mol peptide / mol lipid here vs. the previous work (12), which had 0.5–1.5 μM or 0.019–0.058 mol peptide / mol lipid for magainin 2 and 1.0–5.0 μM or 0.025–0.124 mol peptide / mol lipid for the magainin derivative). Significantly larger concentrations of both peptides were required to achieve the same amount of leakage from stratum corneum vesicles than bacterial vesicles. Another difference is that in the previous study, the timeframe of leakage for the synthetic peptide was much shorter than for magainin 2 (12). The opposite trend in the data presented here suggests that although the additional positive charge on the magainin derivative facilitates faster insertion for bacterial vesicles with large negative charge, it has the opposite effect when the negative charge is reduced and zwitterion content is increased, as in stratum corneum vesicles.

Application to Transdermal Drug Delivery

These results suggest possible applications in transdermal drug delivery. The ability of magainins to disrupt the vesicles used in this study suggests that magainins may be able to disrupt lipid bilayers found in the skin's stratum corneum, thereby facilitating transdermal delivery of many drugs. Given the specificity of magainin action on negatively-charged membranes absent of zwitterionic or cationic charge (6,7), magainins are expected to selectively affect negatively-charged stratum corneum lipids (8) without damaging viable cells found in the skin, whose membranes contain fewer negatively-charged and more zwitterionic lipids. This could prevent the irritation and toxicity associated with some other chemical enhancers. Experiments testing the effects of magainins on human skin are needed to validate these hypotheses because stratum corneum structure is biophysically more complex than our model system, and issues of irritation and possible immunogenicity must be resolved *in vivo*. Suggesting that magainins may be safe, Magainin Pharmaceuticals tested a topical antibiotic cream (1% Pexiganan Acetate) containing magainins for the treatment of foot ulcers in diabetics. Based on the results of these trials, the FDA concluded that this treatment was safe, although not effective in promoting healing (14).

Our model permeant, calcein, represents an especially difficult compound to transport across lipid bilayer barriers due to its relatively large size (623 Da, 0.6 nm radius) and hydrophilicity (net –4 charge). Most drugs are smaller and less hydrophilic than calcein, suggesting that magainin-mediated enhancement of many drugs should be at least as facile as the enhancement of calcein observed here. Moreover, because magainins are believed to create non-specific transmembrane channels (6,7), magainins could enhance transport of a broad range of different molecules.

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