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The Lipid Dependence of Antimicrobial Peptide Activity Is an Unreliable Experimental Test for Different Pore Models

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Supporting Information

ABSTRACT: Antimicrobial peptides usually kill bacteria by making their membranes permeable. Two main models (barrel-stave and Shai−Matsuzaki−Huang) have been proposed to describe the peptide-induced pores. Although several experimental tests can be exploited to discriminate between these two models, the dependence of peptide activity on lipid properties (intrinsic curvature and membrane thickness) is routinely used for this purpose. Here, we show that, contrary to what is currently accepted, this criterion is unreliable.

Antimicrobial peptides (AMPs) are major components of the innate defense system of many organisms, including humans. They display multiple functions, but their main activity is bactericidal and often achieved by perturbing the permeability of microbial membranes.[1] As this mechanism of action reduces the risk of development of bacterial resistance, AMPs are investigated as a possible solution to the health crisis caused by the spread of drug-resistant bacteria. However, a detailed understanding of their mechanism of pore formation is needed for the development of new molecules with the same activity as AMPs, but with better druglike properties.[2] Although several mechanisms have been proposed, it is now well established that most AMPs form pores according to two main models.[3] The first is the barrel-stave model. In this case, helical peptides initially bind to the membrane surface. After a threshold concentration is reached, peptide helices start to insert into the membrane, perpendicular to its plane. This process is favored by the presence of a transmembrane potential. Inserted peptides then aggregate to form a cylindrical superstructure with a water-filled lumen, like the staves in a barrel. The second, the Shai−Matsuzaki−Huang (SMH) model, can also be termed "carpet", "toroidal pore", or "disordered toroidal pore", depending on subtle details of the pore structure. In this case, peptides bind to the membrane surface, parallel to it, and thus cause a perturbation of its surface tension, which eventually leads to the formation of leaky membrane defects.

For some peptides, it is now well established which of these two models better describes their behavior. For instance, in the case of alamethicin (Alm), many biophysical techniques concur in supporting a barrel-stave mechanism of pore formation.[4,5] One example of an extensively characterized peptide for which the SMH model has been conclusively demonstrated to hold is the wasp venom toxin mastoparan-X (Mpx).[6,7] We recently reported fluorescence and molecular dynamics studies supporting the SMH model for cationic AMP PMAP-23, which belongs to the cathelicidin family.[3,8] However, in many other cases, the specific mechanism of pore formation is under debate, and both models have been proposed to apply for the same peptide.[9,10] For this reason, it is extremely important to define specific properties and appropriate experimental tests to discriminate between the two models.

Several methods can be used to determine the structure of the peptide-induced pores, such as X-ray and neutron diffraction,[11] nuclear magnetic resonance[12] and fluorescence[13] spectroscopies, molecular dynamics simulations,[13] and microscopic techniques.[14] However, a simple criterion commonly used to recognize the SMH model at play is the dependence of peptide activity on the so-called "intrinsic curvature" of the lipids forming the membrane.[15−21] Lipids are characterized by a negative intrinsic curvature, like phosphatidylethanolamine (PE), if the cross-sectional area of the polar headgroup is smaller than that of the tails. The opposite is true for positive curvature lipids, like lysophosphatidylcholine. Molecules with a negative intrinsic curvature favor a concave shape of the lipid aggregate in which they are embedded and therefore promote an inverted hexagonal phase. Positive curvature lipids, on the other hand, prefer convex shapes and lead to the formation of micellar structures.[22] However, when these lipids are included in a bilayer (which is planar on a molecular scale), their presence leads to a strain that destabilizes the membrane.[23] Peptides acting according to the SMH model have an effect similar to that of positive curvature lipids: they insert close to the membrane surface, thus favoring a convex curvature of the bilayer leaflet to which they bind. When a threshold peptide concentration is reached, the strain caused by this insertion is relaxed via the formation of membrane defects and re-equilibration of peptide and lipid molecules between the two leaflets. This mechanism of membrane destabilization is counteracted by negative intrinsic curvature lipids, like PE,

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that therefore inhibit the activity of peptides working according to the SMH model.

To test if this criterion is indeed able to discriminate between the two models of pore formation, we compared the peptide-induced leakage of a fluorescent tracer (carboxyfluorescein) caused by Alm, Mpx, or PMAP-23 in vesicles containing PE or phosphatidylcholine (PC) (Figure 1). Surprisingly, both Alm

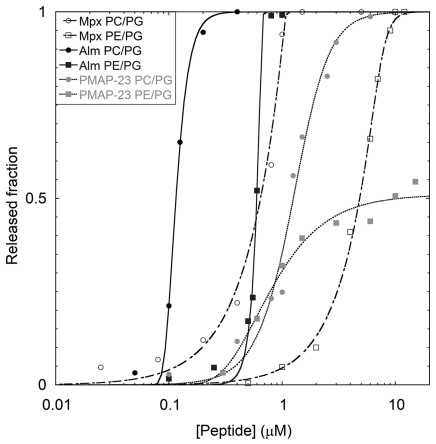

Figure 1. Peptide-induced liposome leakage in membranes with different intrinsic curvatures. The fraction of liposome contents released 20 min after peptide addition is reported. Palmitoyloleoyl phospholipids, phosphatidylglycerol (PG) content of 33%.

and Mpx were inhibited by PE to a similar extent, irrespective of their mechanism of pore formation. PE inhibited PMAP-23 as well, but in this case, it caused an incomplete leakage even at the highest concentration tested, rather than a shift in the values of active concentrations.

A second lipid property that has been used to discriminate between different pore formation models is bilayer thickness. If the length of the peptide chain is different from the thickness of the membrane (a situation termed "hydrophobic mismatch"),[24] insertion of the peptide into the transbilayer orientation causes a local distortion of the membrane. Obviously, this phenomenon has an effect on the aggregation equilibrium that is involved in the formation of barrel-stave channels. For this reason, a dependence of peptide activity on membrane thickness has been considered as an indication in favor of this mode of pore formation, and against the SMH model.[19,25−27] To test if this criterion is correct, we compared the activities of Alm and Mpx in membranes of different thicknesses, namely, dipalmitoleoyl- (DP), dioleoyl- (DO), and dieicosenoyl- (DE) PC (Figure 2), comprising 16, 18, and 20 carbon atoms, respectively, in their aliphatic chains, and a single unsaturated bond. Surprisingly, the activity of Mpx is affected by membrane thickness much more than that of Alm. PMAP-23 could not be tested in this experiment, because its activity against neutral membranes is negligible,[28] as for most cationic AMPs, and anionic lipids with several different tail lengths are not commercially available.

Our findings, while in contrast with the previously held belief, can be easily rationalized. With regard to the unexpected effect of PE on Alm activity, a variation in peptide−membrane binding could in principle be involved, because it has been reported that this lipid reduces the affinity of Alm for membranes.[29] However, under the experimental conditions used for Figure 1, the Alm:lipid molar ratios are always below

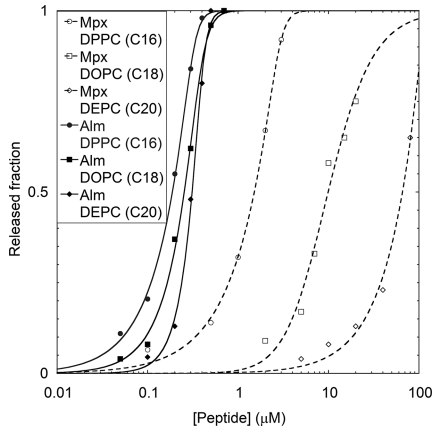

Figure 2. Peptide-induced liposome leakage in membranes with different thicknesses. The fraction of liposome contents released 20 min after peptide addition is reported. The number of carbon atoms in the lipid tails is reported in the inset.

1:50, and therefore, it is likely that most of the peptide is membrane-bound. However, the effect of PE on Alm activity can be explained also through the two-state mechanism of barrel-stave pore formation, which was proposed by Huang and co-workers on the basis of an extensive series of experiments on planar membranes, where the effects of PE lipids were also analyzed.[30−32] Alm is initially bound to the surface of the membrane, and only when a threshold concentration is reached does a significant fraction of its molecules insert into the bilayer and aggregate to form channels. In this view, the negative curvature of PE would reduce the extent of membrane perturbation caused by the superficially associated peptides, and therefore, it would increase the concentration threshold for pore formation. Therefore, the effect of PE would not be very different from what is envisaged in the SMH model. Another possible explanation might be provided by the peculiar interactions of PE headgroups: this lipid contains a primary amine (lacking in PC), which allows it to form strong hydrogen bonds with phosphate or CO groups in other lipids.[33] This H-bond network is responsible for the melting temperature of PE lipids being higher than those of their corresponding PC analogues.[33] It could also explain the effect observed here, because it might increase the energy needed to insert a peptide in the bilayer, or to open a pore.

The unexpected strong dependence of Mpx activity on bilayer thickness can be explained in terms of what Wimley termed "interfacial activity".[1] To cause leakage, a charged, surface-bound, peptide must cause a local defect in the membrane. This effect can be obtained if, at some point, the peptide is able to interact with the headgroups of the lipids of the opposing leaflet of the membrane. This interaction is facilitated by the peptide-induced local perturbation of the membrane order, but clearly it takes place more easily in thinner membranes. Therefore, the observation of a peptide activity dependent on membrane thickness cannot rule out the SMH mode of pore formation.

It is worth mentioning that our liposome leakage results might appear in contrast with those of the conductivity experiments performed on Alm in planar membranes. Those studies indicated that PE favors the formation of larger Alm pores[34] and that the pore size increases with an increase in bilayer thickness.[35] By contrast, our experiments with vesicles showed that the extent of Alm-induced leakage decreases in the

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presence of PE, and with an increase in membrane thickness. This apparent contradiction can be explained by considering that leakage from a vesicle depends not only on the size of pores but also on their number, which in turn is influenced by water−membrane partition and aggregation equilibria.[36] Because experiments with liposomes take all these factors into consideration, they are closer to the actual conditions under which these peptides exert their antimicrobial activity. However, the experiments with planar membranes provide an important confirmation that, in bilayers with a lipid composition similar to that employed in this study, Alm actually conforms to the barrel-stave mechanism.

In conclusion, the data presented here show that the effects of lipid intrinsic curvature and membrane thickness on peptide activity cannot be considered reliable tests for unraveling the mode of pore formation by AMPs. Unfortunately, this observation means that a thorough structural characterization of peptide−membrane interaction, by spectroscopic, diffractometric, simulative, and microscopic approaches, is needed in each specific case to convincingly assess the mechanism of pore formation. For instance, only peptides acting according to the SMH model are expected to produce significant effects on membrane order and dynamics.[3] On the other hand, direct peptide interaction with the deep region of the phospholipid tails, in the hydrophobic core of the bilayer, is a clear-cut hallmark of the barrel-stave mechanism.[37,38] This can be used to rule out the SMH model, which predicts that peptides always remain associated with the phospholipid headgroups, even when forming pores.[39]

■ ASSOCIATED CONTENT

Supporting Information

Materials and methods. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

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