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The role played by lipids unsaturation upon the membrane interaction of the Helicobacter pylori HP(2–20) antimicrobial peptide analogue HPA3

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Abstract The HPA3 peptide is an analogue of the linear antimicrobial peptide, HP(2–20), isolated from the N-terminal region of the *Helicobacter pylori* ribosomal protein, able to interact with zwitterionic lipid membranes and generate pores. Herein we focused on the importance of the degree of unsaturation of lipid acyl chains on HPA3 peptide-membrane interactions. Electrophysiology experiments carried out in reconstituted lipid membranes formed from phosphatidylcholines with one (1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine−POPC) and two monounsaturated acyl chains (1,2-dioleoyl-sn-glycero-3-phosphocholine−DOPC) demonstrate that the lesser degree of the packing density of membrane lipids encountered in DOPC-based planar membranes greatly enhances the electric activity of pores created by the HPA3 peptide. Data derived from fluorescence spectroscopy experiments demonstrate that upon interaction with the bilayer, the HPA3 peptide translocates to the trans-side of the membrane. From the same experiments, we demonstrate that in the case of DOPC-based planar membranes, the net amount of HPA3 peptide which passes across the membrane and re-dissolves in the trans solution is almost 22% greater than POPC-based membranes. Such data further emphasize the modulatory role played by lipid acyl chain in determining antimicrobial peptides-lipids interactions, and demonstrate that small differences in unsaturation degree can impose a sizeable influence on HPA3 peptide activity.

Keywords Electrophysiology · Fluorescence · Lipid unsaturation · Antimicrobial peptide · H. pylori

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Introduction

Antimicrobial peptides (AMPs) represent a class of small proteins synthesized by living organisms of all types for the purpose of fast killing of Gram-positive and-negative bacteria, fungi, parasites, enveloped viruses, and tumor cells (Zasloff 2002; Tossi 2005), and are strongly considered as future therapeutic agents (McPhee and Hancock 2005; Koczulla and Bals 2003). The lipid bilayer is known to exhibit important roles in allosteric regulations of various membrane proteins via its elastic manifestations, and over the years numerous formulations have been invoked to capture the essence of such mechanisms, such as: the intrinsic lipid curvature (Gruner 1985), bilayer-protein hydrophobic mismatch (Mouritsen and Bloom 1984), bilayer deformation energy (Huang 1986), acyl chain packing (Fattal and Ben-Shaul 1993), lateral pressure profile, and lipid packing stress (Bezrukov 2000).

According to the theory of elastic bilayer deformations, the hydrophobic coupling between an embedded peptide and the bilayer, induces a bilayer deformation and the associated deformation energy will be the sum of contributions from bilayer compression, which varies with the depth of the deformation in each monolayer and the area-compressibility modulus (K_a), and monolayer bending which varies with the monolayer curvature and the bending modulus (k_c). These mechanical parameters are strong functions of the lipid membrane composition (Gruner 1985; Mouritsen and Bloom 1984). Elastic properties of bilayers alter the membrane susceptibilitiy to AMPs binding even from the incipient phase of this process since in order to incorporate a peptide into a lipid matrix, an increase in the surface area of the bilayer must occur to accommodate the peptide. Knowing that the mechanical work to expand the bilayer surface is proportional to K_a, alterations in the cohesive interactions among the bilayer lipids will reflect in altered K_a values, which will modulate the AMP-bilayer interactions.

Unsaturation of the lipid hydrophobic tails is known to regulate the packing density of membrane lipids, as well as the membrane transversal curvature stress mainly via changes imposed on the lipid monolayers' intrinsic curvature. By using the constant pressure insertion assay in Langmuir monolayers, it has been proven that an increase in the unsaturation degree led to progressive enhancement of the protegrin PG-1 insertion (Ishitsuka et al. 2006). In a different study, the binding of alamethicin was assessed in membranes composed of palmitoyloleoyl PC (POPC) (one double bond) and dilinoleoyl PC (DLnPC) (four double bonds), and these binding affinities were compared to those obtained for DOPC (two double bonds); results obtained under such conditions strongly suggested that the binding affinity of alamethicin decreases as the level of lipid unsaturation increases (Lewis and Cafiso 1999).

Recently, we investigated the mechanism of action of the HPA3 peptide (Mereuta et al. 2008), an analogue of the linear antimicrobial peptide, HP(2–20), isolated from the N-terminal region of the *Helicobacter pylori* ribosomal protein (Park et al. 2008). The HP(2-20) peptide was shown to display several important functional characteristics, such as: it is bactericidal, neutrophil chemoattractant, and it activates phagocyte NADPH oxidase to produce reactive oxygen species. Moreover, HP(2–20) and its analogues specifically recognize common immune elicitors, including chitin, peptidoglycan, and LPS, thus becoming very promising candidates for the development of new peptide-based antibiotic agents.

In this work, we focused on the importance of the degree of unsaturation of lipid acyl chains upon the HPA3-membrane interactions. Our electrophysiology experiments carried out in reconstituted lipid membranes formed from phosphatidylcholines with one (POPC) and two monoun-saturated acyl chains (DOPC) demonstrate that the lesser degree of the packing density of membrane lipids present in DOPC-based membranes enhances the electric activity of pores created by the HPA3 peptide. To this end and in an attempt to mechanistically explain this behavior, we suggest that the decreased, in-plane lipids cohesive interactions manifested in the case of DOPC-based planar membranes as compared to POPC ones, may entail among others an augmentation of the HPA3 membrane insertion. Data derived from fluorescence spectroscopy experiments un-equivocally demonstrate that upon interaction with the lipid bilayer, HPA3 peptides translocate to the trans-side of the membrane. From the same experiments we inferred that in the case of DOPC-based planar membranes, the net amount of HPA3 peptide which passes across the membrane and re-dissolves in the aqueous trans solution is about 22% greater than POPC-based membranes.

Materials and methods

Electrophysiology experiments were carried out in the folded bilayer membranes system as described before (Mereuta et al. 2008), and represented schematically in Fig. 1. A

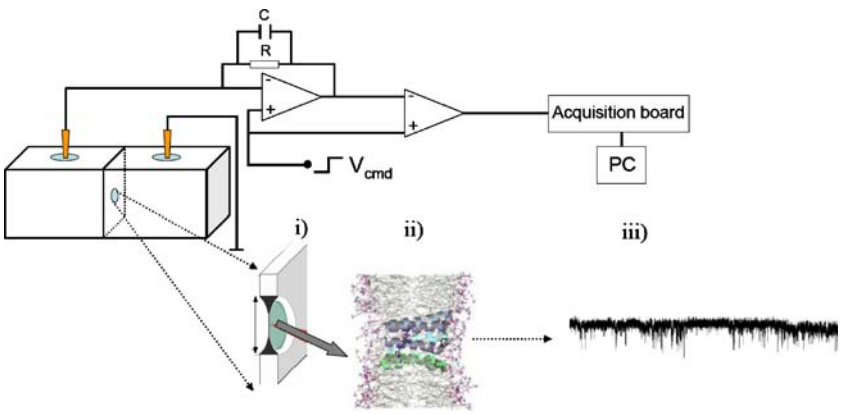

Fig. 1 The general representation of the set-up used in our electrophysiology experiments, which shows schematically the lipid bilayer chamber, and the general diagram of the amplifier and recording blocks. For simplification purposes, in (i) we show a zoom-in of the Teflon partition which accommodates the lipid bilayer (ii), which serves as a membrane support for recording the electric activity of HPA3-induced pores (iii)

symmetrical lipid bilayer was formed either from 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine (POPC, Avanti Polar Lipids, Inc., USA) or 1, 2-dioleoyl-sn-glycero-3-phosphocholine (DOPC, Avanti Polar Lipids, Inc., USA) dissolved in pentane (Fig. 2, panel a). To avoid possible issues related to lipids oxidation, fresh lipid samples were made in-between experiments. Both chambers contained 0.5 M sodium chloride buffered at a pH value of 7.2 in 10 mM BIS-TRIS propane (Sigma-Aldrich, Germany). All experiments were performed at a room temperature of ~ 25°C. The insertion of the purified HPA3 peptide (AKKVFKRLEKLFSKIWNWK) was achieved by adding to the grounded cis chamber of the bilayer cuvette corresponding amounts of the peptide from stock solutions made in water, to achieve concentrations of 1 μM (for experiments with POPC-based bilayers) or 0.5 μM (for experiments with DOPC-based bilayers). These particular concentrations were chosen based on trial experiments, during which we sought to arrive at conditions in which the HPA3 would not destroy the membrane during the course of experiments, yet allow us to optimally record electrical currents induced by it and achieve a measurable amount of the transferred peptide to the trans-side of the membrane. Currents from the bilayer chamber were detected and amplified with a resistive headstage patch-clamp amplifier (EPC 8, HEKA, Germany), and data acquisition was performed with a NI USB 6251, 16-bit acquisition board (National Instruments, USA) at a sampling frequency of 30 kHz.

To evaluate the extent to which HPA3 peptides translocate across the lipid membrane (either POPC– or DOPC-based), at the end of the experimental protocol (i.e., after 5 min, with an applied holding potential of - 50 mV) we drew 800 μL buffer samples from the cis and the trans side of the bilayer chamber, and subjected them to steady-state fluorescence analysis. Emission spectra of the HPA3 peptide were recorded using a FluoroMax-4 spectrofluorometer (Horiba Jobin Yvon, USA) in quartz cuvettes with 0.5 cm path length. The fluorescence intensity of Trp residues of HPA3 was evaluated at 353 nm upon excitation at 285 nm, and the fluorescence intensity spectra of buffer solutions without peptide (i.e., controls) were subtracted from those recorded in the presence of the HPA3 peptide. The evaluation of the relative amount of HPA3 peptide found in either the trans or cis side of the bilayer was quantified by calculating the percent change of maximum fluorescence intensity, as

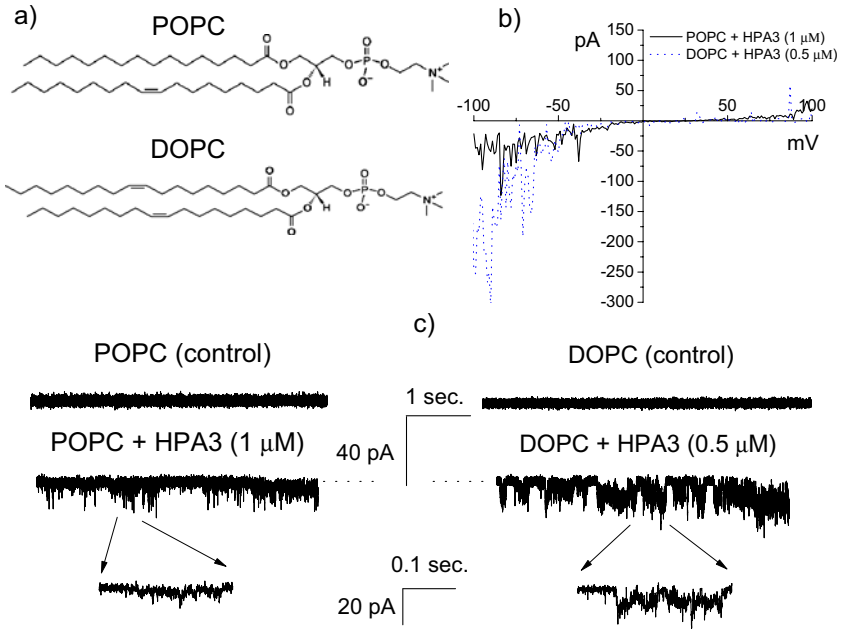

Fig. 2 **a** The molecular structure of the lipids used throughout this study: 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine (POPC) and 1,2-dioleoyl-sn-glycero-3-phosphocholine (DOPC). **b** I-V diagrams which characterize macroscopically transport properties of HPA3 pores inserted in lipid membranes made of lipids containing one monounsaturated acyl chain (POPC) and two monounsaturated acyl chains (DOPC). By considering the higher electric activity of the HPA3 peptides in DOPC lipid membranes as compared to that measured in POPC-based membranes, these recordings illustrate the higher pore-forming propensity of the HPA3 peptide in DOPC lipid membranes. **c** Selected traces showing current fluctuations measured across POPC- and DOPC-based membranes at −50 mV, in the absence (control) and presence of the HPA3 peptide. In the case of POPC lipid membranes, 1 μM of the HPA3 peptide gives rise to mostly single-channel, toroidal-like (Mereuta et al. 2008) aggregates (see the magnified trace inset). For planar lipid membranes made of DOPC, less concentrated amounts of the HPA3 peptide (0.5 μM) were seen to generate a much elevated electric activity at a similar potential difference. The dotted baseline represents the 'closed' state of HPA3-induced channels

compared to values measured at a similar wavelength, inferred from the initial emission spectra of cis buffer solutions with the peptide added, as used at the beginning of each experiment. Throughout fluorescence experiments, we noted that the sum of the fluorescence intensities recorded after 5 min on the cis and trans sides is much smaller than the initial fluorescence intensity measured on the cis side. This was a consistent observation in all our experiments, and we believe that unspecific adsorption of the peptide onto the bilayer chambers over time may be the reason for this discrepancy.

Results and discussion

The original traces derived from electrophysiology experiments are shown in Fig. 2, which reveal the modulatory role played by the degree of unsaturation of lipids acyl chains upon HPA3 affinity towards planar lipid membranes. The representative I–V diagrams drawn for bilayers made of lipids containing one monounsaturated acyl chain (POPC) and two monounsaturated acyl chains (DOPC) (Fig. 2, panel b) clearly show the higher pore-forming propensity of the HPA3 peptide in DOPC lipid membranes. That is, by working at the half concentration (0.5 μM), the membrane-inserted HPA3 peptides lead to a considerable higher electric activity of the DOPC membrane as compared to the case of POPC-based membranes for which, in order to optimally record the electric activity of the inserted HPA3, a peptide concentration of 1 μM was chosen.

In Fig. 2 panel c, we present representative traces showing current fluctuations measured through HPA3 channels embedded on such membranes, measured at −50 mV. As it is seen, in the absence of the peptide (control), the current across the lipid membrane reflect only inherent, mostly thermal noisy contributions whereas 1 μM of the HPA3 peptide generates ion channels-like aggregates in the POPC lipid membranes. However, for lipid membranes made of the DOPC, the less concentrated HPA3 peptide (0.5 μM) was seen to generate a much elevated electric activity at a similar potential difference, and judged from the enhanced heterogeneity of current amplitude values multi-channel aggregates were being formed under such conditions. We suggest that as a direct consequence of the enhanced degree of unsaturation present in DOPC acyl chains as compared to POPC ones, a correspondingly decrease of the in-plane cohesive interactions among the lipids ensues, favoring the process of HPA3 insertion. At this point however one cannot rule out a concerted interplay of changes involving the lateral pressure profile, area compressibility modulus, hydrophobic thickness and bending modulus of the DOPC-based lipid membrane, which eventually makes it more prone to energetically accommodate the incoming HPA3 monomers.

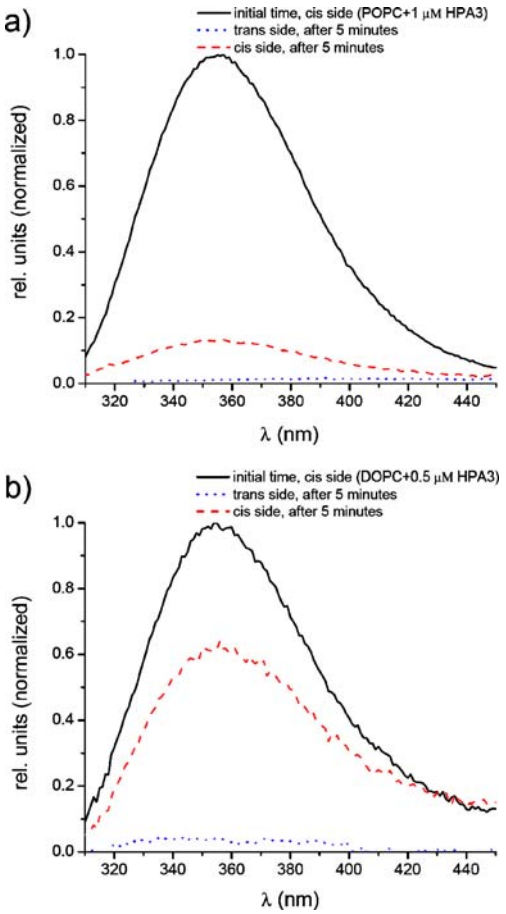

Fig. 3 **a** The normalized fluorescence intensity of Trp residues of HPA3, on samples just added to the cis side of POPC-based lipid membranes (1 μM - continuous line), and samples collected from the trans (dotted line) and cis side of the membrane (*dashed line*) under conditions which ensured continuous pores formation, and promoted HPA3 translocation across the membrane to its trans monolayer (i.e., a −50 mV potential difference applied for about 5 min). **b** Data collected under similar experimental protocols as in **a**, except for that the lipid membranes used were made of DOPC, and the initial concentration of HPA3 added on the cis side of the membrane was 0.5 μM

In Fig. 3, panel a, we show the representative normalized emission fluorescence spectrum of the buffer solution collected from the cis-side of a bilayer membrane made of POPC, where the HPA3 peptide was initially added at a concentration of 1 μM (continuous line). Interestingly, by working under conditions that ensured continuous pores formation and therefore promoted the HPA3 translocation across the membrane to its trans side (e.g., a negative potential difference applied for about 5 min), the spectrum of the trans-side buffer solution shows evidence for the presence of the HPA3 peptide (Fig. 3, panel a, dotted line). This constitutes direct proof supporting our hypothesis that following membrane 'toroidal' pores disintegration, HPA3 monomers translocate across the membrane to the trans side, and subsequently re-dissolves into the buffer solution. This result indirectly substantiates the paradigm according

to which certain antimicrobial peptides, and HPA3 in particular, may exert their cytotoxic role not only by short-cutting the transmembrane electrolyte transport via the formation of transmembrane pores, but also through a non-lytic mechanism which possibly aims at intracellular, non-membrane targets, for which peptide translocation across various lipid membranes is crucial (Brogden 2005). Similar experiments carried out on lipid membranes made of DOPC revealed that the HPA3 peptide not only interacts more easily with such membranes, but the transferred amount of peptide across the membrane seemingly increases (Fig. 3, panel b). Four independent experiments carried out on separate lipid bilayers showed that under our working conditions, the relative percent amounts of the HPA3 peptide which translocate across the lipid membrane as it has been assessed via fluorescence spectroscopy, were 4.6±1.7 (average ± SEM) for lipid membranes made of DOPC and 3.6±1.9 (average ± SEM) for lipid membranes made of POPC.

It must be stressed that previous data have highlighted the fact that phospholipids with polyunsaturated acyl chains play an important role in regulating structure and function of membrane proteins. In one such representative example, polyunsaturated lipids have been shown to modulate the membrane-disruptive action of melittin, whereby fluorescence parameters (e.g., intensity, emission maximum, polarization, lifetime) and acrylamide quenching of melittin incorporated in membranes were proven to be dependent on the degree of unsaturation of lipids in membranes (Raghuraman and Chattopadhyay 2004). Other data have thoroughly demonstrated that elastic membrane properties depend among others, on the lipid hydrocarbon chain composition (Needham and Nunn 1990). Moreover, it has been shown that lipid polyunsaturation reduces energy requirements for elastic deformation of the bilayer and with the introduction of two or more unsaturations, the bending modulus decreases substantially; in addition, acyl chain packing stress was shown to increase with hydrocarbon chains unsaturation (Olbrich et al. 2000; Bernd et al. 1997; Szule et al. 2002).

Nevertheless, a general clarification on the intricate paradigm of how the fatty acid structure of the lipids alters the peptide-induced membrane perturbation for particular classes of antimicrobial peptides is still lacking, in the sense that particular conclusions drawn on this issue for particular classes of peptides cannot be easily extrapolated to other apparently similar peptides. In simpler terms, this could be rationalized by the fact that overall peptide-bilayer interactions influence differently and peptide-specific the binding and membrane permeabilization induced by antimicrobial peptides. Structural features of peptides which work in conjunction with lipid membrane composition and are determinants for the lytic activity, selectivity, and mode of action of a given peptide (e.g., hydrophobic and hydrophilic angles, charge, hydrophobic moment and intrinsic hydrophobicity), may also offer distinct pathways for the mechanisms by which membrane lipids fulfill their non-specific regulatory role.

In this regard, it is worth mentioning that despite its richness in value, data gathered so far merely emphasizes the complexity in interpretation and generalization of the interplay between bulk properties of the lipid matrix, and the behavior of amphipathic antimicrobial peptides on lipid bilayers. To exemplify, for melittin which apparently possesses a similar pore-forming mechanism as the peptide studied herein, it has been established that the equivalent tension at the threshold concentration for pore formation is lower in DOPC lipid membranes (~8.0 pN/nm) as compared to POPC membranes (~12.7 pN/nm). In addition, it has been established that the fraction of peptide molecules in the 'inserted' membrane state in which transmembrane pores form even in the absence of an applied potential difference, is lower in POPC than DOPC (Huang 2006). It must be noted however that the values for the bending modulus (k_c) have been determined experimentally and do not vary much among POPC, SOPC, and DOPC (Rawicz et al. 2000; Kucerka et al. 2005).

It has been long argued that since the propensity of melittin-induced pores is somewhat similar for membranes made of phospholipids with polyunsaturated hydrocarbon chains, characterized by smaller values of the bending modulus (e.g. DLinPC) and lipids with monounstaturated chains (e.g., DOPC), k_c values by themselves are poor predictors for the degree of susceptibility of membranes towards interacting with peptides (Allende et al. 2005). In direct connection to our work, we note that the leakage for the membrane-lytic peptide melittin was shown to be lower for DOPC (diC18:1PC) and DLinPC (diC18:2PC) as compared to SOPC (C18:0/C18:1PC), although the compression modulus (K_a) is nearly the same for SOPC, DOPC, and DLinPC, whereas the bending (k_c) modulus is smaller by a factor of two for DLinPC as compared to SOPC or DOPC (Allende et al. 2005). In the same work, authors found that melittin partitioning was somewhat similar for lipids with 1, 2, or 4 double bonds per molecule.

In a more recent paper, the effect of the lipid acyl chain structure was tested on the activity of δ-lysin, a 26-residue peptide that forms an amphipathic helix when bound to lipid membranes (Pokorny et al. 2008). Extremely interesting, authors provided solid proof that the bilayer area-compressibility modulus (K_a) remains essentially constant for a series of phospholipids containing cis-unsaturated acyl chains in both the sn-1 and sn-2 positions. Even more surprisingly, it has been proven that dye release from liposomes made of POPC and SOPC takes place two orders-of-magnitude faster than from those composed of a

di-monounsaturated lipid, having a comparable bending modulus (k_c). At the same time, authors found that the δ-lysin binding to DOPC is strongest (K_D~30 μM), followed by POPC (K_D~60 μM) and di18:3PC (K_D ~100 μM).

Thus, in conjunction to our data, it can be clearly concluded that bulk properties of the lipid bilayer, such as the area-compressibility and bending modulus, as well as the membrane lateral pressure profile are probably not sufficient to describe unequivocally the different behavior of amphipathic peptides, and HPA3 in particular, toward bilayers composed of phospholipids with various degrees of hydrocarbon chain unsaturation.

To our knowledge this is a rather new approach, whereby by employing electrophysiology and fluorescence techniques we were able to investigate on the same system, i.e. a reconstituted planar membrane, kinetic and transport properties of an antimicrobial peptide. In relation to data presented herein, the relevance of our work may be appreciated by that although the dynamics and magnitude of the leakage induced by specific AMP's are known to depend among others on mechanical properties of the target membrane, a consistent, unified mechanism to fully explain the relationships between binding and leakage induced by linear antimicrobial peptides with distinct chemical and physical properties, and elastic manifestations of lipid membranes with various degrees of unsaturation, is still lacking.

In summary, the results presented in this work emphasize that acyl chain structure remains a key factor which modulate the HPA3 peptide-lipid interactions, and as a direct consequence of the enhanced degree of unsaturation present in DOPC acyl chains as compared to POPC, a decrease of the in-plane cohesive interactions among the lipids ensues, leading to an enhancement of the HPA3 insertion in planar lipids made of DOPC lipids and augmented transmembrane translocation. This data adds new proof which emphasizes the existence of supplementary mechanisms through which AMPs and in particular the HPA3 peptide manifest various degrees of selectivity against various cells, besides those conferred by their amphipathicity, hydrophobicity and cationic charge, based on variations in the composition of each particular cell membrane. As the same time, our data brings into focus the still unresolved paradigm of roles played by unsaturated lipids in the function of membrane-active peptides.

Further experiments involving phospholipids with polyunsaturated acyl chains, in conjunction with micelle-forming amphiphiles known to controllably change the monolayer equilibrium curvature and therefore complementarily modulate the energetics of peptides insertion are underway, in a more comprehensive attempt to shed new light into the influence of membrane composition in AMP dynamics. This will eventually help pave the way towards transforming HPA3 peptides into potent candidates for the development of novel antibiotic agents, with increased specificity and toxicity for particular target membranes.

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