Biophysical Chemistry 210 (2016) 35–44

Contents lists available at ScienceDirect

Biophysical Chemistry

journal homepage: http://www.elsevier.com/locate/biophyschem

Investigations of the synergistic enhancement of antimicrobial activity in mixtures of magainin 2 and PGLa

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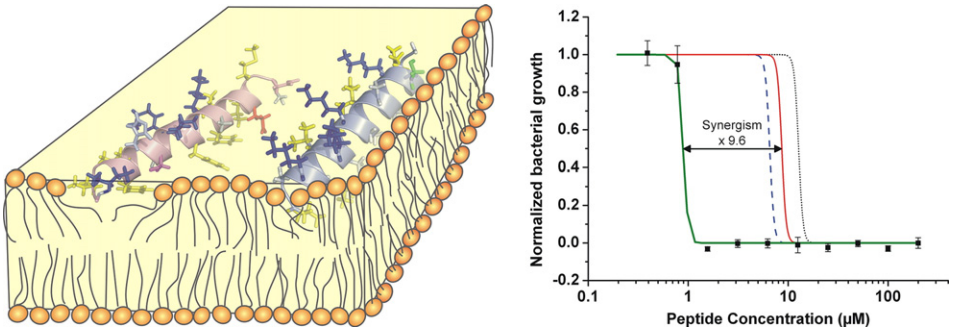
HIGHLIGHTS

- PGLa orients parallel to the surface of membranes made from *E. coli* lipids in the absence or presence of magainin 2.
- Magainin strongly disorders the phospholipid head groups of *E. coli* lipids.
- Synergism does not require pronounced direct/covalent peptide–peptide interactions.
- Lack of correlation between the tendency of PGLa variants to span thin membranes and their synergistic activity

GRAPHICAL ABSTRACT



ARTICLE INFO

Article history:
Received 30 March 2015
Received in revised form 28 May 2015
Accepted 1 June 2015
Available online 9 June 2015

Keywords:
Amphipathic helix
Antimicrobial activities
Membrane topology
Peptide–lipid interactions
Solid-state NMR
Supported lipid bilayer

ABSTRACT

Magainins are antimicrobial peptides isolated from the African clawed frog *Xenopus laevis*. They interact with bacterial membranes where they cause channel formation and membrane disruption. When added as a cocktail magainin 2 and PGLa are considerably more efficient when compared to the corresponding amounts of individual components. In order to investigate this synergistic interaction of PGLa and magainin a number of magainin variants have been prepared and investigated in biological and biophysical assays. In particular we report on the antimicrobial activities and solid-state NMR investigations of magainins that have been extended by a carboxyterminal GGC tripeptide to form covalently linked dimers. Notably, when the formation of the covalent linkage is prevented by exchanging the cystein by serine or alanine no loss in efficiency was observed indicating that the covalent interaction is not necessary for synergistic interaction. In a next step peptides labelled with ^{15}N and ^{2}H were reconstituted into oriented membranes and their topology studied by solid-state NMR spectroscopy. The tendency of some of these peptides to adopt membrane-spanning alignments does not correlate with their synergistic activities in antimicrobial assays. In contrast, the stable alignment of PGLa parallel to the surface of membranes made of *Escherichia coli* lipid extracts is strongly suggestive that the peptides develop synergistic activities when in an in-planar configuration. Notably, the phospholipid head groups of these samples show a high degree of disturbance suggesting that the synergistic interactions between the magainin peptides could be mediated through the lipid phase.

Abbreviations: CI, combination index; di-C10:0-PC, 1,2-didecanoyl-*sn*-glycero-3-phosphocholine; DMPC, 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine; DMPG, 1,2-dimyristoyl-*sn*-glycero-3-phospho-(1′-*rac*-glycerol); ITC, isothermal titration calorimetry; MIC_n, minimal concentration to inhibit n% of bacterial growth; MH, Mueller Hinton; NBD, 4-fluoro-7-nitrobenz-2-oxa-1,3-diazole; NMR, nuclear magnetic resonance; OD, optical density; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PG, phosphatidylglycerol; POPC, 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine; POPE, 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphoethanolamine; POPG, 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phospho-(1′-*rac*-glycerol); RET, resonance energy transfer; r.h, relative humidity.

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http://dx.doi.org/10.1016/j.bpc.2015.06.002
0301-4622/

1. Introduction

The spread of multi-resistant pathogens has become an increasing threat to human health where immediate actions are needed. Bactericidal and fungicidal molecules are abundant in nature and promise that new pharmaceuticals can be developed by mimicking the mechanism of action of such antimicrobial compounds [1–3]. Host defence peptides are one class of antimicrobial macromolecules that have indeed served as lead compounds for the creation of more easy to prepare and/or more efficient analogues [4,5]. Peptides identified from frogs and other amphibians were among the first sequences to be described for their antimicrobial activities [6,7]. These linear peptides are characterized by hydrophobic and a great number of cationic residues which confer a highly amphipathic character. When tested in biological assays they exhibit a broad spectrum of antimicrobial activities. Virucidal and tumoricidal characteristics have also been detected for magainins and other host defence peptides [8,9].

When magainin peptides associate with phospholipid membranes they have been shown to adopt amphipathic α-helical conformations with the cationic residues accumulating on one face of the helix and hydrophobic residues opposite [10–12]. Based on biophysical investigations it has been suggested that the helical domain intercalates into the membrane interface and acts like a wedge thereby destabilizing the membrane packaging [13]. As a consequence the phospholipid bilayers become leaky, the electric conductance increases and the transmembrane electrochemical gradients collapse, which ultimately result in cell killing by these and other amphipathic peptides (reviewed in e.g. [4,14]). The notion that membranes rather than proteinaceous receptors are the main targets of these peptides is supported by observations that enantiomers, retromers or retroenantiomers of magainins all exhibit the high antibiotic and pore-forming activities of the parent L-peptides [15]. Once the peptides have crossed the membrane barrier intracellular targets may also become important for some peptides (for a review see [16]).

A number of biophysical investigations have been performed with magainins [11,17–21], magainin analogues [22–24] and related cationic antimicrobial peptides [25,26] which revealed interesting details about their interactions with membranes. These studies show that the peptides preferentially intercalate into the bilayers at orientations parallel to the membrane surface in agreement with their strongly amphipathic and cationic character [4]. Notably, such biophysical data correlate well with their activities observed in antibacterial assays [27], where the lipid-dependent membrane interactions of the aurein frog peptides parallels nicely their activities against bacterial species in biological experiments [28]. Therefore, when insights from biophysical investigations were combined with structure–activity correlations (e.g. [29]) a number of mechanisms of action were proposed to describe pore formation in membranes either in vitro or in bacteria [4]. Such models include the formation of toroidal pores [30,31], the 'carpet model' [14] or the transient and step-wise increases in membrane conductivity due to stochastic fluctuations in peptide density at the membrane interface [4]. The various supramolecular arrangements of peptides and lipids have recently been summarized in a phase diagram that takes into account that Soft Membranes Adapt and Respond, also Transiently, to external stimuli (SMART model) [32]. Within this model one supramolecular assembly can transform into another shape and this depends on a variety of factors such as the peptide-to-lipid ratio, lipid composition, temperature, buffer composition and pH or other environmental factors. Within the phase diagram of the SMART model the peptides can cause lysis, formation of transient channels and under some conditions even increase the stability of lipid bilayers [13].

In this paper we follow-up an interesting additional observation namely the synergistic enhancements of activities of antimicrobial peptides when added in combination, which also occurs in cocktails of magainin 2 and PGLa, two peptides that are produced and stored together in the frog skin. Notably the peptides' activities are increased not only in biological assays but also when the release of fluorophores from model membranes is monitored. Indeed the addition of equimolar mixtures of these two peptides causes an increase in efficiency by almost an order of magnitude when compared to the individual components. Synergism has also been observed for other amphipathic peptides as well as for mixtures involving conventional antibiotics [12,33–36].

So far only a few biophysical or structural investigations have been published and the molecular interactions that cause synergistic enhancements are poorly understood. For example, using ^{15}N solid-state NMR spectroscopy the membrane alignment of PGLa and magainin 2 was monitored. In phosphatidylcholine membranes, where both fatty acyl are saturated, the tendency of PGLa to adopt transmembrane alignments increased in particular in the presence of magainin 2, when at the same time the latter remains surface oriented [37,38]. Importantly, both peptides maintain orientations parallel to the membrane surface in bilayers made of (partially) unsaturated phospholipids (e.g. POPC or POPC/POPG). Because the latter are thought to represent more closely the natural composition of biological membranes than the saturated phosphatidylcholines this result seems to represent better what happens in a natural environment [38,39].

Previously PGLa and magainin 2 variants were prepared with GGC extensions at either the carboxy- or the amino-terminus as well as with protecting groups neutralizing the charges of both termini [40]. Mixtures of these sequences were incubated in the presence and absence of PC/PG vesicles under high pH conditions favouring cystine bridge formation. Notably, when associated with membranes the parallel heterodimer of the two peptides preferentially formed [40]. This preformed dimer was found equally active than the mixture of wild type peptides when tested against bacteria, when at the same time the covalent linkage much enhanced calcein release activity from egg-PC vesicles [41]. Although such data are suggestive that specific interactions between PGLa and magainin 2 are responsible for their synergistic activities it has proven difficult to identify unique and strong interaction sites on both peptides [34,42]. Therefore, we further investigated the role of sequence additions that have been suspected to help in dimer formation and thereby enhance synergistic activities [40] as well as variants thereof. These sequences were tested in antimicrobial assays and the data compared with their tendency to adopt transmembrane alignments. Because the in-plane to transmembrane equilibrium of PGLa is most easily shifted into its transmembrane state in thin membranes, investigating ^{15}N labelled PGLa in di-C10:0-PC by ^{15}N solid-state NMR spectroscopy provides a sensitive test for such shifts in topological equilibria [38]. Therefore, we analyzed how PGLa and the modified PGLa sequences align in such thin membranes in the absence and presence of magainin 2 peptides and how their tendency to adopt more transmembrane alignments correlates with their potential to interact synergistically.

2. Materials and methods

The phospholipids (di-C10:0-PC, *Escherichia coli* lipid extract) were purchased from Avanti Polar Lipids (Alabaster, AL) and used without further purification.

2.1. Peptide synthesis and purification

The peptide sequences shown in Table 1 were prepared by solid-phase synthesis using a Millipore 9050 automatic peptide synthesizer and fmoc chemistry. For NMR investigations PGLa was labelled with ^{15}N at the Ala-14 and with $^{2}H_3$ at the Ala-10 position using commercial fmoc-protected amino acids (Euriso-top, Paris, France or Isotec® Sigma-Aldrich, St Quentin Fallavier, France).

The peptides were purified by reverse phase HPLC (Gilson, Villiers-le-Bel, France) using a preparative C18 column (Luna, C18–300 Å–5 μm, Phenomenex, Le Pecq, France) and an acetonitrile/water gradient.