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The influence of mild acidity on lysyl-phosphatidylglycerol biosynthesis and lipid membrane physico-chemical properties in methicillin-resistant *Staphylococcus aureus*

Reg P. Rehal[a], Helene Marbach[a,1], Alasdair T.M. Hubbard[a,d], Anam A. Sacranie[a], Federica Sebastiani[c,2], Giovanna Fragneto[c], Richard D. Harvey[a,b,*]

[a] Institute of Pharmaceutical Science, King's College London, London, UK
[b] Institute of Pharmacy, Martin-Luther-Universität Halle-Wittenberg, Halle (Saale), Germany
[c] Institut Laue Langevin, 71 Avenue des Martyrs, BP 156, 38100 Grenoble, France
[d] Nuffield Department of Clinical Medicine, University of Oxford, Oxford, UK

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ABSTRACT

The increased biosynthesis of lysyl-phosphatidylglycerol in *Staphylococcus aureus* when cultured under conditions of mild acidity and the resultant increased proportion of this lipid in the plasma membrane of the bacterium, alters the physico-chemical properties of lipid bilayers in a manner which is itself dependent upon environmental pH. Clinically relevant strains of *S. aureus*, both methicillin susceptible and resistant, all exhibited increased lysyl-phosphatidylglycerol biosynthesis in response to mild environmental acidity, albeit to differing degrees, from ~30% to ~55% total phospholipid. Polar lipid extracts from these bacteria were analysed by ^{31}P NMR and reconstituted into vesicles and monolayers, which were characterised by zeta potential measurements and Langmuir isotherms respectively. A combination of increased lysyl-phosphatidylglycerol content and mild environmental acidity were found to synergistically neutralise the charge of the membranes, in one instance altering the zeta potential from −56 mV to +21 mV, and induce closer packing between the lipids. Challenge of reconstituted *S. aureus* lipid model membranes by the antimicrobial peptide magainin 2 F5W was examined using monolayer subphase injection and neutron diffraction, and revealed that ionisation of the headgroup α-amine of lysyl-phosphatidylglycerol at pH 5.5, which reduced the magnitude of the peptide-lipid interaction by 80%, was more important for resisting peptide partitioning than increased lipid content alone. The significance of these results is discussed in relation to how colonising mildly acidic environments such as human mucosa may be facilitated by increased lysyl-phosphatidylglycerol biosynthesis and the implications of this for further biophysical analysis of the role of this lipid in bacterial membranes.

1. Introduction

The mildly acidic conditions encountered at a number of human epithelia constitute part of the intrinsic barrier to bacterial colonisation and infection. The fact that these epithelial surfaces are colonised by a number of different species of commensal bacteria suggests that these organisms have evolved mechanisms for tolerating the localised level of acidity and other intrinsic defences exhibited by their hosts (Hornef et al., 2005). In the case of opportunistic pathogens such as *Staphylococcus aureus*, the ability to survive under the pressure of such epithelial defences may also provide some clues as to their ability to invade soft tissues and establish infections.

The link between resistance to the human antimicrobial peptides (AMP) which form part of the innate immune defences at epithelia and changes in the plasma membrane phospholipid composition of *S. aureus* has long been established (Li et al., 2007). Increasing biosynthesis of the cationic lipid lysyl-phosphatidylglycerol (L-PG) via the upregulation of the membrane-integral

* Corresponding author at: Institute of Pharmacy, Martin-Luther-Universität Halle-Wittenberg, Halle (Saale), Germany.
E-mail address: richard.harvey@pharmazie.uni-halle.de (R.D. Harvey).
[1] Current affiliation: Functional Microbiology, Institute of Microbiology, Department of Pathobiology, University of Veterinary Medicine Vienna, Veterinärplatz 1, AT-1210 Vienna, Austria.
[2] Current affiliation: CR Competence AB, Lund, Sweden & Department of Physical Chemistry, Lund University, Lund, Sweden.

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lysyl transferase MprF, facilitates resistance to cationic AMP *in vitro* via the putative neutralisation of the plasma membrane charge as the proportion of L-PG reaches parity with anionic lipids (mostly phosphatidylglycerol) (Andra et al., 2011; Staubitz et al., 2004). Mildly acidic environments also increase the biosynthesis of L-PG in *S. aureus*, and may play a more important part in its functional role than has hitherto been considered (Denich et al., 2003; Nesbitt and Lennarz, 1968). Since the L-PG headgroup (Fig. 1) possesses three ionisable groups with distinct pK_a values (Tocanne et al., 1974), the local pH of the lipid will determine whether or not it is likely to form ion-pairs with adjacent anionic lipids to facilitate plasma membrane neutralisation. The pK_a of the L-PG headgroup α-amine has been determined to be ∼7.0 making plasma membrane neutralisation more likely in mildly acidic environments (Tocanne et al., 1974), which may explain why earlier *in vitro* assessments of the role of L-PG in AMP resistance yielded equivocal results (Khatib et al., 2016; Kilelee et al., 2010).

Biophysical assessment of the role of L-PG in resistance to cationic antimicrobials has been hampered by the highly labile nature of the lipid under neutral and mildly alkaline conditions (Danner et al., 2008), as this means that it is readily hydrolysed when subjected to vesicle manufacturing procedures such as ultrasonication and analysis techniques such as x-ray diffraction. This has led to the synthesis of stable analogues of L-PG (Cox et al., 2014; Rehal, 2014) which can be used more effectively in biophysical experiments with long measurement timescales, which aim to elucidate the physical mechanisms of membrane defence facilitated by L-PG in synthetic membranes. In this study, we have attempted to circumvent the problem of L-PG instability by using freshly prepared polar lipid extracts (analysed using ^{31}P NMR) from different clinically relevant methicillin resistant *S. aureus* (MRSA) strains, in rapid biophysical assays performed to assess the physical effects of L-PG in reconstituted lipid membranes and the putative roles of membrane lipid composition and environmental pH in AMP resistance.

We have examined the effect of both L-PG content and pH on MRSA lipid extracts in membrane mimetic monolayers and bilayers using a combination of Langmuir isotherms and zeta-potential measurements. The degree to which such systems can resist interaction with the model AMP magainin 2 F5W at pHs mimicking those of the skin (pH ∼5.5) and blood compartments of the body (pH 7.4), was determined by adapting the monolayer technique to measure peptide partitioning after subphase injection, and by assessing the depth of peptide penetrating into MRSA lipid bilayers using neutron diffraction. In addition to these physico-chemical studies we also conducted a phenotypic analysis of the effects of mild acidity on L-PG biosynthesis in our MRSA strains, using ^{31}P NMR lipidomics. Our findings show that pH not only influences membrane lipid composition, but it also directly affects membrane charge, rigidity and AMP interaction.

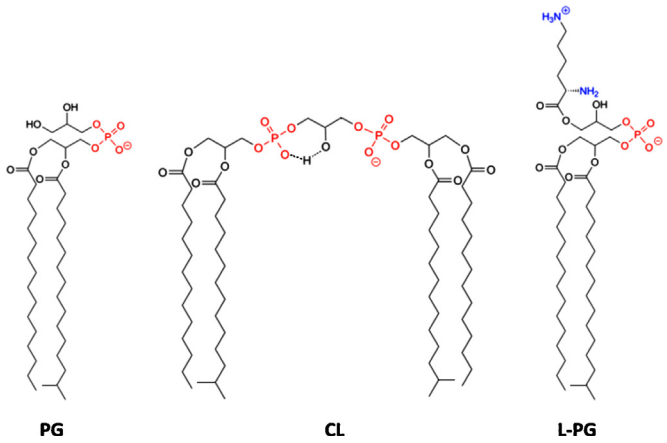

Fig. 1. Representative chemical structures of the three main phospholipids found in the plasma membrane of *S. aureus*, phosphatidylglycerol (PG), cardiolipin (CL) and lysyl-phosphatidylglycerol (L-PG).

2. Materials and methods

2.1. Materials

Brain heart infusion (BHI) media was used for all bacterial cultures and was purchased from Oxoid, UK. Concentrated hydrochloric acid (12.4 M, 38.0%), ethanol (99.9%), d-chloroform with 0.03% (v/v) tetra-methyl silane (≥99.8%D atom), ethylenediaminetetraacetic acid (≥98.0%), tris(hydroxymethyl)aminomethane (>99.0%), glacial acetic acid, sodium sulphate (>99.0%), deuterium oxide (99.9%) and sodium chloride ACS reagent (≥99.0%), were all purchased from Sigma-Aldrich, UK, and used as supplied. Methanol, chloroform and ethanol were purchased from Fisher Scientific, UK, and used as supplied. 1,2-*O*-dipalmitoyl-*sn*-glycero-3-phospho-(1′-*rac*-glycerol) sodium salt (DPPG), 1,2-*O*-dipalmitoyl-*sn*-glycero-3-[phospho-*rac*-(3-lysyl(1-glycerol))] chloride salt (DPL-PG) and 1,1′,2,2′-tetramyristoyl cardiolipin ammonium salt (TMCL) were all purchased with >99.0% purity from Avanti Polar Lipids, USA.

2.2. Bacterial strains

The five *S. aureus* clinical isolates used in this study (Table 1) were kindly provided by the Centre for Clinical Infection and Diagnostics Research (CIDR), at Guy's and St. Thomas' NHS Foundation Trust Hospital in London, UK (GSTT). All these strains were isolated from bacteraemia patients hospitalised between 2001 and 2009. The strains were selected on the basis of CIDR in-house testing and data assessment, which identified their clonal group assignment (multi-locus sequence type and Spa type), together with their methicillin resistance status and vancomycin minimum inhibitory concentrations (MIC).

2.3. Phospholipid extraction and quantification

Each strain of *S. aureus* (Table 1) was pre-cultured in 50 ml BHI broth for 18 h at 37 °C with continuous shaking at 100 rpm. A 1 ml aliquot from each specific pre-culture was then used to inoculate 400 ml of BHI broth with an initial pH of either 7.4, or 5.5 (adjusted with concentrated hydrochloric acid prior to sterilisation). The culture was then incubated for 18 h at 37 °C with constant shaking at 100 rpm. After incubation, the bacterial suspension was centrifuged on a Beckman Coulter J2-21 (Beckman Coulter, UK) at 10 000 rpm for 20 min at 4 °C and the resulting pellet was washed twice with 50 ml of 150 mM sodium chloride solution adjusted to either pH 5.5 or 7.4 (depending on the initial culture

Table 1
Selected genotypic and phenotypic characteristics of the clinically-isolated *Staphylococcus aureus* strains used in this study.

Strain	Sequence type[a]	Spa type[b]	Vancomycin MIC[c] (μg/mL)
S. aureus 476	ST1	T183	<0.5
MRSA G32	ST239	T032	>1.5
MRSA G33	ST22	T032	<0.5
MRSA H64	ST36	T018	<0.5
MRSA H66	ST36	T018	<0.5

[a] Based on multi-locus sequence typing genetic fingerprinting.
[b] Based on the sequence of the polymorphic region of the Protein A gene.
[c] The minimum inhibitory concentration.