Dye-release assay for antimicrobial peptide activity using a competitive lipid environment

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Abstract

A dye-release method investigating the effect of a competitive lipid environment on the activity of two membrane-disrupting antimicrobial peptides (AMP), maculatin 1.1 and aurein 1.2, is presented. The results support the general conclusion that AMP have higher affinity for negatively charged membranes, such as encountered in bacterial membranes, rather than the neutral membrane surface found in eukaryotic cells but only within a competitive lipid environment. Indeed, in a single model membrane environment, both peptides were more potent against neutral vesicles than charged vesicles. The approach was also used to probe the effect of pre-incubating the peptides within a neutral lipid environment and then introducing charged lipid vesicles. Maculatin was shown to traffic from the neutral lipid bilayers where pores had already formed to the charged membrane bilayers. This result was also observed in charged to charged bilayers but, interestingly, not from neutral to neutral lipid interfaces. Aurein was able to translocate from either lipid environment, indicating a weaker binding to lipid membranes, and a different molecular mechanism for lysis of lipid bilayers. Competitive lipid environments could be used to assess other critical parameters that modulate the activity of membrane peptides or proteins.

Introduction

In vivo delivery of membrane-active peptides or proteins to their target occurs with the possibility of binding to several different membrane interfaces. For instance, in the case where an antimicrobial peptide (AMP) is used against a bacterial infection, several membrane types are present, such as those of the host cells, symbiotic bacteria and pathogenic bacteria. The classic approach to study membrane-active peptides or membrane-protein functions is to use *in vitro* and isolated systems, which lack this competitive membrane lipid environment.

For instance, loss of membrane integrity, such as observed in pore formation, is often characterized with dye release assays, where vesicles mimicking the lipid composition of target membranes and containing a self-quenching dye, are used to probe the affinity and potency of membrane-active peptides or proteins (Shai 1999; Shai et al. 1991; Wang et al. 1998). This method is relatively easy to setup and requires little material and, therefore, is widely used.

An adaptation of the dye release assay allowing the simultaneous investigation of different membrane targets is presented herein. Maculatin 1.1 (Mac1) and aurein 1.2 (aurein), two antimicrobial peptides (AMP) expressed in the skin secretions of the *Litoria* genus of Australian tree frogs (Chia et al. 2000; Rozek et al. 1998; Rozek et al. 2000), have been chosen to demonstrate the advantage of such competitive lipid environments. Both peptides have shown antimicrobial and haemolytic activity but to a varying degree (Chia et al. 2011; Rozek et al. 1998; Sani et al. 2013). Furthermore, the mechanism of action is also specific to each peptide, Mac1 being a pore-forming peptide (Sani et al. 2013) while aurein acts via a detergent-like mechanism (Fernandez et al. 2012). Two types of lipid vesicles were used simultaneously to demonstrate the impact of exposing the AMP to a competitive lipid environment: eukaryotic-like membranes were mimicked with phosphatidylcholine lipids and *Staphylococcus aureus* membrane mimics with a mixture of phosphatidylglycerol and cardiolipin lipids (Sani et al. 2013; White and Frerman 1967). Furthermore, the peptides were also pre-incubated with dye-free vesicles prior to addition of dye-containing vesicles of similar or different lipid compositions in order to assess preferential binding and trafficking of AMP between membranes.

Experimental Procedures

Materials

Maculatin 1.1 (GLFGVLAKVAAHVVPAlAEHF-NH₂; MW 2148) with >95% purity was purchased from Federation Bioscience (Melbourne, Australia). Aurein 1.2 (GLFDIIKKIAESF-NH₂) was obtained from the in-house Bio21 peptide synthesis facility. The peptides were washed in 5 mM HCl solution and lyophilised overnight to remove residual trifluoroacetic acid (Sani et al. 2007). Palmitoyl-oleoyl-phosphatidylcholine (POPC), palmitoyl-oleoyl-phosphatidylglycerol (POPG) and tetraoleoylcardiolipin (TOCL) phospholipids were purchased from Avanti Polar Lipids (Alabaster, USA) and were used without further purification. 5(6)-carboxyfluorescein (CF), Triton X-100 and PD-10 columns were purchased from Sigma (St Louis, USA).

Carboxyfluorescein solution preparation

5(6)-carboxyfluorescein (CF) was initially insoluble in water and hence was dissolved in *ca.* 3 eq. KOH and vortexed until completely dissolved. Appropriate volumes of imidazole and EDTA were added to yield a *ca.* 55 mM CF stock in 40 mM imidazole, 1 mM EDTA before final adjustment to pH 7.4 with KOH and HCl concentrated solutions.

CF encapsulation in large unilamellar vesicles

To prepare CF-loaded large unilamellar vesicles (LUV), lipids were suspended in 1 mL of the CF solution to reach a *ca.* 15 mM lipid solution. The solutions were freeze/thawed five times and then extruded 10 times through an Avanti Mini-Extruder (Alabaster, USA) using 0.1 µm polycarbonate filters to produce LUV of a nominal 100 nm diameter. Samples were extruded above the corresponding main gel-to-fluid chain melting temperatures of the phospholipids. Separation from free dye was obtained by gel filtration using PD-10 desalting column with a 2.5 mL dead volume. 1 mL of each CF-loaded LUV lipid system was eluted under gravity with 40 mM imidazole, 110 mM KCl and 1 mM EDTA (pH 7.4) running buffer.

Approximately 2 mL was collected after passing 2.5 mL of running buffer. CF-free LUV were prepared similarly as CF-loaded LUV except that the lipid mixtures were suspended with 1 mL of the running buffer to reach a *ca.* 15 mM stock concentration. Phospholipid concentrations were determined in triplicate using the phosphorus assay of Anderson *et al.* (Anderson and Davis 1982). Briefly, aliquots of CF-loaded lipid solution, buffer-only (negative control) and CF-free lipid solution (positive control), corresponding to an anticipated 0.1 μ mole of phosphorous, were dried in test tubes at 100°C. 450 μ L of sulfuric acid was then added into each tube and the solutions were heated up to 220°C for 25 min on a heating block. 150 μ L of hydrogen peroxide was then added and the samples were placed on the heating block for another 30 min. 3.9 mL of milli-Q water was added, followed by 500 μ L of ammonium molybdate (VI) and 500 μ L of ascorbic acid, thoroughly mixed between each addition. Each tube was then capped and heated at 100°C for 7 min. Absorbance at 820 nm was recorded and concentrations were determined using a calibration curve obtained using known amount of dry DMPC lipid powder.

CF release measurements in single and competitive lipid environment

Samples were prepared by mixing a 1:1 molar ratio of CF-loaded and CF-free vesicles and appropriate amount of peptide stock solution to produce desired lipid to peptide (L/P) molar ratios under two different conditions:

- (i) No pre-incubation: the peptide was added to a mixture of both dye-free and dye-containing LUV prior to measurements; and
- (ii) Pre-incubation: the peptide was let to interact with dye-free vesicles for 30 min prior to addition to dye-containing vesicles.

Negative controls were made by substituting peptide stock for buffer-only and positive controls by adding 10 μ L of 10% Triton X-100.

Measurements were made on a FLUOstar Optima plate reader (BMG Labtech, USA) using a Falcon96 tissue culture 96-well plate (Fisher Sci., USA). The reading was optimized for the Falcon96 plate format. The excitation wavelength was set at 492 nm and the bottom-read emission was recorded at 520 nm. 10 cycles (120 s each) were performed at 30°C with 5 s of orbital shaking prior each cycle and the fluorescence intensities were then averaged.

The percentage of CF fluorescence was obtained by normalising the averaged intensities (I) against the negative (baseline, I_{min}) and positive (100% release, I_{max}) controls, according to:

$$\% fluorescence = 100 \cdot (I - I_{min}) / (I_{max} - I_{min})$$

The normalised intensities were plotted against the lipid to peptide molar ratio (L/P) and fitted using an empirical logistic function:

$$F = F_{max} + \frac{(F_{min} - F_{max})}{1 + \left(\frac{x}{EC_{50}} \right)^p}$$

where F is the normalized CF intensity at each L/P ratio; F_{min} and F_{max} are fixed at 0% and 100%, respectively; x is the L/P ratio; EC_{50} is the L/P ratio necessary to obtain 50% of maximal fluorescence; and p is a cooperativity factor.

Results

Classic dye-release assay versus competitive lipid environment method

CF release assays were performed by mixing the membrane-active AMP with 50 μ M CF-loaded liposomes and 50 μ M CF-free liposomes at L/P molar ratios ranging from 10000:1 to 5:1. Ten measurements were averaged, normalised and plotted against the L/P ratios. The EC_{50} – the L/P necessary to obtain 50% of maximal CF fluorescence – for each lipid system and antimicrobial peptide were obtained by fitting the experimental data using a logistic function. It is noteworthy that the greater is the EC_{50} , the more potent is the peptide to induce

CF release from the LUV. Fig 1 and Table 1 showed that Mac1 was more potent against POPC vesicles – a relatively simple model for eukaryotic cells – with an EC_{50} of 490 (L/P), while aurein showed lower potency with an EC_{50} of 60 (L/P). However, both peptides were about 8-fold less potent against POPG/TOCL vesicles – a lipid model for *S. aureus* outer-membranes. Interestingly, Mac1 was about 7-8 fold more potent to induce CF release from LUV than aurein in both lipid environments. These results correlate well with *in vivo* observations that report a similar difference between Mac1 and aurein minimum inhibitory concentrations (MIC) against *S. aureus*, 4 μ M (Sani et al. 2013a) and 35 μ M (Rozek et al. 2000), respectively. Surprisingly, Mac1 and aurein reported haemolytic concentration (HC_{50}) is above 100 μ M (Chia et al. 2011) and 65 μ M (Rozek et al. 2000), respectively, which indicate that the peptides are expected to be less potent against POPC than POPG/TOCL, and that Mac1 would be less potent against POPC than aurein. Although red blood cell (RBC) membranes are principally composed of POPC lipids, they also contain a high content of cholesterol and sphingomyelin, which could impact highly on Mac1 and aurein lytic potencies.

The presence of an equal amount of POPG/TOCL vesicles in the CF-loaded POPC lipid environment dramatically reduced the potency of Mac1 and aurein, but the effect was specific to each peptide (Fig. 1, filled symbols). Mac1 potency was reduced 35-fold while the charged lipid environment only reduced aurein potency by 3.5-fold. The reverse situation was also true where the presence of POPC had little effect on the potency of the two peptides to lyse CF-loaded POPG/TOCL membranes. EC_{50} were 2-fold decreased compared to the single environments, indicating that the peptides were predominantly interacting with the negatively charged LUV. These results support the proposition Mac1 and aurein have a greater affinity for anionic lipids, a general consensus for membrane-active AMP, but also revealed that fewer peptides are necessary for disrupting neutral membranes. However, since both peptides

would barely interact with the neutral lipid environment in the presence of a negatively charged lipid membranes, further development is worthwhile pursuing.

Reversibility of the membrane-bound state: pre-incubation method

The reversibility of the lipid-bound state can also be assessed in a competitive environment. When Mac1 was pre-incubated with dye-free POPC vesicles and then combined with CF-loaded POPG/TOCL vesicles, a similar potency (EC_{50} 95 L/P) for charged vesicles was observed, confirming the strong affinity for negatively charged membranes (Fig. 2, Table 2). We find it remarkable that the pore-forming Mac1 could dissociate from the neutral lipid vesicle and migrate to an anionic one, suggesting that electrostatics play a crucial role in the peptide selectivity. As expected, pre-incubating the peptide within POPG/TOCL lipid vesicle showed a net reduction of Mac1 potency (EC_{50} 15 L/P) when added to the POPC lipid environment. Furthermore, the pre-incubation within POPC prior to addition of CF-loaded POPC LUV showed that the peptide was not able to migrate in this situation as the EC_{50} was significantly reduced by about 2-fold, an interesting finding for drug delivery applications. At about L/P of 250:1, POPC membranes appeared to be saturated, and the free Mac1 peptides could then interact with the CF-loaded POPC LUV. Unexpectedly, in the case of pre-incubation with POPG/TOCL LUV prior to addition of dye-loaded POPG/TOCL LUV, a similar EC_{50} (13 vs. 15 L/P) was obtained compared to the direct addition of peptide for the anionic lipid environment, which indicates that Mac1 was not as tightly bound (or deeply buried into the lipid hydrophobic core) in POPG/TOCL compared to POPC membranes.

Pre-incubation of aurein with dye-free LUV showed that the peptide was not as tightly bound to either lipid environment since pre-incubation did not modify the peptide EC_{50} compared to the results obtained without pre-incubation (Table 1).

Discussion

Advantage of the technique

A critical aspect of drug discovery and development is the characterization and optimization of the safety and potency of drug candidates (Muller and Milton 2012). As *in vivo* and clinical assays are often costly in time and resources, *in vitro* assays are used as a primary screening tool, especially for membrane-penetrating peptides (Heitz et al. 2009). Furthermore, the efficiency of membrane-active molecules often depend on the lipid environment and, therefore, many studies investigate the effect of particular lipid compositions to determine the molecular mechanism of the potential drug candidate (Epand et al. 2007; Sani et al. 2011; Sani et al. 2012b).

However, we have shown that the common approach of studying a membrane-active peptide in a single lipid environment could mask the potential of a drug candidate. For instance, Mac1 and aurein potency obtained from dye-release assays using one model membrane system at a time showed a higher toxicity against eukaryotic-like rather than *S. aureus* lipid membrane mimics. This result would probably suppress any intent to develop further these peptides as antibiotic alternatives. But having both model membrane types in a single experiment showed that the peptides barely interacted with eukaryotic-like membrane when the concentration of peptide was below a certain threshold (the amount of peptide required to saturate the *S. aureus* lipid system), which strongly promotes these peptides as plausible drug candidate for further development. Therefore, investigating membrane-active molecules in a competitive lipid environment may be a better model for screening membrane-active drug candidates.

Furthermore, and although not tested in this work, several important properties of the lipid cell membranes can be easily investigated. For instance, competition between micelles and

LUV would assess the role of membrane curvature in modulating the molecular mechanism of membrane-active molecules. In future studies, improved model membranes will be utilized, such as POPC/cholesterol to mimic red blood cells, and conditions varied to evaluate dye leakage using appropriate kinetic models.

Bound-state reversibility

The advantages of using a competitive lipid environment are multiple. For instance, the stability or reversibility of a bound-state can be investigated, as previously described for a single environment system (Pokorny and Almeida 2005; Pokorny et al. 2000; Pokorny et al. 2002). The method allows pre-incubation of the peptide with dye-free LUV before adding to the dye-containing LUV. It showed that Mac1 was able to form pores and then relocate to an anionic lipid interface mimicking a bacterial membrane. This observation can help to understand the behaviour of drugs delivered using lipid-based vehicles, where the drug biodistribution profiles apparently depend on the lipid composition is performed only in a single lipid environment (Arias et al. 2011). The mechanism by which membrane-penetrating peptides form pores in natural membranes that are known to contain many lipid domains can be further investigated using similar assays (Coskun and Simons 2011; Dowhan 1997; Sani et al. 2012a; Singer and Nicolson 1972).

Finally, the presented dye-release assays are a useful tool to compare the molecular mechanism by which the antimicrobial peptide interacts with a particular lipid membrane. For instance, aurein was likely coating the LUV surface and, therefore, could translocate from one lipid environment to another, while Mac1 was likely inserted deeper into the hydrophobic core of lipid bilayers as reflected in the reduced potency upon pre-incubation. However, supporting data, such as solid-state NMR and oriented CD, are needed to confirm peptide location onto or into the lipid membranes.

Conclusion

The dye-release assays presented here using a competitive lipid environment provide a tool to assess affinity and reversibility of membrane-penetrating molecules under various conditions (e.g. lipid composition, lipid phase, curvature effects, *etc.*) and serve as a more realistic *in vivo* model for the screening of membrane-active antimicrobials.

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Table 1 Mac1 and aurein lytic activities ^a in single and competitive lipid environments ^b				
LUV (100 nm)	Mac1		Aurein	
	POPC*	POPG/TOCL*	POPC*	POPG/TOCL*
POPC	490	97	60	17
POPG/TOCL	13	58	4	8
^a EC ₅₀ reported values are the lipid to peptide molar ratio required for increasing the fluorescence by 50% obtained by fitting the experimental data to a logistic function. ^b Aliquots of peptide were added to a fixed LUV concentration of 100 μM made of equal amounts of dye-filled LUV (*) and dye-free LUV of similar lipid composition for single environments while competitive lipid environments were made by mixing dye-filled LUV with dye-free LUV of a different lipid composition.				

Table 2 Effect of pre-incubation ^a on Mac1 and aurein lytic activities in single and competitive lipid environments ^b

LUV (100 nm)	Mac1		Aurein	
	POPC*	POPG/TOCL*	POPC*	POPG/TOCL*
POPC	240	95	50	14
POPG/TOCL	15	35	3	6

^a Mac1 and aurein were incubated with 50 μ M of dye-free LUV for 30 min prior to addition of 50 μ M of dye-filled LUV (*).

^b Activities and lipid environments were obtained as for Table 1.

Figure 1 Single *versus* competitive lipid environments impact on CF-release assay upon incubation with increasing amount of Mac1 (left panel) and aurein (right panel). Single lipid environment made of POPC (open squares) and POPG/TOCL (open circles) were made with 50 μ M dye-encapsulated large unilamellar vesicles (LUV, 100 nm) and 50 μ M of dye-free LUV. Competitive lipid environments were prepared by mixing 50 μ M of POPC LUV and 50 μ M of POPG/TOCL LUV (filled symbols). Curve fitting was achieved using a logistic function (see Experimental Procedures).

Figure 2 Effect of 30 min pre-incubation of Mac1 (left panel) and aurein (right panel) with CF-free POPC (open squares) and POPG/TOCL (open circles) vesicles prior to addition of CF-encapsulated LUV of similar composition. Competitive environments were produced by pre-incubating Mac1 and aurein with CF-free POPC LUV prior to addition of CF-encapsulated POPG/TOCL (filled squares) LUV, and vice-versa (filled circles). Curve fitting was achieved using a logistic function (see Experimental Procedures).

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