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Divalent Metal Ion Triggered Activity of a Synthetic Antimicrobial in Cardiolipin Membranes

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Abstract

One member of a prototypical class of antimicrobial oligomers was used to study pore formation in cardiolipin rich membranes. Using both vesicle dye-leakage assays and small angle x-ray scattering, bilayer remodeling was studied. The results indicate that the presence of negative intrinsic curvature (NIC) lipids is essential for pore formation by this class of molecules: In Gram-positive bacteria, cardiolipin and divalent metal cations like Ca^{2+} and Mg^{2+} are needed. This is completely consistent with the role of phosphatidylethanolamine (PE) lipid in Gram-negative bacteria, where antimicrobial activity is dependent on the negative intrinsic curvature of PE, rather than a specific interaction with PE.

Antimicrobial peptides (AMPs) have broad spectrum killing activity against prokaryotes but not against eukaryotes, and consequently are considered promising antibiotic candidates. However, these peptides are typically large (–up to 80 amino acid residues), structurally complex, and expensive to produce. As a result, much effort has been focused on developing synthetic analogs,[1] or antimicrobial oligomers (AMOs). Recent human clinical data indicate that this is a promising approach.[1h] There is general agreement that these antimicrobial molecules interact with the cell membrane, which can have complex distributions of lipids,[2a–e] including lipids with non-zero intrinsic curvature,[2f] such as unsaturated-phosphatidylethanolamine (PE),[2g] and cardiolipin (CL).[2g–i] Bacterial membranes have a significantly higher population of negative intrinsic curvature (NIC) lipids compared to mammalian membranes, and it was recently shown that these differences are one mechanism by which AMOs can differentially kill bacteria over mammalian cells.[2d, 2e, 2j–l]

The specific relationship between PE and AMO **2** was recently described for *E. coli*.[2d, 2j] While this readily explained the potent activity against Gram-negative bacteria (MIC of 0.1 μg/mL against *E. coli*), it did not explain this AMOs activity against Gram-positive bacteria like *S. aureus* (0.2 μg/mL) since this organism is known to contain no PE lipid.[3] In addition, it was demonstrated that although antimicrobial activity is largely ablated in PE-knock-out strains of *E. coli*,[4] residual antimicrobial activity still remains. Moreover, the previous results specifically implicated PE lipid, although it was hypothesized that NIC lipids were more important than any specific binding between the AMO and PE.

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Supporting Information Available: This material is available free of charge via the Internet at http://pubs.acs.org.

Here, we examine how a prototypical synthetic antimicrobial interacts with cardiolipin (CL), which is the major lipid component in most Gram-positive bacteria. We show the structural requirement needed for membrane activity of AMO **2** is the existence of NIC lipids, in this case CL. A central feature of CL is that its intrinsic curvature is influenced by the bound cations and can be tuned between zero intrinsic curvature ($C_0 \sim 0$) in the presence of monovalent salts and negative intrinsic curvature ($C_0 < 0$) when bound to divalent cations like the biologically prevalent Ca^{2+} or also Mg^{2+} (SI, Figure S1). The presence of NIC lipids is one of the ingredients that facilitate pore formation.[2d–e, 2j–l] AMO **2** activity against CL membranes, with and without divalent metal ions (Ca^{2+}, Mg^{2+}) was evaluated using both calcein dye leakage assays (DLA) and small angle x-ray scattering (SAXS).

The ability of AMO **2** to induce calcein dye leakage from CL vesicles depends strongly on the presence of divalent cations. For example, leakage increased from 5% without divalent cations to 60% in the presence of 2.0 mM Mg^{2+} or 1.0 mM Ca^{2+} ions (Figure 1A, 1B). Importantly the presence of 2.0 mM Ca^{2+} ion alone caused no leakage of entrapped calcein dye (SI, Figure S2). Further, the presence of Ca^{2+} ions have no influence on the leakage activity of 20/80 PG/PE vesicles caused by AMO **2** (Figure 1C). These three data sets highlight the necessity of NIC lipids for AMO **2** to induce calcein release. For the PG/PE vesicles which already have a large volume fraction of NIC lipids, the addition of Ca^{2+} does not enhance leakage. In contrast, AMO **2** has limited activity toward CL vesicles until a reasonable concentration of divalent cations is added to the solution. Because most Gram-positive bacteria's plasma membranes are not composed solely of CL lipid, we also examined the dependence of AMO **2** induced leakage in PG (phosphatidylglycerol)/CL vesicles. Again, leakage was dependent on the presence of divalent cations as shown in Figure 1D. Not surprisingly, a higher concentration of divalent cation was required to exhibit dye leakage in this PG/CL system compared to pure CL vesicles.

To further explore the relationships between AMO **2** and CL-rich membranes, we employed SAXS. Recent work showed that the induction of an inverted hexagonal H_{II} phase in PE-rich membranes by AMOs is correlated with dye leakage and bacterial growth inhibition.[2d, 2j] Here, we map out the structural tendency of CL-rich membranes to re-organize in the presence of AMO **2** and Mg^{2+} ions. Figure 2A shows SAXS data for pure CL in the presence of increasing [Mg^{2+}]. Consistent with previous results,[2i, 5] CL can form an inverted hexagonal phase at sufficiently high [Mg^{2+}] concentrations (1–4 mM) in the absence of the AMO.

As Gram-positive bacterial membranes contain a mixture of CL and PG lipids, SAXS experiments were also performed on mixed lipid compositions. The addition of anionic PG lipids to a CL membrane is expected to decrease the tendency to form negative curvature by reducing the local concentration of NIC CL. SAXS data (SI, Figure S5, SAXS data without AMO's and other control experiments are described in the SI) is consistent with this expectation as DOPG/CL vesicles with 4.0 mM [Mg^{2+}] show no peaks corresponding to the inverted hexagonal phase in contrast to Figure 2A. Meanwhile, Figure 2B shows SAXS data from complexes formed by AMO **2** and DOPG/CL = 32/68 vesicles in the presence of different [Mg^{2+}] concentrations (0 mM, 0.5 mM, 4.0 mM), with AMO to lipid molar ratios fixed at A/L = 1/5. Consistent with the DLA results which showed some leakage [(See Figure 1D], AMO **2** can induce a phase with hexagonal symmetry without Mg^{2+} ions as indicated by peak positions at the characteristic ratio of 1:√3:2. However, the presence of divalent Mg^{2+} increases the structural tendency to form this phase, as evidenced by stronger diffraction peaks. Also consistent with this, the phase with hexagonal symmetry forms at significantly lower [Mg^{2+}] in the presence of AMO **2** than for pure CL vesicles (Compare Figure 2A and 2B, specifically [Mg^{2+}] = 0.5 mM).

Electron density reconstructions (SI, Figure S6) from the above SAXS data confirm that an inverted hexagonal H_{II} phase is formed, as well as allow an estimate of the number of AMO and Mg^{2+} ions in the H_{II} water channels. From the reconstructed electron density profiles, we find the calibrated average electron density at the hydrophilic surface to be 0.58, 0.57, and 0.55 (e/Å^3) for complexes formed at [Mg^{2+}] of 0, 0.5, and 4.0 mM, respectively. For complexes with a high A/L ratio of 1/5 and without Mg^{2+} ions, we estimate ~ 4 AMO **2** molecules per 4 nm length of the water channel (thickness of a lipid bilayer) embedded near the hydrophilic region of the membrane, using a procedure described previously.[2j] From the reconstructed electron density profiles of complexes formed at high [Mg^{2+}] and pure CL vesicles with no added AMO, we estimate that each CL molecule binds ~ 0.6 Mg^{2+} ions (See SI).

This information was used to examine the self-assembly of AMO **2** with PG/CL membranes in the presence of Mg^{2+}. Using the working hypothesis that the number of bound Mg^{2+} ions per CL for DOPG/CL = 32/68 is the same as that of CL vesicles, it was estimated that the number of AMO **2** molecules per 4 nm of channel length is ~ 3.6 and ~ 3.2 for complexes at [Mg^{2+}] of 0.5 mM and 4 mM. Interestingly this observed channel occupancy of AMO **2** molecules are quite close to that found in DOPG/DOPE = 20/80 at high A/L ratios.[2j] These results suggest that, with the help from curvature generating divalent Mg^{2+} ions, CL-rich membranes have ~ 3 AMO **2** molecules embedded into their hydrophilic surface lining per 4 nm length of water channel. These results are also consistent with recent work that shows that bacterial mutants with cardiolipin-rich membranes can be killed by AMO **2** in the presence of Mg^{2+} ions.[2j] This suggests that CL, in the presence of Mg^{2+}, acts as a NIC lipid and functions as a structural substitute for PE during the membrane interaction by AMO **2**. It is likely that both are able to play a structural role in forming the negative curvature necessary for the circumferential barrel of the transmembrane pore.[2d]

These results indicate that the presence of NIC lipids is important for pore formation by this class of AMO molecules: In Gram-positive bacteria, CL and divalent metal cations like Ca^{2+} and Mg^{2+} are needed. This is completely consistent with the role of PE lipid in Gram-negative bacteria, where AMO antimicrobial activity is dependent on the NIC of PE, rather than a specific interaction with PE. These results lead to the conclusion that influencing curvature either directly, or indirectly by targeting NIC lipids, can be a new approach for antibiotic design.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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References

1. (a) Liu DH, DeGrado WF. J. Am. Chem. Soc. 2001; 123:7553–7559. [PubMed: 11480975] (b) Arnt L, Nusslein K, Tew GN. J. Polym. Sci. Part A: Polym. Chem. 2004; 42:3860–3864.(c) Lienkamp K, Madkour AE, Musante A, Nelson CF, Nusslein K, Tew GN. J. Am. Chem. Soc. 2008; 130:9836–9843. [PubMed: 18593128] (d) Tew GN, Liu DH, Chen B, Doerksen RJ, Kaplan J, Carroll PJ, Klein ML, DeGrado WF. Proc. Natl. Acad. Sci. U. S. A. 2002; 99:5110–5114. [PubMed: 11959961] (e) Som A, Vemparala S, Ivanov I, Tew GN. Biopolymers. 2008; 90:83–93. [PubMed: 18314892] (f) Sambhy V, Peterson BR, Sen A. Angew. Chem. Int. Ed. 2008; 47:1250–1254.(g) Porter EA, Wang XF, Lee HS, Weisblum B, Gellman SH. Nature. 2000; 404:565–565. [PubMed: 10766230]

(h) Scott RW, DeGrado WF, Tew GN. Curr. Opin. Biotechnol. 2008; 19:620–627. [PubMed: 18996193]

2. (a) Zasloff M. Nature. 2002; 415:389–395. [PubMed: 11807545] (b) Pouny Y, Rapaport D, Mor A, Nicolas P, Shai Y. Biochemistry. 1992; 31:12416–12423. [PubMed: 1463728] (c) Steiner H, Andreu D, Merrifield RB. Biochim. Biophys. Acta. 1988; 939:260–266. [PubMed: 3128324] (d) Yang L, Gordon VD, Mishra A, Som A, Purdy KR, Davis MA, Tew GN, Wong GCL. J. Am. Chem. Soc. 2007; 129:12141–12147. [PubMed: 17880067] (e) Som A, Tew GN. J. Phys. Chem. B. 2008; 112:3495–3502. [PubMed: 18293958] (f) Cullis PR, Hope MJ, Tilcock CPS. Chemistry and Physics of Lipids. 1986; 40:127–144. [PubMed: 3742670] (g) Cullis PR, Dekruijff B. Biochim. Biophys. Acta. 1978; 513:31–42. [PubMed: 31173] (h) Dekruijff B, Verkleij AJ, Leunissenbijvelt J, Vanechteld CJA, Hille J, Rijnbout H. Biochim. Biophys. Acta. 1982; 693:1–12. [PubMed: 7150583] (i) Rand RP, Sengupta S. Biochim. Biophys. Acta. 1972; 225:484–492. [PubMed: 4333431] (j) Yang LH, Gordon VD, Trinkle DR, Schmidt NW, Davis MA, DeVries C, Som A, Cronan JE, Tew GN, Wong GCL. Proc. Natl. Acad. Sci. U. S. A. 2008; 105:20595–20600. [PubMed: 19106303] (k) Epand RF, Schmitt MA, Gellman SH, Epand RM. Biochim. Biophys. Acta. 2006; 1758:1343–1350. [PubMed: 16564494] (l) Epand RF, Martinou JC, Fornallaz-Mulhauser M, Hughes DW, Epand RM. J. Biol. Chem. 2002; 277:32632–32639. [PubMed: 12082098]

3. (a) Tew GN, Clements D, Tang HZ, Arnt L, Scott RW. Biochim. Biophys. Acta. 2006; 1758:1387–1392. [PubMed: 16626628] (b) Nusslein K, Arnt L, Rennie J, Owens C, Tew GN. Microbiol. 2006; 152:1913–1918.

4. (a) Rietveld AG, Chupin VV, Koorengevel MC, Wienk HLJ, Dowhan W, Dekruijff B. J. Biol. Chem. 1994; 269:28670–28675. [PubMed: 7961817] (b) Dechavigny A, Heacock PN, Dowhan W. J. Biol. Chem. 1991; 266:5323–5332. [PubMed: 2002065]

5. (a) Cullis PR, Verkleij AJ, Ververgaert PHJT. Biochim. Biophys. Acta. 1978; 513:11–20. [PubMed: 102344] (b) Vasilenko I, Kruijff BD, Verkleij AJ. Biochim. Biophys. Acta. 1982; 684:282–286. [PubMed: 6799000] (c) Powell GL, Marsh D. Biochemistry. 1985; 24:2902–2908. [PubMed: 4016078]

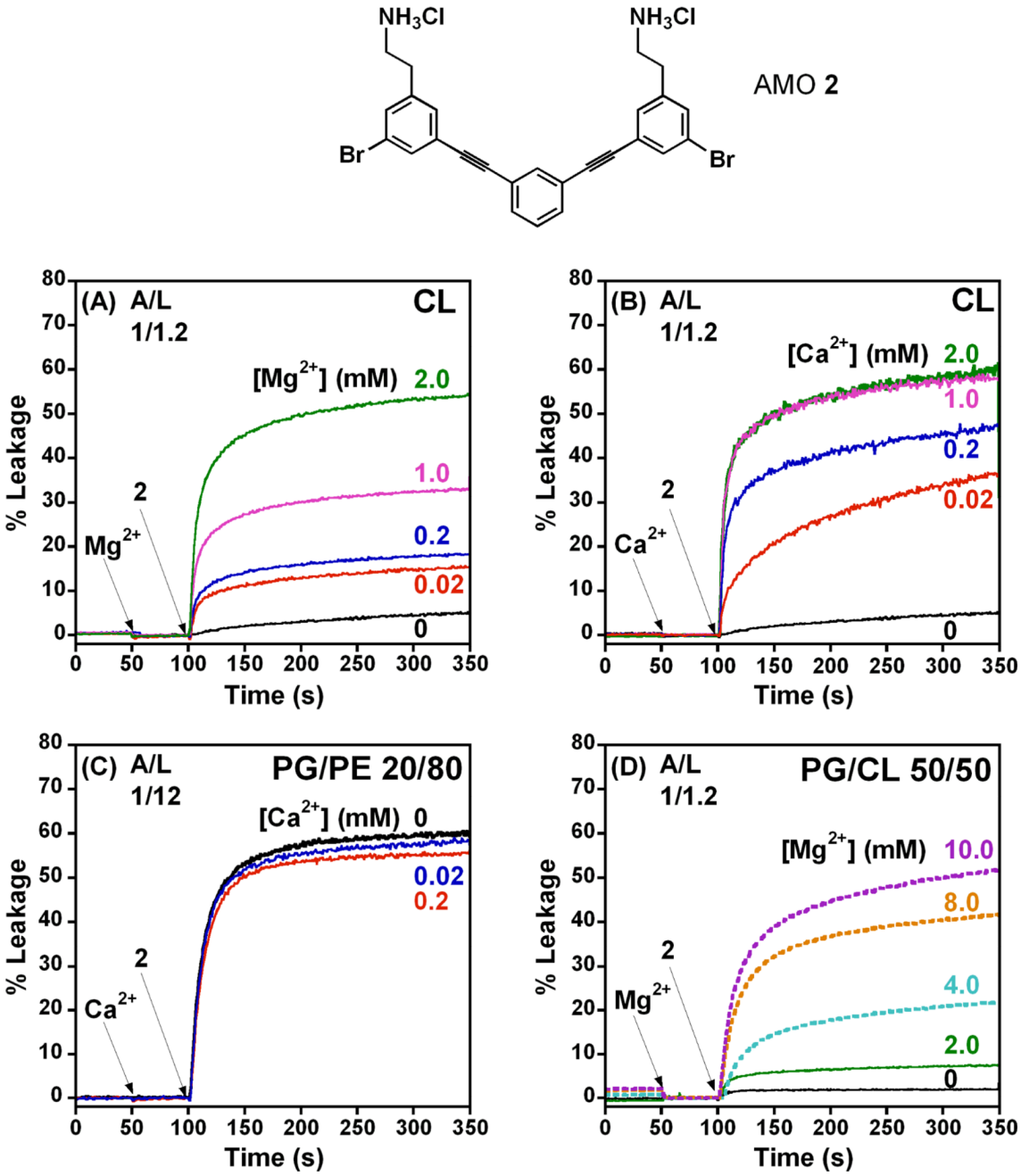

Figure 1.

AMO **2** (2.5 μg/mL for A, B and D, 0.25 μg/mL for C) induced calcein dye leakage from (A, B) 100% CL vesicles, (C) 20/80 PG/PE vesicles, (D) 50/50 PG/CL vesicles with 0 mM, 0.02 mM, 0.2 mM, 1.0 mM, 2.0 mM, 4.0 mM, 8.0 mM, and 10.0 mM (A, D) Mg^{2+}, and (B, C) Ca^{2+}. Final lipid concentration was 5.0 μM for assay.

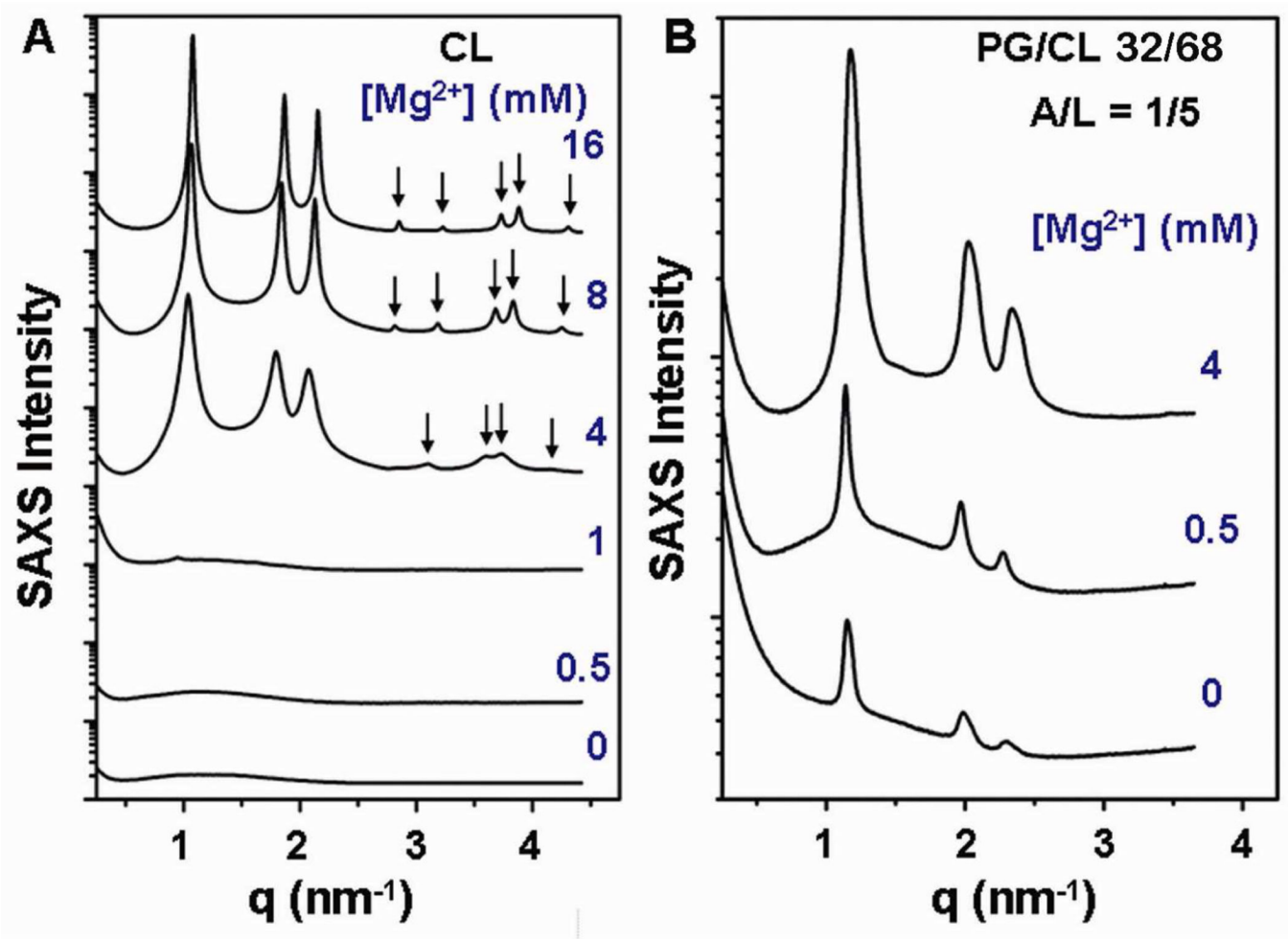

Figure 2.

(A) Synchrotron SAXS data show that, at sufficiently high [Mg^{2+}] (between 1–4 mM), Mg^{2+} ions can induce SUVs composed of CL to form an inverted hexagonal structure. (B) Synchrotron SAXS data show that AMO **2** induces SUVs composed of DOPG/CL = 32/68 into a hexagonal structure at reduced concentrations of divalent Mg^{2+}ions.