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Aust. J. Chem. **2003**, *56*, 1031–1034

Electrostatically Controlled Interactions of Mouse Paneth Cell α-Defensins with Phospholipid Membranes

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Antimicrobial peptides of the innate immune systems of many organisms are known to interact with lipid membranes, with electrostatic interactions playing an important role. We have studied the interactions of the mouse α-defensin, cryptdin-4, and its precursor, procryptdin-4, with phospholipid model membranes in the form of vesicles. Both peptides induce 'graded' leakage of vesicle contents, however procryptdin-4 exhibits only minimal membrane disruptive activity. Vesicles containing a higher fraction of anionic lipid are more susceptible to peptide-induced leakage. Electrophoretic mobility measurements at several vesicle compositions reveal a correlation between the surface potential of vesicles and the peptide-induced vesicle leakage.

Manuscript received: 30 April 2003.
Final version: 3 July 2003.

Antimicrobial peptides (AMPs) are innate immune components of all organisms investigated.[1] Seemingly ubiquitous among the animal and plant kingdoms, these molecules are likely to have served as weapons of the host defence system throughout evolution. The bactericidal activity of AMPs is considered to result from their deleterious interactions with the plasma membranes of these pathogens. The interactions are facilitated by electrostatic attractions between the generally basic peptides and bacterial membranes, which are typically more negatively charged than those of eukaryotic cells. In this work, we use techniques from colloid and interface science to investigate whether the membrane perturbation properties of an AMP that is secreted into the small intestinal lumen and its precursor are correlated with the surface charge of the membrane.

The AMP of interest is a member of the mammalian α-defensin peptide family. α-Defensins are amphipathic peptides with molecular masses of about 3.5 kDa, having a β-sheet structure stabilized by three disulfide bonds. Human (HNPs) and rabbit (NPs) neutrophil α-defensins have been isolated and studied using model membrane systems.[2–5] Despite having similarities in secondary structure, important differences in HNP and NP behaviour toward membranes have been observed. For example, HNPs exist as dimers in aqueous solution and form long-lived pores in vesicular membranes;[4] in contrast, NPs are monomeric and generate only transient membrane defects.[2]

Here in particular we focus on α-defensins secreted by the Paneth cells of mouse small bowel. Several isoforms of these α-defensins, termed cryptdins, have been isolated from the mouse small intestinal tract. Antibacterial activity assays have been conducted with at least the first six cryptdins of this series,[6] and the most potent of these, cryptdin-4 (Crp4), is the subject of this study. Crp4 possesses a large net molecular charge of +8.5 at pH 7.4, arising from its high content of arginine residues.[6] In mouse Paneth cells, cryptdins derive from inactive precursors by matrix metalloproteinase-7-mediated proteolytic activation before secretion into the intestinal lumen.[7] Existing as a net neutral molecule, the natural precursor to Crp4, proCrp4, lacks activity in bactericidal assays in vitro.[5] A second objective of this study is to compare the membrane perturbation properties of Crp4 and its precursor to those reported for the human and rabbit neutrophil defensins.

We utilized unilamellar vesicles in a series of experiments designed to characterize the peptide–membrane interactions. Membrane perturbation is quantified using a simple leakage assay in which vesicle-encapsulated fluorophores (8-aminonaphthalene-1,3,6-trisulfonic acid, ANTS) are released across the membrane into the peptide-containing solution; the fluorophore is co-encapsulated with quencher (*p*-xylene-bis-pyridinium bromide, DPX) and fluoresces upon dilution into the extravesicular medium.[8] The mode of leakage is determined using a fluorescence requenching technique utilizing this same fluorophore/quencher system.[4] Electrophoretic mobility measurements, allowing subsequent calculation of the surface potential of the vesicles, were conducted using a Coulter DELSA 440SX, which

 10.1071/CH03110 0004-9425/03/101031

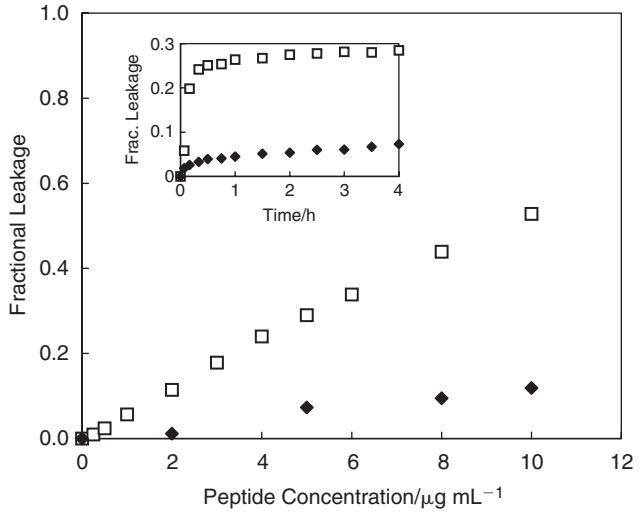

Fig. 1. Crp4- and proCrp4-induced leakage from phospholipid vesicles as a function of peptide concentration. Vesicles composed of POPG at a concentration of 74 μM were exposed to Crp4 (□) and proCrp4 (♦) at several concentrations. Fluorescence was measured at intervals for 4 h and normalized against the maximum fluorescence obtained once vesicles were solubilized by Triton X-100. Inset: The biphasic kinetic behaviour of fluorophore release.

operates on the principle of laser Doppler velocimetry. Vesicles composed of various ratios of the anionic lipid 1-palmitoyl-2-oleoyl-phosphatidylglycerol (POPG) and the neutral lipid 1-palmitoyl-2-oleoyl-phosphatidylcholine (POPC) were fabricated using a standard rehydration–extrusion technique.[9] Using dynamic light scattering, the diameter of these vesicles was determined to be 101 ± 0.9 nm with a polydispersity index of 0.036 ± 0.008.

Recombinant Crp4 and proCrp4 were prepared using the pET-28a vector to express the proteins in *E. coli* as described.[10] After CNBr cleavage of the recombinant fusion proteins, Crp4 and proCrp4 were purified to homogeneity using C_{18} reversed-phase HPLC as judged by analytical RP-HPLC and acid–urea polyacrylamide gel electrophoresis, and molecular masses of peptides were validated by MALDI-TOF MS.

The experimental details of the vesicular leakage assays were as follows. The fluorescent solution contained within the vesicles consisted of 17 mM ANTS, 60.5 mM DPX, 31 mM NaCl, and 10 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), while the solution in which these vesicles were suspended contained 130 mM NaCl and 10 mM HEPES. These equi-osmolar solutions, both at 25°C, were buffered to pH 7.4 with NaOH. Solution conditions were identical for leakage and electrokinetic experiments.

Figure 1 displays the leakage of vesicle contents induced by Crp4 and proCrp4 as a function of peptide concentration after four hours of peptide exposure. As shown in the inset, the vesicle leakage process is time-dependent with biphasic kinetic behaviour in which a rapid rate of release is observed over the first hour; the leakage rate then slows and plateaus to a value of less than 1% per hour. We note that the time dependence of the leakage process is similar to that induced by neutrophil α-defensins from humans and rabbits.[3,4]

The most significant observation is the potency of Crp4 relative to that of its precursor. Their membrane disruptive activities are consistent with binding of Crp4 and proCrp4 to vesicles using a lipid/polydiacetylene mixed vesicle colorimetric assay. They are also consistent with the relative bactericidal activities of the two peptides and support the hypothesis that membrane/peptide interactions are at the root of AMP activity.[5]

Leakage from large unilamellar vesicles (LUVs) may be by a 'graded' or 'all-or-none' process. In graded leakage, all vesicles release a portion of their contents; in an all-or-none process, some vesicles relinquish all their contents, but other vesicles remain unperturbed and experience complete retention. A requenching assay using the ANTS/DPX system differentiates these modes.[4] The assay allows one to determine the quenching factor of fluorophores remaining in the vesicles after peptide perturbation: A lack of change relative to that in unperturbed vesicles indicates an all-or-none release. We find that leakage produced by both Crp4 and proCrp4 occurs as a graded release (data not shown). Hence, leakage induced by the mouse-derived Crp4 peptide occurs in a similar fashion to that induced by NPs,[2] but HNPs generate an all-or-none response.[4]

Leakage experiments of the type performed in this work can be used to speculate on the mechanism of membrane perturbation. Previous work has shown that HNPs exist as non-covalent dimers, which are deduced to aggregate to form 20 Å pores made of 6–8 assembled dimers. These long-lived pores are consistent with the all-or-none response. On the other hand, the kinetics of the leakage data for Crp4, coupled with the graded mode of release, are similar to the behaviours of at least four other peptide systems (alamethicin,[11] magainin II,[12] melittin,[13] as well as NP[2]) and are consistent with a transient pore-formation mechanism. Briefly, this mechanism entails peptide binding to the outer monolayer of the vesicle membrane, aggregation and insertion of the peptide into the membrane to form a pore, and translocation of the peptide to the inner monolayer of the membrane upon pore closure. The pores created by this mechanism, which serve as channels for leakage, are short-lived and the process of pore-formation stops after a fraction of peptide has translocated across the membrane. The proCrp4 peptide also generates graded, biphasic release, supporting the possibility that the relatively minor leakage induced by this peptide is also associated with pore formation.

In the specific case of Crp4 and its precursor, we find a small but constant rate of leakage after the rapid regime. The mere incorporation of peptide into the membrane may cause minor defects or fluctuations in lipid packing that provoke the sustained residual leakage. The recent equilibrium binding studies of Satchell and coworkers have shown that Crp4 tends to reside at the lipid–water interface;[5] disruption of lipid headgroup packing may be responsible for the long-duration residual leakage observed. Though the leakage data presented for Crp4 and proCrp4 are consistent with

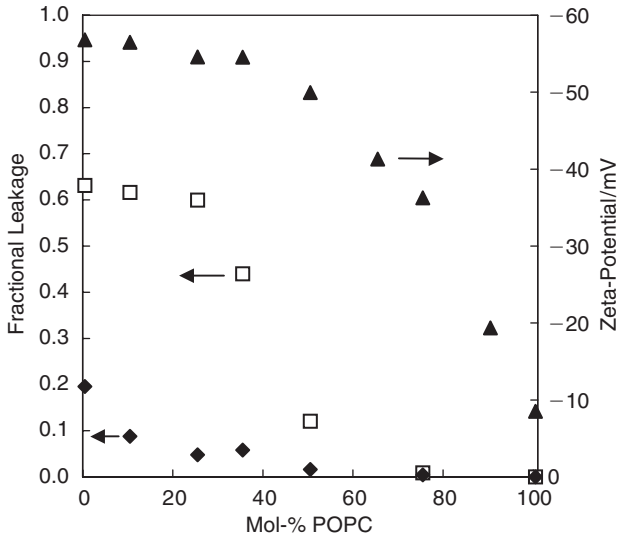

Fig. 2. Membrane composition dependence of induced leakage and membrane zeta-potential. Several vesicle compositions from 100% POPG to 100% POPC were prepared and incubated with peptide Crp4 (□) and proCrp4 (♦). Fractional leakage values after 4 hours of exposure are reported. Zeta-potentials of vesicles (▲) were calculated from electrophoretic mobility measurements.

transient pore formation, additional experiments are clearly required to confirm this mechanism.

Figure 2 shows how membrane composition affects the extent of membrane leakage (namely, peptide activity). In particular, at fixed peptide concentrations, the extent of leakage is shown for membranes containing different ratios of POPG and POPC. Vesicles richer in the anionic lipid POPG are more susceptible to peptide-induced leakage. Even proCrp4, which induces only slight leakage, demonstrates a dependence on membrane composition, exhibiting greater activity toward those vesicles of greatest negative charge. The dependence of peptide activity on membrane composition has also been seen in studies with human and rabbit α-defensins.[2,4] Binding and leakage studies with those defensins showed that addition of neutral lipids to anionic vesicles weakened peptide activity and produced a similar trend to the one shown in Figure 2.

To elucidate the role of electrostatics in the process of peptide-induced membrane perturbation, we characterized the exact charging characteristics of the POPG/POPC membranes used in the leakage studies. Electrokinetic measurements of vesicle mobilities were carried out, allowing the zeta-potential of the membranes to be determined in the same salt solutions (without peptide) used in the leakage experiments. In particular, electrophoretic mobility μ was measured and related to the zeta-potential ζ using the Hückel relationship, Equation (*1*)

$$\mu = \frac{2\varepsilon\varepsilon_0\zeta}{3\eta} f_1(\kappa a) \qquad (1)$$

where ε is the dielectric constant of water, ε_0 is the permittivity of a vacuum, η is the viscosity of water, κ is the Debye–Hückel parameter, a is the vesicle radius (50 nm in these experiments), and $f_1(\kappa a)$ is Henry's correction factor that accounts for deformation of the applied field around the vesicle.[14] Results are shown in Figure 2. Zeta-potential values begin at about −10 mV for completely zwitterionic vesicles and ultimately reach an asymptotic value of approximately −60 mV above a POPG content of 50 mol-%. In short, due to screening by ions in solution, the membrane charge is nearly constant, at the upper limit, for systems containing 50% or more of the anionic lipid. This behaviour and associated zeta-potential measurements have been reported on similar systems.[15,16]

As shown in Figure 2, in comparing the peptide-induced leakage response to the membrane charge (zeta-potential), a distinct correlation is observed. To better analyze this correlation, we estimate the surface potential of the membranes ψ_0 from their measured zeta-potentials using a simplification of the Poisson–Boltzmann expression, Equation (*2*).[14]

$$\tanh\left(\frac{ze\zeta}{4kT}\right) = \tanh\left(\frac{ze\psi_0}{4kT}\right)\exp(-\kappa x) \qquad (2)$$

Here z is the valence of electrolyte in solution, e is the electron charge, k is Boltzmann's constant, T is the absolute temperature, and x is the distance of the hydrodynamic plane of shear from the membrane surface (assumed to be 0.2 nm).[17] It is also insightful to estimate the actual membrane charge density (details can be found in various texts[18]). At a zeta-potential of −60 mV, where all lipids constituting the vesicle membrane are POPG molecules, the corresponding surface potential and actual membrane charge density are −82 mV and $-6.2\,\mu C\,cm^{-2}$, respectively. This effectively means that only about 27% of the total number of lipids within the membrane bear a charge. These values are comparable to zeta-potential and charge-density values of phosphatidylglycerol vesicles reported by Wiese et al.[15]

Membrane leakage correlates directly with surface potential. Because it seems reasonable that greater leakage is the result of a greater membrane surface concentration of peptide, let us assume that the leakage observed is proportional to the local concentration of peptide at the membrane surface. Due to electrostatic interactions between the negatively charged membrane and the positively charged peptide, the concentration of peptide at the membrane surface is enhanced over that in the bulk by the exponential Boltzmann factor, $\exp(z_p e\psi_0/kT)$, where z_p is the net charge of the peptide. Hence a linear relationship is predicted between the natural logarithm of membrane leakage and surface potential. As shown in Figure 3, the behaviour of bactericidal Crp4 follows this prediction. Interestingly, the slope yields an effective peptide charge of about +3.5, roughly half the actual charge. This may result from localized interactions between the membrane and a subset of charges on the peptide molecule.

Similar to that of Crp4, the activity of proCrp4 depends on membrane charge, however the simple predicted trend is not as closely followed (especially as POPC content goes to zero, as can be seen in both Figs. 2 and 3). The proCrp4 peptide is identical to Crp4 except for an extra 39 amino acid propiece at the *N*-terminus. This propiece carries a −8.5 charge at pH 7.4, completely offsetting the +8.5 charge of the other end of the molecule.[6] Since proCrp4 is a net neutral molecule, it is perhaps surprising that membrane charge

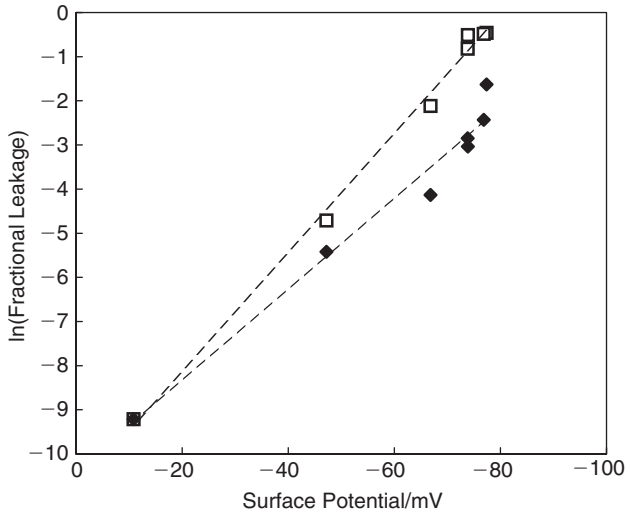

Fig. 3. Correlation of membrane surface potential with induced leakage. Dashed lines represent a prediction based on a simple electrostatic binding mechanism: Crp4 (□) and proCrp4 (♦).

does indeed regulate the perturbation potential of the peptide. Even though the anionic charges of the propiece likely associate with cationic charges of the mature peptide,[5,19] our results show that the molecule bears an overall dipole moment. The fact that proCrp4 does not strictly adhere to the simple prediction is probably related to its larger size and charge distribution.

In summary, we have characterized the membrane perturbation properties of Crp4 and its precursor. We find that mouse Crp4, a peptide secreted into the small intestinal lumen, behaves similarly to rabbit neutrophil α-defensins, causing a graded mode of vesicle leakage as opposed to an all-or-none release observed for human neutrophil α-defensins. The peptide-perturbed membranes eventually reseal, reaching a condition of negligible leakage. One possible explanation for this phenomenon is that leakage may occur during the time it takes for the peptide to distribute itself between the inner and outer membrane leaflets, possibly through the formation of transient pores. Although the exact mechanism of membrane perturbation is still not well understood, it is clear that the process is lipid-specific: Membranes richer in anionic lipid are more susceptible to peptide-induced leakage. Explicit measurements of membrane surface potential reveal a high correlation between extent of leakage and membrane charge. A simple model accounting for electrostatic effects on surface binding captures the trends observed for the systems studied, offering insight into the role of electrostatics on peptide–membrane interactions. Finally, and most importantly, the results of these model membrane systems are in accord with biological assays of peptide activity. We are currently pursuing related studies investigating the membrane perturbation properties of various Crp4 mutants (for example *N*-terminal mutants[20] as well as *C*-terminal mutants). Model membrane studies are in excellent agreement with biological assays, suggesting that membrane studies provide a useful platform for understanding the mechanisms of AMP activity.

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