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Surface behaviour and peptide–lipid interactions of the antibiotic peptides, Maculatin and Citropin

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Abstract

Surface behaviour of Maculatin 1.1 and Citropin 1.1 antibiotic peptides have been studied using the Langmuir monolayer technique in order to understand the peptide–membrane interaction proposed as critical for cellular lysis. Both peptides have a spontaneous adsorption at the air–water interface, reaching surface potentials similar to those obtained by direct spreading. Collapse pressures (Π_c, stability to lateral compression), molecular areas at maximal packing and surface potentials (ΔV) obtained from compression isotherms of both pure peptide monolayers are characteristic of peptides adopting mainly α-helical structure at the interface. The stability of Maculatin monolayers depended on the subphase and increased when pH was raised. In an alkaline environment, Maculatin exhibits a molecular reorganization showing a reproducible discontinuity in the Π–A compression isotherm. Both peptides in lipid films with the zwitterionic palmitoyl-oleoyl-phosphatidylcholine (POPC) showed an immiscible behaviour at all lipid–peptide proportions studied. By contrast, in films with the anionic palmitoyl-oleoyl-phosphatidylglycerol (POPG), the peptides showed miscible behaviour when the peptides represented less than 50% of total surface area. Additional penetration experiments also demonstrated that both peptides better interact with POPG compared with POPC monolayers. This lipid preference is discussed as a possible explanation of their antibiotic properties.

Keywords: Antibiotic peptide; Lipid–peptide interaction; Peptide adsorption; Monolayer stability; Peptide monolayer

1. Introduction

Maculatin 1.1 (GLFGVALKVAAHVVPAIAEHF-NH_2) and Citropin 1.1 (GLFDVIKKVASVIGGL-NH_2) are antibiotic peptides from Australian tree frogs. The amphipathicity associated with their sequence allows these molecules to be soluble in aqueous solution and adopt mainly α-helical structures in a membrane environment [1,2], and interact with the biological membranes in which they perform their antibiotic effect [3–5]. Two main mechanisms have been proposed to explain the cellular lysis performed by these peptides as a direct peptide–membrane interaction: either, by the oligomerization of the peptides at the interface with channel formation across the bilayer, or by aggregation parallel to the membrane interface solubilizing/disrupting the lipids [6]. The aim of the present work is to study the surface properties of above mentioned antibiotic peptides by using the Langmuir-monolayer technique at the air–water interface. The information obtained from these experiments of peptide adsorption, lipid monolayer penetration, and peptide–lipid interaction at the interface is useful in order to understand the mechanism by which these molecules spontaneously interact with membrane lipids exerting their antibiotic properties. Our earlier studies have shown that these peptides lyse bacterial membranes [7] but have little effect on neutral/zwitterionic bilayers [8].

2. Materials and methods

2.1. Chemicals

1-Palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC) and 1-palmitoyl-2-oleoyl-*sn*-glycero-3-[phospho-rac-(1-glycerol)] (POPG) were purchased from Avanti Polar Lipids Co (Birmingham, AL) and used without further purification.

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Maculatin 1.1 [GLFGVLAKVAAHVVPAIAEHF-NH_2] and Citropin 1.1 [GLFDVIKKVASVIGGL-NH_2] were commercially synthesised by Chiron Mimotopes (Melbourne, Australia) from L-amino acids via standard N-α-Fmoc methods. The purity of the peptides, as judged by HPLC and MS, was greater than 90%.

2.2. Monolayer studies

Monolayer experiments were performed at room temperature. The subphase was NaCl 145 mM at the specified pH. Adsorption and penetration experiments were done by injecting the peptides from their aqueous solution (~ 0.36 mM) into 18 ml of subphase contained in an 18 cm^2 trough. Lipids were dissolved in chloroform/methanol (67:33, v/v) solution. Pure peptide monolayers were formed by direct spreading from trifluoroacetic acid (0.2% in water)/chloroform/methanol (5:62.5:32.5, v/v) solutions by using a microsyringe. The total surface area of the Teflon trough was 80 cm^2 with a 75 ml volume of the subphase. Spreading solvent was allowed to evaporate for at least 5 min before compression was started, at a rate of 43 cm^2/min. For lipid–peptide mixed monolayers, peptide and lipid were premixed in the desired proportions from their respective pure solution, and then directly spread on the surface. The surface pressure (Π) (Wilhelmy method via platinized-Pt plate), the area enclosing the monolayer, and the surface potential (ΔV) (via milliVoltmeter with air-ionizing ^{241}Am plate and calomel electrode pair) were automatically measured (with the control unit Monofilmmeter with Film Lift, Mayer Feintechnique, Gottingen, Germany). The data were recorded continuously and simultaneously with a double channel X-YY recorder. The behaviour of mixed lipid–peptide monolayers was analysed by comparing the experimental force–area curve with the theoretical isotherms for the corresponding films in which no interactions between the molecules are assumed (cf. Refs. [16,18]). The immiscible behaviour between the film forming molecules in the mixed monolayer was determined according to the surface phase rule (cf. Refs. [16,18]). For a mixed film, in which individual components have differentiated collapse pressure, the surface phase rule determines lateral miscibility if one collapse point is observed in the mixture. Otherwise, two collapse points that are well determined in the mixed isotherm trace indicates lateral inhomogeneity with some film forming amphiphile molecules separating out from the rest of film components when they are under compression. Fig. 3 shows both cases that were emphasized by arrows.

3. Results

3.1. Peptide adsorption

The highest values of surface pressure (Π_{eq}) and surface potential (ΔV_{eq}) obtained by adsorption experiments into a lipid-free interface were 24 mN/m and 510 mV for Maculatin and 26 mN/m and 500 mV for Citropin (Fig. 1). These values are close to those obtained by direct spreading (see below), indicating that both peptides adopt a similar conformation/arrangement at the interface, independently of the method in which the monolayer is formed.

The thermodynamic tendency of a peptide to partition into a lipid-free interface can be estimated by calculating the free energy of the peptide adsorption process, evaluated by the Eq. (1):

$$\Delta G_{ads} = -RT \ln \frac{C_i}{C_s} \quad (1)$$

where C_i and C_s are the molar concentrations of the adsorbed peptide at the interface and subphase, respectively, T is the working temperature (~ 295 K) and R is the gas constant [9–12]. C_i is calculated from the molecular area obtained from the compression isotherm of pure peptide monolayers (see below), taking into account a total surface area of 18 cm^2. A mean monolayer thickness equivalent to the peptide length of about 3.15 nm for Maculatin and 2.4 nm for Citropin (0.15 nm of axial rise per residue for an α-helix structure, see Table 1) is estimated for each of the peptides since their respective surface molecular areas are compatible with an α-helical conformation with its long axis perpendicular to the interface (see below).

Table 1 shows similar negative values of adsorption free energy with more than 10^5 times of peptide accumulation at the interface. These results indicate that the spontaneous process of peptide adsorption at the interface is quite favourable, as found for other surface-active lytic peptides such as melittin [14].

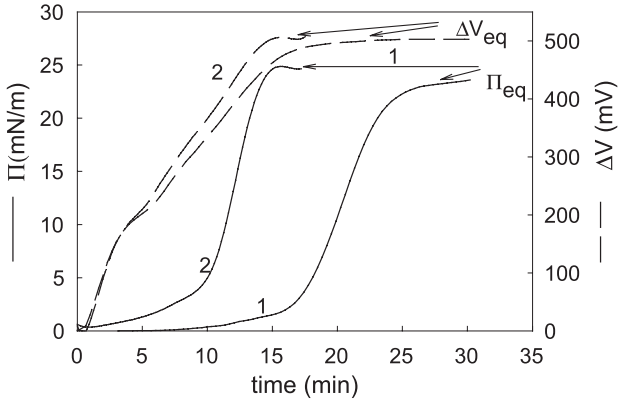

Fig. 1. Time-course adsorption of Maculatin and Citropin into lipid-free surface. Maculatin 200 nM (1) and Citropin 400 nM (2) interacting with air–NaCl 145 mM interface. Surface pressure (Π) solid line and surface potential (ΔV) dashed line. Π_{eq} and ΔV_{eq} correspond to the maximal equilibrium surface pressure and surface potential acquired by peptide adsorption.

Table 1
Adsorption free energy of Maculatin and Citropin antibiotic peptides

Peptide	C_s (nM)	Π_{eq} (mN/m)	Molecular area (nm^2/molecule)	ΔG_{ads} (kcal/mol)	C_i (nM)
Maculatin	200	23	2.80	− 8.1	1.9×10^8
Citropin	400	26	1.80	− 8.1	3.8×10^8

C_s = peptide bulk concentration in the subphase. C_i = interfacial peptide concentration assuming an α-helix perpendicular to the interface as indicated in the text. Π_{eq} = maximal equilibrium surface pressure acquired by peptide absorption.

3.2. Pure peptide monolayers

The compression isotherms (Π–A) and the change in the surface potential (ΔV–A) of pure peptide films of Maculatin and Citropin at different pH of the subphase are shown in Fig. 2(A) and (B), respectively. At pH 6 Maculatin had a molecular area of 2.80 nm^2/molecule and a surface potential of 560 mV at the highest surface molecular packing (at the collapse pressure point, Π_c). The Π_c for Maculatin was close to 24 mN/m at pH 6 (Fig. 2(A)). This value is in agreement with the behaviour of other peptides adopting an α-helical structure perpendicular at the air–water interface [2,14,15] and in keeping with the recent studies done for this peptide using ^{15}N-NMR [8]. The collapse pressure is interpreted as a measure of the film stability to remain as a monomolecular layer under compression [11,14–16]. Interestingly, we observed a dependence of Maculatin film stability on the pH of the subphase. Fig. 2(A) shows the increase of Π_c from 24 mN/m at pH 6 to 32 mN/m at pH 11, decreasing to 18 mN/m at pH 2. Of note is the discontinuity in the isotherm at 10 mN/m in alkaline medium. This could result from some form of molecular rearrangement under lateral stress (higher surface pressure) probably brought about by the deprotonation of amine side-chains of basic residues in the peptide. This discontinuity should not be interpreted as a lower collapse point since the lateral pressure and surface potential further increase upon compression. Compression isotherms of Citropin gave a molecular area at maximal lateral packing of about 1.90 nm^2/molecule, a Π_c of 28 mN/m and a surface potential of 495 mV (at pH 6, Fig. 2(B)). The surface molecular area and film stability are also compatible with an α-helical conformation of the peptide also perpendicular to the interface. The α-helical conformation for these peptides in bilayers has been recently reported [8]. Even when the Langmuir-balance studies do not give direct data of peptide conformation, the surface cross-sectional molecular area is an unquestionable *surface parameter* to deduct the orientation that these highly asymmetric molecules may acquire at the interface, as it was accurately used for many years for lipids (cf. Refs. [11,16]). For an α-helical conformation the expected length for both peptides would be 3.15 and 2.4 nm (0.15 nm of axial rise per residue) for Maculatin and Citropin, respectively. If we estimate an average of 1.5 nm for a helix diameter (0.5 nm for the helix core plus 0.5 nm for the side chains extent surrounding the core), the surface molecular cross-sectional area would be 4.70 and 3.60 nm for a complete parallel orientation to the surface. These values are far from the values of 2.80 and 1.80 nm found for Maculatin and Citropin, respectively (Fig. 2 and Table 1). The theoretical value of a cross-sectional surface molecular area of an α-helix oriented perfectly perpendicular to the interface would be around 1.75 nm^2 (calculated with the values given above). This value is rather closer to experimental surface areas found for both antimicrobial peptides at maximal lateral packing.

Contrary to Maculatin, the stability of monolayers of Citropin is independent of subphase pH. However, the dipolar arrangement at the interface is modified since the surface potential decreased as the pH of the subphase was raised.

3.3. Lipid–peptide monolayers

The monolayer technique is a powerful tool to measure lipid–peptide interactions [1,11,15]. This unique technique allows working with a known lipid–peptide ratio at the interface. The methodology also permits the study of interfaces with high proportions of protein-covered surface similar to that found in natural membranes [15]. In our experiments, we used peptide mole fraction proportions equivalent to 25%, 50% and 75% of area covered by the peptide at the mixed interface; thus, the peptide mole

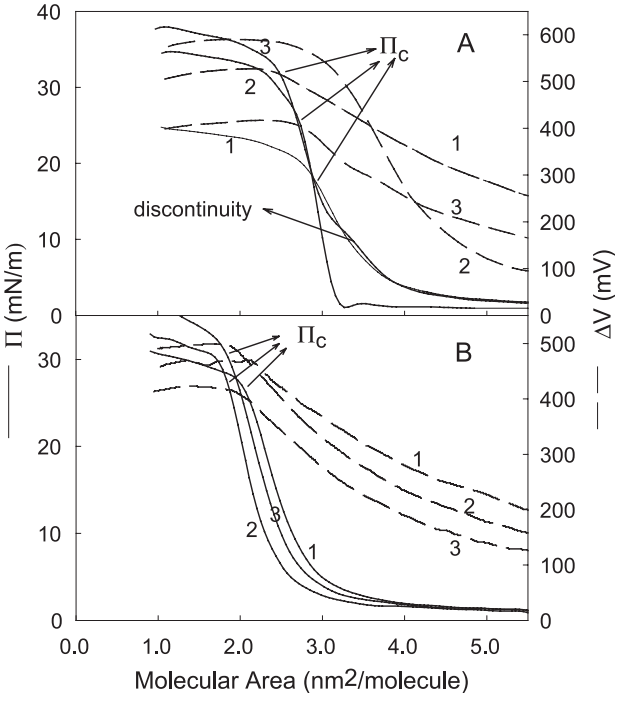

Fig. 2. Surface behaviour of pure Maculatin and Citropin monolayers. Π–A (solid line) and ΔV–A (dashed line) isotherms of Maculatin 1.1 (A) or Citropin 1.1 (B) at pH 2 (1), pH 6 (2) and pH 11 (3) for the subphase. Upper arrows indicate the collapse pressure (Π_c) of peptide films. Lower arrow in (A) indicates the discontinuity of Π–A Maculatin isotherm at pH 11.

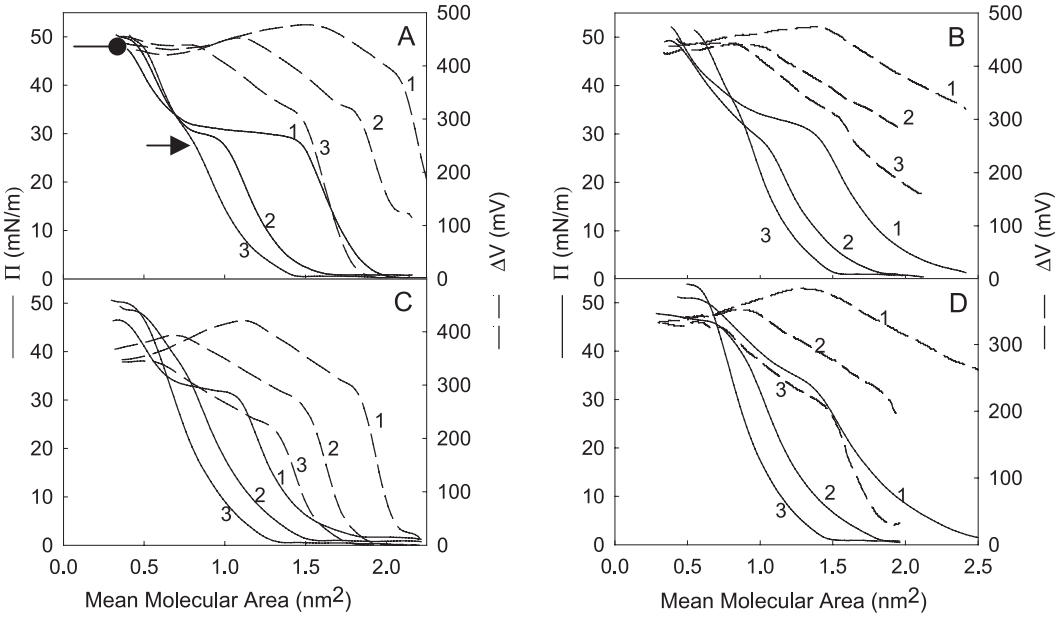

Fig. 3. Surface behaviour in mixed lipid–peptide interfaces. Π–A (solid line) and ΔV–area (dashed line) isotherms of: POPC–Maculatin (A); POPC–Citropin (B); POPG–Maculatin (C); POPG–Citropin (D). The corresponding peptide area proportion in the mixed monolayer is 75% (1), 50% (2) and 25% (3). Subphase, 145 mM NaCl pH 6. Lower and upper arrows indicate in (A) the region of lateral pressure where the first lower and the higher collapse points take place, respectively. Isotherm traces of (A) are clearly example of mixed film immiscibility. Isotherm traces 2 and 3 of (C) and (D) are clearly example of lipid–peptide miscibility (compare with trace 1).

fraction is lower than that of the lipid due to the dissimilar individual molecular areas of the lipids compared with those obtained for Maculatin and Citropin.

Two lipids were used as a model of biological membranes: (a) POPC is a zwitterionic lipid that has a liquid-expanded phase state at room temperature in the interface [11], and is frequently used to mimic eukaryotic cell membranes; and (b) POPG is a negatively charged lipid with an equivalent liquid-expanded phase to POPC and is frequently used to mimic bacterial Gram-positive membranes [4].

The characteristics of lipid–peptide compression isotherms of both peptides mixed with POPC at all lipid–peptide proportions and pH studied are indicative of an immiscible behaviour, i.e. a lateral peptide rich phase separates from the lipid-enriched phase with two observable collapse pressures (see the surface phase rule, [16,17]). This two-phase formation can be deduced from the first discontinuity in the Π–A mixed isotherm obtained at pressures near to those attained for pure peptides, see Fig. 3(A) and (B).

By contrast, mixed films of both peptides with POPG are miscible when the peptides occupy a proportion of surface area covered lower than 50%. Under these conditions, only one Π_c is seen in the Π–A isotherm (see Fig. 3(C) and (D)). The evidence that opposite-charge interaction is stabilizing the lipid–peptide interface (particularly with POPG) becomes apparent, since at pH ≥ 10.5 the mixed interface became immiscible, similar to the behaviour observed for POPC at pH 6 (Fig. 4). Maculatin retained the discontinuity in the Π–A isotherm at around 10 mN/m, seen in the pure peptide, even when mixed with any of the lipids studied

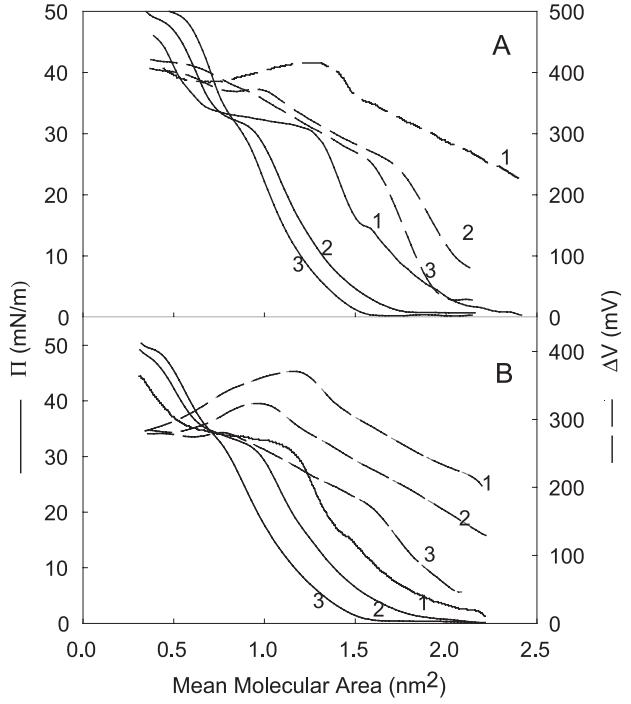

Fig. 4. Influence of alkaline medium on Maculatin–lipid interaction. Π–A (solid line) and ΔV–area (dashed line) isotherm of POPC–Maculatin (A) and POPG–Maculatin (B). The corresponding peptide area proportion in the mixed monolayer is 75% (1), 50% (2) and 25% (3). Subphase, 145 mM NaCl pH 11.

(particularly evident at high peptide proportions, see Fig. 4(A) and (B)). This is an interesting result showing a differential lipid–peptide interaction depending on the net charge of the peptide. The isotherms were reproducible after recompression when the first compression was stopped below 20 mN/m (data not shown), indicating that no desorption of the peptide or monolayer into the subphase takes place during compression.

When the experimental mean molecular area of the mixed films was compared with those assuming ideal mixing behaviour, no significant deviations were seen (Fig. 5). Only POPG–Maculatin mixed monolayers showed negative deviation (less than 20%, see Fig. 5(D)) due to attractive lipid–peptide interactions.

These findings indicate that both peptides are essentially preserving the biophysical properties of the lipids at the interface with a moderate affinity for anionic charged lipids. Correspondingly, the lipids are not altering the conformation of the peptides at the interface since the molecular areas and surface potentials were not substantially modified. The peptides have considerable surface activity with high lateral surface stability and, thus, they are able to become part of the lipid interface. The interface would be expected to have large peptide patches at the membrane provoking lateral discontinuities and, probably, local chaos in membrane permeability, i.e. a long-range lipid–peptide stable interface with high local disorder. This behaviour is in contrast with that observed for hydrophobic signal sequence peptides [15].

3.4. Penetration of Maculatin and Citropin into lipid monolayers

Penetration experiments measure the ability of peptides of being incorporated into a pre-organized lipid interface [1,11,13,16,18]. Considering the initial pressure of the lipid monolayer, Π_i (density of the lipid array), C_s (subphase peptide concentration), and $\Delta\Pi_{max}$, that is the maximum change in surface pressure acquired by the interface as a consequence of peptide interaction (insertion), it can be seen that the peptides interact with both lipids tested (POPC and POPG). However, a more obvious interaction was observed when the monolayer was initially composed of the anionic POPG (Fig. 6). The cutoff surface pressure point is the highest lateral packing in which no further penetration is observed, and is calculated by extrapolating the linear dependence of $\Delta\Pi_{max}$ with Π_i, as revealed in Fig. 6. Clearly, for both peptides penetration into POPG monolayers has a cutoff surface pressure around 15–20 mN/m higher than for POPC monolayers, indicating a higher spontaneous interaction of the both basic peptides with the anionic lipid. The lateral packing of natural membranes is to some extent an average value, estimated to fall in the range of 25–35 mN/m [19,20]. The final value of surface pressure due to the peptide interaction was within this limit for POPC but was well above for POPG. The observed changes in surface pressure were higher in POPG than POPC monolayers, indicating a higher interaction of the basic peptides with the anionic lipid. As seen previously for the mixed lipid–peptide study, no substantial changes in molecular area, neither in the peptide nor lipid, occurred at the lipid–peptide interface. Hence, the higher changes in surface pressure for POPG films observed for both peptides penetrating into organized anionic lipids can be interpreted as a greater amount of peptide being able to self-incorporate into the membrane compared to POPC. In other words, the anionic interface further facilitates the peptide interaction and/or membrane incorporation. This may explain the selective antimicrobial activity of these

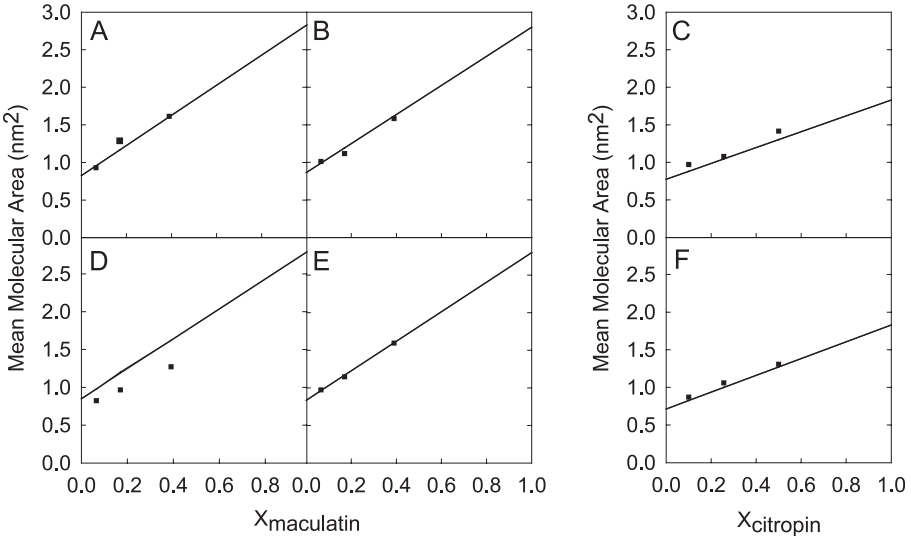

Fig. 5. Lateral interaction in mixed lipid–peptide monolayers. Mean molecular area (square) compared with ideal behaviour (line) for POPC–Maculatin at pH 6 (A) or pH 11 (B); for POPG–Maculatin at pH 6 (D) or pH 11 (E); for POPC–Citropin pH 6 (C) and for POPG–Citropin at pH 6 (F).

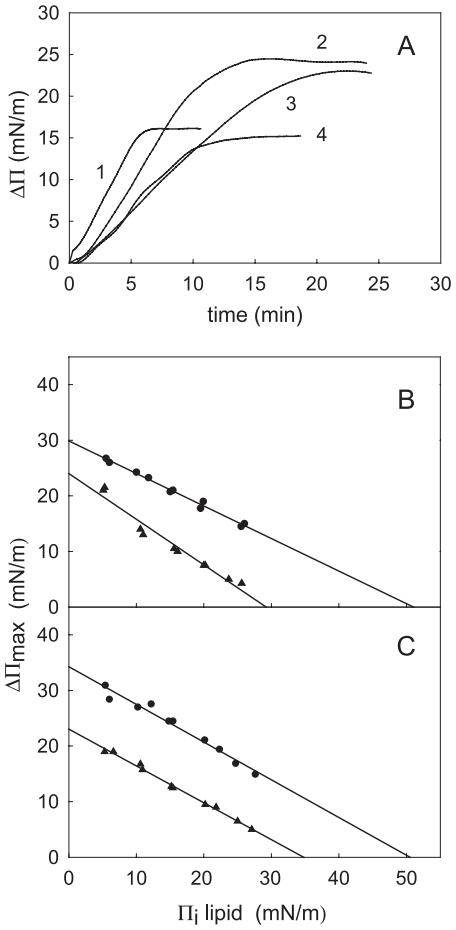

Fig. 6. Maculatin and Citropin penetration into POPC and POPG monolayers. (A) Kinetics of the surface pressure increase related to the penetration of Maculatin and Citropin 200 nM into POPC (1 and 4 respectively) and POPG (2 and 3 respectively) monolayers at initial pressure of 10 mN/m. (B and C) Maximum increase in surface pressure ($\Delta\Pi_{max}$) as a function of Π_i of POPC (triangle) and POPG (circle) upon injection of Maculatin and Citropin 200 nM, respectively. Subphase, 145 mM NaCl, pH 6.

peptides [3,5]. Very often, uncharged phosphatidylcholine and sphingomyelin are the more relatively concentrated lipids in the external monolayer of mammalians cell membranes [21], whereas the anionic POPG was proposed to mimic the negatively charged Gram-positive bacterial membrane [4]. Furthermore, the concentration of POPG in the outer leaflet monolayer of eukaryotic cell membrane is low [21].

4. Discussion

The surface properties of pure Maculatin 1.1 and Citropin 1.1 monolayers are consistent with an α-helical structure adopted by the peptides at the air–saline (NaCl 145 mM, pH 6) interface with a main orientation perpendicular to the interface at high lateral packing compatible with biomembranes. The α-helical conformation for these peptides in a membrane-like environment has been reported with ATR-FTIR, NMR and CD [3,5,8]. The stability of Maculatin increases as consequence of the deprotonation of basic residues. The higher stability might be due to some changes in the secondary structure of the peptide. This change could involve a decrease in the helix content in favour of some β-sheet conformation. This is indirectly concluded since it is known that the stability of pure peptide monolayers in a β-sheet configuration is higher than for an α-helix [1,2,15].

The higher cross-sectional molecular area found for Maculatin compared with Citropin (Fig. 2 and Table 1) should not be ascribed only to its longer sequence. It is rather would be a consequence of the proline 15 that imposes a conformational constrain tilting the last N-terminal residues from the main long axis of the helix as proposed recently [8].

As observed for other helical peptides [1,2,14], both antimicrobial peptides act immiscible when mixed with POPC. By contrast, the peptides mixed with the anionic POPG have a miscible behaviour (for peptide area proportion lower than 50%). Frequently, antibiotic peptides are positively charged. This property and appropriate peptide amphiphilicity seem to be the two main molecular requirements for high affinity peptide incorporation into anionic lipid interfaces. The incorporated peptides, in turn, structure themselves into a multimolecular arrangement that is toxic towards bacterial cells. Lipid penetration experiments gave further evidence confirming this hypothesis.

Transient lesions at the planar membrane have been proposed to form when peptides are incorporated, altering the biophysical properties of the membrane [22], and helix multimers may have a destabilizing effect on the membrane integrity [23]. Our monolayer results reveal that the lipid–peptide arrangements at the interface have a *high lateral stability*, indicating that the mixed interface supports surface pressures equivalent to natural membranes. Thus, independent of the mechanism by which they exert their toxicity, postulated to be either "carpet" or "channel forming" [6], the peptide mode of action is not occurring by a "literal" lipid membrane solubilization. The long-range interfacial organization is maintained and, from a surface point of view, the peptide–POPG mixed interfaces behave as a pure lipid with almost identical stability. The peptide incorporation does not occur with membrane destabilization or lipid solubilization and, once incorporated, the peptide remains at the interface, becoming a part of it. Thus, the probable mechanism of toxicity may be due to local disruption of transverse hydrophobic/hydrophilic sealing of the membrane caused by specific peptide accumulation at the interface, being more effective in negatively charged membranes. Differences in behaviour of the 21-residue Maculatin compared to the 16-residue Citropin may be due to the helix-breaking proline residue in the former. Further studies of peptide analogues in mixed lipid monolayers and bilayers are proposed.

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