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Pore-forming properties of iturin A, a lipopeptide antibiotic

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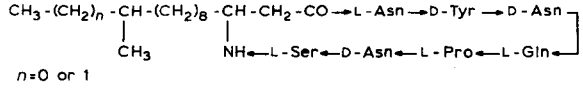
The addition of iturin A, a lipopeptide antibiotic extracted from *Bacillus subtilis*, to a bimolecular lipid membrane (BLM) increases dramatically its electrical conductance. For very low concentration of iturin A, discrete conductance steps are observed which are assigned to the formation of conducting pores. The characteristics of these pores depend on the lipid content of the BLM and they change with time. Cholesterol considerably increases the lifetimes of open states. The pores are slightly anion versus cation selective. These first observations unable us to briefly discuss the pore-forming properties of lipopeptides.

Introduction

Iturin A belongs to a family of lipopeptides extracted from the culture media of different strains of *Bacillus subtilis* and which exhibit antibiotic properties [1]. In all the structures determined up to now [2,3] a cycle containing seven peptide residues with the constant chiral sequence LDDLLDL is closed by a β-aminoacid, hydrophobic tail of which includes 10 to 17 carbon atoms. The formula of iturin A determined by Peypoux et al. [2] is the following:



It has a broad antifungal spectrum whereas its antibacterial activity is limited to a few species [4]. All these lipopeptides are unsoluble in water and their biological activity seems to be closely related to their interactions with cytoplasmic membranes [4]. Although the existence of interactions with lipids has been displayed [5], the mechanism of their action at the molecular level remains unknown. In this paper, we demonstrate the capacity of iturin A to induce the formation of conducting pores in BLM (bimolecular lipid membranes). This study is carried out in parallel with a NMR and theoretical conformational analysis reported elsewhere [6].

Materials and Methods

The device used to prepare BLM is shown in Fig. 1. BLM are formed on a Parafilm septum. The aperture in the Parafilm sheet is made by electrically heating a thin metallic wire. Hole diameters between 0.1 and 0.4 mm have been used.

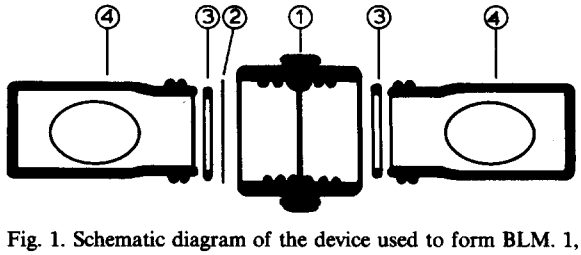


Fig. 1. Schematic diagram of the device used to form BLM. 1, SVL clamp; 2, Parafilm sheet; 3, joins; 4, glass cells.

Planar membranes are formed from lipid vesicle suspensions [7] routinely in 1 M KCl/10 mM phosphate buffer (pH 7.7). Using Parafilm, the pretreatment of the septum is not necessary. Solvent-free membranes stable for hours were prepared by using an hydrophobic material (Parafilm) for the septum and egg phosphatidylcholine vesicles for forming the bilayer.

The electrical measurements are made via a pair of Ag/AgCl electrodes. A Keithley current amplifier (model 427, Keithley Instruments) is used and the information stored on a microcomputer Apple II (Apple computer).

For the determination of the 'zero-current potential', the current-voltage relationship (I–V) is first established in symmetrical unbuffered 1 mM KCl. Then, the salt concentration on one side of the BLM is progressively raised by adding small amounts of concentrated KCl under stirring. An equal volume of 1 mM KCl is added on the other side. Egg phosphatidylcholine (egg-PC) is prepared by M. Charlier in our laboratory, according to Ref. 9. Cholesterol is from Prolabo and dimyristoylphosphatidylethanolamine (DMPE) from Sigma. KCl, analytical grade is from Merck. Iturin A is prepared as previously described [10].

Results

Effect of iturin A on the conductance of BLM

In the absence of iturin A, the conductance of egg phosphatidylcholine BLM is around 10^{-8} S · cm^{-2}. After addition of few amounts (final concentration 0.01 ng/ml) of iturin A (ethanolic solution) in the two compartments, a dramatic increase in the conductance of the membrane is observed (up to 10^{-3} S · cm^{-2}). In symmetrical KCl solutions, the current-voltage relationship (I–V) is linear.

When only traces of iturin A are present, the current membrane fluctuates in a step-like fashion (Figs. 2A and 3). These current jumps are apparently due to the formation of conducting pores. The macroscopic increase of the membrane conductance observed for higher amounts of iturin A is attributed to the simultaneous opening of many pores.

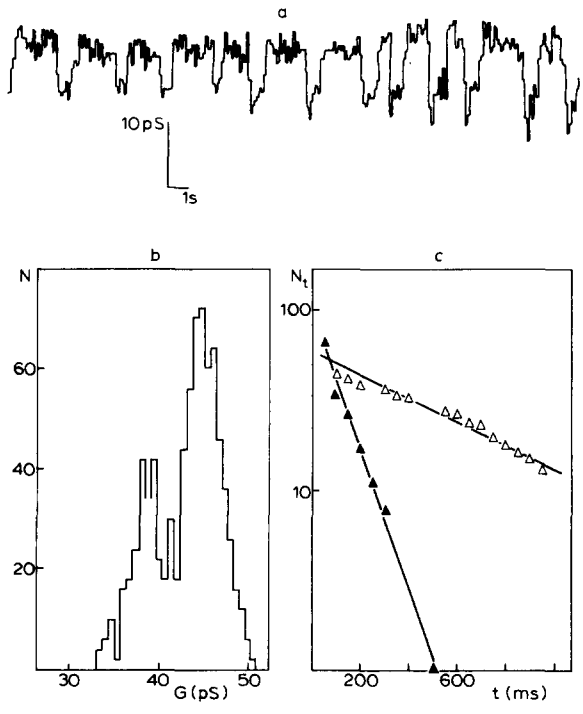


Fig. 2. Membrane current fluctuations induced by iturin A in egg-PC BLM. The BLM is made from egg-PC vesicles in 1 M KCl/10 mM phosphate buffer (pH 7.7). Traces of iturin A are present on the two sides of the BLM. The applied voltage is 100 mV. (a) Current trace. (b) Conductance histogram (N: number of events). (c) Cumulative frequency of dwell-times in open (△) and closed (▲) states. The representation is semi-logarithmic. The graph shows the number of individual dwell-times longer than t. The straight lines indicate the best fit to the distributions. Total number of events $N_T = 67$.

Lipid dependence of the current fluctuations induced by iturin A

When the BLM is formed only with egg phosphatidylcholine, the amplitude of the current fluctuations are variable. The lowest value observed is 7 pS in symmetrical 1 M KCl and is independent of the applied voltage. The mean dwell time in open state is around 1 s ($t = 986$ ms, average lifetime of 66 events at 100 mV).

When the BLM is formed with a mixture egg-PC/DMPE (8 : 2), the appearance of the current fluctuations changes with time. In Fig. 3, a typical record shows this evolution. First, the current fluctuations are rather irregular, and the transitions between open and closed states are slow. Then, they become more and more regular and the

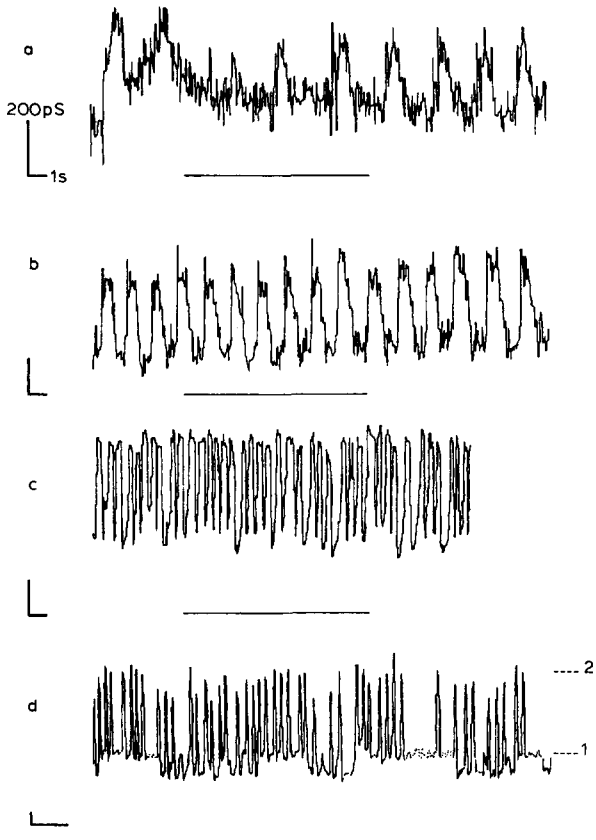


Fig. 3. Membrane current fluctuations induced by iturin A in PC/PE (8:2) BLM. Evolution with time. The BLM is made from egg-PC/DMPE (8:2) vesicles in 1 M KCl/10 mM phosphate buffer (pH 7.7). Traces of iturin A are present on both sides of the BLM. Time after formation of the BLM: (a) 40 min, (b) 55 min, (c) 80 min, (d) 105 min.

transitions are faster (Fig. 4 shows the cumulative frequency distributions of open and closed states 40 min and 100 min after the formation of the BLM). In parallel we observe an increase in the conductance steps from 100 pS (10 min after the formation of the BLM) to 900 pS (100 min after). At this time we can distinguish two levels of conductance: level 1 (230 pS) and level 2 (900 pS).

When cholesterol is present in the BLM (egg-PC/cholesterol 4:1 or 2:1) the pores remain open almost all the time and we observe on the record the sum of several pores that open ones after the others (Fig. 5). In some rare cases closing of the pores occurs but the dwell-time in open state is very long (generally more than 1 min).

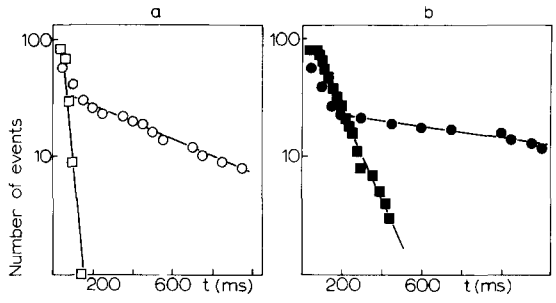


Fig. 4. Distribution of the dwell-times in open and closed state for conducting pores in PC/PE BLM. Evolution with time. The BLM is made from egg-PC/DMPE (8:2) vesicles in 1 M KCl/10 mM phosphate buffer (pH 7.7) Traces of iturin A are present on the two sides of the BLM. The applied voltage is 11 mV. (a) Cumulative frequency of dwell-times in open state: 40 min after the formation of the BLM (○) and 105 min after the formation of the BLM (level 2) (□). (b) Cumulative frequency of dwell-times in closed state 40 min (●) and 105 min (■) after the formation of the BLM. The graphs show the number of individual dwell-times longer than t. The representation is semi-logarithmic. The total numbers of events recorded are 58 for (○,●) and 81 for (□, ■).

Ion selectivity

The ion selectivity is calculated according to the Goldman-Hodgkin-Katz equation [11].

$$V_m = \psi_1 - \psi_2 = \frac{RT}{F} \ln \frac{P_c a_2 + P_a a_1}{P_c a_1 + P_a a_2} \quad (1)$$

(P_c and P_a: permeability to cation and anion, respectively, a: KCl activity. The subscript 1 refers to the more concentrated side and the subscript 2 to the more dilute side. R is the gas constant, T the absolute temperature and F the Faraday constant).

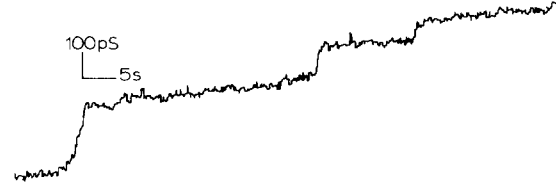


Fig. 5. Membrane current fluctuations induced by iturin A in cholesterol containing BLM. The BLM is made from egg-PC/cholesterol (4:1) vesicles in 1 M KCl/10 mM phosphate buffer (pH 7.7). Traces of iturin A are present on the two sides of the BLM. The applied voltage is 45 mV.

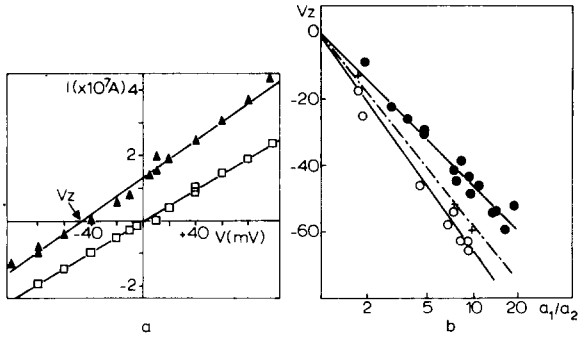


Fig. 6. Ion selectivity induced by iturin A in egg-PC BLM. (a) Current-voltage relationship in symmetrical 1 mM KCl (□) and in a 10-fold KCl gradient (▲) (dilute side positive). (b) 'Zero-current potential' V_z as a function of the logarithm of the ratio a_1/a_2 of the KCl activities in the two compartments. (- - -) theoretical Nernst relation $(-(RT/F)\ln(a_1/a_2))$; (+) measured in the absence of BLM; (○) BLM of egg-PC; (●) BLM of egg-PC in the presence of iturin A.

The membrane potential V_m is deduced from the 'zero-current potential' V_z by the relation:

$$V_m = V_z + \frac{RT}{F}\ln\frac{a_1}{a_2}$$

Fig. 6 shows variations of the reversal potential versus the logarithm of a_1/a_2.

In the absence of iturin A, the egg phosphatidylcholine BLM behaves as a cation selective membrane, probably by adsorbing cations at the lipid-water interface. When the BLM contains iturin A, the selectivity is inversed: iturin A induces a slight selectivity for anions ($P_{Cl^-}/P_{K^+}=$ 1.7).

Discussion

The main result of this study is the ability of iturin A to increase the permeability of lipid membranes by forming conducting pores, characteristics of which depend on the lipid composition of the BLM and change in course of time. These pores present a low selectivity for anions.

Preliminary measurements show that this property exists for other members of the Iturin family, specially for Bacillomycins L and D and also for another natural lipopeptide, peptidolipin NA (Heitz, F., personal communication). Therefore, it seems that the ability to form pores in lipid bilayers is general for this class of compounds. This ability seems to be not completely unspecific since the characteristics of the pores vary with the structure of lipopeptides.

An analogy exists with several other antibiotics interacting with membranes (for a review see Ref. 12). However, except for the well characterized molecular channels of gramicidin A [13], the mechanism of action of such compounds remains under debate. According to a generally received assumption, pores correspond to transient structures formed by selfassociation of the active compound [14] or by its association with the lipids [15]. The organization and the dynamics of such structures have been never elucidated. Perturbations of the cohesion of the lipid bilayer have been also considered [16,17] by analogy to those appearing around the phase transition of phospholipids [18].

Iturin A exhibits the characteristic properties of amphiphilic compounds. Its solubility is restricted to a few solvents and it has a propensity to self associate. It destroys the lipid vesicular structures to form smaller mixed assemblages [19]. Considering these properties and the dependence of the characteristics of the pores on both the structure of the lipopeptide and on the lipids, one can assume that the conducting structures should involve mixed aggregates fluctuating in course of time. The evolution with time would correspond to an evolution of the concentration of the lipopeptide within the BLM until an optimum is reached leading to a regularly fluctuating structure. Cholesterol strongly influences such a process by increasing the lifetime of the open state. The same behavior has been observed with a toxin [20] and with amphotericin B [21]. For this polyene two modes of action have been proposed: formation of a stoechiometric complex with cholesterol and modification of the physical state of the membrane [16,17].

The presence of cholesterol is not an obligatory condition for the formation of pores in BLM or for antibiotic activity since iturin A is able to induce changes of permeability in cholesterol free membranes.

Conclusion

It is premature to give an accurate description of the mechanism of action of iturin A on BLM

and membranes. Nevertheless, on the basis of the present results, mixed structures in which both lipopeptides and lipids participate specifically seem to play a primordial role. Useful informations will be now provided by the NMR conformational analysis. The existence of a stable conformation of iturin A in homogeneous solutions has been recently proved [6]. The study of interactions between lipopeptides and lipids is now undertaken. The possibility to vary the peptide sequence by using different compounds in the Iturin family should facilitate the elucidation of the mode of action of these antibiotics.

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