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ARTICLE

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Magainin 2 channel formation in planar lipid membranes: the role of lipid polar groups and ergosterol

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Abstract Magainin 2, a polycationic peptide, displays bactericidal and tumoricidal activity, presumably interacting with negatively charged phospholipids in the membrane hosts. In this work, we investigate the role played by the lipid head-group in the interactions and self-association of magainin 2 during pore formation in lipid bilayers. Two methods are used: single-channel and macroscopic incorporation into planar lipid membranes. Single-channel incorporation showed that magainin 2 did not interact with zwitterionic membranes, while the addition of negatively charged dioleoylphosphatidylglycerol to the membrane leads to channel formation. On the other hand, magainin 2 did not form channels in membranes made up of dioleoylphosphatidylserine (DOPS), although the addition of ergosterol to DOPS membranes leads to channel formation. This finding could indicate that ergosterol may be a possible target of magainin 2 in fungal membranes. Further support for this hypothesis comes from experiments in which the addition of ergosterol to palmitoyloleoylphosphatidylcholine membranes induced channel formation. Besides the role of negatively charged membranes, this study has shown that magainin 2 also forms channels in membranes lacking heads, such as monoolein and oxidized cholesterol, indicating an interaction of magainin 2 with acyl chains and cholesterol, respectively. This finding provides further evidence that peptide binding and assembly in lipid membranes is a complex process driven by electrostatic and/or hydrophobic interactions, depending on the structure of the peptide and the membrane composition.

Keywords Ion channel · Polycationic peptides · Lipid-peptide interaction · Ion selectivity · Sterols

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Introduction

Magainins belong to a family of linear 23-residue peptides produced by the skin of the African frog, *Xenopus laevis*. Magainin 2, which is a member of this family, is a polycationic peptide with a net positive charge of 4, a high degree of helicity and an angle subtended by the polar face of 180° (Matsuzaki 1999). It has been extensively studied for its killing action on various microorganisms, as well as on tumor cells (Westerhoff et al. 1989a, 1989b; Cruciani et al. 1991; Grant et al. 1992). It does this by permeabilizing their membranes, either in a detergent-like manner or by making well-structured channels, as has been found in liposomes, patch clamp cells or planar lipid membranes (PLMs) (Duclohier et al. 1989; Cruciani et al. 1991; Bechinger 1997). The appealing interest of this peptide lies in the fact that it can easily overcome the bacterial cell barrier, and thus the electrochemical gradient and other functions of cells can be compromised.

Numerous experimental approaches, such as NMR (Hirsh et al. 1996; Bechinger 1997), Raman spectroscopy (Williams et al. 1990), fluorescence (Matsuzaki et al. 1994, 1995a, 1995b, 1995c, 1998), differential scanning calorimetry (Wieprecht et al. 1999), lamellar X-ray diffraction and OCD spectra (Huang 2000) and electrophysiological studies on PLMs and cells (Duclohier et al. 1989; Cruciani et al. 1991, 1992; Haimovich and Tanaka 1995) have been used in order to gain insight into the mechanism of action of magainin on membranes.

Matsuzaki et al. (1998) proposed a model whose fundamental steps are: (1) electrostatic interaction between negatively charged membranes and magainin 2, followed by its rapid helical induction; (2) formation of

transient pores made up of a transbilayer aggregate of 4–5 membrane-spanning helices and lipids with a diameter of 2 nm and a lifetime of 12 ms; and (3) the peptide-lipid supramolecular complex collapses, inducing a rapid flip-flop of the membrane lipids.

More recently, Huang (2000) proposed a two-state model in which at low peptide to lipid (P/L) ratios the peptide is essentially associated with the lipid head-groups in a functionally inactive state. Once a threshold P/L value has been reached, the peptide forms a multiple pore state.

There is broad consensus (Cruciani et al. 1992; Matsuzaki et al. 1995a; Ludtke et al. 1996) that the interaction of polycationic magainin 2 with neutral phospholipids, such as palmitoyloleoylphosphatidylcholine (POPC), is rather weak, and that electrostatic interaction with negatively charged lipids seems to be a primary step for the permeabilizing action exerted at the bacterial membrane (Zasloff 1987; Zasloff et al. 1988; Duclohier et al. 1989; Matsuzaki et al. 1989; Westerhoff et al. 1989a, 1989b; Cruciani et al. 1992), although Wieprecht et al. (1999), by means of isothermal titration calorimetry, found that these peptides show an affinity for POPC with a K value of 10^4, while Williams et al. (1990) found that a high concentration of magainin 2 amide also lyses the POPC membrane. So the different lipid composition of membranes could be the discriminating factor between bacterial and eukaryotic cells. However, in prokaryotic cells, some relevance has been shown by sterols in the incorporation of proteins and bacteria (Gatfield and Pieters 2000). Moreover, evidence exists that fungal membranes are rich in ergosterol, while some tumor cell membranes are rich in negatively charged components, such as phosphatidylserine (Löffler et al. 2000).

PLMs (for a review, see Tien and Ottova 2001) are a convenient tool to use for protein or peptide incorporation, as they display various advantages. In particular:

1. In the case of channel incorporation, lifetimes orders of magnitude larger than those observed with liposomes (Matsuzaki et al. 1998) can be measured.
2. The range of P/L ratio investigated is an order of magnitude lower than that observed with liposomes and as regards this variable the PLM technique complements the studies with liposomes.

In this work, we report a systematic investigation of the interaction of magainin 2 with PLMs in the hope that studies conducted with different techniques and model system can help to explore different aspects of the same issue regarding peptide action on membranes, with the aim of evaluating its pharmacological and biotechnological applicability. In particular, we:

1. Intend to support the previous findings on channel formation with statistically more significant data (Duclohier et al. 1989; Cruciani et al. 1991, 1992; Haimovich and Tanaka 1995).
2. Further clarify the role of the lipid head-group and ergosterol in pore formation.
3. Look for the presence of channel-mediated activities in hydrophobic membranes without a polar head, such as monoolein and oxidized cholesterol, in order to evaluate the importance of hydrophobic interactions between the apolar phase of the amphipathic peptide and the acyl chains of lipids.

Materials and methods

Planar membrane experiments

Two different techniques were used to study the interaction of magainin 2 with lipid bilayer membranes: alternating current measurements (AC method) and single-channel measurements. In both cases, Teflon chambers were used, with two aqueous compartments connected by small circular holes. The hole diameter was 1.3 mm for the AC method and 0.2 mm for the single-channel experiments. The AC method has been described in detail elsewhere (Gallucci et al. 1996; Micelli et al. 2000, 2002). A generator mixing 1 Hz of variable amplitude (Vs) and 1 kHz of 2 mV signals applied the voltage to a Pt electrode situated in one compartment of the cell. The output voltage, acquired through a second Pt electrode in the other compartment, was electronically amplified and filtered in order to separate the two frequency components. The data were collected via a PC computer interfaced with two voltage-frequency converters and stored on floppy disk for further analysis.

In single-channel experiments, the membrane current was monitored with an oscilloscope and recorded on a chart recorder for further analysis. The *cis* and *trans* chambers were connected to the amplifier head stage by Ag/AgCl electrodes in series with a voltage source and a highly sensitive current amplifier. The single-channel instrumentation had a time resolution of 1–10 ms, depending on the magnitude of the single-channel conductance. The *cis*-side compartment, where the magainin 2 was added, had a positive polarity. In some experiments, magainin 2 was added to both compartments facing the membrane without affecting the results. Perfusion was performed by simultaneously withdrawing the bathing media and adding fresh fluid.

PLMs were formed across the holes by the "painting" technique, as has been described elsewhere (Benz et al. 1978). Membranes were formed with: palmitoyloleoylphosphatidylcholine (POPC), or a mixture of POPC and ergosterol (Erg) (75:25 w/w), or dioleoylphosphatidylserine (DOPS), or DOPS and cholesterol (75:25 w/w), or DOPS and Erg (75:25, w/w), or POPC and dioleoylphosphatidylglycerol (DOPG) (85:15, w/w), or monoolein dissolved in *n*-decane (4% w/v) or oxidized cholesterol (OxCh), obtained following the method used by Tien et al. (1966). The salts used in the experiments were of analytical grade. The aqueous solutions were used unbuffered and had a pH of 7; the temperature was 24 ± 2 °C.

Data analysis

The single-channel data were obtained from at least two or four experiments (more than 100 single channels for each experiment) performed on different days. A histogram of the current amplitude distribution for each experiment was constructed and fitted by a gaussian distribution function (GraphPad Prism, version 3.0; GraphPad Software http://www.graphpad.com). The distribution of a single-channel lifetime was fitted with a single- or two-exponential function (GraphPad Prism, version 3.0). Results are expressed as mean ± SE unless otherwise specified.

Chemicals

Salts and other basic chemicals were bought from Merck (Darmstadt, Germany; analytical grade). The phospholipids POPC and

DOPS were purchased from Avanti Polar Lipids (Alabaster, Ala., USA) and DOPG, monoolein, ergosterol and cholesterol from Sigma (Munich, Germany), and were used without further treatment. Gramicidin and protease were purchased from Sigma, magainin2 from American Peptide (Calif., USA) and *n*-decane from Fluka.

Results

Formation of ion channels

PLMs of different composition were used to see whether magainin 2 is able to form channels, and under what experimental conditions. With this aim, POPC and DOPS membranes were used as zwitterionic and negatively charged membranes, respectively, POPC:DOPG and DOPS:Erg as mixed membranes with diluted charges, POPC:Erg as mixed neutral membranes and, finally, monoolein and OxCh membranes as neutral membranes with a different hydrophobic core and almost total absence of polar head.

First of all, we tested the conductance and capacitance of each membrane, before magainin 2 addition, by applying a voltage of ± 100 mV for 10–15 min under stirring, to ensure that the membrane was stable. In a large number of experiments with POPC or DOPS membranes, carried out in different periods over the year, we were unable to observe any current fluctuations for many hours using a wide range of applied voltages after magainin 2 addition (1×10^{-7} g/mL). In other experiments in DOPS membranes, the magainin 2 concentration was increased stepwise until a final value of 4.83×10^{-6} g/mL was reached. Again, no channels were observed. To test whether membrane thickness was inhibiting magainin 2 channel formation, gramicidin A (1×10^{-10} M) was added. Shortly after gramicidin addition, we observed stepwise fluctuations of the current, which are indices of channel formation (results not shown). This result is in accordance with other findings (Matsuzaki et al. 1998) in which a very high P/L ratio was required to permeabilize the liposome membrane.

By contrast, there was clear evidence of non-random discrete current fluctuations in mixed membranes of POPC:DOPG, DOPS:Erg or POPC:Erg, and in monoolein and OxCh membranes registered only after magainin 2 addition. These current jumps were compatible with channel-type openings and closures with different conductance levels and lifetimes. To verify whether these current fluctuations were due to magainin 2, control experiments were carried out in which protease was added to the medium before magainin 2 addition. In this case, no channels were observed.

Figure 1 shows a typical example of single-channel recordings while Fig. 2 shows the corresponding histograms of the distribution of magainin 2 channel conductance at a peptide concentration of 1×10^{-7} g/mL in mixed, monoolein and OxCh bilayers in a 0.5 M KCl medium, and with an applied voltage of $+160$ mV ($+140$ mV for the POPC:Erg membrane). As shown in Fig. 1, the occurrence of single-channel activity sometimes occurred in highly variable steps. Furthermore, we also observed alternating periods of paroxystic channel activity followed by quiescent periods, and open times interrupted by brief closures. All the histograms show single-peaked conductance distributions (Fig. 2). The central value of conductance (Λ_c) obtained by the gaussian best-fit, reported in Table 1, characterizes the conductance state of magainin 2 in various membranes.

These results indicate that magainin 2 molecules are able to aggregate to form channels in mixed, in monoolein and in OxCh membranes, despite the differences in conductance. In fact, in our experimental conditions, magainin 2 is unable to form channels in

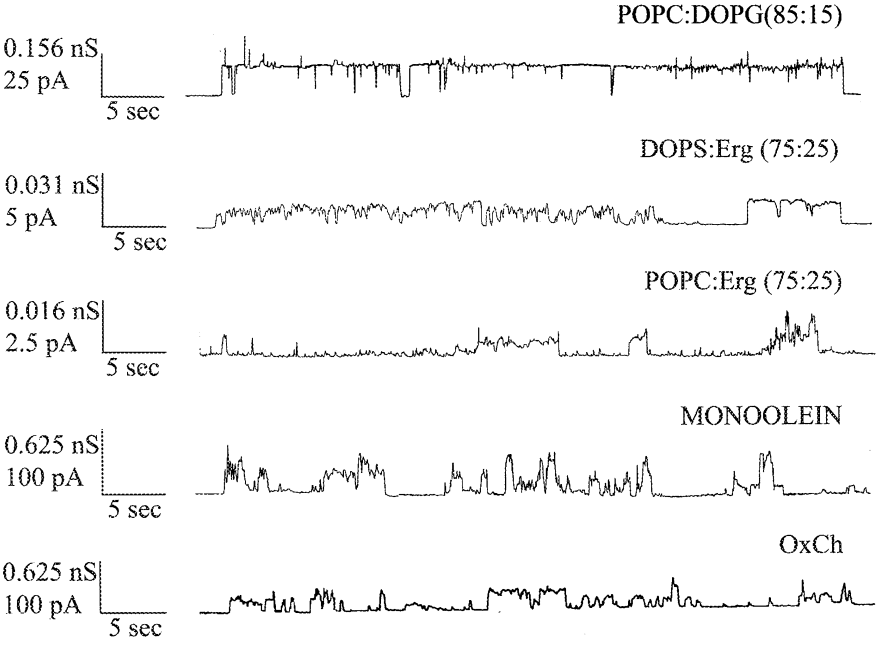

Fig. 1 Single-channel traces of magainin 2 channel conductance in PLMs of different composition. Experiments were performed in the presence of magainin 2 (10^{-7} g/mL) added to the *cis* side; the voltage was $+160$ mV, the aqueous phase contained 0.5 M KCl (pH 7) and $T=22\pm2$ °C. Each trace represents a fragment of the recording obtained in an independent experiment

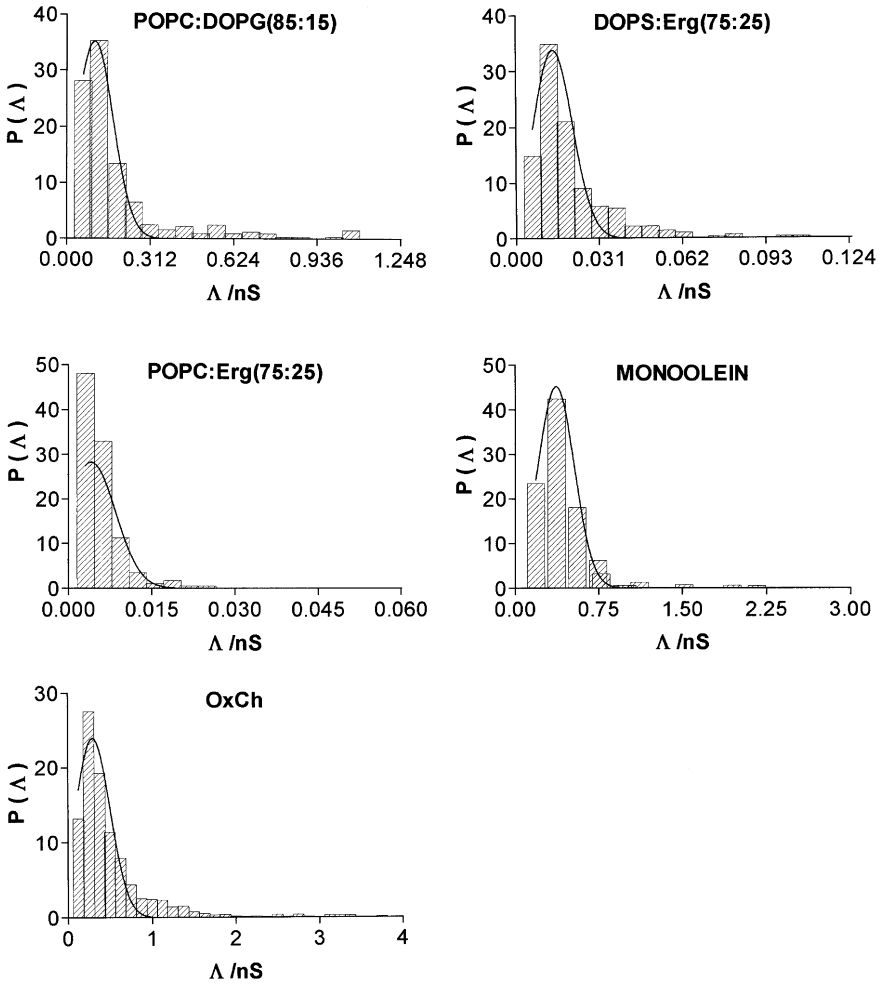

Fig. 2 Amplitude histogram of magainin 2 channel conductance. The histogram of the probability, $P(\Lambda)$, for the occurrence of a given conductivity unit was fitted by a gaussian, which is shown as a *solid curve*

Table 1 The mean conductance fitted by gaussian distribution (Λ_c) and the total number of magainin 2 channels in different PLMs

Membrane	Λ_c (nS) ± SE	No of channels
POPC:DOPG (85:15)	0.103 ± 0.005	560
DOPS:Erg (75:25)	0.013 ± 0.001	507
POPC:Erg (75:25)	0.0041 ± 0.003	378
Monoolein	0.360 ± 0.007	777
OxCh	0.294 ± 0.012	1512

membranes made up of POPC or DOPS. The addition of DOPG to the POPC solution used to form membranes results in channel activity, indicating that negatively charged lipids play an important role in the electrostatic interaction between magainin 2 and the membrane, as proposed by other authors (Cruciani et al. 1992; Matsuzaki et al. 1995c; Wenk and Seelig 1998). On the other hand, in monoolein and OxCh membranes, magainin 2 shows a higher channel formation activity. This may be compatible with a larger number of molecules assembling in the channel formation.

It is interesting to note that the addition of ergosterol, a constituent of fungal membranes, to a DOPS membrane determines channel formation, which, however, has a lower mean conductance. It cannot be excluded that this effect could partially be due to the dilution of the negative charge at the DOPS/aqueous interface of the membranes.

The addition of ergosterol to POPC membranes also allows magainin 2 to form channels, although their conductance was three times lower than that found in DOPS:Erg membranes. Finally, in DOPS:cholesterol membranes, magainin 2 failed to form channels. These results further indicate the role played by the lipid composition of the membrane, and in particular a possible target function of ergosterol in fungal membranes.

Moreover, to test if the single-channel conductance is a linear function of the ionic strength, we performed experiments at 0.1 M and 1 M KCl in membranes made up of POPC:DOPG. The central value of conductance versus the ionic strength is shown in Fig. 3. As can be seen from the linear and quadratic best-fit, the single channel conductance behavior is not linear.

Single-channel lifetimes

The lifetime of single channels is another parameter that can be used to characterize a channel. Single-channel current recordings with a conspicuous number of

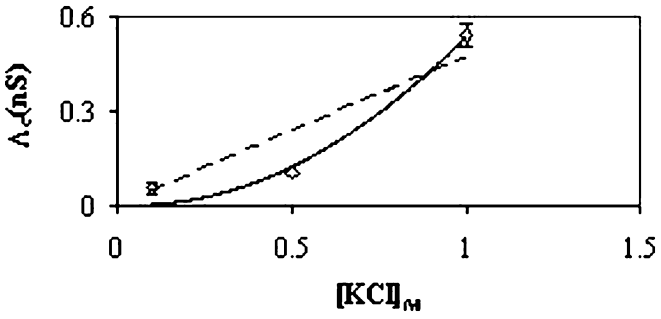

Fig. 3 Single-channel conductance dependence on ionic strength in POPC:DOPG (85:15) membranes. Experiments were performed in the presence of magainin 2 (10^{-7} g/mL) added to the *cis*-side; the voltage was +160 mV (pH 7) and $T = 22 \pm 2$ °C. The *superimposed lines* are the results of the linear best-fit (*dashed line*), where the slope = 0.47 and $r^2 = 0.844$, and of the quadratic best-fit (*full line*), where $y = 0.583x^2 - 0.047x$ and $r^2 = 0.978$

channels were analyzed to obtain cumulative open-state lifetime distributions. The results of analyzing magainin 2 at $V = 160$ mV and 0.5 M KCl are shown in Fig. 4. In relation to membrane composition, the distribution of the open times has been found to follow a single-exponential or a two-exponential function. The equation used is:

$$N = A_1 \exp(-t/\tau_1) + A_2 \exp(-t/\tau_2) \quad (1)$$

where N is the number of channels that remain open for a time equal to or greater than a certain time t, A_1 and A_2 are the zero time amplitudes, and τ_1 and τ_2 are related to the fast and slow components of the time constant. The single-exponential distribution is included in the formula ($A_2 = 0$). In order to choose between the two models, we performed an appropriate statistical test (F-test, Graphpad Prism 3). The results obtained indicate that analysis of the open-time distributions gives a statistically significant better description ($P < 0.05$) of the single-exponential than the two-exponential function only for magainin 2 channels formed in POPC:DOPG and in POPC:Erg membranes; in all other cases, analysis of the open-time distributions gives a statistically significant better description ($P < 0.05$) with the sum of the two exponentials. τ_1 ranges between 0.6 and 1.8 s, while τ_2 ranges between 4.3 and 12.73 s.

When the ionic strength is lowered (0.1 M KCl), POPC:DOPG membranes seem to show a second channel population with a longer time constant (Table 2).

Occurrence of single-channel events

The occurrence frequency represents the mean number of openings in a period of 60 s, obtained from the total

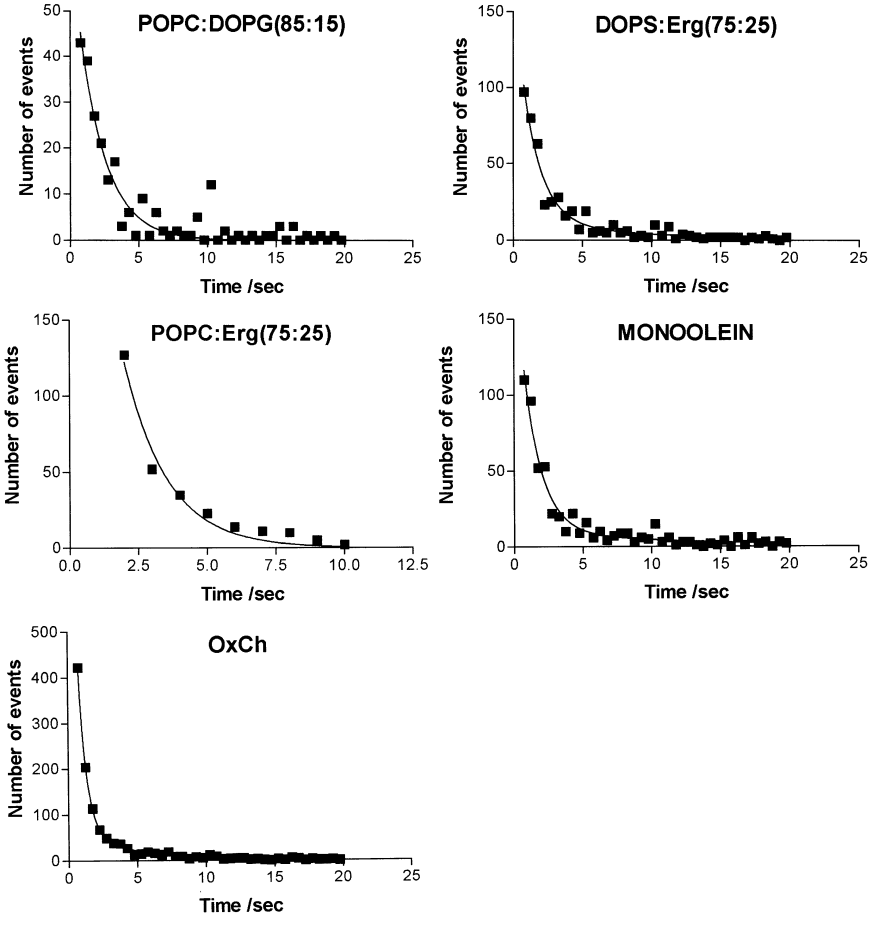

Fig. 4 Frequency histograms of single magainin 2 channel open time in PLMs of different composition. The analysis was performed on the same experiments reported in Fig. 1. The time constants, τ_1, τ_2 were determined by fitting the data points obtained as described in the text. For magainin 2 channels formed in POPC:Erg membranes, the fitting of the data points was performed excluding the first two data points due to the poor resolution of these very low channel conductances

number of records. The frequency of magainin 2 channel opening and the half-life of channels for different membranes used is reported in Tables 2 and 3. It can be observed that the occurrence frequency changes in relation to the membrane lipids considered.

Voltage-dependent pore formation

It has been shown that magainin 2 interacts with membranes containing negatively charged phospholipids, indicating that the initial interaction of the peptide with negatively charged lipids is driven by the surface potential. Channel formation by magainin 2 has been found to be potential dependent, i.e. in all the membranes tested a threshold potential of about ±160 mV is required to induce channel activity, although a lower potential was needed for negatively charged membranes. This result is in accordance with other authors' findings (Cruciani et al. 1992). On the other hand, if there is a transmembrane electric potential, this can lower the free energy of the insertion state relative to the surface adsorption state for peptides possessing a dipole moment, such as α-helix peptides.

Current-voltage (*I-V*) relationships of the single-channel currents were measured in the range –180 to +200 mV (–200 to +200 mV) in PLMs made up of POPC:DOPG (OxCh). Figure 5 shows a symmetrical behavior of the current-voltage relationship.

Ion selectivity

The ion channel selectivity of magainin 2 was measured by means of reversal potential (V_{rev}) and *I-V* relationships for POPC:DOPG and OxCh membranes. About 60 min after magainin 2 addition on the *cis* side, the membrane conductance reached a virtually stable value; then the salt concentration on the *cis* side of the membrane was raised to 200 mM by the addition of

Table 2 The mean conductance fitted by gaussian distribution (Λ_c), the occurrence (channels/min) and the fitted lifetimes (see text) of single-channel events (s) in different POPC:DOPG membranes at different ionic strengths. The surface potential (ψ) was calculated by means of the formula: $4A^2\sigma^2/C = \exp(-ze\psi/kT)$ where k is the Boltzmann constant, T is temperature, e is electronic charge, z is the valence of the symmetrical electrolyte solution, σ is the charge density, ψ is the surface potential, C is the bulk aqueous electrolyte concentration and $A = 1/(8N\epsilon_r\epsilon_0 kT)^{1/2}$, where N is Avogadro's number, ϵ_r the dielectric constant and ϵ_0 the permittivity of free space (McLaughlin 1977)

KCl (M)	ψ (mV)	Λ_c (nS) SE	Channels/60 s SD	τ_1 (s)	τ_2 (s)
1	–17.22	0.54 0.04	1.75 0.07	1.6	–
0.1	–48.22	0.054 0.02	4.44 0.23	1.14	5.06

Table 3 The occurrence (channels/min) and the fitted lifetimes of single-channel events (s) in different PLMs

Membrane	Channels/60 s SD	τ_1 (s)	τ_2 (s)
POPC	–	–	–
DOPS	–	–	–
POPC:Erg (75:25)	0.34 0.06	1.6[a]	–
DOPS:Erg (75:25)	2.90 0.76	1.3	8.7
POPC:DOPG (85:15)	2.60 0.95	1.8	–
Monoolein	1.20 0.32	1.3	12.7
OxCh	7.50 1.88	0.6	4.3

[a]See legend to Fig. 3

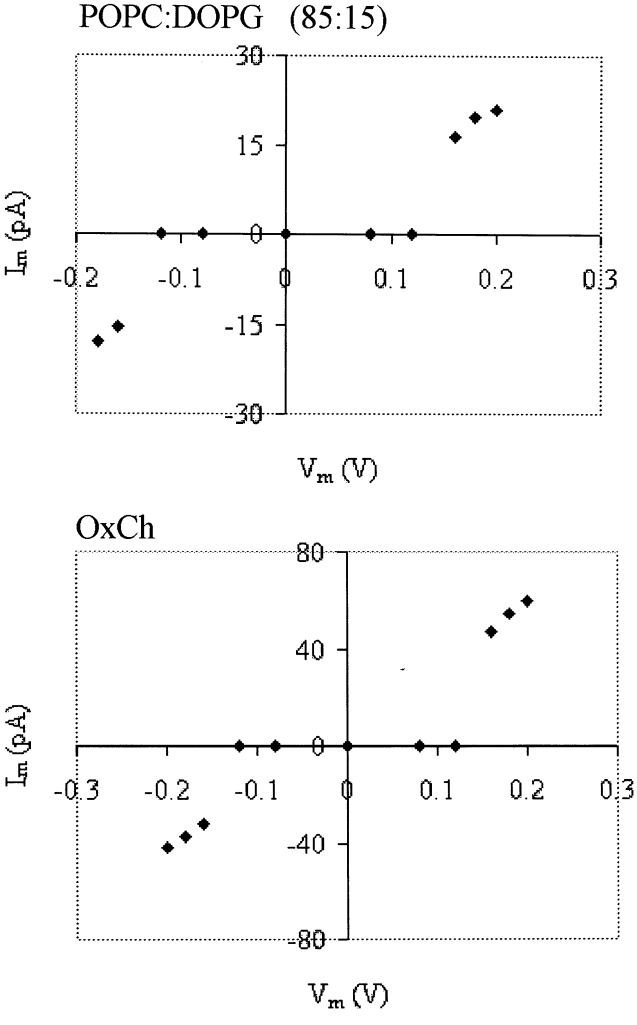

Fig. 5 Current-voltage relationship for magainin 2 channels. The current-voltage relationship was symmetrical but a threshold potential of at least ±160 mV is required to induce channel activity. Experimental conditions: KCl (0.5 M) and magainin 2 (10^{-7} g/mL) was present on both sides of the membrane

concentrated salt solution. A twofold concentration gradient was used, with 0.2 M KCl on one side (*cis*) and 0.1 M KCl on the other side (*trans*), because the reversal potential is not linearly correlated with the salt gradient at elevated salt concentrations.

Single-channel currents were observed when the holding potential reached ±140 and ±120 mV in OxCh and POPC:DOPG membranes, respectively. The V_{rev} were determined by changing the holding potential of ±2 mV step by step, and the potential at which the current is zero was taken as the reversal potential for the open channel. The mean V_{rev} was –12.3 mV and –16.3 mV for OxCh and POPC:DOPG membranes, respectively (Fig. 6). The permeability ratio was calculated using the Hodgkin-Goldman-Katz equation (Hille 1984). The P_{K}^{+}/P_{Cl}^{-} ratio was 5.4 and 3.4 for OxCh and POPC:DOPG membranes, respectively.

Approximately the same result was obtained with the *I-V* curve (Fig. 6), where the measured amplitude of the channel events at each membrane potential was used. In fact the V_{rev} was –11.9 mV for OxCh membranes and –16.6 mV for POPC:DOPG membranes; the selective ratio P_{K}^{+}/P_{Cl}^{-} was 5.3 and 3.5 for OxCh and POPC:DOPG membranes, respectively.

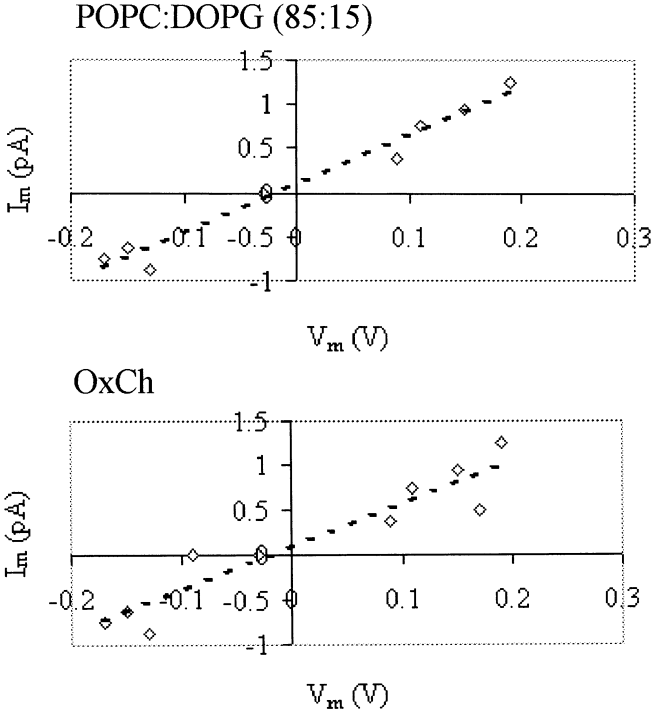

Fig. 6 The magainin 2 channel is cation-selective. The amplitude of the magainin 2 channel current (pA) is plotted as a function of the transmembrane potential (V). Each point along the *I-V* curve represents the mean value of at least three readings at the amplitude of the current at the potential indicated. Conductance was determined by linear regression of current values from ±160 (±120 mV) for POPC:DOPG (OxCh) membranes, respectively, and was 0.065 nS (0.33 nS) in asymmetrical solutions (200 mM KCl *cis* and 100 mM KCl *trans*). Intercepts were –16.6 and –11.9 mV for magainin 2 channels incorporated in POPC:DOPG and OxCh membranes, respectively, and were used to estimate P_K/P_{Cl}

Macroscopic conductance measurements

The conductance measurements were performed at the same magainin 2 concentration used for single-channel experiments, but at a lower applied potential (80 mV), by means of the AC method (Gallucci et al. 1996; Micelli et al. 2000, 2002) on POPC:DOPG, OxCh and DOPS:Erg membranes.

Depending on the nature of the membrane, the increase occurred in membrane total current and in capacitance (Fig. 7) at variable times following magainin 2 addition to the solutions bathing a black membrane. This means that magainin 2 molecules inserted and

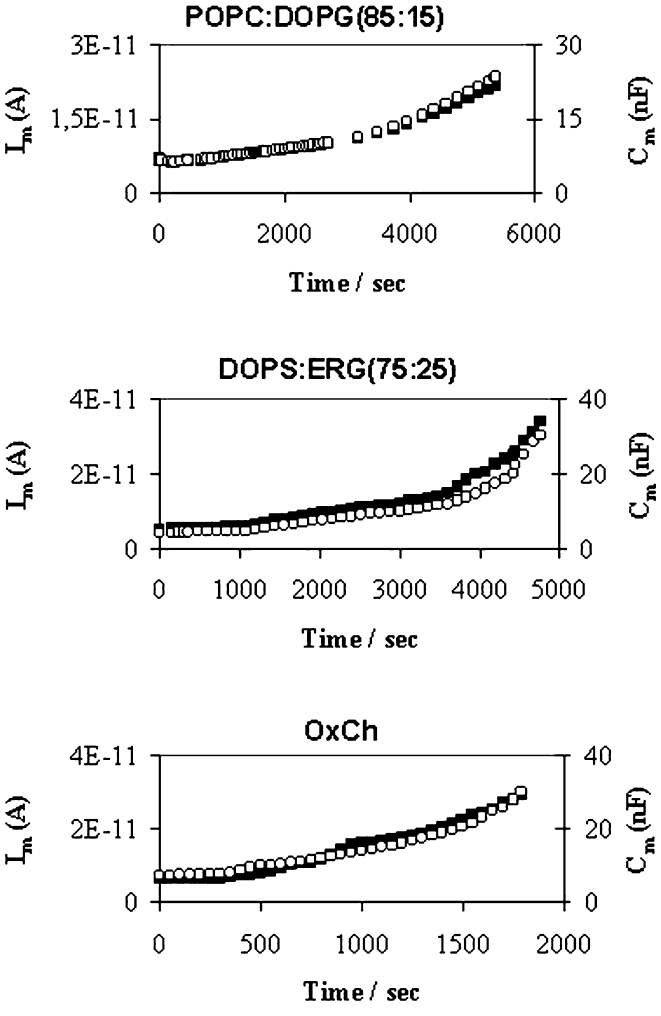

Fig. 7 Time course of the membrane total current (I_m) and capacitance (C_m) during magainin 2 incorporation into POPC:DOPG, DOPS:Erg and OxCh membranes. Magainin 2 concentration was 10^{-7} g/mL. The aqueous phase contained 0.5 M KCl. Electrical resistance and capacitance of the measuring circuit were $R_l = 1$ MΩ and $C_l = 20$ nF, $f = 1$ Hz, hole diameter $\phi = 1.3$ mm, temperature 22 ± 2 °C

aggregated into membranes in order to form pores in a cooperative manner. In a few experiments the current reached a steady state, though this behavior cannot be generalized because rupture of the membrane occurs after a certain time. The relative percent of variation [i.e. (final value – basal value)/basal value×100] of the value of currents and capacitances between the basal (before drug addition) and the final values are shown in Fig. 8.

Discussion

To explain the bactericidal and tumoricidal effect displayed by magainin 2, the interactions of positively charged peptide residues with negatively charged phospholipids such as DOPG and DOPS [the major components of bacterial and some tumor cells, respectively (Duckworth et al. 1974; Wilkinson 1988; Connor et al. 1989)] have been invoked as the first event determining anchoring to the membranes (Matsuzaki et al. 1997). On the other hand, a large body of literature testifies the role of negatively charged lipids in organizing folded proteins by electrostatic interaction with positive domains on the proteins (de Kruijff 1994). On reconstituted membranes, such as liposomes (Matsuzaki et al. 1995c, 1996), magainin 2 has shown leakage activity of the entrapped compounds, in which pore formation and lipid flip-flop are coupled in a translocation that generates a simultaneous ion and lipid flux. On the other hand, on black lipid membranes made up of negatively charged lipids, conductance increases were found when magainin 2 or magainin 1 was used, indicative of pore formation, with selectivity towards cations for the former (Cruciani et al. 1992) and towards anions for the latter (Duclohier et al. 1989).

The results presented in this work provide further statistically significant support to the pore-like structure made by magainin 2, found with the voltage-clamp technique in planar membranes and with the patch-clamp technique in BALB/3T3 or SRD/3T3 cells (Cruciani et al. 1992; Haimovich and Tanaka 1995).

It is worth noting that in our experiments, as in the ones cited above, a threshold potential is required to induce magainin 2 incorporation. This potential is required to reduce the energy of incorporation because of the high internal tension of planar lipid membranes as compared to liposomes (Beschiaschvili and Seelig 1992). However, hydrophobic interaction seems to play an important role in magainin 2 incorporation and channel lifetime, suggesting that an appropriate matching between peptide length and bilayer thickness facilitates channels with an increased number of oligomers, as the results with monoolein and OxCh membranes indicate.

Moreover, assuming that the pores formed in black lipid membranes by magainin 2 are similar to the pores formed in bacterial membranes, there is no certainty that these are the cause of the killing action, because this action is based on many properties shown by bacterial membranes, including high membrane potential (Bakker and Mangerich 1978), conductance, life spanning time of channel, interactions with other components of the cell besides lipids, functional reserve of the cell, impairment of membrane-associated functions such as ionic unbalance, nutrient transport and energy failure of the cell (homeostasis).

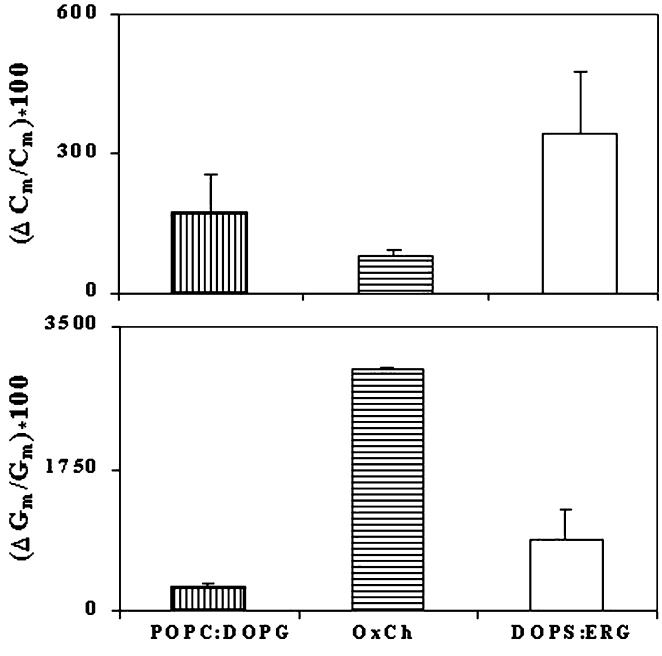

Fig. 8 The relative variations of the membrane current and capacitance before and after magainin 2 incorporation into POPC:DOPG, DOPS:Erg and OxCh membranes

Role of the membrane polar head

Magainin 2 activity, as proved by channel formation, shows less propensity to interact with artificial membranes made up of zwitterionic lipids such as POPC, a result which corroborates other authors' findings (Cruciani et al. 1992; Matsuzaki et al. 1995a; Ludtke et al. 1996). This lack of action has been invoked to explain the preservation of red blood cells from hemolysis (Matsuzaki 1999). Moreover, the addition of 15% of negatively charged lipid DOPG to POPC induces a conspicuous number of channels. On the other hand, magainin 1 formed channels in POPC:DOPE (7:3) planar membranes (Duclohier et al. 1989).

Assuming the channel to be a water-filled hole spanning the membrane in which the ions' mobility is similar to that of the bulk solution, its conductance is given by:

$$g = \pi r^2 \Lambda c/d \qquad (2)$$

where r is the radius of the channel, d the length, Λ the equivalent conductivity of the ions in the channel and c the concentration. Assuming the average thickness of a lipid bilayer made up of POPC:POPG (monoolein) to be

5 nm (3 nm), a pore area of 0.1 nm^2 (0.3 nm^2) could be estimated. It is worth underlining, however, that the calculation of pore area by means of conductance, assuming the channel to be a water-filled hole, is not a straightforward rule (Smart et al. 1997), although the values found are in the range of values calculated by means of another technique by Matsuzaki et al. (1994).

Moreover, by lowering the ionic strength the occurrence frequency of channels increases significantly (Table 2) and two lifetime constants are observed. The presence of a larger τ, as is well known, is indicative of channel stabilization. These observations are in agreement with the model proposed, involving electrostatic attraction as a first step in the cascade of events leading to channel formation.

On the other hand, in membranes with a virtual absence of the polar head, such as monoolein membranes, where a good matching exists between α-helix length of peptide (26.9–35 Å) (Ludtke et al. 1996; Gesell et al. 1997; Matsuzaki et al. 1997) and bilayer thickness (~30 Å) (Sperotto and Mouritsen 1988;Wiener and White 1992), respectively, the first step (electrostatic interaction) is absent, and the hydrophobic side-chain of magainin 2 penetrates deeper into the apolar core of the membrane, relieved as it is by the constraint of interacting with the polar heads of zwitterionic or negatively charged phospholipids.

The conductance and the stability, as indicated by a higher τ_2, of the magainin 2 channel are higher than in POPC:POPG membranes, in agreement with the theoretical study of Sperotto (1997) that a good matching facilitates the assembly of larger oligomers. Furthermore, the matching of peptide length to lipid thickness may play a determinant role in the energetics of transmembrane transitions (Vogt et al. 2000).

Owing to its amphipathic structure, magainin 2 can interact with both hydrophobic and hydrophilic counterparts and its channel structure will depend upon the physical state of the lipid bilayer in which it is incorporated.

It is puzzling to observe that cationic selectivity is maintained in both monoolein or OxCh membranes, which lack a polar head, indicating that lipids are not involved in the selectivity of the channel. Another example of cation selectivity by basic peptides is given by the S4 segment of the sodium channel (Tosteson et al. 1989; Brullemans et al. 1994).

Wieprecht et al. (1999) have shown that magainin 2 displays a larger hydrophobic partition constant for POPC than for DOPG membranes, and this was explained assuming that, in DOPG membranes, magainin 2 accumulates in the aqueous/lipid water interface by electrostatic interaction, but the transfer to the membrane surface shows a low binding constant ($K = 50\ M^{-1}$).

Taking into account all our results, one is tempted to conclude that the differences in channel-forming activity for different lipid surfaces could be caused either by electrostatic force (as is the case for POPC: DOPG membranes) or by hydrophobic force (as could be the case for monoolein and OxCh membranes) or by the specificity of membrane components such as ergosterol.

The lethal activity displayed by magainin 2 in yeasts, fungi, and some tumor cells could reflect the selective affinity to the lipid membrane's composition and its concentration. Fungal cells and some tumor cells display a preponderance of ergosterol and DOPS, respectively, and the results obtained with membranes made up of these different lipids support this concept. In fact, magainin 2 in both zwitterionic membranes (POPC) and negatively charged membranes (DOPS) does not manifest any significant channel activity, while the addition of ergosterol leads to channel formation. On the other hand, sterols present in the plasma membrane represent a target for some antibiotics and toxins that exert their action by means of membrane permeabilization; examples are polypeptide toxins, such as streptolysin-O and pneumolysin (de Kruijff 1990), or the polyene antibiotics, nystatin and amphotericin B (Norman et al. 1972; Marty and Finkelstein 1975; de Kruijff 1990; Langlet et al. 1994) or the cyclic lipodepsipeptides, syringomycin and syringotoxin (Dalla Serra et al. 1999). Moreover, some relevance has been shown in the incorporation of proteins and bacteria by sterols (Gatfield and Pieters 2000).

Macroscopic incorporation

The macroscopic incorporation of magainin 2 into lipid membranes, followed by a current increase, indicative of pore formation, highlights some aspects of the peptide partition into the membrane. According to Boggs et al. (2001), the kinetics of incorporation of magainin 2 into lipid membranes shows a cooperative shape and could mimic the natural behavior of peptide interaction with plasma membranes. This cooperative mechanism of magainin 2 incorporation into PLMs seems to follow the same pattern already observed for other peptides such as salmon calcitonin (Stipani et al. 2001), gramicidin (Gallucci et al. 2002), alamethicin (Mak and Webb 1995) and mitochondrial porin (Zizi et al. 1995; Gallucci et al. 1996; Micelli et al. 2000, 2002), which has been interpreted by assuming that pre-inserted molecules of peptide/protein in the membrane facilitated further peptide/protein insertion.

Considering a mean of seven molecules of magainin 2 per channel (Huang et al. 2000), and the mean area occupied by lipid molecules of ~58 $Å^2$, from the conductance at the end of the experiments a rough approximation of the number of channels incorporated into the bilayer can be calculated (~370). From this calculation or simply considering the surface ratio between lipids and the magainin 2 channel, assuming a magainin 2 diameter of 84 Å (Yang et al. 2001), a very low P/L ratio of about 10^{-9} is obtained; therefore, a cooperative effect is also present at this very low P/L ratio.

Moreover, the partition of magainin 2 into lipid membranes modifies the other parameter characterizing the electrical properties of the bilayer, i.e. the capacitance. This modification is slow no matter whether the magainin 2 is added to one or both sides of the solution bathing the membrane or whether the membranes were formed in the solution in which the peptide was previously added. The capacitance increase is likely due to a decrease in the thickness of the membrane; a decrease in thickness has been demonstrated using other techniques (Yang et al. 2001).

Taken together, the single-channel results, the macroscopic incorporation and the findings of other authors lead us to the conclusion that the pre-eminent variables of magainin 2 interaction with membranes are cooperativity, peptide/lipid ratio and the physicochemical structure of lipids and that as well as electrostatic interaction, hydrophobic interaction also seems to play a significant role.

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