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Membrane re-arrangements and rippled phase stabilisation by the Cell Penetrating Peptide Penetratin

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Abstract

Cell penetrating peptides are promising vectors for molecular drug delivery in eukaryotic cells. Despite of their discovery 20 years ago, the mechanisms of peptide membrane crossing are still controversial. The different suggested penetration mechanisms reflect the high sequence and structural diversity of cell penetrating peptides. The fundamental step for peptide penetration into the cytosol is the crossing of the membrane lipid barrier at the level of the plasma membrane or the endosomes. Therefore, the study of the peptide-lipid interaction is the key for peptide penetration mechanisms understanding. In order to study the changes in lipid organisation induced by the cell penetrating peptide penetratin, several experiments by three different physicochemical approaches were performed. X-ray diffraction data shows that penetratin is able to induce membrane phase separation and lipid rearrangements observed by inter-lipid distances. These changes are accompanied by a temperature stable behaviour of some of the induced membrane domains. The membrane environment fluorescent probe laurdan showed that, in DMPC and DMPC/DMPG membranes, the peptide induces de-packing of lipids. Calorimetric analyses shows that penetratin favours the gel phase to gel-like rippled phase transition. Overall, the data suggest both, that the rippled phase is a heterogeneous structure formed by gel-like and fluid-like coexisting components, and that the penetratin-induced membrane heterogeneity could be important for membrane destabilisation during cell penetration.

Keywords:

(lipids packing, membrane organisation, penetratin, rippled phase)

Abbreviations:

Chol: cholesterol, CPP: cell penetrating peptide, DMPC: 1,2-Dimyristoyl-sn-glycero-3-phosphocholine, DMPG: 1,2-Dimyristoyl-sn-glycero-3-phosphoglycerol, DPPC: 1,2-Dipalmitoyl-sn-glycero-3-phosphocholine, DPPG: 1,2-Dipalmitoyl-sn-glycero-3-phosphoglycerol, DSC: differential scanning calorimetry, GUV: giant unilamellar vesicle, LUV: large unilamellar vesicle, MLV: multilamellar vesicle, PC: L- α -phosphatidylcholine, PG: L- α -phosphatidyl-DL-glycerol, SAXD: small angle X-ray diffraction, SM: sphingomyeline and WAXD: wide angle X-ray diffraction.

1. Introduction

Cell penetrating peptides (CPPs) are potential therapeutic vectors for delivery of molecules inside eukaryotic cells. Such peptides (*i.e.* TAT, penetratin) are usually rich in basic amino acid residues. Penetratin, a peptide derived from the homeodomain transcription factor Antennapedia was described to successfully carry active molecules inside cells and is one of the most studied CPPs [1-3].

Membrane binding and cell penetration of CPPs are modulated by the physicochemical properties of both the membrane and the peptide [4]. Cell penetration is known to use two pathways: the receptor- and metabolic energy-independent pathway, and the endocytic pathway [5, 6]. However, to reach the cytosol and the nucleus after cellular or "physical" endocytosis, the peptides must cross the plasma membrane or the endosomal membrane barrier. Therefore, a direct interaction with membrane lipids seems to be the most important factor for their translocation into the cytosolic or nuclear compartments.

Several mechanisms for CPP membrane translocation have been proposed including an "electroporation-like" mechanism [7], neutralisation of arginine residues by guanidinium-phosphate complex formation [8], inverted micelles [9] and also a direct translocation through the membrane bilayer [10] (for reviews see [11-15]. Experiments with model membranes showed that membrane translocation in large unilamellar vesicles (LUVs) is dependent on membrane potential including pH gradient and is modulated by the lipid composition [16-19]. However, one report showed that membrane potential is not strictly necessary for translocation in LUVs [20], and in giant unilamellar vesicles (GUVs), membrane translocation had also shown to be membrane potential-independent [21]. Membrane curvature and/or membrane tension which are higher in LUVs than in GUVs could be responsible of these differences.

Using membrane models, we have previously shown that penetratin and different basic peptides induce membrane invaginations, which results in the formation of tubular structures [22-24]. Therefore, membrane negative curvature induced by basic peptides could be crucial for the mechanism of internalisation by tube formation ("physical endocytosis") [22] and inverted micelles. Moreover, the capacity of peptides to induce tubes and both negative or positive curvature was also observed in membranes of biological origin [25, 26]. Besides the capacity of peptides to modify the arrangements of membrane phospholipids, peptide

structural changes might be important for membrane translocation. Penetratin has been extensively studied showing to acquire α -helix, β -sheet and coil conformations in different conditions [27-34].

The membrane domains and raft microdomains concepts have been developed in the last fifteen years, and the role of lipid arrangements in cellular membranes functions and organisation have been demonstrated [35]. The role of membrane microdomains in CPP binding and uptake is not well understood. Moreover, the role of membrane lipid charges in the penetratin binding ability has been a subject of controversy. Several studies have suggested that negatively charged phospholipids are required for binding [29, 30, 36], but others have shown that zwitterionic phospholipids are also able to bind the peptide [27, 37]. It has also been shown that cholesterol (Chol) (an essential component of the so called membrane "rafts" microdomains) modulates CPP binding to phosphatidylcholine/phosphatidylglycerol (PC/PG) membranes [28]. Moreover, we demonstrated that membranes in a liquid ordered raft-like phase seems to block or reduce the peptide-induced effect on membrane curvature in comparison with membranes in the liquid disordered phase [23, 38]. The mechanisms involved in the peptide interaction with different membrane domains and the modifications of membrane lipids organisation by CPPs are therefore important factors for the development of vector-mediated therapeutic strategies at both, the peptide (vector) and the lipid membrane (target) levels. Therefore, we have investigated the influence of the CPP penetratin on membranes with different phospholipid composition mimicking different microdomains of the eukaryotic plasma membranes. The influence of the membrane state on penetratin induced phospholipid organisation was studied by X-Ray diffraction, laurdan fluorescence and differential scanning calorimetry (DSC). The results indicate that penetratin is able to induce rearrangements of membrane lipids that favour phase separation and membrane heterogeneity as shown specifically by the stabilisation of the membrane rippled phase. The implications of the data in penetratin membrane activities are discussed.

2. Materials and Methods

2.1. Materials

Egg yolk L- α -phosphatidylcholine (PC), egg yolk L- α -phosphatidyl-DL-glycerol (PG), sphingomyeline (SM) and cholesterol (Chol) were purchased from Sigma. 1,2-Dimyristoyl-sn-glycero-3-phosphocholine (DMPC), 1,2-Dipalmitoyl-sn-glycero-3-phosphocholine (DPPC), 1,2-Dimyristoyl-sn-glycero-3-phosphoglycerol (DMPG) and 1,2-Dipalmitoyl-sn-glycero-3-phosphoglycerol (DPPG) were purchased from Genzyme. Penetratin (RQIKIWFQNRRMKWKK) was synthesised using Boc solid phase strategy and was purified by HPLC as previously described [22]. Laurdan was from molecular probes.

2.2. Preparation of membranes

Multilamellar vesicles (MLVs) for X-ray diffraction were obtained as previously described [23]. Briefly, by dissolving 20 mg of lipids in the appropriate proportions in a mixture of chloroform and methanol, 2/1 (v/v), followed by solvent evaporation under nitrogen. Final traces of solvent were removed in a vacuum chamber attached to a liquid nitrogen trap for 3-4 hours. Lipid films were hydrated with 25 μ l of buffer 10 mM HEPES pH 7.4 containing 2 mg of penetratin and mixed extensively. The lipid dispersion was thoroughly stirred, sealed under argon and kept until examination at 4°C.

Large unilamellar vesicles (100 nm diameter LUVs) were prepared by extrusion in 0.5 mM HEPES buffer (pH 7.4) as described in [39]. The lipids were added at the desired proportions to obtain a suspension of 1 mg ml⁻¹ LUVs in buffer. Laurdan was present at 0.1% lipid weight ratio.

MLVs for DSC experiments were performed as follows: 2 mg of lipids on chloroform were dried and subjected to vacuum for 10 minutes. Then, 2 ml of buffer were added in the presence or the absence of penetratin at the desired Peptide/Lipid Molar Ratio (P/L) (1/50 or 1/10). The samples were strongly stirred to obtain the suspension of MLVs (1 mg ml⁻¹) ready to use for the CSC calorimeter. For the experiments performed with the DSC 822 calorimeter, the MLV suspensions (2 mg ml⁻¹) were centrifuged in a Beckman centrifuge at 100 000g. The MLV pellets were recovered in a final volume of 40 μ l (MLV final concentration of 50 mg ml⁻¹).

2.3. Fluorescence spectroscopy

Fluorescence measurements were performed with a Jasco fluorimeter as previously described [40]. The excitation and emission band-pass were set at 5 nm. Spectra were recorded 7 min after addition of penetratin to LUVs in 0.5 mM HEPES buffer (pH 7.4). Laurdan emission spectra were recorded from 400 to 650 nm using a 365 nm excitation wavelength in the absence or presence of penetratin at different penetratin/lipid molar ratios. The temperature was regulated with a Peltier device.

The generalised polarisation (GP) was calculated with the equation:

$$GP = (I_{430} - I_{485}) / (I_{430} + I_{485})$$

And the ΔGP was defined as $GP_{\text{final (after penetratin addition)}} - GP_{\text{initial (before penetratin addition)}}$.

2.4. X-ray diffraction

Small angle and wide angle X-ray diffraction (SAXD, WAXD) measurements were performed at the Synchrotron Radiation Source of Spring8 (Japan) following the protocol described in [23]. Briefly, the samples (~20 μ l) were deposited between two thin mica windows and mounted on a programmable temperature stage. Samples were exposed for 30 seconds recordings. Spacing distances were determined from circular integration of 2-D images using FIT2D program (Andy Hammersley) and silver behenate was used as the x-axis calibration standard. Peak analysis was performed with Origin and PeakFit 4 programs.

2.5. Differential scanning calorimetry (DSC)

Calorimetry was performed on a high-sensitivity Differential Scanning Calorimeter (Calorimetry Sciences Corporation, CSC) and a Mettler-Toledo DSC 822 calorimeter. A scan rate of 1 $^{\circ}\text{C min}^{-1}$ was used and a delay of 10 min between sequential scans in a series was set for thermal equilibration. Indium and zinc (purity higher than 99.9%) were used for temperature and enthalpy calibration of the DSC device. Data analyses were performed with

the programs provided by the constructors. The total lipid concentrations used were 1 and 50 mg ml⁻¹ for the CSC and the Mettler-Toledo calorimeters, respectively. For peptide concentrations corresponding to peptide/lipid molar ratio (P/L) 1:10, no thermal events were observed over the temperature range of 0-100°C. This indicates that the endothermic events observed in this study arise solely from phase transitions of the phospholipids vesicles. For the CSC calorimeter a minimum of three heating and cooling scans were performed.

2.6. Statistical analysis

The statistical significance of differences between experiments was determined by the Student's t-tests with GraphPad Prism software. For differences before and after peptide addition we used paired test, and for differences between different conditions the unpaired test was performed. Differences were considered statistically significant at $P < 0.05$. Graphs are given as bar plots \pm standard error (SE).

3. Results

3.1. Membrane lipids rearrangements studied by X-ray diffraction.

The penetratin effects on phospholipid phase separation and its thermal stability were studied by Small Angle X-ray diffraction (SAXD) in model membranes mimicking different domains of cellular plasma membranes. PC mimics the liquid disordered (Ld) membrane domains. PC/PG (9/1) mimics Ld domains but in the presence of a small proportion of negative lipids. This domains are present in several cancer cells. SM/Chol (1/1) mimics the liquid ordered (raft) domains. SM/Chol/PG (4/5/1) mimics the raft domains in the presence of negative charges, and finally, PC/SM/Chol (1/1/1) membrane mimics the overall heterogeneous plasma membrane with coexisting liquid ordered and liquid disordered domains. We previously showed that penetratin was able to induce phase separation in fluid membranes but not in liquid ordered membranes. Here we studied in more detail the temperature dependent variations on d-spacing, *i.e.* the distance between two membranes including the water layer (Figure 1). The variation of the two first diffraction orders of diffractograms for PC as well as the variation of the corresponding d-spacing distances are represented as a function of the

temperature in Figure 1. As can be seen, for PC in the absence of penetratin, the d-spacing corresponding to one lamellar structure (L1) decreases with increasing the temperature. When penetratin is added to PC, diffraction peaks with higher d-spacing corresponding to two different lamellar structures (L2 and L3) were then observed (Figure 1B). As previously reported [41], the electron density profiles show that the two SAXD peaks correspond to two different lamellar phases and not only to an increase of the hydration layer due to peptide accumulation. The L2 of similar thickness as the L1, but the L3 layer being thicker by about 0.5 nm [41]. This indicates that a real phase separation took place. As can be seen, the peak width is broadened. As for pure PC (L1), when the temperature increases, the d-spacing of the L2 phase decrease. On the contrary, for the L3 phase a thermal stability was observed (Fig. 1B and 1C).

The d-spacing obtained from SAXD experiments are gathered in Table 1 for the 5 MLV of different composition at 25 °C with and without penetratin.

In SM/Chol and PC/SM/Chol membranes, penetratin showed only moderate effect in phase separation (PC/SM/Chol as published previously [41]) and thermal stability (SM/Chol in Figure 1C). However, as for PC, PC/PG and SM/Chol/PG, penetratin showed different lamellar structures with an increase in d-spacing. For comparison, table 1 shows the d-spacing at 25°C. It is evident the increase of d-spacing in liquid disordered phases and in the presence of PG as well as phase separation. After Penetratin addition the observed tendency was that the peptide did not modify the thermal behaviour of the thinner phase (L2), but induced thermal stability of the thickest phase (L3, d-spacing of about 8.7 nm).

The penetratin effect on the lateral phospholipid organisation and on their thermal stability was studied by Wide Angle X-ray Diffraction (WAXD). In the absence of penetratin, for the five different MLVs studied (three examples are shown in Figure 2), the WAXD peaks were very symmetric and shifted to higher d-spacing with the increase of the temperature. At 25°C the spacing distances were in the range of 4.39 to 4.63 Å (Figure 2A-C and Table 1S of supplementary material). With the exception of SM/Chol liquid ordered MLVs (Figure 2F and Table 1S), the presence of penetratin induced, in all cases, the splitting of the symmetric WAXD peak into a signal composed by two or three different contributions (Figures 2D,E and Table 1S). The analysis of WAXD signal for PC in the presence of penetratin at 25 °C showed to be formed by the contribution of three peaks at 4.72, 4.42 and 4.15 Å and for

PC/SM/Chol two peaks at 4.70 and 4.47 Å. This fact reveals a rearrangement of head groups in interaction with penetratin as previously suggested [42]. These distances could be consistent with lipid organisation in a quasi hexagonal or a distorted triangular lattice [43-46]. Moreover (with the exception of SM/Chol), the position (spacing) of peaks remained thermally more stable compared to the peaks in the absence of the peptide, especially for the contribution around 4.7 Å (Figures 2G,H,I). Two particular cases were observed: the spacing changes at low temperature from 5 to 15°C for PC and from 5 to 25°C for PC/SM/Chol (Figures 2G and 2H) and at higher temperature the spacing remains stabilised by the peptide.

Overall, these data show that penetratin induces stronger changes in liquid disordered membranes compared to the liquid ordered (raft) domains.

3.2. Changes in membrane lipids packing induced by penetratin.

In order to study the effect of penetratin on membrane lipids compactness we used the environment probe laurdan. We showed previously that penetratin was able to induce phospholipid rigidity in PG and PE but not in PC membranes [34]. Herein, and in order to better understand the observed effect of penetratin on head group organisation (WAXD data) we studied the interaction of penetratin on well characterised DMPC membranes which organise in different phases: at 9 °C (gel phase), at 19 °C (rippled phase) and 30 °C (liquid phase) in the absence or in the presence of a negatively charged phospholipid (DMPC and DMPC/DMPG (9/1) respectively).

Figure 3A shows an example of laurdan spectra of DMPC/DMPG LUVs at 30°C in the presence and in the absence of penetratin. In DMPC membranes, penetratin showed a moderate effect in lipid packing depending on the lipid phases. As shown in figure 3B at low P/L ratio (1/50), penetratin only showed a decrease in lipids packing in the liquid phase (30 °C) and at higher P/L ratio (1/10), the effect in lipid compactness was stronger in the gel (9 °C), rippled (19 °C) and liquid (30 °C) phases.

In the presence of DMPG, a negative charged lipid that increases the affinity of penetratin to membranes, the penetratin effect in lipid packing was stronger. As shown in figure 3C, the decrease in compactness was observed for both P/L ratios but it was clear that the peptide-induced changes are stronger in liquid phase and are smaller in the rippled phase. In order to

further study the effect of penetratin on lipid organisation, we characterised the thermal lipid phase transitions: gel-rippled and rippled-liquid transition temperatures by DSC.

3.3. Penetratin induced changes in thermal transitions of the membrane.

The results from the laurdan fluorescence and X-ray diffraction reveal that penetratin induces a different organisation in local regions of the phospholipid membranes. Thus, to clarify the penetratin effect on thermal stability on different lipid phases, we performed calorimetric experiments with DMPC, and DMPC/DMPG (9/1) membranes in the absence and in the presence of penetratin at 1/10 and 1/50 peptide/lipid molar ratios. The thermograms showed the typical two transition peaks (Fig 4A). The temperature and enthalpy of transition values are reported in Table 2. For DMPC MLVs, a small peak for the pre-transition temperature (T_{pre}) (transition from $L\beta'$ gel to the rippled $P\beta'$ phases) was observed starting at 12.5 °C, and for the transition temperature (T_m) ($P\beta'$ to $L\alpha$ liquid state) at 23.6 °C, values in agreement with several reports [47-49]. After the addition of penetratin, there was no significant variation for the transition temperatures at low peptide concentration. However, for higher concentration, penetratin induced a significant shift in the pre-transition temperature to a lower value (11.7 °C). To figure out the effect of a negative charge in the membranes, MLVs were prepared with DMPC/DMPG (9/1). The transition temperatures in the absence of the peptide remained stable as compared with DMPC alone. Nevertheless, the penetratin effect was similar but more pronounced compared to DMPC membranes. The peptide induced a stronger shift of the pre-transition peak resulting in a decrease of the pre-transition temperature (10.5 °C) (Figure 4B and table 2). In addition, for DMPC/DMPG membranes we observed a very small but constant and significant increase in the transition temperature (Figure 4B). These effects were observed at low and high concentration of penetratin, but were stronger at higher penetratin concentration (1/10 peptide/lipid molar ratio) with a temperature lowering of 1.9 °C for the $L\beta'$ - $P\beta'$ phase transition.

Concerning the enthalpy, we observed a significant decrease for MLVs with penetratin at high concentration during the pre-transition. This decrease in enthalpy is probably due to the increase in the peptide-induced heterogeneity of the sample by the introduction of different lamellar structures into the system.

In order to further study the peptide effect in the membranes, we prepared DPPC/DPPG membranes since the presence of DMPG induced stronger effects with DM-lipids. The results presented in Table 2 show a similar behaviour for DPPC/DPPG compared with DMPC/DMPG MLVs. Penetratin at higher concentration reduce the pre-transition temperature of 2.1 °C. We observed as well a lowering in enthalpy for the $L\beta'$ - $P\beta'$ phase transition.

4. Discussion

Studies of CPP-membrane interaction present a high appositeness in pharmaceutical application. To better elucidate the CPP capacity to translocate through membranes and then promote an appropriate biological response, the understanding of membrane lipid rearrangement induced by CPPs is relevant. This work is focused on penetratin peptide, which have been shown to induce membrane invaginations resulting in the formation of tubular structures [22, 23, 38]. The peptide induces negative curvature of membrane, which could be crucial for cellular internalisation by tube and inverted micelles mechanisms [22]. Penetratin also had shown to acquire α -helix, β -sheet and coil conformations in different conditions [27-34, 50, 51].

To get more insight into the lipid rearrangements induced by penetratin, we analysed the physicochemical properties of membrane lipids after the addition of penetratin. Specifically, we characterised lipid rearrangements by X-ray diffraction, lipid packing by laurdan fluorescence and lipid phase transition by differential scanning calorimetry.

To perform the X-ray diffraction study, we chose different membranes. PC to mimic liquid disordered external leaflet domains, PC/PG (9/1) to study the effect of negative charge and SM/Chol (1/1), SM/Chol/PG (4/5/1) and PC/SM/Chol (1/1/1) to study pure raft, and heterogeneous cholesterol containing membranes. We observed, with the exception of SM/Chol membranes (raft), that penetratin induced membrane phase separation. This fact confirmed the precedent data showing that penetratin was unable to induce membrane tubulation in liquid ordered membranes [23]. A second interesting point from these experiments was the observation that after Penetratin addition, the resulting lamellar phases showed different temperature dependence. Particularly the lamellar phase of higher d-spacing showed to be more resistant to change the d-spacing in function of temperature. These

penetratin effects were confirmed by WAXD analyses. The peptide induced a change in the WAXD signal from one single peak (probably a hexagonal lattice) to a peak containing three different contributions suggesting a distorted hexagonal organisation. Since WAXD experiment reflects the head polar group arrangement of phospholipids, we demonstrated that penetratin induce a re-organisation of lipids within the membranes. As for SAXD we observed that after a temperature increase, the lipid distances for one of the contributions induced by penetratin (around 4.7 Å) were more stable than the two others. In summary, the results from WAXD and SAXD experiments are correlated because after the penetratin addition, there were appearance of thermally stable peak in both, the polar head group distance (WAXD) and the lipid bilayer thickness (SAXD).

To study the lipid organisation thoroughly, we measured the compactness of the membrane lipids by laurdan fluorescence. Synthetic lipids were used here (DMPC and DMPC/DMPG) to obtain data of penetratin interaction on specific well-characterised membrane phases, which are stable at different temperatures (gel $L\beta$, rippled $P\beta'$ and fluid $L\alpha$ phases). The laurdan environment polarity increases with the increase of the polar head groups distance, producing a red shifted spectrum. Our data showed that penetratin induce a quite small change on DMPC membranes. In fact, the peptide decreases compactness only at a high peptide/lipid ratio. The changes were very small on the gel and rippled phases but stronger for the liquid phase. In the presence of 10% DMPG, which increases the affinity of penetratin for the membrane, the effect of penetratin on lipid packing was clear. We observed that penetratin induces a strong reduction of the lipids packing in the liquid phase. On the other hand, the effects were moderate for the gel phase and even smaller for the rippled phase. These results allow us to suggest that the interaction of penetratin with the $P\beta'$ phase is naturally occurring. In other words penetratin adapts easily to this organisation inducing only a small change in lipid packing, since in the other phases penetratin trigger stronger changes in membrane compactness when interacting with them.

Then to better understand the penetratin effect on the different lamellar phases (gel, rippled and liquid), we performed DSC experiments, which allowed the study of the influence of the peptide on the transition temperatures between these different phases.

As shown in table 2, the peptide decreased considerably the pre-transition temperature and slightly increased the transition temperature. As for the laurdan experiments, this

perturbations in temperature were stronger for DMPC/DMPG than for DMPC membranes. These results were also observed for DPPC/DPPG membranes. Therefore, the peptide clearly favours the transition through the rippled phase in a way to stabilise it. A very similar behaviour was also observed for the peptide S4(13)-PV with DPPC membranes [52].

The whole data presented here shows that penetratin is able to separate lamellar phases in a membrane and that these new phases acquire new thermal properties. Penetratin induces thermal stability of the "the novo" formed phases and the de-packing of saturated lipids. Moreover, the lipid packing experiments showed that the peptide perturbs the less the rippled phase which is in agreement with the calorimetry studies showing that the peptide seems to favour the rippled phase. Two models have been suggested for the lipid organisation of the rippled phase. The first proposed that the lipids were tilted with respect to the normal of the membrane with periodic ripples [53]. However, Tenchov [54] reported data showing the existence of two different $P\beta'$ phases, one being metastable. More recently, two groups published experimental and simulation data suggesting that the $P\beta'$ phase could be organised as two structurally related structures, one typically in the tilted gel state coexisting with a second more fluid responsible of the ripples within the lamella [44, 45]. Our data is consistent with this hypothesis since penetratin seems to prefer the rippled phase and at the same time induces de-packing and phase separation. The capacity of the penetratin to modify the organisation of membrane lipids could be an important factor involved in its capacity to cross the plasma membrane. Notice that the penetratin effect reported does not indicate that the rippled phase is important for membrane translocation (this phase is not present in cellular membranes). We suggest that the penetratin-induced membrane heterogeneity when interacting with an homogeneous membrane could be involved in cell penetration. The stabilisation of the rippled phase deduced by the low effect on membrane compactness and the effect on transition temperatures support this hypothesis on the importance of membrane heterogeneity, because the rippled phase is an heterogeneous structure in which gel and liquid contributions coexist. How this membrane heterogeneity would be involved in cellular peptide penetration? The penetratin would take advantage of the presence of mismatches, defects or discontinuities on the membrane between the pre-existent or the peptide-induced membrane phases (i.e. membrane domains) of different thickness and physical properties. Further experiments with biological membranes will be necessary to test this interesting hypothesis.

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Tables

Table 1. SAXD d-spacing from MLVs at 25°C in the absence and presence of penetratin.

	d-spacing of MLVs alone (nm) ^a	d-spacing of MLVs + penetratin (nm) ^a	
MLVs	Phase L1	Phase L2	Phase L3
PC	5.98	7.07	8.70
SM/Chol (1/1)	6.59	6.77	-
PC/SM/Chol (1/1/1)	6.71	6.69	5.96
PC/PG (9/1)	6.11	6.91	8.73
SM/Chol/PG (4/5/1)	6.41	-	8.20

^a; Spacing values were obtained as the average of 3 to 5 orders.

Table 2. Thermodynamic parameters obtained by DSC for the interaction of penetratin with different MLVs^a.

MLV	P/L	T _{pre} (°C)	ΔH _{pre} (kJ/mol)	T _m (°C)	ΔH _m (kJ/mol)
DMPC	0	12.5 ± 0.37	2.7 ± 0.19	23.6 ± 0.24	21.0 ± 1.6
	1/50	12.4 ± 0.38	2.6 ± 0.23	23.6 ± 0.24	22.0 ± 1.0
	1/10	11.7 ± 0.33 *	2.2 ± 0.18 *	23.5 ± 0.15	19.4 ± 1.4
DMPC/DMPG	0	12.4 ± 0.19	1.9 ± 0.25	23.6 ± 0.15	18.0 ± 1.0
	1/50	11.5 ± 0.34 *	1.2 ± 0.16 *	23.7 ± 0.16 **	18.0 ± 1.4
	1/10	10.5 ± 0.34 ***	1.0 ± 0.18 **	23.7 ± 0.16 *	19.5 ± 1.6
DPPC/DPPG	0	31.8 ± 0.30	1.9 ± 0.12	40.6 ± 0.17	22.7 ± 1.4
	1/50	32.0 ± 0.10	1.5 ± 0.20 ***	41.0 ± 0.10 *	21.7 ± 1.7 *
	1/10	29.7 ± 0.30 ***	1.1 ± 0.06 ***	40.5 ± 0.10	20.4 ± 0.9 *

a; Values are mean values ± standard error. Experiments were performed from 7 to 11 times for DMPC and DMPC/DMPG membranes and from 3 to 5 experiments for DPPC/DPPG MLVs. Statistical significance were performed by comparison of membranes alone and in the presence of penetratin. (*p<0.05. **p<0.01. ***p<0.001).

Figure legends

Figure 1. Penetratin effects on membrane phase separation and thermal stability by SAXD. A; diffractograms of PC MLVs at different temperatures (5°C continuous line. 15°C dotted line. 25°C dashed line. 35°C dotted-dashed line and 45°C bi-dotted-dashed line). Only the two first reflexions of the lamellar phase are shown. Notice the temperature-dependent d-spacing shift of the lamellar phase (L1). B; Diffractograms of the PC MLVs in the presence of penetratin at different temperatures as in (A). Notice the two lamellar phases: one thermal sensitive shifting to the right (L2, horizontal arrow) and one thicker thermally stable (L3, vertical arrows). C; d-spacing of different lamellar phases in function of the temperature. (□)

SM/Chol (Δ) SM/Chol plus penetratin. (\blacksquare) PC phase L1. (\blacktriangle) phase L2 of PC plus penetratin. (\blacktriangledown) phase L3 of PC plus penetratin.

Figure 2. WAXD profile as a function of the temperature in the presence and absence of penetratin. A; PC. B; PC/SM/Chol. C; SM/Chol/PG. D; PC in the presence of penetratin. E; PC/SM/Chol in the presence of penetratin. F; SM/Chol in the presence of penetratin at different temperatures (5°C continuous line. 15°C dotted line. 25°C dashed line. 35°C dotted-dashed line and 45°C bi-dotted-dashed line). Notice the presence of three different contributions (arrows in D and E). G. H and I; spacing of the different contributions as a function of temperature in the absence of penetratin (\circ , dotted line) and the presence of the peptide. peak a (\blacksquare). peak b (\blacktriangle) and peak c (\blacktriangledown). G; PC. H; PC/SM/Chol and I; SM/Chol/PG.

Figure 3. Penetratin changes in membrane lipids compactness. A); Laurdan fluorescence spectrum of DMPC/DMPG (9/1) LUVs at 30 °C in the absence (continuous line) and the presence of penetratin at P/L ratio 1/10 (dotted line). B); Delta GPs of laurdan fluorescence spectra of DMPC. C); Delta GPs of laurdan fluorescence spectra of DMPC/PG (9/1). For B and C, membranes after penetratin addition at two different P/L (Peptide/Lipid) molar ratios and at three temperatures corresponding to the membranes in the gel (9 °C), rippled (19 °C) and liquid/phases (30 °C). The experiments were performed from 17 to 19 times. Bars represent the values with SE: *: $p > 0.05$. **: $p > 0.01$. ***: $p > 0.001$. Stars at the base of the bars are related to the difference between GP before and after addition of penetratin. Stars with lines are related to the comparison between different conditions.

Figure 4. Penetratin effects on the transition temperatures of DMPC/DMPG membranes. A); DSC profile of MLVs before and after addition of penetratin at P/L 1/50 molar ratio. B; Detail of the pre-transition temperatures (gel $L\beta$ to rippled $P\beta'$) of MLVs in the absence and the presence of penetratin at P/L 1/10. C; Detail of the transition temperatures (rippled $P\beta'$ to liquid $L\alpha$) of MLVs in the absence and the presence of penetratin at P/L 1/50. MLVs before (continuous lines) and after penetratin addition (dotted lines). The endothermic signals are pointing up.







