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INTRINSIC FLUORESCENCE STUDY OF LIPID-PROTEIN INTERACTIONS IN MEMBRANE MODELS

BINDING OF MELITTIN, AN AMPHIPATHIC PEPTIDE, TO PHOSPHOLIPID VESICLES

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Summary

Melittin is a small peptide extracted from bee venom, which has a direct lytic activity on living cells, and equally disrupts the liposome structure. In agreement with a previous work of Mollay, C. and Kreil, G. ((1973) Biochim. Biophys. Acta 316, 196—203), it is shown that intrinsic fluorescence of the only tryptophan of melittin is very sensitive to the binding to phospholipids. The observed blue shifts, from 352 to 333 nm, upon addition of lipid vesicles, indicate that the Trp residue is going from a polar to a non-polar environment, and clearly show that melittin displays hydrophobic interaction with zwitterionic or negative phospholipids, whatever the ionic strength or pH.

The hydrophobic nature of the interactions is confirmed by the sensitivity of the fluorescence intensity of the Trp residue to the phase transitions of phosphatidylserine, dimyristoyl and dipalmitoyl phosphatidylcholine, which implies a close contact between this residue and aliphatic chains.

It is also shown that the length of aliphatic chains has no significant effect on binding, but that their fluidity is a critical parameter. Binding is indeed much less efficient when aliphatic chains are in their crystalline state, below the phase transition temperature.

Binding is strongly dependent on the net electrical charge borne by the vesicles. The effect of a pH increase, or of an addition of dicetylphosphate to phosphatidylcholine vesicles leads to the conclusion that binding is enhanced by an increase of the net negative charge of the lipid bilayer. This result is illustrated by the fact that melittin is totally bound when the lipid to protein molar ratio is equal to 3 or 4 in the case of phosphatidylserine, and about 25 for phosphatidylcholines. Phosphatidylserine vesicles can then bind up to 8 times more melittin than do phosphatidylcholines.

In conclusion, both electrostatic and hydrophobic forces have to be con-

sidered as important binding parameters: the first step could be an ionic interaction between Lys and Arg residues of melittin and negative groups of phospholipids, either phosphate or carboxylic, the second step being the insertion of hydrophobic residues within the bilayer, this involving at least the Trp residue, but probably all the hydrophobic part of the peptide.

Introduction

Lipid-protein interactions control many functions of biological membranes, and their study still remains essential for a better understanding of the structure-function relationship in these systems. Natural membranes being very complicated, one way commonly followed is to study much more simple model systems, such as liposomes interacting with purified proteins. Several studies have already been published dealing principally with soluble or extrinsic proteins which display electrostatic interactions with the polar head groups of phospholipids, but also, to a lesser extent, with integral or intrinsic proteins which penetrate into the lipid bilayer and display hydrophobic interactions with the aliphatic chains of phospholipids [1—3].

According to Habermann [4], melittin, a small peptide extracted from bee venom, would belong to this kind of amphipathic proteins, its sequence displaying an hydrophobic moiety (from 1 to 20) followed by a strongly basic C terminal peptide. Sessa et al. [5] have shown that melittin, which has a direct lytic activity on living cells, disrupts the liposome structure in a similar way. More recently, Mollay et al., by looking at the intrinsic fluorescence of the only tryptophan of melittin, have confirmed the hydrophobic nature of its interactions with lecithins [6] and phosphatidylethanolamine [7]. They have also shown that melittin stimulates the action of bee venom phospholipase A_2.

The effect of melittin binding on the fluidity of lipid bilayers has also been investigated by several physical methods [8,9] and especially by electron spin resonance [10—12]. However, due to the difficulties arising from the use of spin labels in the presence of proteins, the interpretation of the results in terms of changes of the chains dynamics was not always possible.

In this paper, our purpose was to determine more precisely soi ıe of the pertinent parameters governing the binding. For this purpose, we have studied, by means of intrinsic fluorescence, the effects on the binding (i) of the nature of the polar head group of the phospholipid, (ii) of the length and fluidity of the aliphatic chains, and (iii) of the ionic strength and pH of the medium.

Materials and Methods

Melittin was purchased either from Sigma Chemical and Co. or Serva Feinbiochemica (Heidelberg), and was used without further purification. In 20 mM, phosphate buffer, pH 7.5, it moved by gel chromatography on a Sephadex G-50 column as a tetramer, in agreement with previous authors [4]. It contained some phospholipase activity, but no significant amount of lyso compounds appeared during the time of experiments as was checked by thin-layer chromatography. Moreover, control experiments were done in a buffer solution

containing 1 mM EDTA, which totally inactivates phospholipase A_2 [13], and they gave the same results.

Natural phosphatidylcholine was purified from hen egg yolks according to the classical procedure of Singleton et al. [14]. Lysolecithin, synthetic phosphatidylcholines, and phosphatidylserine (pig brain) were supplied by Sigma and P.L. Biochemicals; by thin-layer chromatography, some small amounts of lyso compounds were detected in synthetic phosphatidylcholines. Phosphatidylserine contained some oxidation products.

The selected phospholipids were suspended in buffer solution and sonicated for about 15 min under nitrogen atmosphere, at temperatures just above their melting point (4° C for egg phosphatidylcholine, 25° C for phosphatidylserine and synthetic short chain lecithins, 45° C for dipalmitoyl phosphatidylcholine). As it was proved by Huang [15] and by Hauser et al. [16], it mainly resulted in single shelled vesicles which generally were used directly. When necessary, large size particles were pelletted by centrifugation. The binding experiments were performed directly in the fluorescence cells, by adding some microliters of the liposomes solution to a freshly prepared solution of melittin (2 ml), the peptide concentration being generally around 10 μM.

In the case of vesicles of dimyristoyl phosphatidylcholine or egg phosphatidylcholine charged with dicetylphosphate or stearylamine, co-lyophylisation in benzene of an appropriate mixture of the two components was first done, then vesicle preparation was carried out as described above.

After sample preparation, the true lipid concentration was checked by radioactivity measurements, small amounts of [^{14}C]phosphatidylcholine being added (<1%) before lyophilisation. Counting was done with an SL 30 Intertechnique multichannel liquid scintillation spectrometer.

Fluorescence experiments were performed on a FICA 55 MK II differential spectrofluorometer, which automatically recorded corrected excitation or emission spectra. All the spectra, unless otherwise mentioned, were obtained at 25° C, with an excitation wavelength of 280 nm, excitation and emission slits being 7.5 nm. The wavelength shifts were estimated by checking the wavelength of the middle-point at half-height of the spectrum.

The studied samples were generally rather turbid, since the binding of melittin to phospholipids is followed by a breakdown of the single shell vesicular structure into onion-like liposomes [5]. This could be a source of artefact, either by a direct contribution of the exciting light to the emission spectrum, or by a decrease of the observed intensity due to scattering of incident and fluorescent beams and depending on the apparatus configuration. We have verified that these scattering effects could be neglected in the experimental conditions used. First, fluorescence spectrum of a non interacting peptide such as GlyTrpGly was totally unchanged by the addition of lipid vesicles up to turbidity values similar to those generally encountered with melittin. Moreover, as it will be described later, isoemissive points were observed in some instances. This rules out a general perturbation of the spectra as more lipids were added.

Results

Binding of melittin to lysolecithin. Fig. 1a shows the fluorescence spectra of melittin in the presence of increasing amounts of lysolecithin. As it can be seen,

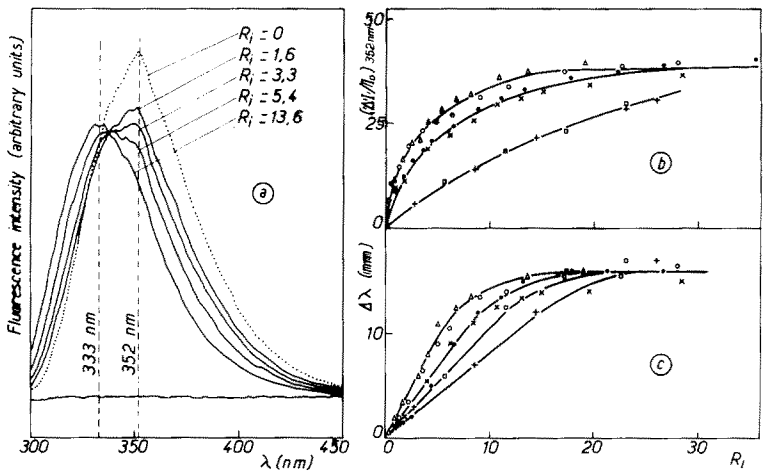

Fig. 1. Binding of melittin of lysolecithin. (a) Effect of addition of lysolecithin on the emission spectrum of melittin at pH 9.1. R_i is the lipid to protein molar ratio. (b) Relative decrease of the fluorescence intensity at 352 nm versus R_i, at various pH values. (c) Apparent wavelength shift. Experimental conditions: melittin concentration 3.6 μM. +———+, pH 4.6; □———□, pH 6.1; ●———●, pH 8.0; X———X, pH 7.0; △———△, pH 9.1; ○———○, pH 10.3. pH values were adjusted by addition of NaOH or HCl solutions to a 20 mM, phosphate buffer pH 7.0.

the wavelength of the emission maximum shifts from 352 to 333 nm as the lipid to protein molar ration (R_i) increases, and one can notice, for R_i values >2, the existence of an isoemissive point, centered around 340 nm.

The direct plot of the apparent wavelength shift, or of the relative decrease in fluorescence intensity at 352 nm (Fig. 1b,c) clearly shows that, after an important effect at the beginning of lysolecithin addition, a plateau is reached for R_i values between 10 and 25, depending on the pH.

At last, it must be mentioned that binding also occurs when ionic strength of the medium is increased up to 1 M NaCl.

Binding of melittin to synthetic phosphatidylcholines. Some spectra obtained by stepwise additions, at 44° C, of dipalmitoyl phosphatidylcholine vesicles to a melittin solution are represented on Fig. 2a. In this case, a blue shift is still observed from 352 to 333 nm, but there is a large increase of the fluorescence intensity, and the isoemissive point occurs at 369 nm.

In Fig. 2b and c are summarized the results obtained with various phosphatidylcholines of different chains lengths: dilauryl (C_{12}), dimyristoyl (C_{14}), and dipalmitoyl (C_{16}) phosphatidylcholines. Both the wavelength shifts and the relative increases of the fluorescence intensity at 333 nm are quite similar for these three phospholipids, as long as binding experiments are done above their phase transition temperature, i.e. at 25° C for dilauryl and dimyristoyl phosphatidylcholine, and at 44° C for dipalmitoyl phosphatidylcholine. Moreover, a 10-fold increase of the melittin concentration has no effect on the binding curve obtained with dimyristoyl phosphatidylcholine.

On the contrary, when an experiment is carried out below the transition temperature, as it can be seen in Fig. 2b and c in the case of dipalmitoyl phosphatidylcholine at 25° C, binding curves are much less steep, and the plateau is reached for a larger R_i value, of about 100 instead of 25.

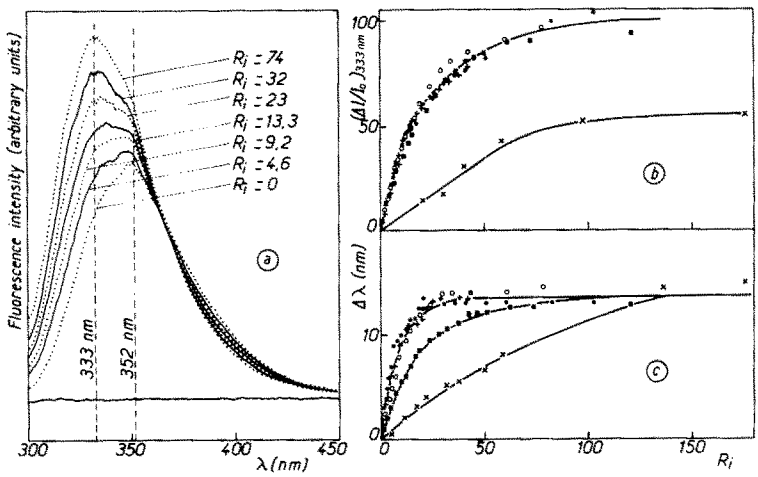

Fig. 2. Binding of melittin to synthetic phosphatidylcholines. (a) Effect of dipalmitoyl phosphatidylcholine vesicles addition on the emission spectrum of melittin at 44°C. (b) Relative increase of the fluorescence intensity at 333 nm, versus R_i = [lipid]/[protein], upon addition of various synthetic phosphatidylcholines. (c) Apparent wavelength shift. Experimental conditions: phosphate buffer 20 mM, pH 7.5; melittin concentration 3.5 μM; temperature 25°C. +——+, dilauryl phosphatidylcholine; ○——○, dimyristoyl phosphatidylcholine; X——X, dipalmitoyl phosphatidylcholine. Melittin concentration 35 μM; temperature 25°C. ●——●, dimyristoyl phosphatidylcholine. Melittin concentration 3.5 μM; temperature 44°C. ■——■, dipalmitoyl phosphatidylcholine.

Binding of melittin to phosphatidylserine. Binding of melittin to phosphatidylserine also leads to a blue shift of the emission spectrum, but in this case, the fluorescence intensity at 352 nm decreases by 70% and the isoemissive point is now observed at 320 nm (Fig. 3). Moreover, as in the previous case, the binding curves are independent of the melittin concentration, at least between 3.4 and 25 μM, and we checked that an increase in ionic strength up to 1 M NaCl has no effect on the fluorescence spectrum of the complex. On the contrary, pH variations are followed by important effects: the higher the pH value, the steeper the binding curves (Fig. 3b and c).

The drastic decrease in intensity observed in this case seems to be inconsistent with results previously obtained with phosphatidylcholines, since one could expect that the environment of the Trp residue is not very different from one lipid bilayer to another. In fact, it seems that the observed decrease cannot be related to an effect of the environment, but is rather due to a quenching of fluorescence by some oxidation products contained in the phosphatidylserine used. This is supported by recent experiments performed with a phosphatidylserine free of oxidation products available from Lipids Products (Nutfield, U.K.). On binding with melittin, we then observed the expected increase in intensity at 333 nm, paralleled by blue shifts similar to those shown in Fig. 3.

Binding of melittin to phosphatidylcholine vesicles charged with dicetylphosphate or stearylamine. Addition of dicetylphosphate or stearylamine to zwitterionic dimyristoyl phosphatidylcholine produces lipid vesicles bearing variable negative or positive electrical charges. This leads to the striking effects on the binding curves shown in Fig. 4. An increase of the net negative charge is followed by an apparent increase of the affinity of melittin for the lipid bilayer.

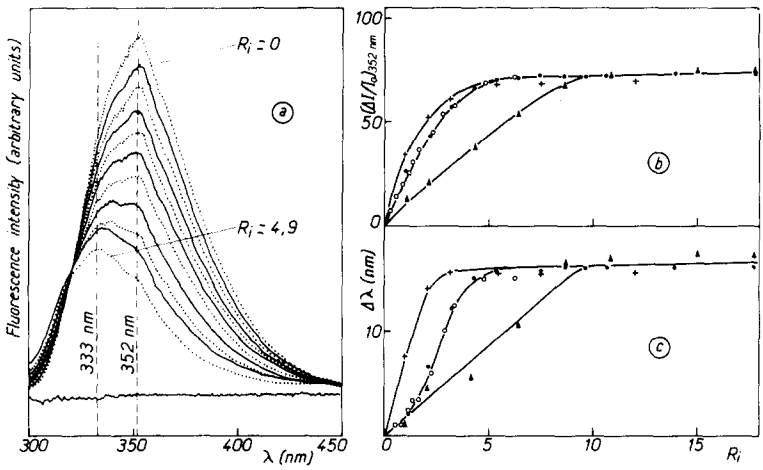

Fig. 3. Binding of melittin to phosphatidylserine. (a) Effect of addition of phosphatidylserine vesicles on the emission spectrum of melittin at pH 8.0 for protein concentration 25 μM; the successive lipid to protein molar ratio being R_i = 0, 0.3, 0.6, 0.9, 1.2, 1.4, 1.7, 2.3, 2.9, 3.4, 4.9. (b) Relative decrease of the fluorescence intensity at 352 nm versus R_i at various pH values. (c) Apparent wavelength shift. Experimental conditions: melittin concentration 3.4 μM: ▲——▲, pH 3.1; ●——●, pH 8.0; +——+, pH 10.1. Melittin concentration 25 μM: ○——○, pH 8.0. pH values were adjusted by addition of NaOH or HCl solutions to a 20 mM phosphate buffer.

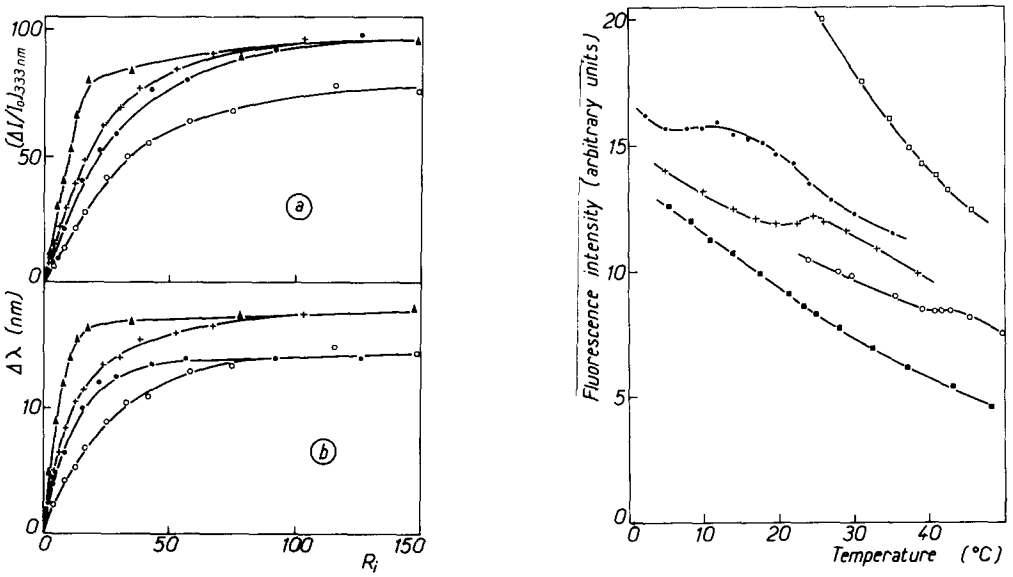

Fig. 4. Binding of melittin to dimyristoyl phosphatidylcholine vesicles charged with stearylamine or dicetylphosphate. (a) Relative increase of fluorescence intensity at 333 nm versus the lipid to protein molar ratio R_i. (b) Apparent wavelength shift. Binding of melittin (20 mM phosphate buffer pH 7.5 at 25° C [melittin] = 5 μM) with: ●——●, pure dimyristoyl phosphatidylcholine; ○——○, dimyristoyl phosphatidylcholine + 15% (molar fraction) stearylamine; +——+, dimyristoyl phospatidylcholine + 5% dicetylphosphate; ▲——▲, + 15% dicetylphosphate.

Fig. 5. Effect of temperature on the fluorescence intensity of melittin measured at the wavelength of the emission maximum. ■——■, melittin alone; ●——●, melittin phosphatidylserine complex (R_i = 30); +——+, melittin dimyristoyl phosphatidylcholine complex (R_i = 100); ○——○, melittin dipalmitoyl phosphatidylcholine complex (R_i ≈ 85); □——□, fluorescence intensity of a non interacting peptide GlyTrpGly in the presence of dipalmitoyl phosphatidylcholine.

But addition of stearylamine, which is positively charged, produces the opposite effect.

Similar experiments performed with egg phosphatidylcholine vesicles charged with different amounts of dicetylphosphate or stearylamine allowed to observe the same effects. Moreover, experiments with 15% dicetylphosphate have also been done in the presence of 1 M KCl. The fluorescence spectrum of pure melittin in solution is then shifted to 349 nm, but as negatively charged vesicles are added, the final value reached is always the same, 333 nm.

Effect of temperature on the fluorescence intensity of melittin bound to various phospholipid vesicles. The changes in the fluorescence intensity of melittin either free in solution or bound to phosphatidylserine, dimyristoyl and dipalmitoyl phosphatidylcholine vesicles are plotted versus temperature in Fig. 5. In the studied temperature range, from 5 to 45°C, the fluorescence intensity of free melittin decreases monotonically. But in the case of complexes with phosphatidylserine, dimyristoyl and diplamitoyl phosphatidylcholine, an increase is observed around 10, 23 and 41°C, respectively, i.e. in the temperature ranges of the phase transitions of pure phospholipids.

The emission maxima of the fluorophore are temperature independent for all the studied systems. When lipid-protein interactions occur, the emission maxima are observed at 333 nm indicating that the protein remains totally bound, even below the transition temperature of the concerned lipid.

In order to test a possible effect of turbidity changes on the observed phenomena, experiments were carried out with the dimyristoyl phosphatidylcholine complex at three different concentrations. The same relative fluorescence changes were obtained in every cases. Furthermore, when a tripeptide such as GlyTrpGly which does not interact with phosphatidylcholines, is used instead of melittin, its fluorescence spectrum which is totally unaffected by the addition of dipalmitoyl phosphatidylcholine vesicles, is also absolutely insensitive to the phase transition of this phospholipid (Fig. 5). This clearly shows that the previous results cannot be related to turbidity changes.

Discussion

The first conclusion which arises from the above results is that intrinsic fluorescence is a very sensitive technique for studying lipid-protein interactions, as has already been shown by previous results obtained with melittin itself [6,7], and also with apolipoproteins [17—19], intrinsic membrane proteins [20,21] or amphipathic peptides [22,23]. The observed blue shifts in the melittin emission spectrum on addition of lipid vesicles, which indicate that the Trp residue is passing from a polar to a non-polar environment, clearly show that melittin displays hydrophobic interactions with all the phospholipids used, whatever the nature of the medium (ionic strength or pH).

The existence of isoemissive points indicates that, in some instances, free and bound proteins have well defined fluorescent characteristics. Assuming that binding can be described as a simple equilibrium reaction and that binding sites are independent, this allows the use of fluorescent data to calculate an association constant (K_a), and a number of binding sites (n), where n is the number of lipid molecules per molecule of bound melittin at an infinite protein concentra-

tion. However, as it has been mentioned, binding curves are independent of the melittin concentration in the micromolar range in the case of synthetic phosphatidylcholines and phosphatidylserine (Figs. 2 and 3). According to the classical scheme, this leads to the conclusion that the association constant is very large ($>10^6$ M^{-1}), and cannot be determined from fluorometric titration experiments. In these conditions, the R_i value for which the plateau is reached is a direct estimation of the number of binding sites n. It is found to be about 25 for synthetic phosphatidylcholine, and about 3 or 4 for phosphatidylserine, at physiological pH. However, it should be pointed out that such a simple interpretation implying well defined binding sites could be totally inadequate to describe lipid-protein interactions. Furthermore, binding experiments being performed by adding melittin to preformed phospholipid single shell vesicles, results in binding sites located on the inner face of vesicles being inaccessible to the protein; this could lead to inaccurate n values. However, the important increase of the turbidity of samples which is observed during binding experiments indicates that melittin induces a general breakdown of the vesicular structure, in agreement with the previous results of Sessa et al. ref. 5, and with the lytic properties of melittin. So, melittin is probably able to bind on both sides of the lipid bilayer.

The large difference between the numbers of lipid molecules bound per molecule of melittin at saturation in the case of phosphatidylcholines and phosphatidylserine strongly suggests that the binding of melittin to phospholipids is charge dependent. Two further results tend to confirm this conclusion. First, as can be seen in Fig. 4, an increase of the net negative charge of phosphatidylcholine vesicles by addition of dicetylphosphate seems to enhance the affinity of melittin for vesicles. It is then useful to point out that a very low level of negatively charged impurities can notably enhance the binding and so be a source of artefacts. Furthermore, a similar effect is observed in the case of phosphatidylserine vesicles when the pH value if raised from 3.1 to 10.1, which again results in an increase of the net negative charge of vesicles (Fig. 3b and c). These results can be explained very simply by the fact that melittin, which is a basic polypeptide, is positively charged. It follows that electrostatic attraction promotes the interactions of melittin with negatively charged vesicles. The importance of this kind of interaction on the formation of lipid-protein complexes has already been shown in the case of a partially integrated membrane protein like cytochrome b_5 [20,24].

On the contrary, the length of the aliphatic chains of phospholipids does not seem to be a critical parameter for the interaction of melittin with liposomes. The observed binding curves are indeed quite similar for dilauryl, dimyristoyl and dipalmitoyl phosphatidylcholines (Fig. 2b and c). Only the fluidity of the lipid bilayer has to be considered, the binding of melittin to dipalmitoyl phosphatidylcholine being weaker when the experiment is done below 41°C, i.e. below the transition temperature of this phospholipid (Fig. 2b and c). It should be noticed that this result is in good agreement with those previously obtained on binding of apolipoprotein-alanine [18] or cytochrome d-b_5 [24] to synthetic phosphatidylcholines. This apparent decrease in the binding of melittin below the transition temperature can be explained (i) by a weaker affinity of melittin when lipids are in a crystalline state or, more probably, (ii)

by a drastic decrease of the binding rate as it has already been proposed by Mollay and Kreil [6] and Pownall et al. [18].

The detection by the protein fluorophore of the melting phenomena occurring in the surrounding lipids is again a proof that Trp is embedded into the hydrophobic core of the bilayer. It has already been shown that intrinsic fluorescence of two integral proteins, namely cytochrome b_5 [20] and C_{55}-isoprenoid alcohol phosphokinase [21], is sensitive to lipid phase transitions. Another conclusion which can be drawn is that the transition temperatures are not significantly affected by the presence of bound melittin, at least for the lipid to protein molar ratio we used. This is in agreement with the calorimetric data obtained by Mollay [8], and with the results of Papahadjopoulos et al. [2], who showed that the transition temperatures of phospholipids are not modified when mainly hydrophobic interactions are involved in the formation of the lipid-protein complex. However, in order to ascertain the lack of effect of melittin on the transition temperatures, more extensive studies are currently developed by looking the phospholipid behaviour when the amount of bound protein is increased up to saturation values.

The results presented agree fairly well with what is actually known about interactions between amphipathic proteins and lipid bilayers. However, there are some apparent discrepancies between our results and those obtained by Mollay and Kreil [6] and Sessa et al. [5], and it is now necessary to discuss them in more detail.

Concerning the work of Mollay and Kreil [6], several points have to be considered; (i) we never observed the kinetic effects mentioned by these authors on binding of melittin to phospholipids; (ii) they obtained with lysolecithin a blue shift of only 10 nm of the emission spectrum of melittin, from 350 to 340 nm; (iii) they detected absolutely no binding of melittin to distearoyl phosphatidylcholine below the transition temperature. These two last discrepancies can be simply explained by the fact that Mollay and Kreil used for their experiments a very small lipid to protein molar ratio ($R_i \leqslant 5$), whereas a complete binding of melittin requires an R_i of about 10 in the case of lysolecithin, and larger than 100 for dipalmitoyl phosphatidylcholine below the transition temperature. In this latter case, an R_i ratio equal to 5 leads indeed to a shift practically undetectable, smaller than 1 nm (see Fig. 2c).

On the other hand, it should be noticed that Mollay and Kreil performed binding experiments by addition of an ethanol solution of phospholipids to an aqueous solution of melittin. The slow kinetics they observed could then be due to a different state of phospholipids, which were not arranged in single shell vesicles.

The results we presented here seem to contradict the conclusions of Sessa et al. [5] that the charge of liposomes has no effect neither on the ability of melittin to induce leakiness in liposomes, nor on its binding to monomolecular lipid films. In fact, leakage experiments have been performed by Sessa et al. using very large lipid to protein molar ratio, ranging from 10^5 to 10^3; with such R_i values, it is evident from our results that melittin is totally bound, whatever the liposomes net charge. So, it is not surprising that, in these conditions, the same lytic effects were observed either for positively or negatively charged liposomes. Furthermore, in agreement with this interpretation, and with our results,

is the fact, also described by Sessa et al., that liposomes with net negative charges competed approximately 10-times more efficiently for the lytic activity of melittin on erythrocyte than did liposomes with positive charges.

In summary, we want to emphasize the necessity of full range titration experiments to investigate lipid-protein interactions. In addition to the concentration of reactants, the lipid to protein molar ratio R_i is a very important parameter, and it has to be varied from very small, here less than one, to very high values. This allowed us to detect the charge effects and to show the important difference on the maximum "stoichiometry" for the different classes of phospholipids used. Finally, taking into account the R_i values allowed an unique understanding of the various experiments carried out until now by different authors.

In conclusion, it seems that a simple two-step mechanism can be proposed for the binding of melittin to phospholipid bilayers. The first step could be a direct electrostatic interaction between basic residues of melittin and the phosphate and/or the carboxyl groups of the polar head of lipids. This would lead to a close proximity between hydrophobic residues and aliphatic chains which could then interact, depending on the fluidity of the bilayer. Afterwards, hydrophobic interaction would prevail, and prevent the release of the peptide from the membrane by an increase in ionic strength. Further studies are still necessary to ascertain this scheme, but it seems that such a mechanism can probably account for interactions between phospholipids and most of the amphipathic proteins.

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