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ARTICLE

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Melittin-induced leakage from phosphatidylcholine vesicles is modulated by cholesterol: a property used for membrane targeting

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Abstract Melittin, an amphiphathic peptide, affects the permeability of vesicles. This can be demonstrated using the dye release technique. Calcein, a fluorescent marker, is trapped in large unilamellar 1-palmitoyl-2-oleoyl-phosphatidylcholine (POPC) vesicles and melittin-induced leakage of the dye can be monitored directly by increasing fluorescence intensity. First, we characterized the effect of increasing cholesterol content in the membrane on melittin-induced leakage and our results reveal that cholesterol inhibits the lytic activity of the peptide. Using intrinsic fluorescence of the single tryptophan of melittin and ^{2}H-NMR of headgroup deuterated phosphatidylcholine, we demonstrated that the affinity of melittin for phosphatidylcholine vesicles is reduced in the presence of cholesterol; this is associated with the tighter lipid packing of the cholesterol-containing bilayer. This reduced binding is responsible for the reduced melittin-induced leakage from cholesterol-containing membranes. The pathway of release was determined to be an all-or-none mechanism. Finally, we investigated the possibility of achieving specific membrane targeting with melittin, when vesicles of different lipid composition are simultaneously present. Melittin incubated together with vesicles made of pure POPC and POPC containing 30(mol)% cholesterol can empty nearly all the cholesterol-free vesicles while the cholesterol-containing vesicles remain almost intact. Owing to the preferential interaction of melittin with the pure POPC vesicles, we were able to achieve controlled release of encapsulated material from a specific vesicle population.

Key words Melittin · Permeability · Cholesterol · Lipid-protein interaction · Fluorescence · NMR

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Abbreviations chol cholesterol · POPC 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphatidylcholine · PC phosphatidylcholine · $\Delta\nu_q$ quadrupolar splitting · DPPC 1,2-dipalmitoyl-sn-glycero-3-phosphatidylcholine · HEPES 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid · EDTA ethylenediaminetetraacetic acid · LUV large unilamellar vesicle · Ri incubation lipid/melittin molar ratio

Introduction

Membrane permeability can be affected by different classes of molecules such as surfactants, small peptides and proteins. Recent results have highlighted an interesting feature: the membrane-perturbing ability of these molecules often depends on the composition of the lipid matrix. For example, magainin 1, an antimicrobial peptide, induces leakage only from acidic lipid vesicles (Matsuzaki et al. 1989). The activity of nisin, a lantibiotic, has been found to be inhibited by negatively charged lipids (Garcera et al. 1993). Furthermore, the perturbations induced by some membrane-disrupting surfactants (Nagawa and Regen 1991) and mastoparan (Katsu et al. 1990) were shown to be dependent on the cholesterol content of the membranes.

However, the question of whether such selectivity against lipid composition could be used to target a specific vesicle population when membranes of different compositions are simultaneously present has not yet been addressed. In the present paper, we examined the possibility of achieving membrane targeting leading to controlled release of encapsulated material from specific vesicles using the melittin/lipid model system. Melittin, a cytotoxic agent from the European honey bee Apis mellifera, is a popular model peptide for probing lipid-peptide interactions (for a review, see Dempsey 1990). Melittin, constituted of 26 amino acids with 5–6 positive charges, increases the permeability of biological and model membranes (Dawson et al. 1978; DeGrado et al. 1982; Tosteson et al. 1985; Schwarz et al. 1992; Ohki et al. 1994; Ben-

achir and Lafleur 1995). The amphipathic α-helical structure in the membrane-bound state is well defined (Drake and Hider 1979; Lauterwein et al. 1979; Lavialle et al. 1982). Melittin is a relevant model because it has similarities, with regard to structure and membrane-damaging activity, to antibiotics such as alamethicin and magainin (Portlock et al. 1990; Cornut et al. 1993). Furthermore, the structural similarity of melittin to signal peptides which direct proteins to mitochondria (Schatz 1987) makes it an interesting model in the study of membrane targeting. The ability of melittin to induce membrane damage can be controlled by lipid composition. Vesicle leakage is inhibited by negatively charged lipids (Ohki et al. 1994; Benachir and Lafleur 1995). Micellization caused by the peptide is inhibited by increasing the cholesterol content of a bilayer (Monette et al. 1993; Pott and Dufourc 1995). Therefore, melittin acts as a membrane-disrupting agent which is sensitive to bilayer composition, a prerequisite for examining membrane targeting. In the present study, the parameter used to modulate the lytic activity was the cholesterol content of the vesicles.

Cholesterol (chol[1]), a major component of several biological membranes, affects the physical properties of biological and model membranes (Yeagle 1985). First, cholesterol increases the order of the acyl chains in fluid membranes and, as a consequence, leads to a tighter acyl chain packing, a thicker bilayer and a reduced lipid surface area (Stockton and Smith 1976; Dufourc et al. 1984; Nezil and Bloom 1992). Cholesterol also influences the mechanical properties, leading to an increased resistance against isotropic area dilation and rupture of the bilayer (Needham and Nunn 1990). In addition, cholesterol reduces the permeability of fluid phospholipid bilayers to small molecules (Demel et al. 1972; Bittman et al. 1984).

First, we examined whether the power of melittin to induce permeability changes in large unilamellar phosphatidylcholine vesicles was dependent on cholesterol content. We also investigated if the pathway of melittin-induced leakage was modified by the presence of cholesterol in the membrane. Afterwards we explored the possibility that melittin is capable of discriminating between vesicles with different lipid compositions. In other words, we examined the possibility of targeting and causing leakage in vesicles of a given composition in the presence of other lipidic vesicles; as a consequence, there would be the specific release of the material trapped in the targeted vesicles. The experimental technique made use of the self quenching properties of calcein, a fluorescent marker, trapped in large unilamellar 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphatidylcholine (POPC) vesicles. Its leakage can be monitored directly by increasing fluorescence intensity (Allen 1984).

Because the increase of chain compactness of cholesterol-containing fluid bilayers could also influence the affinity of the peptide for the membrane, we characterized the binding of melittin using two different techniques. First, the intrinsic fluorescence of the single tryptophan of melittin is affected by the polarity of the environment. The different polarity of the aqueous medium and the bilayer induces a shift in the tryptophan maximum emission from 354 to 338 nm upon binding (Dufourcq and Faucon 1977). This shift was used to quantify melittin binding to the lipid bilayers. Second, head group deuterated phosphatidylcholine (PC) was used to monitor the binding of melittin at the bilayer interface in the presence of cholesterol. It has been shown that the quadrupolar splitting ($\Delta\nu_q$) of PC deuterated on the α position of the choline group ([α-^{2}H]-PC) is a sensitive probe of the charge density of the membrane interface (Altenbach and Seelig 1984; Macdonald and Seelig 1987; Roux et al. 1989). It is therefore possible to monitor the binding of charged species at the membrane interface by looking at variations of $\Delta\nu_q$ of such lipids; this approach has been used to examine the binding of ions (Akutsu and Seelig 1981; Altenbach and Seelig 1984 and 1985; Seelig et al. 1987) and melittin (Dempsey and Watts 1987; Dempsey et al. 1989; Kuchinka and Seelig 1989).

Materials and methods

Materials

POPC and 1,2 dipalmitoyl-sn-glycero-3-phosphatidylcholine (DPPC) were purchased from Avanti Polar Lipids (Birmingham, AL, USA). Cholesterol and 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) were obtained from Sigma (St. Louis, MO, USA). Melittin was purified from bee venom (Sigma) by ion exchange chromatography on SP-Sephadex C-25, and desalted according to the high-performance liquid chromatography procedure described by Lafleur et al. (1987). Calcein (2,4-bis-[N,N′-di(carboxymethyl)aminomethyl]fluorescein) was purchased from Molecular Probes, Inc. (Eugene, OR, USA) and used without further purification. Ethylenediaminetetraacetic acid (EDTA) was bought from Aldrich (Milwaukee, WI, USA). [α-^{2}H]-DPPC was a generous gift from Dr. Michel Roux.

Preparation of LUV's

Lipids were dissolved in benzene and lipid mixtures were obtained by mixing appropriate volumes of the stock solutions. They were lyophilized from benzene and then hydrated with a dye-containing buffer (80 mM calcein, 100 mM HEPES, 5 mM EDTA, 30 mM NaCl, adjusted to pH = 7.4 with 5 M NaOH) to give a liposomal suspension of approximately 10 mM. The lipid suspension was freeze-thawed five times from liquid nitrogen to room temperature followed by extruding the dispersion 10 times through two stacked polycarbonate filters of 100 nm pore size (Nuclepore, Pleasanton, CA, USA) using a LiposoFast low pressure extruder from Avestin (Ottawa, ON, Canada) to obtain large unilamellar vesicles (LUV's). The calcein-containing vesicles were separated from free calcein by exclusion chromatography using a column filled with Sephadex G-50 fine gel swollen in an isosmotic buffer

(100 mM HEPES, 5 mM EDTA, 150 mM NaCl, adjusted to pH = 7.4 with 5 M NaOH). Both dye-containing and isosmotic buffers had an osmolality of 400 mOsm/kg. It should be noted that the osmotic contribution of the fluorophore must be counterbalanced by some outside solute when using the dye release technique. Previously, we have demonstrated the occurrence of potential artifacts if this procedure is neglected (Benachir and Lafleur 1996). Phospholipid concentrations were determined according to the Fiske-SubbaRow phosphorus assay (Fiske and SubbaRow 1925).

Leakage experiments

The eluted LUV dispersion was diluted into the isotonic buffer to obtain a final lipid concentration of about 10–20 μM in the cuvette. The high concentration (80 mM) of the encapsulated marker led to self-quenching of its fluorescence, resulting in low background fluorescence intensity of the vesicle dispersion (I_B). Melittin was added to the lipid dispersion leading to the release of calcein into the medium. This leakage of the dye was monitored by measuring the increasing fluorescence intensity. After a rapid release of the probe, occurring over a period of about 2 minutes, the fluorescence intensity remains almost constant; the fluorescence intensity used to calculate the release (I_F) was measured when the plateau was reached. The experiments were normalized relative to the total fluorescence intensity (I_T), measured after complete disruption of all the vesicles by Triton X-100 (0.1 vol%). The percentage of released calcein is calculated according to:

$$\% \text{ release} = 100\ (I_F - I_B)/(I_T - I_B) \quad (1)$$

A reproducibility of 5% was obtained for three independent measurements. Melittin is, under these conditions, predominantly in the monomer form because the concentration of the added melittin solution was about 1 μM (Quay and Condie 1983). Exact melittin concentration was determined at 280 nm using $\varepsilon = 5570\ M^{-1}\,cm^{-1}$.

Mechanism assays

The self-quenching efficiency of the vesicle-entrapped calcein can be used to determine whether melittin-induced leakage follows an all-or-none event from individual vesicles or a graded release from all the vesicles. The experimental details have been described elsewhere (Benachir and Lafleur 1995). Briefly, the calibration curve for fluorescence self-quenching efficiency of trapped calcein was first determined for various concentrations of trapped calcein. The self-quenching efficiency (Q) was calculated according to:

$$Q = (1 - (I_B/I_T)) \times 100 \quad (2)$$

where I_B is the background fluorescence of the trapped calcein at a given concentration and I_T the total fluorescence after addition of Triton X-100. The second step was the determination of the self-quenching efficiency of trapped calcein after incubation with melittin. LUV's were prepared with a high entrapped calcein concentration (25 mM). After removal of the free fluorophore, they were diluted at a final concentration of 50 μM into the cuvette. Different amounts of melittin were added and the releases were calculated according to Eq. (1). As soon as the fluorescence intensity reached its plateau, the mixtures were passed down a Sephadex G-50 column to remove released calcein. The portion containing the vesicles was collected, diluted and its fluorescence measured before and after addition of Triton-X 100. The self-quenching efficiency was calculated according to Eq. (2).

Binding studies

a) Fluorescence. Intrinsic fluorescence of the single tryptophan of melittin was used to quantify the binding of melittin to lipid vesicles. Melittin was suspended at a concentration of 5 μM in a 20 mM HEPES, 5 mM EDTA, 100 mM NaCl buffer, pH = 7.4 adjusted with 5 M NaOH. LUV's of POPC at 10 mM and of POPC/30 (mol)% chol at 20 mM were prepared in the same buffer. Lipid aliquots were added stepwise to the melittin solution. An emission spectrum with an excitation wavelength of 280 nm was recorded after each addition of lipid. Blanks without melittin were also recorded and subtracted from the emission spectrum to eliminate the Raman band of water and diffusion effects. The emission wavelength was measured at the middle point at half height of the band in the emission spectrum. The percentage of bound melittin was obtained by normalisation relative to the maximum shift observed from free melittin in solution (354 nm) to completely bound melittin (338 nm).

b) NMR. A mixture of 10 mg [α-^{2}H]-DPPC and 20 mg DPPC was colyophilized from a benzene:MeOH (95:05) solution. DPPC/chol samples were also colyophilized from benzene prior to hydration. About 30 mg of dried lipid were hydrated in 1 ml buffer (10 mM HEPES, 5 mM EDTA, 100 mM NaCl at pH = 7.4). The sample was then heated and vortexed. Melittin was added to the hydrated multilamellar dispersion to obtain the desired lipid-to-protein molar ratio (Ri). The sample was vortexed, frozen and thawed prior to data acquisition. ^{2}H-NMR spectra were acquired on a Bruker AM-300 spectrometer with a home-built probe equipped with a 10-mm solenoid coil. The quadrupolar echo sequence was used with a 90 ° pulse varying from 4.1 to 5.7 μs and a interpulse delay of 50 μs. After the second pulse, 8192 points were acquired with a dwell time of 8 μs. A recycling delay of 0.2 s was used and the number of scans was typically 45 000.

Fluorescence experiments

Fluorescence measurements were performed on a SPEX Fluorolog-2 spectrometer. The fluorescence intensity of

calcein was monitored using an excitation wavelength of 490 nm, an emission wavelength of 513 nm and a response time of 0.5 s. The excitation and emission bandpath widths were set at 2.2 and 1.9 nm, respectively. The excitation wavelength of melittin was 280 nm. The spectrometer was equipped for sample stirring.

Results

Cholesterol affects melittin-induced release

Melittin induces permeability changes of lipid bilayers as assessed by following the release of calcein trapped in LUV's. The percentage of released calcein was calculated according to Eq. (1). In the case of POPC vesicles, increasing the amount of melittin induces an enhanced calcein release up to an Ri of about 150, at this ratio all the calcein is released (Fig. 1). This is in quantitative agreement with previous results (Ohki et al. 1994; Benachir and Lafleur 1995). Since cholesterol modifies the physical properties of bilayers, we investigated whether these changes affect the capacity of melittin to induce permeability changes. Our results show that introducing different amounts of cholesterol in POPC vesicles decreases the capacity of melittin to induce the release of calcein. For example, to induce 50% release of calcein, four times more melittin is required for POPC vesicles containing 30 (mol)% cholesterol than for pure POPC vesicles. For an equivalent release, an intermediate value of 1.5 times is required for vesicles containing 15 (mol)% cholesterol. Therefore, the lytic power of melittin is inhibited by the presence of cholesterol in the phosphatidylcholine bilayers and it appears that the extent of calcein leakage is dependent on the concentration of cholesterol in the membrane, up to 30 (mol)%.

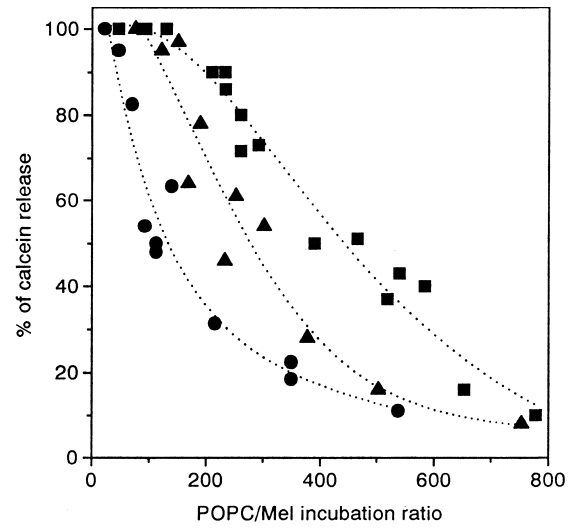

Fig. 1 Inhibition of the lytic power of melittin by cholesterol. Percentage of calcein release versus lipid/melittin molar ratio for (■) pure POPC, (▲) POPC/15 (mol)% chol and (●) POPC/30 (mol)% chol

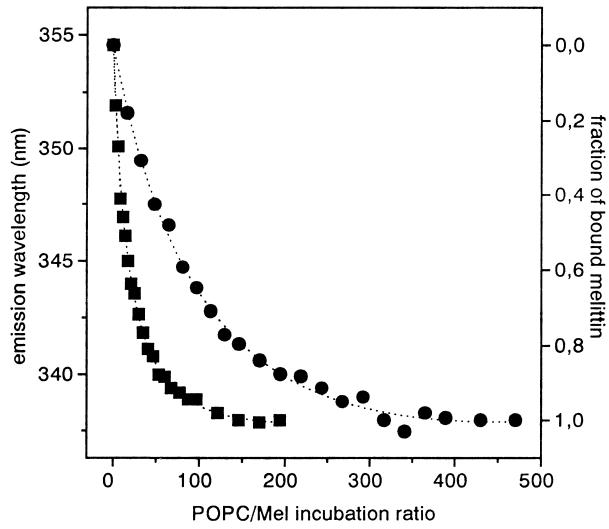

Fig. 2 Binding of melittin to lipid vesicles as measured by the shift of the fluorescence band maximum of the single tryptophan of melittin, for (■) POPC and for (●) POPC/30 (mol)% chol

Binding of melittin to lipid bilayers

In the previous section, we have demonstrated that melittin-induced release from phosphatidylcholine vesicles is inhibited in the presence of cholesterol. Considering the well-known rigidifying effect of cholesterol (Stockton and Smith 1976; Dufourc et al. 1984; Nezil and Bloom 1992), it is possible that this reduced release arose from a reduced affinity of melittin for cholesterol-containing bilayers. We investigated the binding of melittin using the intrinsic fluorescence of the single tryptophan of melittin and head group labelled PC. First, the binding of melittin can be quantified by the blue shift of the maximum emission wavelength of its single tryptophan upon binding to lipid vesicles. Figure 2 shows the shift observed when increasing amounts of lipid are added to a melittin solution. Free melittin exhibits a maximum emission wavelength at 354 nm, whereas melittin completely bound to POPC or POPC/30 (mol)% chol vesicles shows a maximum at 338 nm. As indicated by the plateau, complete binding is achieved at an Ri of 125 for the cholesterol-free vesicles and of 350 for the cholesterol-containing vesicles. Therefore, approximately three times more lipid is required to bind melittin completely in the presence of cholesterol. By assuming that the shift of the emission maximum varies linearly with the proportion of bound melittin, we can calculate the percentage of bound melittin from the normalized shift of the emission spectrum. The Ri required to bind 50% of melittin is 15 for POPC and 63 for POPC/30%(mol) chol. These results clearly suggest that the presence of cholesterol strongly reduces the affinity of melittin for the lipid vesicles. The melittin binding curve obtained with POPC

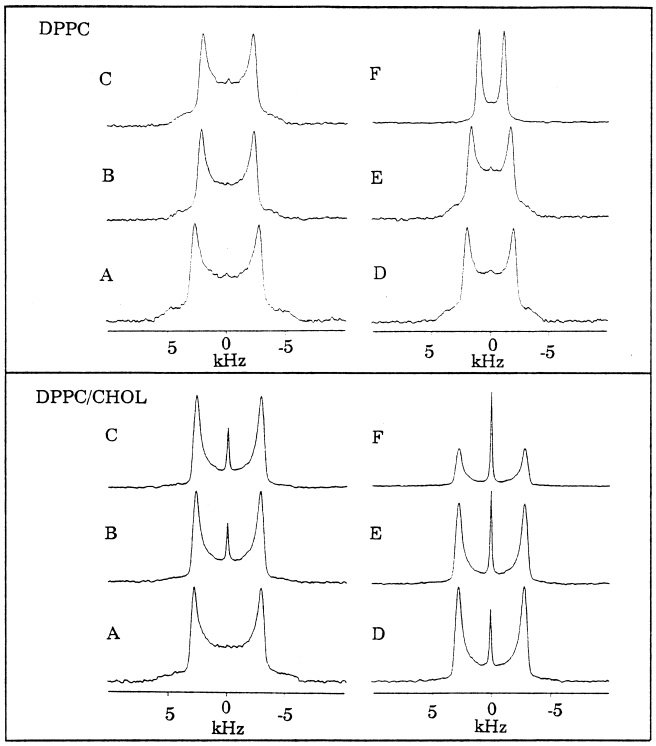

Fig. 3 Deuterium NMR spectra, at 56 °C, for (*top*) [α-^{2}H]-DPPC and for (*bottom*) DPPC-α^2H/35 (mol)% chol A) without melittin and with lipid/melittin incubation ratios of B) 100, C) 80, D) 60, E) 40 and F) 20

is in agreement with previous results obtained with a similar salt content, by circular dichroism (Beschiaschvili and Baeuerle 1991).

The binding of melittin to vesicles was also studied by ^{2}H NMR of head group deuterated PC. In Fig. 3, we present the deuterium spectra obtained from a multilamellar [α-^{2}H]-DPPC suspension, at 56 °C. At this temperature, DPPC is in the liquid-crystalline phase. The binding of melittin to this lipid should be comparable to that with POPC because it was reported that acyl chain unsaturation has no significant influence on the affinity of melittin for PC membranes (Subbarao and MacDonald 1994). The spectrum obtained from the pure phosphatidylcholine corresponds to a single powder pattern, suggesting that both α-deuterium nuclei have the same mean orientation relative to the symmetry axis of the lipid, in agreement with previous work (Brown and Seelig 1978; Akutsu and Seelig 1981). The top panel illustrates the change in the spectra of DPPC bilayers for increasing melittin proportions. There is a reduction of the quadrupolar splitting in the presence of melittin; $\Delta\nu_q$ goes from about 6 kHz for a pure DPPC bilayer to about 2 kHz for a lipid/melittin complex with an Ri of 20. These results are in agreement with those previously obtained with POPC (Kuchinka and Seelig 1989) and DMPC (Dempsey and Watts 1987; Dempsey et al. 1989). The reduction of $\Delta\nu_q$ has been associated with the binding of melittin at the interface. This effect has been attributed to the reorientation of the choline group as a reaction to the electric field created by melittin charges near the interface. This interpretation is supported by the fact that PC deuterated at the β position of the choline showed an increase in $\Delta\nu_q$ in the presence of melittin (Dempsey et al. 1989; Kunchica and Seelig 1989). The variations of the α and β splittings in opposite ways indicate that they are essentially attributed to a reorientation of the choline group of PC in the presence of melittin. A similar set of experiments was also performed on DPPC containing 35 mol(%) cholesterol (Fig. 3, bottom panel). The spectrum obtained from the dispersion without melittin is similar to that obtained with pure DPPC; the presence of cholesterol (a neutral species) does not significantly influence the quadrupolar splitting of the α deuterium on head group labelled PC (Brown and Seelig 1978). For DPPC bilayers containing cholesterol, the addition of melittin does not significantly affect the quadrupolar splitting of the α-deuterons of PC; for an Ri of 20, the change in $\Delta\nu_q$ is less than 10% relative to the pure lipid. This behavior contrasts with that observed for the cholesterol-free DPPC bilayers. Another difference is that melittin induces micellization of the DPPC/chol membrane even in the fluid phase, at 56 °C, as indicated by the rise of an isotropic component in the ^{2}H-NMR spectrum. This signal has been previously attributed to small lipid/melittin comicelles which are stabilized by the presence of cholesterol over a wide range of temperature (Monette et al. 1993; Pott and Dufourc 1995). Therefore the measured $\Delta\nu_q$ is representative only of the behavior of the phosphatidylcholine remaining in the fluid lamellar structure.

Pathway of melittin-induced dye release

The self-quenching properties of the entrapped dye can be used to distinguish two pathways for the release. First, the all-or-none pathway results in one fraction of the vesicles having released their entire dye content whereas the other fraction is still intact; this implies that the calcein concentration in the intact vesicles corresponds to the initial concentration. Second, the graded release from all the vesicles results in vesicles which have lost, on average, a fraction of their content such that the internal calcein concentration is lowered. Since the self-quenching efficiency depends on the internal calcein concentration (Fig. 4), these two pathways can be distinguished by measuring the self-quenching efficiency of entrapped calcein after incubation in the presence of melittin. The results are depicted in Fig. 4. The residual calcein concentration in POPC vesicles containing 30 (mol)% cholesterol after interaction with melittin remains fairly constant, even for percentages of release as high as 80%. This indicates an all-or-none mechanism for the release of calcein from cholesterol-containing POPC vesicles. These results are in agreement with previous results (Benachir and Lafleur 1995), showing that melittin-induced release of calcein from large unilamellar POPC vesicles follows an all-or-none pathway.

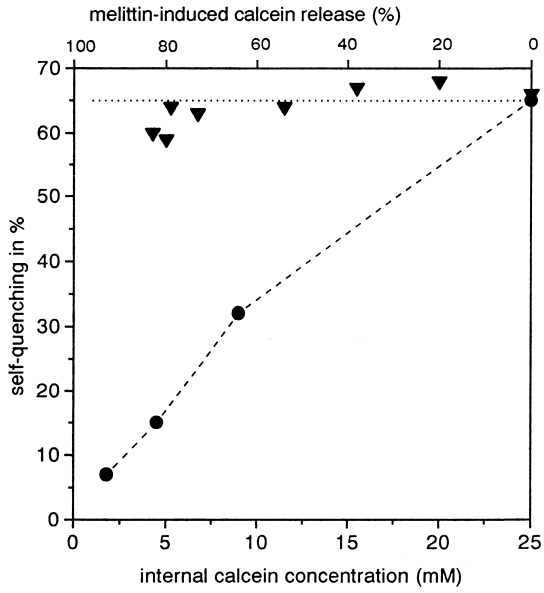

Fig. 4 (●) Self-quenching efficiency of calcein entrapped in POPC/30 (mol)% chol vesicles as a function of internal calcein concentration (lower x-axis). (▼) Self-quenching efficiency as a function of calcein release (upper x-axis) for POPC/30 (mol)% chol. (…) Expected value for an all-or-none release

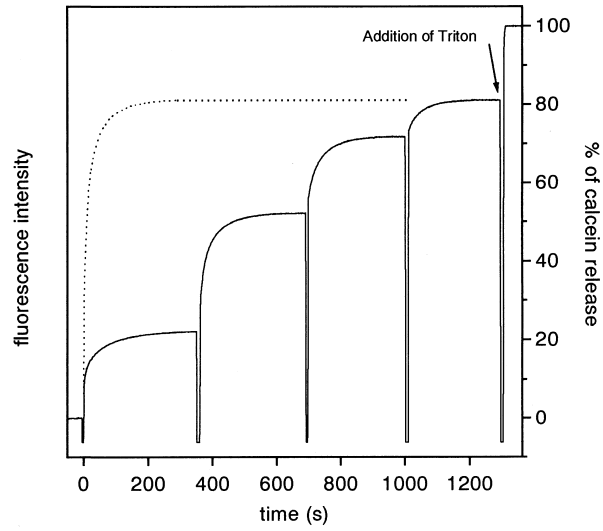

Fig. 5 Independence of calcein release on the number of melittin fractions, N. Melittin amount to get a final lipid/melittin ratio (Ri) of 75 is added in 4 fractions to POPC/30 (mol)% chol vesicles. The *dotted line* corresponds to a single addition leading to the same Ri

Melittin distribution between intact and lysed vesicles

Previously, we have reported that, in the case of POPC vesicles, melittin shows a greater affinity for the lysed vesicles compared to the intact ones (Benachir and Lafleur 1995). In the present paper, we investigated whether cholesterol affects this recognition of the emptied vesicle population by melittin. To address this question, a given amount of melittin was added to lipid vesicles in a different number of aliquots. If melittin distributes equally between the empty and the intact vesicles, the percentage of calcein release should be dependent only on the total amount of melittin added. On the other hand, if melittin has a specific affinity for one of the populations, the percentage of calcein release would change with the number of fractions, N, because, in this case, melittin distribution on the vesicles would not be random. The results of such an experiment are presented in Fig. 5. Melittin was added to the vesicles to get a final Ri of 75. When melittin was added in a single aliquot, a calcein release of 81% was observed. Adding the same amount of melittin but divided into four fractions led to the same percentage of calcein release from the vesicles. The percentage of calcein release after the addition of the first fraction of melittin (corresponding to an Ri of 300) was 22%. The second (cumulative Ri of 150) and the third (cumulative Ri of 100) fractions led to 52% and 72% release, respectively. Comparison with the standard curve for POPC/30 (mol)% chol corresponding to single additions (Fig. 1) indicates that the release is independent on the number of aliquots. These results suggest a random distribution of the toxin on the vesicles or, in other words, that melittin cannot distinguish between lysed and intact vesicles.

Targeting of phospholipid vesicles by melittin

In the first section, we showed that the lytic power of melittin is inhibited by the presence of cholesterol in POPC vesicles. This observation raised the question of whether melittin can actually distinguish cholesterol-free bilayers from cholesterol-containing bilayers. Therefore we examined melittin-induced release when POPC vesicles with and without cholesterol were simultaneously present, in an equimolar ratio. To assess the release from the cholesterol-containing vesicles, they were filled with calcein, whereas the vesicles without cholesterol were calcein-free. Different amounts of melittin were added to the samples containing both populations and the percentage of calcein release was measured. Then the same experiment was performed the opposite way. Calcein was trapped in the cholesterol-free vesicles, whereas the vesicles containing cholesterol were calcein-free. The results are depicted in Fig. 6. Two dose-response curves are presented: one was obtained for the melittin-induced release from POPC vesicles in the presence of dye-free POPC/30 (mol)% chol vesicles whereas the second one reflects the melittin-induced release from POPC/30 (mol)% chol vesicles in the presence of dye-free pure POPC vesicles. First, a comparison between these two curves reveals that the extent of leakage is much more pronounced from the cholesterol-free vesicles than from the cholesterol-containing ones. Actually, for an Ri of 200, nearly all cholesterol-free vesicles have released their content whereas the cholesterol-con-

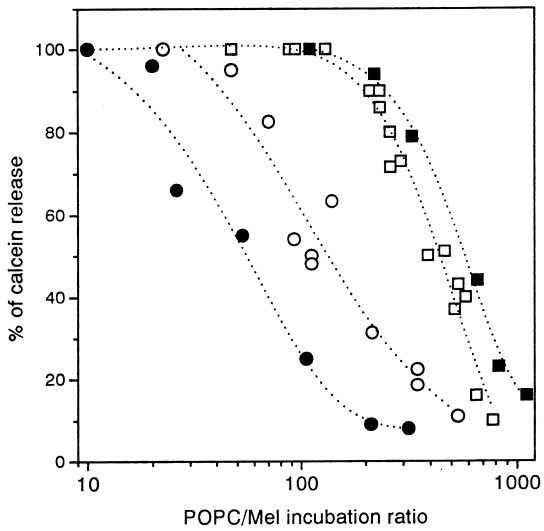

Fig. 6 Melittin-induced release from coexisting POPC and POPC/30 (mol)% chol vesicle populations, in a 1:1 ratio: (■) release from the POPC vesicles, (●) release from the POPC/30 (mol)% chol vesicles. For purpose of comparison, the calcein release induced by melittin from a single vesicle population is reproduced: (❐) from pure POPC vesicles, and (○) from POPC/30 (mol)% chol vesicles

taining vesicles have maintained their integrity. Therefore it is possible to induce, using melittin, the release of trapped material from a specific vesicle population by selecting the appropriate lipid composition of the vesicular containers. In addition,the release curves obtained when two different vesicle populations were simultaneously present can be compared to the curves describing the release from a single population (POPC or POPC/30 (mol)% chol). First, the release curve of the cholesterol-containing population in the presence of cholesterol-free vesicles is shifted towards lower Ri's compared to the single population of POPC/30 (mol)% chol. For example, significant release (≥10%) from the cholesterol-containing vesicles is observed only if the Ri is smaller than 150 when both populations are present, whereas half the vesicles are lysed at the same Ri when there are only cholesterol-containing vesicles. Likewise, at Ri = 50, only 50% of the vesicles are lysed when both types of vesicles are mixed whereas nearly all the vesicles are lysed if only the cholesterol-containing population is present. Therefore the extent of melittin-induced release observed for the cholesterol-containing vesicles is reduced in the presence of cholesterol-free vesicles. Comparing the release curve of the pure POPC population in the presence and the absence of cholesterol-containing vesicles indicates a promotion of the lysis, shifting the release curve towards higher Ri's. One should note that, because the leakage measurements were completed a few minutes after the populations had been mixed, cholesterol exchange between the cholesterol-containing vesicles and the pure POPC vesicles should be insignificant (less than 5%) under our conditions (McLean and Phillips 1984).

Discussion

Our goal was to investigate the possibility of membrane targeting using melittin. The prerequisite for distinguishing between two different membrane populations is a distinct affinity of melittin for each of them. As we have shown, this is achieved by incorporating cholesterol into a pure POPC bilayer. First, we characterized the ability of melittin to induce leakage from pure POPC vesicles and compared this to vesicles containing 15 and 30 (mol)% cholesterol. The presence of cholesterol inhibits melittin-induced leakage from POPC vesicles, and the extent of the inhibition appears to be dependent on cholesterol concentration, up to 30 (mol)%. A similar inhibition has been reported for melittin-induced micellization of multilamellar DPPC bilayers by melittin (Monette et al. 1993; Pott and Dufourc 1995): the disruption of gel-phase bilayers by melittin was inhibited by high concentrations of cholesterol (≥30 (mol)%). It was proposed that the tight lipid packing restricts the penetration of melittin into the bilayer. A similar rationale has been proposed as the origin of the reduced activity of some membrane-perturbing agents in the presence of cholesterol. Mastoparan, a toxin with a strong structural resemblance to melittin, also exhibits a decrease in its power to induce permeability changes in the presence of cholesterol (Katsu et al. 1990). It was associated with the hypothesis that mastoparan cannot intrude efficiently into cholesterol-containing membranes. A similar inhibition of detergent-induced leakage was also obtained when cholesterol was present in the membrane (Nagawa and Regen 1991). Along the same line, a DPPC/chol mixture in the liquid-ordered phase was found to exhibit resistance to Triton X-100 solubilization (Schroeder et al. 1996). In conclusion, our results showing that melittin lytic activity is inhibited by cholesterol reinforce the idea that cholesterol-containing fluid membranes are less susceptible to membrane perturbing agents.

The inhibition of melittin-induced leakage may result from a reduced binding of the peptide to the bilayer. To get insight into the effect of cholesterol on melittin-lipid interactions, we investigated the binding of melittin to the lipid bilayer. First, a reduced affinity of melittin for cholesterol-containing POPC bilayers is concluded from the fluorescence measurements. These experiments show that the maximum shift of the tryptophan fluorescence emission maximum is observed for an Ri = 125 for pure POPC vesicles and an Ri = 350 for cholesterol-containing vesicles. This means that three times more lipid is required to reach complete binding of melittin to the bilayers in the presence of cholesterol. This difference reflects the strongly reduced affinity of melittin for the membrane containing cholesterol.

^{2}H-NMR was also used to study melittin binding to the vesicles by probing the surface charge density. The results show that the addition of melittin to pure phosphatidylcholine bilayers affects the $\Delta\nu_q$ of the [α-^{2}H]-DPPC head group (Fig. 3), as previously shown for other PCs (Dempsey and Watts 1987; Kuchinka and Seelig 1989; Dempsey

et al. 1989). The presence of the positively charged peptide leads to a change in the surface charge density reported by the head group labelled PC. The diminution of $\Delta\nu_q$ observed as a function of melittin concentration is of the same order of magnitude as that obtained for DMPC (Dempsey et al. 1989), the small difference being probably due to a different ionic strength. In the presence of 35 (mol)% cholesterol, the results are drastically different, the $\Delta\nu_q$ being much less sensitive to the presence of melittin. This reduced effect could be associated with the fact that it could be more difficult for the choline head group to reorient in a cholesterol-containing bilayer because of the tighter lipid packing; this would make the $\Delta\nu_q$ a less sensitive "electrometer". This origin can be ruled out based on two results. First, the addition of cholesterol up to 50 (mol)% has been shown to have very little effect on the orientation and conformation of the choline group in the fluid phase (Brown and Seelig 1978). It is therefore unlikely that cholesterol leads to a profound repacking of PC head groups. Second, we have verified that $\Delta\nu_q$ measured for a [α-^{2}H]-DPPC bilayer containing 35 (mol)% cholesterol is sensitive to the presence of Ca^{2+} ions, a species known to reduce the α-splitting of the deuterated PC head group (Altenbach and Seelig 1984). Ca^{2+} ions lead to a reduction in $\Delta\nu_q$ for both cholesterol-free and cholesterol-containing DPPC bilayers: in the presence of 50 mM Ca^{2+}, $\Delta\nu_q$ was 3.8 kHz for DPPC bilayers (a decrease by about 34% relative to that measured in the absence of Ca^{2+}) and 4.5 kHz for DPPC containing 35 (mol)% cholesterol (a decrease by about 24%) (data not shown). The variations of $\Delta\nu_q$ of cholesterol-containing DPPC bilayers measured in the presence of Ca^{2+} ions are not as pronounced but, nevertheless, these changes indicate clearly that the $\Delta\nu_q$ of α-deuterons of the PC head group is still sensitive to the surface charge density even in the presence of high cholesterol content. Therefore, the new results presented in this paper indicate clearly the reduced affinity of the peptide for the remaining large membranes as inferred from the limited change in surface charge density reported by the NMR probe. In conclusion, both techniques show the reduced binding of melittin to cholesterol-containing membranes. This decreased affinity is clearly a factor explaining the limited permeability damages experienced by the cholesterol-containing membranes in the presence of melittin. However, this does not rule out other possible contributions of the inhibition such as less perturbing bound melittin or the need to have more bound peptides to create leaks. Further investigations are needed to verify the existence of these potential contributions.

Melittin-induced release from cholesterol-containing vesicles was examined in more detail. First, the pathway of the leakage was investigated. We demonstrated that the release proceeds mainly by an all-or-none mechanism leading to two different vesicle populations: one having released its entire calcein content, the other one still intact. An all-or-none mechanism was also reported for melittin-induced leakage of calcein from large unilamellar POPC vesicles and from small unilamellar vesicles (Schwarz et al. 1992; Benachir and Lafleur 1995). In addition, the release of haemoglobin from erythrocytes induced by melittin was reported to follow an all-or-none pathway (Tosteson et al. 1985). The mode of action of melittin does not appear to be modified by the presence of cholesterol in the membrane. Second, melittin distribution between intact and empty vesicles was investigated. The peptide was added in a single or in multiple fractions to the lipid vesicles and the extent of release of calcein was compared. After the first addition of melittin, intact and empty vesicles coexist; melittin added subsequently could then distribute randomly between both types of could prefer one of them. For a random distribution, the percentage of release is the same when an equal amount of melittin is added in a single or in multiple aliquots. For cholesterol-containing vesicles, the same percentage of release was found when melittin was added in an single aliquot or if the same amount of melittin was added in four portions. Similar results were obtained with GALA, a synthetic amphipathic peptide, and α-haemolysin, a bacterial protein toxin (Parente et al. 1990; Ostolaza et al. 1993). This behavior contrasts with that of melittin with pure POPC vesicles. Previously, we have reported a decreasing extent of dye release with increasing number of added aliquots, for a given amount of melittin (Benachir and Lafleur 1995). This was associated with the capacity of melittin to discriminate the lysed and the intact vesicles and to target preferentially the lysed vesicles; it appears to be a particular behavior of that system.

Melittin-induced leakage from POPC vesicles with and without cholesterol share some similarities. For both cholesterol-containing and cholesterol-free POPC vesicles, melittin-induced release stops after a few minutes, having reached an extent of release which is regulated by the molar incubation ratio. Furthermore, we observe an all-or-none pathway from vesicles with or without cholesterol. The main difference associated with the presence of cholesterol is a reduced affinity of melittin for the bilayer. Using this difference, we introduce a new feature of melittin; namely its ability to discriminate between cholesterol-containing and cholesterol-free membranes and to induce specifically the release of trapped material in pure POPC vesicles. Because of the preferential interaction of melittin with pure POPC vesicles, melittin perturbs selectively the cholesterol-free vesicles. Actually, for an Ri of 200, the POPC vesicles are completely emptied whereas the cholesterol-containing vesicles remain almost intact. Furthermore, the distribution of melittin between the two coexisting vesicle populations enhances the difference in induced leakage observed between the two populations. Higher proportions of melittin are required to induce an equivalent leakage from the cholesterol-containing population in the presence of pure POPC vesicles than in their absence; to observe a 50% release from the POPC/chol vesicles, an Ri of 110 is required when there is only this type of vesicle whereas an Ri of 60 is required when the vesicles with and without cholesterol coexist. Likewise, the leakage from the POPC vesicles is greater for a given Ri in the presence of POPC/30 (mol)% chol vesicles than in their absence. The Ri needed to have a 50% release from the POPC

vesicles is shifted from 420 for POPC vesicles only to 610 for the coexisting populations. This phenomenon may be simply described by the higher affinity of melittin for pure POPC bilayers compared to that for cholesterol-containing vesicles; the difference in affinity leads to an unequal distribution of bound melittin when the two populations coexist, favouring an increased proportion of bound melittin to the POPC vesicles. We conclude that the membrane properties in the presence of cholesterol are different enough to be recognized by melittin and this phenomenon can be utilized for membrane targeting. To our knowledge, this is the first report of the targeting of coexisting vesicles by a peptide.

Conclusions

Melittin-induced dye leakage from large unilamellar phosphatidylcholine vesicles is inhibited by the presence of cholesterol. As we have shown, greater amounts of melittin are necessary to empty cholesterol-containing vesicles of their contents than pure POPC vesicles. This phenomenon is associated with the reduced binding of the peptide to the cholesterol-containing bilayer. The pathway of leakage in the presence of cholesterol follows an all-or-none mechanism. Our results also suggest that melittin added subsequently cannot distinguish between the lysed and the intact vesicles. Finally, we have demonstrated that melittin is capable of discriminating different membrane populations simultaneously present. The peptide is capable of recognizing cholesterol-containing membranes and this phenomenon can lead to specific release from the pure POPC vesicles. This specificity provides clues in understanding the behavior of similar peptides. The sensitivity of a membrane perturbing agent to cholesterol content may be involved in the antimicrobial activity of amphipathic helical antibiotics. The presence of sterol in plasmic membranes is one property distinguishing eucaryotic cells from eubacteria (Bloom et al. 1991). This difference could have an important implication in the targeting of bacteria by certain antibiotics showing analogy with melittin. Similarly, it is interesting to note that the mitochondrial membranes of mammalian cells generally have a low cholesterol content relative to most intracellular membranes (Daum 1985). These membranes are the target for proteins in the cytoplasm bearing a signal peptide which has structural similarity with melittin. The present study showing the possibility of membrane targeting by an amphipathic peptide based on lipid composition suggests that these ideas should to be examined.

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