Osmotic and pH Transmembrane Gradients Control the Lytic Power of Melittin

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ABSTRACT Transmembrane osmotic gradients applied on large unilamellar 1-palmitoyl-2-oleoyl-phosphatidylcholine vesicles were used to modulate the potency of melittin to induce leakage. Melittin, an amphipathic peptide, changes the permeability of vesicles, as studied using the release of entrapped calcein, a fluorescent marker. A promotion of the ability of melittin to induce leakage was observed when a hyposomotic gradient (i.e., internal salt concentration higher than the external one) was imposed on the vesicles. It is proposed that structural perturbations caused by the osmotic pressure loosen the compactness of the outer leaflet, which facilitates the melittin-induced change in membrane permeability. Additionally, we have shown that this phenomenon is not due to enhanced binding of melittin to the vesicles using intrinsic fluorescence of the melittin tryptophan. Furthermore, we investigated the possibility of using a transmembrane pH gradient to control the lytic activity of melittin. The potency of melittin in inducing release is known to be inhibited by increased negative surface charge density. A transmembrane pH gradient causing an asymmetric distribution of unprotonated palmitic acid in the bilayer is shown to be an efficient way to modulate the lytic activity of melittin, without changing the overall lipid composition of the membrane. We demonstrate that the protective effect of negatively charged lipids is preserved for asymmetric membranes.

INTRODUCTION

Transmembrane osmotic and pH gradients are omnipresent in biological membranes (Maeda and Thompson, 1986; Einspahr et al., 1988; Csonka, 1989; Häussinger and Lang, 1991). They present a convenient way to modulate some properties of membranes without changing their overall composition. Many efforts are devoted to understanding their roles in cellular life. For example, it has been suggested that fusion-fission processes (Akabas et al., 1984; Lucy and Ahkong, 1986) and the activity of mechanosensitive ion channels (Morris, 1990) can be regulated by osmotic forces. Furthermore, osmotic compression can induce an inhibition of electron transport activity, affecting respiration in mitochondria and photosynthesis in chloroplasts (Mathai et al., 1993). To get insight into the importance of gradients across cellular membranes, recent investigations have examined the influence of transmembrane gradients on model lipid vesicles, revealing impressive changes. First, osmotic gradients can induce macroscopic morphological perturbations, including vesiculization, lysis, and shrinkage (Boroske et al., 1981; Döbereiner et al., 1993; Lerebours et al., 1993; Mui et al., 1993). Vesicles respond to osmotic stress by changes in surface area and elasticity, as shown recently (Rutkowski et al., 1991; Ertel et al., 1993; Hallett et al., 1993; Mui et al., 1993). Moreover, the fluidity of the membrane increases with increasing osmotic pressure (Borochov and Borochov, 1979; Surewicz, 1983; Lehtonen and Kinnunen, 1994). Second, pH gradients have been shown to modulate bilayer asymmetry (Hope and Cullis, 1987; Hope et al., 1989); they can be used to load vesicles with charged species (Cullis et al., 1991) or to control fusion processes (Wilschut et al., 1992). Furthermore, their presence can modify the morphology of the vesicles (Farge and Devaux, 1992).

These alterations in membrane structure and properties should have their counterparts in alterations in lipid-protein interactions. The goal of this paper is to demonstrate the influence of transmembrane gradients on lipid/peptide interactions, using the lipid/melittin system. Melittin, a cytotoxic agent from the bee *Apis mellifera*, is a popular model peptide; it is constituted of 26 amino acids with 5–6 positive charges and increases the permeability of biological membranes, as in erythrocytes and ghosts, and model membranes (Dawson et al., 1978; DeGrado et al., 1982; Tosteson et al., 1985; Schwarz et al., 1992; Ohki et al., 1994; Benachir and Lafleur, 1995). Melittin-induced lysis of red blood cells is proposed to be due to a colloid osmotic mechanism: the release of hemoglobin follows the formation of ion channels in the bilayer, leading to osmotic swelling of the cells (Tosteson et al., 1985; Katsu et al., 1988). This model peptide is particularly interesting because its amphipathic α-helical structure in the membrane-bound state is well defined (Drake and Hider, 1979; Lauterwein et al., 1979; Lavialle et al., 1982), and it has structural features and membrane-damaging activity similar to those of several toxic agents, including the lentivirus lytic peptide LLP-1, part of HIV-1 transmembrane protein (Miller et al., 1993; Portlock et al., 1990; Yianni et al., 1986). In addition, melittin shows an analogy with some proteins of the complement system (Bashford et al., 1986; Laine et al., 1988). It is therefore important to understand how melittin interacts with membranes. In this paper, we demonstrate how the leakage induced by melittin from large unilamellar vesicles

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(LUVs) can be modulated by the presence of transmembrane osmotic and pH gradients.

Recently it has been shown that the ability of melittin to induce permeability changes and micellization can be controlled by the composition of the lipid bilayer. Membrane damages caused by the peptide are inhibited by increasing the cholesterol content of the bilayer (Monette et al., 1993; Pott and Dufourc, 1995) or by increasing the negative charge density at its interface (Dempsey et al., 1989; Monette and Lafleur, 1995; Benachir and Lafleur, 1995). At this stage, we extend our investigation by examining whether transmembrane gradients that modify the physicochemical properties of the bilayer modulate, as a consequence, the activity of melittin. First, the response of the bilayer to an osmotic gradient rises the question of whether melittin is capable of recognizing a vesicle under osmotic stress and if osmotic stress may modulate melittin-induced lysis. Second, we have examined the possibility of controlling the lytic activity of melittin by modulating negative charge density using a transmembrane pH gradient. Such a gradient induces asymmetric distribution of unprotonated fatty acids in both leaflets of the bilayer and can be used as a tool to modify the charge density of the external leaflet (Hope and Cullis, 1987). Because melittin activity is controlled by bilayer surface charge density, one can expect that manipulating the pH gradient can be an indirect way of modulating melittin-induced leakage.

From the experimental point of view, LUVs prepared by the extrusion technique were used as a very convenient membrane model. The application of an osmotic stress on the membrane is a relatively straightforward method using a NaCl transmembrane concentration gradient (Hantz et al., 1986; Ertel et al., 1993; Mui et al., 1993). The very low permeability coefficients of Na^+ and Cl^- (Mimms et al., 1981) ensure the stability of the osmotic gradient. Similarly, a transmembrane pH gradient can be easily established with LUVs (Hope and Cullis, 1987; Hope et al., 1989). Our permeability measurement made use of the self-quenching properties of calcein, a fluorescent marker (Allen, 1984), entrapped in LUVs. The addition of melittin to these LUVs causes an increased bilayer permeability and therefore induces the release of calcein monitored directly by increasing fluorescence intensity. In this paper we used the term "lysis" to describe the release of calcein from the vesicles. However, it should be pointed out that no major morphological deformations are caused by the addition of melittin because previous cryo-electron microscopy pictures have shown that, at the melittin proportions used in our study, the lipids remain in vesicular form (Dufourcq et al., 1986).

MATERIALS AND METHODS

Materials

1-Palmitoyl-2-oleoyl-phosphatidylcholine (POPC) was purchased from Avanti Polar Lipids (Birmingham, AL). Cholesterol, palmitic acid (PA), HEPES, 2-(*N*-morpholino)ethanesulfonic acid, nigericin, and valinomycin were obtained from Sigma (St. Louis, MO). 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) and used without further purification. EDTA was bought from Aldrich (Milwaukee, WI).

Preparation of vesicles

The 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 to give a liposomal suspension of approximately 10 mM. The lipid suspension was freeze-thawed five times from liquid nitrogen to room temperature and then extruded 10 times through two stacked polycarbonate filters of 100 nm pore size (Nuclepore, Pleasanton, CA) using a LiposoFast low-pressure extruder from Avestin (Ottawa, ON, Canada) to obtain LUVs.

Generation of a hyposmotic gradient

For osmotic gradient assays, the vesicles were prepared in a buffer containing 80 mM calcein, 100 mM HEPES, 5 mM EDTA, 600 mM NaCl, adjusted to pH 7.4 with NaOH (5 M). The calcein-containing vesicles were separated from the free calcein by exclusion chromatography using a column filled with Sephadex G-50 fine gel swollen in an isosmotic buffer. The isosmotic buffer contained 100 mM HEPES, 5 mM EDTA, 700 mM NaCl, adjusted to pH 7.4 with NaOH (5 M). Both dye-containing and isosmotic buffers had an osmolality of 1500 mOsm/kg. The eluted lipid suspension was diluted 10 times with the isosmotic buffer. The osmotic gradient across the vesicle bilayer was generated by diluting an aliquot of 50 μl of the vesicle suspension directly into the cuvette. This dilution ensured an instantaneous osmotic gradient across the vesicle due to the high permeability of water (Inoue et al., 1985). The osmolality of the buffer in the cuvette was adjusted to obtain the desired transmembrane osmotic gradient; this buffer was made by diluting the isosmotic buffer with an appropriate volume of hyposmotic buffer (100 mM HEPES, 5 mM EDTA, adjusted to pH 7.4 with NaOH (5 M)). The final phospholipid concentration in the cuvette was approximately 10 μM. The exact phospholipid concentration was determined according to the Fiske-SubbaRow phosphorus assay (Fiske and SubbaRow, 1925).

Generation of a hyperosmotic gradient

For hyperosmotic gradient assays, the osmolality of the vesicle interior was kept lower than the osmolality of the outside medium. The vesicles were prepared in a buffer containing 80 mM calcein, 100 mM HEPES, 5 mM EDTA, 300 mM NaCl, adjusted to pH 7.4 with NaOH (5 M). The correspondent isosmotic buffer contained 100 mM HEPES, 5 mM EDTA, 400 mM NaCl, adjusted to pH 7.4 with NaOH (5 M). Both dye-containing and isosmotic buffers had an osmolality of 900 mOsm/kg. The eluted vesicle dispersion was diluted 10 times with the isosmotic buffer. The osmotic gradient was generated by diluting an aliquot of 50 μl of the vesicle suspension directly into a cuvette containing a buffer of 1300 mOsm/kg and 1700 mOsm/kg.

Generation of a pH gradient

For pH gradient assays, the vesicles were prepared in a buffer containing 80 mM calcein, 80 mM 2-(*N*-morpholino)ethanesulfonic acid, 20 mM HEPES, 5 mM EDTA, 5 mM KCl, 20 mM NaCl, adjusted to pH 6.5 with NaOH (5 M). The correspondent isosmotic buffer used for the exclusion chromatography contained 150 mM H_2BO_3, 5 mM EDTA, 5 mM KOH, 140 mM NaCl adjusted to pH 9 with NaOH (5 M). Both dye-containing and isosmotic buffers had an osmolality of 450 mOsm/kg. Thus, a pH gradient of about 2.5 units was generated across the bilayer after the

exclusion chromatography (Hope and Cullis, 1987). The dissipation of the pH gradient was performed by using the combination of nigericin (H^+/K^+ exchanger) and valinomycin (K^+ ionophore) in a concentration of 1 $\mu g/\mu mol$ lipid. It has been shown on similar systems that, under these conditions, a rapid and effective dissipation is achieved (Hope and Cullis, 1987).

Marker release

The high concentration (80 mM) of the encapsulated calcein led to the self-quenching of its fluorescence, resulting in a low background fluorescence intensity of the vesicle dispersion (I_B). The addition of melittin to the stirred dispersion induced the release of calcein. It led to the dilution of the dye into the medium and could therefore be monitored by increasing fluorescence intensity. After a rapid release of the probe, occurring during a short time period of about 2 min, 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 was calculated according to

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

The reproducibility for a given lipid/melittin incubation ratio was ±5% for three independent measurements. Control experiments to ensure the reproducibility were described elsewhere (Benachir and Lafleur, 1995). It should be noted that, under our conditions, melittin was predominantly in the monomer form (Quay and Condie, 1983), because the concentration of the added melittin solution was on the order of 1 μM. The exact melittin concentration was determined by spectrophotometry, using $\epsilon = 5570\ M^{-1}\ cm^{-1}$ at 280 nm.

Binding

Intrinsic fluorescence of the single tryptophan of melittin is sensitive to the polarity of its environment and can therefore be used to quantify the binding of melittin to lipid vesicles (Dufourcq and Faucon, 1977). Melittin was suspended in 2 ml of buffer (100 mM HEPES, 5 mM EDTA, 700 mM NaCl, adjusted to pH 7.4 with NaOH (5 M)) at a concentration of 5 μM. Emission spectra with an excitation wavelength of 280 nm were recorded after stepwise addition of POPC vesicles (suspended in the same buffer, at a concentration of 20 mM). The change in the emission wavelength was measured at the middle point at half-height of the emission spectrum. The percentage of bound melittin was obtained by normalization relative to the maximum shift observed from free melittin in solution (351 nm in isosmotic buffer and 353 nm in hyposmotic buffer) to completely bound melittin (~338 nm for isosmotic and hyposmotic conditions).

Osmolality measurements

Solution osmolalities were determined from freezing point depressions using an Advanced DigiMatic Osmometer (Advanced Instruments). Standards of 290 and 900 mOsm/kg were analyzed before samples.

Spectroscopic method

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 1.3 and 0.5 mm, respectively. The spectrometer was equipped for sample stirring.

RESULTS

Osmotically induced release of calcein

Our goal is to demonstrate the influence of osmotic gradients across vesicles on the lytic properties of melittin. Therefore, we first had to characterize the osmotic stability of the vesicles. It has been shown that vesicles can resist a certain applied hyposmotic gradient, but spontaneous leakage is observed if the osmotic pressure becomes too strong (Ertel et al., 1993; Mui et al., 1993). We have defined, in our conditions, the magnitude of the gradient that POPC LUVs can sustain without showing spontaneous leaking. This permeability measurement as a function of transmembrane osmotic gradient was achieved by the dye release technique, as leakage of trapped calcein can be monitored directly by increasing fluorescence intensity (Fig. 1). A transmembrane hyposmotic gradient of 1200 mOsm/kg applied across POPC vesicles leads to the spontaneous release of entrapped calcein. The release occurs essentially during the first 30 s, and then the fluorescence intensity remains fairly constant. This release is drastically different from the slow and limited leakage observed under isosmotic conditions, as depicted in Fig. 1. The percentage of calcein release obtained when an osmotic stress is applied on the vesicle can be quantified using Eq. 1. In Fig. 2, we present the dependence of calcein release on the strength of the applied osmotic gradient. For a transmembrane gradient below 600 mOsm/kg, there is no significant release of entrapped calcein. However, the application of a greater gradient across the bilayer leads to the spontaneous release of a fraction of trapped calcein. The proportion of released calcein is proportional to the magnitude of the applied hyposmotic gra-

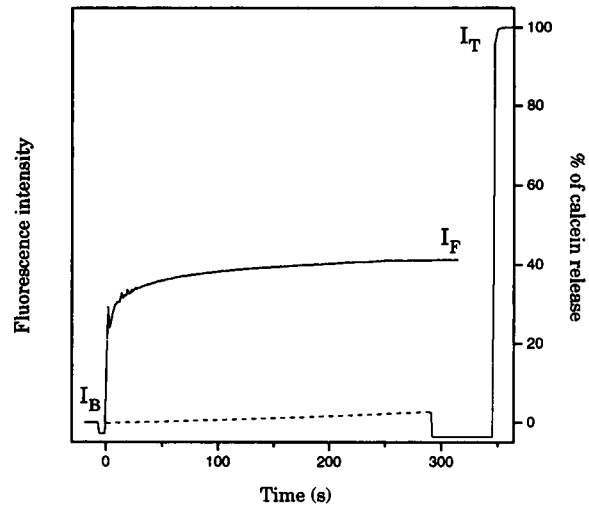

FIGURE 1 Profile of calcein release from large unilamellar POPC vesicles induced by an applied hyposmotic gradient of 1200 mOsm/kg. I_B is the background intensity of the trapped calcein, I_F is the fluorescence intensity after partial release of calcein due to the application of the gradient, and I_T is the maximum fluorescence intensity, after addition of Triton. – – –, Profile of calcein release for POPC vesicles under isosmotic conditions.

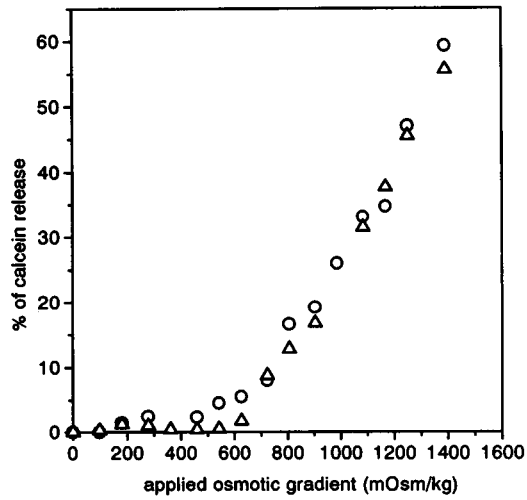

FIGURE 2 Osmotically induced release of calcein from large unilamellar vesicles of POPC (○) and POPC/Chol (△) 30(mol)%. The percentage of calcein release is calculated according to Eq. 1, where I_B is the fluorescence intensity of a sample without gradient. I_B and I_F intensities were measured after a 5-min equilibration period.

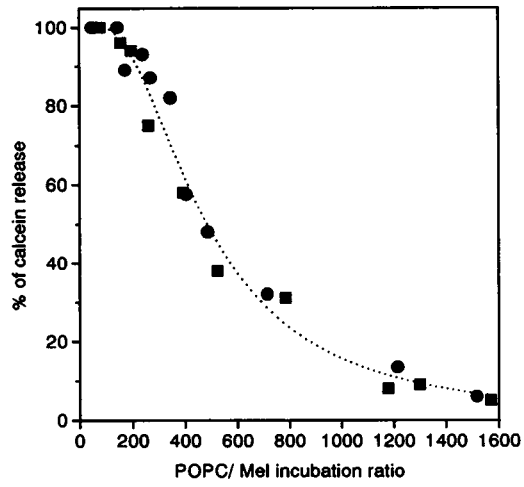

FIGURE 3 Influence of ionic strength on the lytic power of melittin. Percentage of calcein release versus lipid/melittin molar ratio for a buffer containing 700 mM NaCl (■) and 400 mM NaCl (●). The vesicles are under isosmotic conditions.

dient, in agreement with previous results (Ertel et al., 1993). It is important to note that introducing cholesterol into phosphatidylcholine bilayers does not appear to modify significantly their resistance against NaCl gradients. Because of the limited stability of LUVs against osmotic gradients, we have performed our experiments applying gradients smaller than 600 mOsm/kg to work with osmotically stressed vesicles but to avoid spontaneous release.

Influence of the external ionic strength on melittin-induced leakage

We have generated a transmembrane osmotic gradient by keeping the osmolality inside the vesicles constant and by lowering the osmolality of the outside medium. This approach has the advantage that a series of measurements could be done with the same batch of LUVs. It implies, however, that the external salt concentration changes, and the variation of the ionic strength of the medium could play a role in the interaction between melittin and phosphatidylcholine bilayers. To exclude effects due to differences in ionic strength, we have investigated whether the lytic power of melittin was dependant on salt concentration, ranging from 400 mM to 700 mM NaCl, under isosmotic conditions, i.e., equivalent internal and external osmolality. This range corresponds to that used for the experiments in the presence of gradients: lowering the external salt concentration from 700 mM to 400 mM NaCl leads to a gradient of 600 mOsm/kg, the maximum osmotic gradient that can be applied without spontaneous release, as determined in the previous section. The percentage of calcein release as a function of the lipid/melittin molar ratio is shown for the two limiting ionic strengths (Fig. 3). As expected, the percentage of calcein release increases with the amount of added melittin, and the measurements are in good quantitative agreement with previous results (Benachir and Lafleur, 1995). As indicated by the two sets of measurements, the ionic strength of the solution between 400 mM and 700 mM NaCl clearly has no influence on the permeability changes induced by melittin.

The transmembrane hyposmotic gradient

The impact of osmotic gradients on the potency of melittin in inducing permeability changes has been investigated. Transmembrane hyposmotic NaCl gradients were imposed on the vesicles, and the lytic activity of melittin on these stressed vesicles was examined (Fig. 4). The presence of osmotic pressure across the POPC vesicles results in an enhanced capacity of melittin to induce leakage, as shown by the shift of the curve toward higher lipid/Mel incubation ratios. In other words, a given amount of melittin causes a greater release from stressed vesicles than from vesicles under isosmotic conditions. For POPC vesicles containing 30(mol)% cholesterol, the impact of the osmotic gradient is even more pronounced. For example, the POPC/Mel incubation ratio (R_i) required to induce 50% of release is 150 under isosmotic conditions. Applying a transmembrane hyposmotic gradient of 500 mOsm/kg across the bilayer results in the increase of this R_i to 600; this means that four times less melittin is necessary to induce an equivalent release. As seen in Fig. 4, the enhanced release appears to be dependent on the magnitude of the transmembrane gradient. It should be noted that control experiments have shown that the calcein release measured for a given R_i was reproducible over a period of 1 h after the creation of the gradient. This result suggests that the stress induced by the osmotic perturbation and causing the enhanced melittin-induced lysis is maintained for a certain time period.

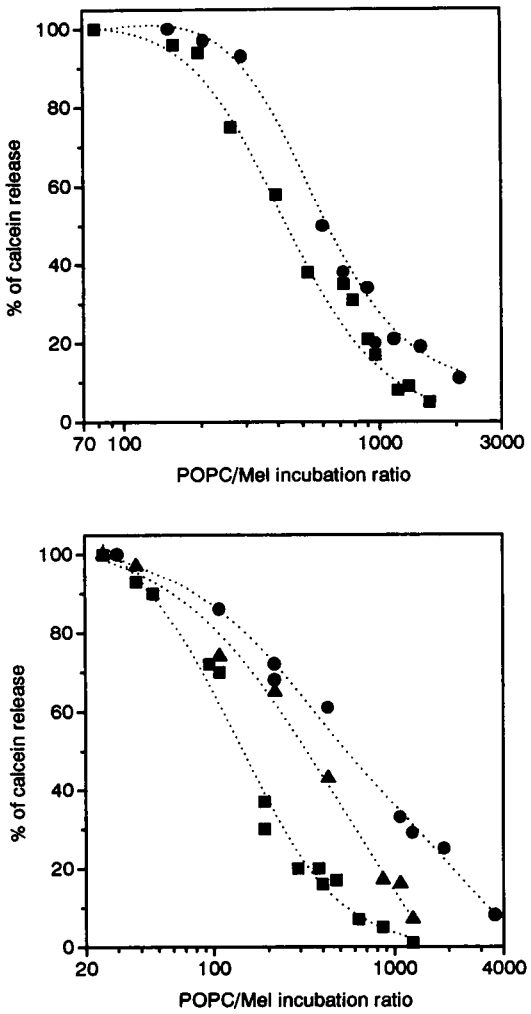

FIGURE 4 Dependence of melittin-induced release of calcein on the presence of an osmotic gradient for (*top*) pure POPC with a gradient of (■) 0 mOsm/kg (isosmotic) and (●) 500 mOsm/kg (700 mM NaCl inside, 450 mM NaCl outside) and for (*bottom*) POPC/30(mol)% Chol with a gradient of (■) 0 mOsm/kg (isosmotic), (▲) 200 mOsm/kg (700 mM NaCl inside, 600 mM NaCl outside), and (●) 500 mOsm/kg.

Because of the osmotically induced structural perturbations in the bilayer, one can assume that the origin of this promotion may be attributed to enhanced melittin binding to the vesicles. The binding of melittin can be assessed by the blueshift in the maximum emission wavelength of its single tryptophan upon binding to lipid vesicles (Dufourcq and Faucon, 1977). We quantified the binding of melittin to POPC/Chol 30(mol)% vesicles under isosmotic and hyposmotic conditions. The results are presented in Fig. 5. The addition of an aliquot of lipid leads to the binding of melittin to the vesicles. Increasing the amount of lipid increases the fraction of bound melittin up to an effective total binding as indicated by the presence of a plateau. The binding curves in isosmotic and hyposmotic conditions are very similar, indicating that the binding of melittin is not influenced by osmotic gradients.

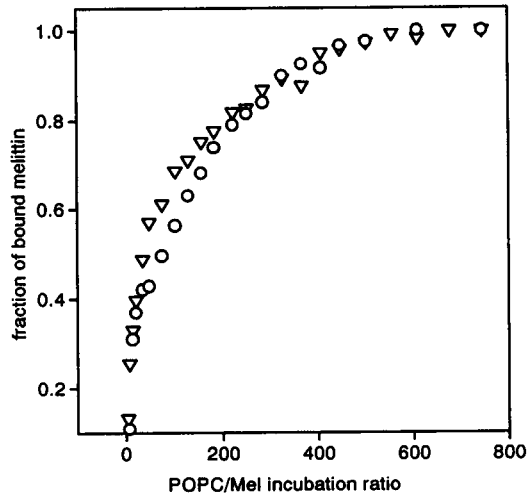

FIGURE 5 Binding of melittin to (▽) POPC/Chol 30(mol)% vesicles under isosmotic conditions and to (○) POPC/Chol 30(mol)% vesicles with a 600 mOsm/kg hyposmotic gradient. The percentage of bound melittin was calculated based on the normalized shift of the intrinsic tryptophan fluorescence (see Materials and Methods).

Influence of a hyperosmotic transmembrane gradient

In the previous section we have shown that the interaction between melittin and POPC vesicles is modulated by hyposmotic gradients. We have examined whether an inverse transmembrane gradient (i.e., a hyperosmotic gradient) would also influence the lytic power of melittin. For a hyperosmotic gradient, the salt concentration of the external medium exceeds that of the trapped solution; this can be experimentally performed by raising the outer osmolality while keeping the inner osmolality constant. In opposition to hyposmotic gradients, spontaneous release caused by hyperosmotic salt gradients is not easily achieved. Vesicles can be exposed to enormous hyperosmotic gradients without disturbing significantly their integrity. The permeability of the membrane is only slightly perturbed (spontaneous release of about 10%) by an osmotic pressure as high as that caused by a 3000 mOsm/kg gradient (data not shown). This behavior is different from the extensive leakage observed for POPC small unilamellar vesicles (SUVs) in the presence of hyperosmotic gradients (Lerebours et al., 1993); however, this difference can be due to the fact that SUVs have been shown to be under tension, even in isosmotic conditions (De Kruijff et al., 1975). In parallel with the weak effect of hyperosmotic gradients on spontaneous leakage, permeability changes induced by melittin do not appear to be sensitive to the presence of hyperosmotic gradients. The percentage of melittin-induced release has been measured for POPC vesicles in the presence of a hyperosmotic gradient of 400 and 800 mOsm/kg across the bilayer; the release curve was, within the experimental error, identical to that obtained for isosmotic conditions (Fig. 2). This behavior contrasts sharply with the enhanced released shown by vesicles in the presence of hyposmotic gradients.

Effects of negative charge density on the outer face

Recently it has been shown that the lytic power of melittin is inhibited by the presence of negative charges at the vesicle surface (Benachir and Lafleur, 1995); for example, a decreased lytic activity of melittin was observed when unprotonated palmitic acid (up to 30(mol)%) or POPG (up to 50(mol)%) was present in phosphatidylcholine bilayers. In that study, the negatively charged lipids were distributed uniformly between the two leaflets of the bilayer. Because it is straightforward procedure to create asymmetric vesicles by using a pH gradient (Hope and Cullis, 1987; Hope et al., 1989), we have examined the possibility of modulating the lytic activity of melittin by modifying the negative charge density on the surface, not by changing the overall lipid composition, but by creating an asymmetric transbilayer distribution of unprotonated palmitic acid (PA^-). Unprotonated fatty acids distribute asymmetrically in the bilayer in response to a transmembrane pH gradient, according to the Henderson-Hasselbach equilibrium (Hope and Cullis, 1987), assuming that the unprotonated fatty acid can flip rapidly across the bilayer (Kamp and Hamilton, 1992):

$$\frac{[PA^-]_{out}}{[PA^-]_{in}} = \frac{[H^+]_{in}}{[H^+]_{out}}. \quad (2)$$

This implies that the asymmetric transbilayer distribution of PA depends directly on the magnitude of the pH gradient.

In Fig. 6, we show how melittin-induced release of calcein can be modulated by altering the surface charge density of vesicles as a consequence of a pH gradient. A gradient of 2.5 pH units was applied to POPC vesicles containing 15(mol)% PA, acidic pH inside. This results in an almost quantitative distribution of PA in the outer leaflet ($[PA^-]_{out} \approx 320\ [PA^-]_{in}$ according to Eq. 2). Therefore, the negative surface charge density of the external leaflet of these vesicles corresponds approximately to that of a vesicle whose overall PA concentration is 30(mol)%. Melittin-induced release of calcein has been measured for these asymmetrical vesicles and is compared with the lytic activity of melittin observed with vesicles containing an overall PA concentration of 15 and 30(mol)%, in the absence of a pH gradient. For these latter measurements, the experiments were done using borate buffer (pH 9) inside and outside; most of the fatty acids were deprotonated (the pK_a of PA in a PC bilayer is estimated at 8.5; Schullery et al., 1981), and the external environment to which melittin is exposed is the same for both the vesicles with and without pH gradient. As seen in Fig. 6, the extent of leakage observed for the asymmetrical vesicles containing an overall PA concentration of 15(mol)% is very similar to that measured for symmetrical vesicles containing 30(mol)% PA; a significant inhibition of melittin-induced release is observed compared to the vesicles with an overall PA concentration of 15(mol)%, in the absence of the pH gradient. In addition, if the pH gradient is dissipated by the addition of ionophores, the release curve is shifted toward higher R_i and becomes similar to that obtained from vesicles containing 15(mol)% PA distributed uniformly in both leaflets. It should be noted that a pH gradient on pure POPC vesicles did not have any influence on the lytic activity of melittin (data not shown).

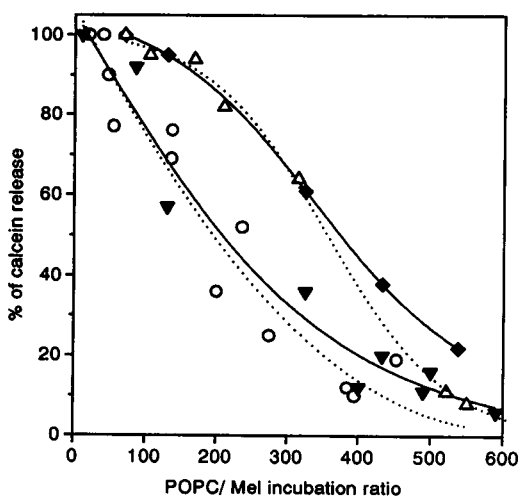

FIGURE 6 Melittin-induced calcein release for (△) POPC/PA 15(mol)% (pH 9 inside and outside), (○) POPC/PA 30(mol)% (pH 9 inside and outside), (▼) POPC/PA 15(mol)% with a pH gradient (pH 6.5 inside, pH 9 outside), (◆) POPC/PA 15(mol)% after dissipation of the pH gradient.

DISCUSSION

Osmotic stress is recognized by melittin

First of all, the behavior of vesicles under osmotic stress should be briefly discussed to define the system to which melittin is added. Our results, in agreement with previous papers (Ertel et al., 1993; Mui et al., 1993), show that a certain hyposmotic gradient can be applied to POPC vesicles without causing leakage. It is proposed that the gradient leads to water influx into the vesicles, causing them to swell (Rutkowski et al., 1991; Hallett et al., 1993). When the applied osmotic gradient is greater than a critical value, the vesicles release a part of their content to reduce the tension in the membrane and then reseal. It has been proposed that the membrane resides in a strained state as a result of the vesicle fighting back the residual osmotic pressure (Ertel et al., 1993). The release of the trapped material has been shown to be due to lysis and not to permeation (Hallett et al., 1993; Mui et al., 1993). The time release profile of carboxyfluorescein (CF) obtained by Mui et al. (1993) has shown that, after a rapid release phase (which lasts a few minutes), only limited additional release occurs; this has been nicely reproducible in our hands with calcein. Moreover, the release of the vesicle content as a function of the applied osmotic gradient that we have measured (Fig. 2) is in quantitative agreement with results obtained with 100 nm DOPG vesicles prepared by extrusion in a buffer containing 100 mM CF, 600 mM NaCl, 20 mM sodium (3-(*N*-morpholino)propanesulfonic acid), pH 7.4 (Hallett et al., 1993); a

critical applied osmotic gradient of about 800 mOsm/kg is observed. Our data are also in agreement with CF release from EggPC:Chol vesicles containing 100 mM CF, 600 mM NaCl, 60 mM HEPES, pH 7.4 (Mui et al., 1993). However, the release is considerably different from that observed by the same authors on the same lipid system but with vesicles prepared in 2.35 M NaCl. It appears that such a high salt concentration inside the vesicles has a considerable effect on their behavior with regard to osmotic stress. It has been suggested that the internal salt concentration of vesicles prepared in high-salt buffer could be significantly different from that of the external milieu (Lerebours et al., 1993), and this may be at the origin of the discrepancy. It is noteworthy that the spontaneous release observed in the presence of cholesterol in the membrane is very similar to that measured in the absence of cholesterol. To examine the ability of melittin to recognize vesicles under osmotic stress, we have applied gradients below the critical osmotic gradient to maintain the integrity of the membrane itself and to exclude osmotically induced permeability changes in the bilayer; therefore the applied osmotic gradient did not exceed 600 mOsm/kg.

Our results demonstrate clearly that the osmotic perturbation of phosphatidylcholine bilayers leads to the promotion of the lytic activity of melittin. For example, the lipid/melittin ratio for which a 50% release is observed is increased by 300% (from 150 to 600) when a hyposmotic gradient of 500 mOsm/kg is applied across POPC/30(mol)% Chol membranes. This is, to our knowledge, the first time that melittin sensitivity to stress created by a hyposmotic gradient is reported. The osmotic perturbation of the bilayer recognized by melittin suggests the existence of some osmotically induced changes in membrane physicochemical properties. Previous studies using fluorescence (Borochov and Borochov, 1979; Lehtonen and Kinnunen, 1994) and spin probes (Curtain et al., 1983; Surewicz, 1983) have shown that membrane fluidity increases in the presence of hyposmotic gradients. In parallel, several studies using dynamic light scattering have shown that the vesicle size increases under hyposmotic conditions, reflecting an increased molecular surface area; this increase in lipid area due to vesicle swelling has been reported in a range of 5% to 25% (Sun et al., 1986; Hantz et al., 1986; Hallett et al., 1993). These two concomitant modifications are actually linked if one considers that the introduction of structural defects due to *trans/gauche* isomerization leads to an increase in both fluidity and molecular area. We propose that the stretching of the external leaflet induced by the osmotic swelling destabilizes the vesicles and, as a consequence, facilitates melittin-induced lysis.

Several factors that may actually favor the interaction of melittin with the stressed vesicles can be proposed. First, swelling of the vesicles increases the molecular area of the lipids, and consequently, the binding of melittin may be enhanced, leading to a greater calcein release. However, we have shown, using intrinsic fluorescence of melittin, that the binding of the peptide to the bilayer is not affected by the presence of an osmotic gradient. Second, the disorder induced in the acyl chains by the hyposmotic gradient (Curtain et al., 1983; Surewicz, 1983) may affect the vesicle stability. This enhanced disorder should lead to the thinning of the bilayer because there is a direct correlation between chain order and bilayer thickness. It is possible that the osmotically thinned membrane is more easily perturbed by melittin and therefore a given proportion of melittin would cause greater perturbations and increased leakage. Third, the disordering of the lipid acyl chains observed when an osmotic gradient is applied leads to the potential exposure of the apolar core to the aqueous medium. This phenomenon is proposed to be at the origin of an additional interaction: the hydrophobic interaction (Bailed et al., 1990). The increase in membrane tension caused by an osmotic gradient can lead to considerable hydrophobic interaction, and it has been proposed that this may favor vesicle adhesion (Bailed et al., 1990) and fusion (Akabas et al., 1984; Woodbury and Hall, 1988). A similar interaction between the stretched bilayer surface and the hydrophobic face of melittin may favor its insertion into osmotically stressed vesicles and be at the origin of the osmotically enhanced leakage of the vesicles.

A similar rationale has been proposed for the increased permeability due to amphipathic lipids in the presence of a gradient (Naka et al., 1992). These molecular harpoons, as referred to by the authors, can, like melittin, recognize stressed membranes and induce a greater CF leakage from POPC/33(mol)% Chol vesicles. Similar to our results, the membrane-disrupting power of the surfactant increases with an applied osmotic stress. However, it is rather difficult to compare the sensitivity of melittin toward osmotic stress with the surfactants because the lytic activity of melittin is much more pronounced: melittin induces a 50% dye release for a lipid/melittin ratio of 150, whereas it requires 0.1 phospholipid/surfactant. We observed an increase of the lipid/melittin ratio by a factor of about 4 when the hyposmotic gradient is increased from 0 to 500 mOsm/kg across POPC/Chol vesicles. Comparatively, a factor of 2 is reported for one of their membrane-disrupting surfactants and for Triton X-100 for a similar gradient. Two other surfactants were examined, and it appears that the sensitivity to osmotic gradient is more pronounced for surfactants that are inactive in the absence of a gradient. To conclude, our study reveals that melittin, an amphipathic helical peptide, can recognize, as some surfactants, an osmotically stressed bilayer.

We have shown that the promotion of the lytic activity of melittin by an osmotic gradient applied to cholesterol-containing POPC vesicles is considerably greater compared to that promoted in pure POPC vesicles. Substantial changes in the elastic properties of SOPC vesicles by incorporating cholesterol in the bilayer were reported (Needham and Nunn, 1990). In the range investigated here, increased cholesterol concentrations lead to an increased area compressibility modulus K, which reflects the resistance of the membrane to isotropic area dilation, as well as an increased

tensile strength, which reflects its resistance to rupture. It is therefore rather surprising that cholesterol-containing vesicles show a greater sensitivity to osmotic stress with regard to melittin lysis. At this point, we can suggest few arguments to rationalize this phenomenon. First, the structural defects induced by the osmotic gradient may be more significant for cholesterol-containing vesicles because the smaller headgroup of the sterol could lead to a greater exposure of the outer leaflet to the water interface. This may favor the penetration of the peptide into the membrane due to an enhanced favorable hydrophobic interaction, as discussed previously. Second, because the cholesterol-containing phosphatidylcholine bilayer is less flexible than the pure POPC bilayer, it is possible that the vesicle cannot compensate easily the effect of ion gradients by size or shape changes. The residual osmotic pressure experienced by the membrane may then be closer to the applied osmotic pressure.

It should be noted that there is some controversy concerning the residual osmotic pressure experienced by the vesicle relative to the applied osmotic pressure (Ertel et al., 1993; Mui et al., 1993). It has been proposed that water inflow resulting from the applied gradient causes primarily a change in vesicle shape to maximize the volume/area ratio rather than a swelling of the vesicle (Mui et al., 1993). In that case, the water influx dilutes the internal content, and the residual osmotic pressure should be lower than that expected for the applied osmotic gradient. Actually, based on the increased of trapped volume, it was proposed that the residual osmotic pressure across EggPC/Chol membranes was nil for applied gradient osmotic below 1600 mOsm/kg. In these experiments, the inner salt concentration was extremely high (2.35 M) and, as mentioned previously, appears to influence the dye release process. The residual gradient in our experiments can be lower than the applied one, but the ability of melittin to recognize stressed vesicles indicates that the residual osmotic stress experienced by the vesicles is not negligible; it is unlikely that the enhanced lytic activity of melittin is due to a change of vesicle shape, without alteration of the membrane. Supporting our conclusions, applied osmotic gradients on the same order of magnitude (i.e., $\leq$600 mOsm/kg) have also been shown to affect the behavior of fluorescent probes (Borochov and Borochov, 1979; Lehtonen and Kinnunen, 1994) and to cause the swelling of the vesicles (Rutkowski et al., 1991; Hallett et al., 1993).

Imposing a hyperosmotic gradient on POPC vesicles (such that the vesicle cavity is at a lower salt concentration than the dispersion medium) has a different effect on both spontaneous leakage and melittin-induced leakage compared to a hyposmotic gradient. First, spontaneous leakage is very modest for salt gradients as high as 3000 mOsm/kg. Second, melittin-induced leakage does not seemed to be affected by hyperosmotic gradients in the range investigated. Osmotic shrinkage experiments on both biological and model membranes have shown an increased chain packing (Takahashi et al., 1986; Lehtonen and Kinnunen, 1994). One could assume that this effect may restrict melittin insertion into the bilayer and thus reduces calcein release. However, under our conditions, melittin does not exhibit an osmotic sensitivity toward hyperosmotic stress. Hyperosmotic gradients have some effects on membranes, such as in cold shock hemolysis (Takahashi et al., 1986), but the conclusions presented in this paper do not provide explanations regarding their origins.

Transmembrane pH gradient as a tool for controlling melittin activity

It has been shown previously that the potency of melittin in inducing the release of trapped material can be controlled by a negative surface charge density of vesicles (Benachir and Lafleur, 1995). Here we demonstrate that the protective effect of negatively charged lipids is preserved for asymmetric membranes for which the fatty acid molecules are mainly distributed in the external leaflet. The asymmetric charged lipid distribution is created by the application of a transmembrane pH gradient (Hope and Cullis, 1987). A pH gradient of 2.5 units (inside pH 6.5, outside pH 9) results in a quantitative distribution of PA in the outer leaflet (Eq. 2). A basic pH is used to ensure the deprotonation of the fatty acid, taking into account that the pK_a of an acid inserted in a lipid bilayer is estimated to 8.5 (Schullery et al., 1981). For such vesicles in the presence of the pH gradient, the negative surface charge density is approximately 2 times higher than for vesicles with the same overall composition but without the pH gradient for which the distribution of PA is uniform in both leaflets. Consequently, the melittin-induced release of entrapped dye is decreased for vesicles whose PA molecules are quantitatively flipped in the outer leaflet compared to vesicles with the same amount of PA equally distributed in both leaflets. Actually, the leakage curve obtained for vesicles containing 15(mol)% PA in the presence of a pH gradient is similar to that obtained with vesicles containing 30(mol)% PA in the absence of pH gradient. When the pH gradient across the membrane containing 15(mol)% PA is dissipated using ionophores and, as a consequence, the random PA distribution is restored, the leakage curve becomes similar to that obtained for vesicles with 15(mol)% PA in isopH conditions. These results are rationalized considering that melittin added to preformed vesicles senses only the charges of the outer leaflet. This hypothesis is supported by the study of melittin-induced hemoglobin release from erythrocytes (DeGrado et al., 1982). These authors propose that the rapid phase of the release is strictly due to melittin interacting with the outer surface of the plasmic membrane. The fact that a transmembrane pH gradient applied to pure POPC vesicles has no influence reinforces the proposed coupling between the change in charge distribution and melittin-induced lysis. Our findings offer the possibility of controlling the potency of a lytic agent by modulating surface charge density through a transmembrane pH gradient, without changing the

overall composition of the membrane. The creation of an asymmetrical bilayer using a pH gradient has also been shown to control Ca^{2+}-induced fusion of phospholipid vesicles containing free fatty acids (Wilschut et al., 1992). These two results highlight the fact that membrane asymmetry must be taken into account in phenomena for which an asymmetrically distributed component is involved.

CONCLUSIONS

The potency of melittin in changing the permeability properties of membranes was assessed by the release of trapped calcein from large unilamellar POPC vesicles. Transmembrane osmotic and pH gradients were used to modulate its lytic activity. First, melittin has the ability to recognize osmotic stress, and we have shown that an applied osmotic gradient leads to the promotion of the lytic activity of melittin. We proposed that this enhanced leakage is due to the stretching of the external leaflet, leading to structural defects at the surface that ease the perturbation of the bilayer by the peptide. From these results, it is clear that, in leakage experiments, the osmotic contribution of the fluorophore has to be counterbalanced by some solute outside; otherwise, an osmotic stress may influence the leakage measurements. This prerequisite has been stated previously (Allen, 1984), and experimental results presented in this paper highlight potential artifacts that can be obtained if it is neglected. Furthermore, imposing hyperosmotic salt gradients across POPC LUVs did not perturb in a significant manner their integrity, and melittin did not display any osmotic sensitivity regarding hyperosmotic stress. We have also presented the finding that the potency of melittin in inducing permeability changes can be regulated by a transmembrane pH gradient. The distribution of palmitic acid in both leaflets dictated by the magnitude of the pH gradient across the vesicle can be used as a tool to modulate negative surface charge density. The protective effect associated with negatively charged lipids is maintained for asymmetric membranes.

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