ELSEVIER

Biophysical Chemistry 58 (1996) 75–85

Biophysical Chemistry

Pore formation induced by the peptide melittin in different lipid vesicle membranes

Sybille Rex *

Department of Biophysical Chemistry, Biocenter of the University of Basle, Klingelbergstr. 70, CH-4056 Basle, Switzerland

Received 18 January 1995; revised 9 May 1995; accepted 16 May 1995

Abstract

We investigated the interaction of the peptide melittin with differently sized vesicles consisting of various lipid compositions. This system was characterized by dynamic light scattering to estimate the size of vesicles. For SUV we obtained a radius of 12 nm, for LUV 53 nm. The pore forming process of melittin in vesicles was investigated by efflux of encapsulated fluorescent dyes at a self-quenching concentration. The influence of the following parameters on efflux and pore formation was estimated: lipid composition (POPC and DOPC), vesicle size (SUV and LUV) and size of the encapsulated dye (carboxyfluorescein and FITC-dextran). We found that under similar conditions vesicles of DOPC give always less leakage than vesicles of POPC independent of the fluorescent dye. For SUV and LUV we have obtained a different leakage behaviour at identical surface concentrations of melittin (if the same partition coefficient is assumed). From efflux measurements with different dyes we concluded that 6–20 molecules of melittin are necessary to form a pore. The possibility that not pore formation but fusion is the mechanism of melittin induced efflux was disproved by fusion experiments using a resonance energy transfer assay.

Keywords: Marker efflux; Phosphatidylcholine; Carboxyfluorescein; FITC-dextran; Fusion; Dynamic light scattering

1. Introduction

The peptide melittin is the main component of the bee venom of the european honeybee *Apis mellifera* consisting of 26 amino acid residues with the sequence Gly–Ile–Gly–Ala–Val–Leu–Lys–Val–Leu–Thr–Thr–Gly–Leu–Pro–Ala–Leu–Ile–Ser–Trp–Ile–Lys–Arg–Lys–Arg–Gln–Gln–NH_2 [1] and a net charge of +5 to +6 at neutral pH. Due to the amphipathic character it is very well soluble in water and associates with membranes very fast [2]. The structure of melittin in a crystallized form, in aqueous solution as well as in membranes [3], has been extensively investigated by means of various methods like X-ray crystallography [4], nuclear magnetic resonance [5], circular dichroism [6], fluorescence [7,8] and raman spectroscopy [9]. Nevertheless melittin is still an object of further research [10].

In aqueous solution melittin has a random coil structure and is monomeric at low concentrations. In membranes it is α-helical. Melittin shows a wide range of possibilities to interact with biological as well as artificial membranes, e.g. it causes hemolysis of cells [11] and leakage of entrapped dyes in lipid

* Tel.: 0041-61-267-2179; fax: 0041-61-267-2189; e-mail: rex@ubaclu.unibas.ch.

SSDI 0301-4622(95)00087-9

vesicles [12], it induces bilayer micellation [13] and fusion [14], and it shows voltage-gated channel formation [15]. But in most cases the molecular mechanisms are still unknown and give rise to further suggested discussions. Therefore melittin is a suitable and interesting model for the investigation of membrane–protein interactions [16].

Biological membranes consist of different lipids and other components. To eliminate possible contributions stemming from the inserted components we used here model membranes of one lipid only. We restricted ourselves to vesicle membranes consisting of pure phospholipids either POPC or DOPC. Results from SUV (small unilamellar vesicles) are often controversially discussed as model systems for cells because of their small size and bilayer defects due to the high curvature. Keeping in mind this argument we used also LUV (large unilamellar vesicles) of about 100 nm diameter to get closer to cell-like structures.

Two basic steps in the interaction of melittin with membranes can be distinguished: (1) partitioning between aqueous and membrane phase and (2) aggregation in the membrane (pore formation). In this paper we will focus especially on the second step which was investigated by efflux measurements. A fluorescent dye at a self-quenching concentration is encapsulated in vesicles and separated from the dye in the outer medium by gel filtration. After interaction of melittin with the vesicles the dye is released due to pore formation. The increase in the fluorescence intensity is measured.

The term 'pore' we define as a structure that facilitates the transport of certain molecules through the membrane. That means the diffusion coefficient in a membrane is much less than the diffusion coefficient in a pore. Such a pore can for instance be a water-filled channel of a well-defined, irreversibly formed structure, as it is known from alamethicin [17,18]. Or as another possibility a pore can be understood to be a cluster of different numbers of monomers reversibly formed.

To test whether the experimental conditions have an influence on the pore forming process we have chosen the following three parameters: first the lipid composition, second the vesicle size and third the size of the entrapped fluorescent dye. The third parameter will give us an idea how many monomers are necessary to build up a pore. With our experiments we show how the pore formation is changed by the mentioned parameters when all the other parameters are constant.

The question was raised whether the vesicles are disrupted after interaction with melittin. Here we can show by means of fusion experiments and dynamic light scattering (DLS) that this is not the case. Especially in the case of SUV it was tested that the melittin-induced efflux of an entrapped dye is not caused by a fusion process but by pore formation.

2. Material and methods

2.1. Peptide solution

Synthetic melittin (not contaminated with phospholipase A2) was purchased from Bachem Feinchemikalien (Bubendorf BL, Switzerland) and used without further purification. Melittin stock solutions were made by solving 1 mg of the peptide in a few (1–5) ml water. The concentration was determined by UV spectroscopy using an absorption coefficient of 5570 M^{-1} cm^{-1} at 280 nm [8]. The stock solution was divided into smaller portions and stored at −20°C.

2.2. Buffer solution

The standard buffer was composed of 10 mM HEPES (N-(2-hydroxy-ethyl)-piperazine-N′-2-ethanesulfonic acid) from Bioprobe, Chemie Brunschwig AG, Basle, Switzerland), 107 mM NaCl, 1 mM $Na_2EDTA \cdot 2H_2O$ (both supplied by Merck, Darmstadt, Germany) and approximately 5–6 mM NaOH for setting the pH to 7.4 at 20°C. The buffer was then stored at −20°C.

2.3. Dye solutions

2.3.1. Carboxyfluorescein

The fluorescent dye 5(6)-carboxyfluorescein (CF; mixed isomers, MW = 376, 99% pure by HPLC) has been a product of Sigma Chemical Co. (St. Louis MO, USA). We prepared an aqueous stock solution of 50 mM CF with 10 mM HEPES, 10 mM NaCl, 1 mM $Na_2EDTA \cdot 2H_2O$ and approximately 134 mM

NaOH in order to get a pH of 7.4 at 20°C. The dye solution was stored at 4°C in the dark. The concentration was determined by UV spectroscopy using an absorption coefficient of 72000 M^{-1} cm^{-1} at 492 nm [19].

2.3.2. FITC-dextran

As a second fluorescent dye FITC-dextran (with the commercial name FD10, MW = 9400, 0.008 mol fluoresceinisothiocyanate (FITC)/mol glucose) from Sigma Chemical Co. (St. Louis MO, USA) has been used. The dye solution was composed of 7.4 mM FITC-dextran, 7.4 mM HEPES, 37 mM NaCl, 0.75 mM $Na_2EDTA \cdot 2H_2O$ and approximately 9 mM NaOH to set the pH to 7.4 at 20°C. The dye solution was stored at 4°C in the dark.

The size of a FITC-dextran molecule was estimated by dynamic light scattering. We measured a hydrodynamic radius, R_H, of 2–3 nm which is in good agreement with the literature [20,21] but we found also another population with a very large radius R_H of 60–90 nm. This fraction was, however, separated from the vesicles by gel filtration.

2.4. Osmometric measurements

In order to prevent osmotic stress on the vesicles the osmolarities of the solution inside (dye solution) and outside (buffer) of the vesicles have been checked by means of a vapor pressure osmometer (Knauer, Berlin, Germany). The dye solutions and the buffer were adjusted to be isoosmolar.

2.5. Lipid vesicles

2.5.1. Lipid films

The lipids 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphatidylcholine (POPC) and 1,2-di-oleoyl-*sn*-glycero-3-phosphatidylcholine (DOPC) in chloroform, as obtained from Avanti Polar Lipids, Inc. (Alabaster AL, USA) have been used without further purification. Lipid films free of chloroform were made for all vesicle preparations. Usually we dried 10 mg of a lipid by rotary evaporation and then placed it overnight under an oil pump vacuum. The films were stored at −20°C under argon in order to prevent oxidation processes. The lipid concentration was determined by phosphate analysis [22].

2.5.2. Preparation of SUV loaded with dye

To a dried lipid film 1 ml of the dye solution was added, vortexed and bubbled by nitrogen. The SUV were prepared by ultrasonic irradiation (for 45 min) under a nitrogen atmosphere using a microtip sonicator (MSE ultrasonic disintegrator) with a cooling bath of 10°C. The resulting clear solution was centrifuged at 10000 min^{-1} for 10 min to remove titanium particles. Then we passed the supernatant over a Sepharose CL4B column (1 × 30 cm, Pharmacia, Uppsala, Sweden) – equilibrated with the standard buffer, detection at 490 nm – so that the smaller loaded vesicles (with a radius of about 12 nm) are separated from external dye and from an apparent fraction of larger vesicles having a radius of about 60 nm. The obtained SUV were used for experiments on the same day.

2.5.3. Preparation of LUV loaded with dye

By means of the extrusion technique [23] we prepared LUV with a diameter of 100 nm. A dried lipid film was dispersed in 2 ml dye solution and vortexed until all lipid was removed from the tube wall. Then a freeze–thaw cycle was repeated five times. Subsequently, the suspension was extruded ten times through a polycarbonate membrane of 100 nm pore size (Nuclepore Corporation, Pleasanton CA, USA) under approximately 15 bar nitrogen pressure. The resulting stock solution was stored at 4°C under argon.

In the case of CF the external dye was separated from the vesicles by gel filtration over a Sephadex G50 column. In the case of FITC-dextran a Sephacryl S-300 HR column (both 1 × 30 cm, Pharmacia, Uppsala, Sweden) was applied. Both kinds of vesicles were detected at 490 nm.

2.5.4. Determination of the vesicle size

Vesicle sizes have been examined by determination of the hydrodynamic radius obtained with dynamic light scattering [24,25]. We used a commercial apparatus (ALV-125 laser light scattering goniometer equipped with a He–Ne laser (λ = 632 nm) and an ALV-5000 correlator) available in the Institute of Physical Chemistry at this university. The measurements were done under a scattering angle of 90° at 20°C. The probes for DLS have been prepared in the same way and concentration range as for efflux

measurements in order to have identical conditions. Cylindrical quartz cuvettes (1 cm diameter) especially made for light scattering measurements were used. The measured autocorrelation function was fitted by a CONTIN-analysis (built-in-software in ALV-5000).

2.6. Efflux measurements

All measurements were done on a Jasco FP 777 spectrofluorometer (Jasco, Japan Spectroscopic Co., Tokyo) with excitation at 480 nm (slit 1.5 nm), emission at 518 nm (slit 5 nm) and usage of a cut-off filter at 495 nm. The 1 × 1-cm quartz cuvettes – continuously stirred and held at 20°C – contained a final volume of 2 ml buffer with vesicles. In this state we measured the initial fluorescence signal F_0 before each run was started by adding a certain amount of the melittin stock solution. In contrast to earlier statements [26] we found it to be advisable to add melittin to the vesicles because otherwise adsorption effects of melittin on the glass walls of the cuvette would falsify the peptide concentration. For SUV the melittin concentration ranged from 0.1 to 5 μM and the lipid concentration from 20 to 80 μM. In the case of LUV the melittin concentration ranged from 0.01 to 0.5 μM and the lipid concentration from 20 to 160 μM.

Then we followed continuously the increasing fluorescence signal $F(t)$ within 20 min (see Fig. 1). The final signal F_∞ (all dye released at $t \rightarrow \infty$) has been determined after the addition of about 50 μl of a 10% (w/w) Triton X-100 solution (Merck, Darmstadt, Germany) whereby the vesicles are completely lysed and mixed micelles formed. The F_0 and F_∞ values have been corrected for dilution due to the addition of melittin and Triton X-100, respectively.

In control experiments done without melittin the loaded vesicles showed a small spontaneous efflux (SUV: 5% fluorescence increase after 20 min, LUV: 1%). We did not correct our data for that effect.

2.7. Fusion measurements

Fusion between labeled and unlabeled small unilamellar vesicles of POPC was measured by the resonance energy transfer assay [27]. The labeled vesicles contained 0.6 mol% each of N-(lissamine rhodamine B sulfonyl)-1,2-di-oleoyl-*sn*-3-phosphatidylethanolamine (Rh-DOPE) and N-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)-1,2-di-oleoyl-*sn*-3-phosphatidylethanolamine (NBD-DOPE). The two lipids were a product from Avanti Polar Lipids. The vesicles have been prepared as described above but instead of a dye solution the standard buffer has been used. Both vesicle solutions were given separately over a Sepharose CL4B column to separate the fraction of smaller from the fraction of larger vesicles. The pure POPC vesicles were detected at 260 nm and the labeled vesicles at 465 nm.

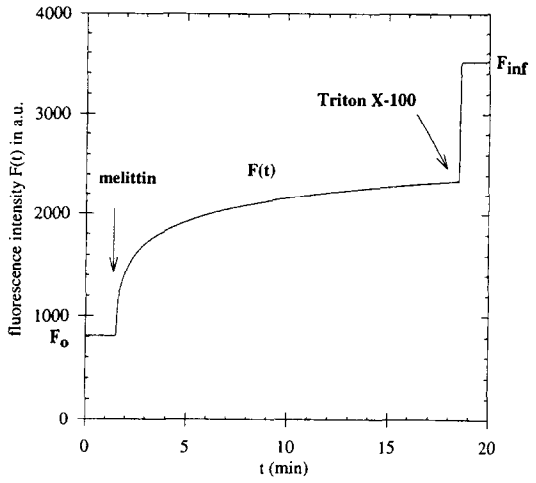

Fig. 1. Typical time course of the fluorescence signal during an efflux measurement. F_0 is the initial fluorescence value of the filled vesicles before addition of melittin (no dye is released), F_∞ indicates the final fluorescence value after addition of Triton (all dye is released). By means of both these values the efflux function $E(t)$ and the leakage can be calculated from the time course $F(t)$. For details see Material and methods and Theoretical consideration.

For the fusion measurements we used a Jasco FP 777 spectrofluorometer with excitation at 465 nm (slit 1.5 nm), emission at 530 nm (slit 10 nm) and a cut-off filter at 495 nm. The 1 × 1-cm quartz cuvettes were stirred continuously and maintained at 20°C. To a final volume of 2 ml (both vesicle solutions in various ratios and buffer) melittin was added. The resulting time course of fluorescence increase $G(t)$ of NBD was measured over 20 min (as in the efflux experiments). The final lipid concentration ranged from 20 to 200 μM and the peptide concentration from 0.5 to 5 μM.

The initial fluorescence value G_0 was measured before addition of melittin. After injection of 50 μl of a 10% Triton X-100 solution each run was stopped and the fluorescence signal G_∞ was obtained. The latter value was subsequently corrected for dilution and the effect of Triton on the quantum yield of NBD-DOPE. A correction of the value G_0 for dilution due to the added melittin has also been done.

In control experiments where melittin was added only to labeled vesicles no increase in the NBD fluorescence could be estimated.

3. Theoretical consideration

3.1. Efflux measurements

The measured raw data $F(t)$ of an efflux experiment were normalized to obtain the efflux function $E(t)$:

$$E(t) = \frac{F_\infty - F(t)}{F_\infty - F_0} \tag{1}$$

with $F(t)$ the fluorescence increase with time t, F_0 the initial fluorescence signal before addition of melittin and F_∞ the final fluorescence signal after addition of Triton (see Material and methods). This function $E(t)$ – described previously in [26,28] – is a measure of the marker content in vesicles and ranges from 1 to 0. The leakage of a dye is calculated due to the relation $(1 - E(t)) \cdot 100\%$ and varies logically between 0 and 100%.

Two modes of dye release can be distinguished: all-or-none release and graded release. In the case of all-or-none release a vesicle is either completely filled or empty after interaction with a peptide. The efflux function $E(t)$ as defined in Eq. 1 can be used for further calculations. In the case of graded release the content of the vesicles vary gradually from completely filled to empty at each time point after the interaction with a peptide. A correction of $E(t)$ leading to the retention function $R(t)$ has to be done where self-quenching of the remaining label is taken into account. The procedure how the all-or-none and graded release can be measured and distinguished, and how this retention function is then derived is described in detail in G. Schwarz and A. Arbuzova (submitted for publication).

As indicated in the introduction there are two basic steps in the interaction of melittin with membranes. First, the peptide associates with the membrane. Second, the bound peptide molecules build aggregates like pores or other diffusion facilitating structures.

The first step – the association – can be described by a partition equilibrium of melittin between the aqueous phase and the membrane. By means of thermodynamics the partition coefficient K_p can be derived which is defined as [2]:

$$K_p \cdot c_f = \alpha \cdot r \quad \text{with} \quad r = \frac{c_{as}}{c_L} \tag{2}$$

where c_f is the concentration of the free peptide, c_{as} the concentration of the membrane associated peptide, α the activity coefficient describing the interaction between the associated melittin molecules (mainly due to electrostatic repulsion of the inherent positive charges) and c_L the lipid concentration. The activity coefficient $\alpha = \alpha(r; z, b)$ can be calculated based on the Gouy–Chapman theory (where z is effective charge number of the peptide and b is a dimensionless parameter determined by the ionic strength in the aqueous solution; they are obtained by fitting the binding curve).

The value of K_p for a special system can be determined by binding measurements. For SUV of POPC the value $K_p = 7 \cdot 10^4\ M^{-1}$ [12] and for SUV of DOPC the value $K_p = 3 \cdot 10^4\ M^{-1}$ (both with $z = 1.85$ and $b = 11.5$) [2] were found in good agreement with other authors [29]. These data were used in our calculation procedure for SUV as well as for LUV.

The variable r is the relevant quantity to compare the efflux function under different conditions. Combination of Eq. 2 with the mass conservation law for melittin: $c_P = r \cdot c_L + c_f$ (c_P is the total peptide concentration) results in:

$$r = \frac{K_p \cdot c_L}{\alpha(r; z, b) + K_p \cdot c_L} \cdot \frac{c_P}{c_L}. \tag{3}$$

Here it is documented that for a certain constant c_P/c_L-ratio the value of r is not necessary the same. The dependence on α should also be taken into account. Therefore comparisons being based upon this ratio would lead to unclear results.

By means of Eq. 3 we can calculate r if the

partition coefficient K_p and the activity coefficient α are known from experiments for the appropriate conditions (lipid composition, vesicle size).

Another relevant quantity for comparing efflux curves is the variable x, the average number of associated melittin molecules per vesicle. x is readily derived from r and n, the number of lipid molecules per vesicle:

$$x = r \cdot n \tag{4}$$

where n was calculated from the hydrodynamic radius R_H of a vesicle (determined by DLS, see Table 1) and from the surface area of a lipid molecule in the inner A_L^{in} and outer half of the bilayer A_L^{out}. An outer hydration layer of 0.5 nm [30] was subtracted from R_H in order to get the real radius of a vesicle. A membrane thickness of 3.7 nm was taken.

For SUV we estimated $n = 3500$ which is based on the vesicle parameters $A_L^{in} = 61$ Å^2 and $A_L^{out} = 74$ Å^2, as established by Huang [30]. For LUV we have obtained $n = 98\,000$ calculated from a surface area of $A_L^{in} = A_L^{out} = 66$ Å^2 for a lipid molecule.

As a third quantity derived from r the surface concentration s of melittin has been calculated. This variable describes the number of peptide molecules normalized for a unit area on the vesicle surface:

$$s = r \cdot \frac{1}{A_L^{out} \cdot \beta} \quad \text{with} \quad \beta = \frac{n_{out}}{n}$$

(β-ratio of the number of lipid molecules in the outer half (n_{out}) to the total number of lipid molecules in a vesicle). For SUV we calculated s by the equation:

$$s = r \cdot \frac{1}{47.4\,\text{Å}^2} \quad \text{with } A_L^{out} = 74\,\text{Å}^2 \text{ and } \beta = 0.64$$

and for LUV we used the relation

$$s = r \cdot \frac{1}{35.6\,\text{Å}^2} \quad \text{with } A_L^{out} = 66\,\text{Å}^2 \text{ and } \beta = 0.54.$$

The second step of the interaction between melittin and vesicle membranes is the pore formation. The general kinetic scheme of this process on a vesicle is:

$$m\ \text{Monomers}(A_1) \rightarrow \text{Pore}(P)$$

As it was recently pointed out in [12] a relation between the efflux function $E(t)$ and the average number of pores per vesicle $p(t)$ can be derived in the all-or-none case:

$$E(t) = e^{-p(t)}.$$

For $p(t)$ an empirical fit function was found:

$$p(t) = v_{pi} \cdot t + \frac{v_{po} - v_{pi}}{k}(1 - e^{-kt}) \tag{5}$$

with v_{po} the initial rate of pore formation, v_{pi} the final rate of pore formation and k the rate coefficient. These three fit parameters are all functions of the quantity r or x, respectively.

An alternative fit function with only two parameters was empirically found for $E(t)$. However, a kinetic model on which this function could be based was not possible to establish up to now. The fit parameters u and v are difficult to interpret in terms of kinetic parameters like rate coefficients. Nevertheless this function is given here:

$$E(t) = (1 + u \cdot t)^{-v} \tag{6}$$

with $\log\,(u(r) \cdot \text{min}^{-1}) = 1.09 + 0.38 \cdot \log\ r$ and $\log\ v(r) = 3.53 + 1.72 \cdot \log\ r$.

Table 1
Hydrodynamic radius of different vesicle types (filled with CF) and the influence of melittin on these vesicles

Type of vesicle	SUV	LUV
Type of lipid	POPC, DOPC	POPC, DOPC
Hydrodynamic radius, R_H	12 ± 1 nm	53 ± 3 nm
After addition of melittin	Fusion	No fusion

3.2. Fusion experiments

From the measured fluorescence data $G(t)$ [1] we can derive a normalized fusion function $H(t)$:

$$H(t) = \frac{G_\infty^* - G(t)}{G_\infty^* - G_0} \tag{7}$$

with $G(t)$ the time course of the fluorescence increase of NBD, G_0 the initial fluorescence signal and G_∞^* – the corrected final fluorescence signal (see Material and methods). $H(t)$ can be interpreted as a

[1] To prevent confusion with the efflux measurements we use here the letters G and H instead of F and E.

measure for the fusion process. And the function $(1 - H(t)) \cdot 100\%$ shows at which percentage this process has been taken place. The fluorescence value of G_∞^* was calculated by the following equation:

$$G_\infty^* = \frac{G_\infty^{korr} \cdot G_{100}}{100\%} \quad (8)$$

where G_{100} is the percentage of fluorescence correspond to 100% fusion. For the ratio of labeled to unlabeled vesicles = 1:1 (which were used here) the value of G_{100} is 50% [31] under the assumption that only two vesicles can fuse together (no multiple fusion takes place) at a certain time t.

We corrected the measured value G_∞ for dilution by Triton ($G_{\infty,\,dilution}$ is obtained) and for the effect of Triton on the quantum yield of NBD by the empirical factor 1.4 [27] as follows:

$$G_\infty^{korr} = 1.4 \cdot G_{\infty,\,dilution}.$$

4. Results

4.1. Dynamic light scattering

Vesicles were prepared under various conditions as described in Material and methods. A fraction of each batch was used for DLS measurements to characterize the size. The vesicle size was independent of lipid composition and encapsulated fluorescent dye. The results are shown in Table 1.

In a first series of experiments we measured the size of the vesicles without melittin. In the case of SUV we found only after application of the Sepharose CL4B column a monodisperse size distribution for the fraction of smaller vesicles and a hydrodynamic radius of 12 ± 1 nm. For LUV we obtained also a monodisperse distribution which was stable over about a week. The hydrodynamic radius could be estimated to be 53 ± 3 nm.

In a second series of DLS measurements we have looked at the influence of melittin on the vesicle size. The addition of melittin to SUV induced an increase in size dependent on the added amount of peptide and the time after which the measurement was carried out. This effect was the first indication for melittin induced aggregation or fusion of SUV under our conditions. For LUV – in contrast to SUV – we did not get any increase in the vesicle size. We added melittin in various concentrations and measured the size during a day but we got no hint for an aggregation or fusion process.

After addition of Triton X-100 the original peak of vesicles disappeared and mixed micelles of 3 nm radius were found. In comparison to Triton, addition of melittin in our concentration range did not cause such a dramatical change of vesicle structure in a detectable extent.

4.2. Fusion experiments

In the literature it was shown [14,32,33] that melittin induces fusion of small unilamellar vesicles but the experimental conditions were not the same as ours or other methods were used. Therefore we investigated this process under our special conditions.

It is not possible to distinguish between aggregation and fusion by DLS measurements. In order to differentiate both processes we performed fusion experiments by using a resonance energy transfer assay. These experiments were done with SUV consisting of POPC. We found that in our case fusion and not only aggregation of SUV is induced by melittin.

To decide if the fusion process is possibly the cause of efflux of a fluorescent dye, a comparison with an efflux measurement under identical conditions was carried out. The result is shown in Fig. 2. It is clearly seen that the time scales of both processes are different. After 15 min the vesicles are fused at about 10% in contrast to the leakage which were nearly completed to 100%. Therefore we concluded that the efflux of a dye is not due to fusion.

4.3. Efflux measurements

The efflux measurements were performed under various conditions. In all experiments the system was kept at pH 7.4 and 20°C. We changed the lipid composition, the vesicle size and the size of the encapsulated dye. From the measured fluorescence increase $F(t)$ the normalized efflux function $E(t)$ was deduced, the leakage $(1 - E(t)) \cdot 100\%$ calculated and compared for one parameter.

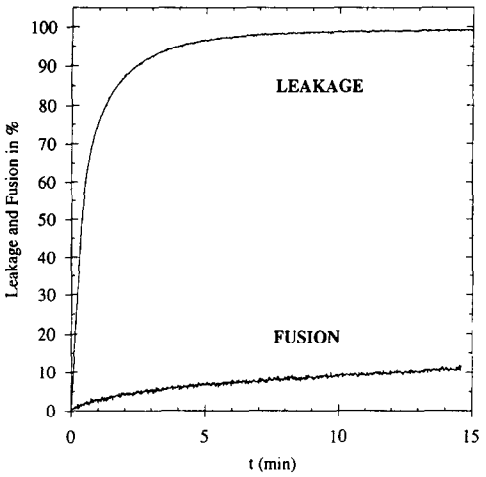

Fig. 2. Comparison between the extent of the fusion process and the leakage of carboxyfluorescein due to pore formation in the same time range of 20 min. The fusion experiment were done with lipid concentration $c_L = 66$ μM and peptide concentration $c_P = 0.8$ μM. In the efflux measurement were $c_L = 55$ μM, $c_P = 0.75$ μM. The ratio of unlabeled to labeled vesicles has been 1:1 in the fusion experiment. The leakage is described by the function $(1 - E(t)) \cdot 100\%$. The fusion is presented by the function $(1 - H(t)) \cdot 100\%$. For calculation of $E(t)$ and $H(t)$ in detail see Theoretical consideration.

4.3.1. Lipid composition

We used large unilamellar vesicles loaded with carboxyfluorescein. In Fig. 3a we compared the leakage of vesicles consisting of POPC and DOPC. As it is readily seen the vesicles of DOPC give less leakage than vesicles of POPC if the same number of

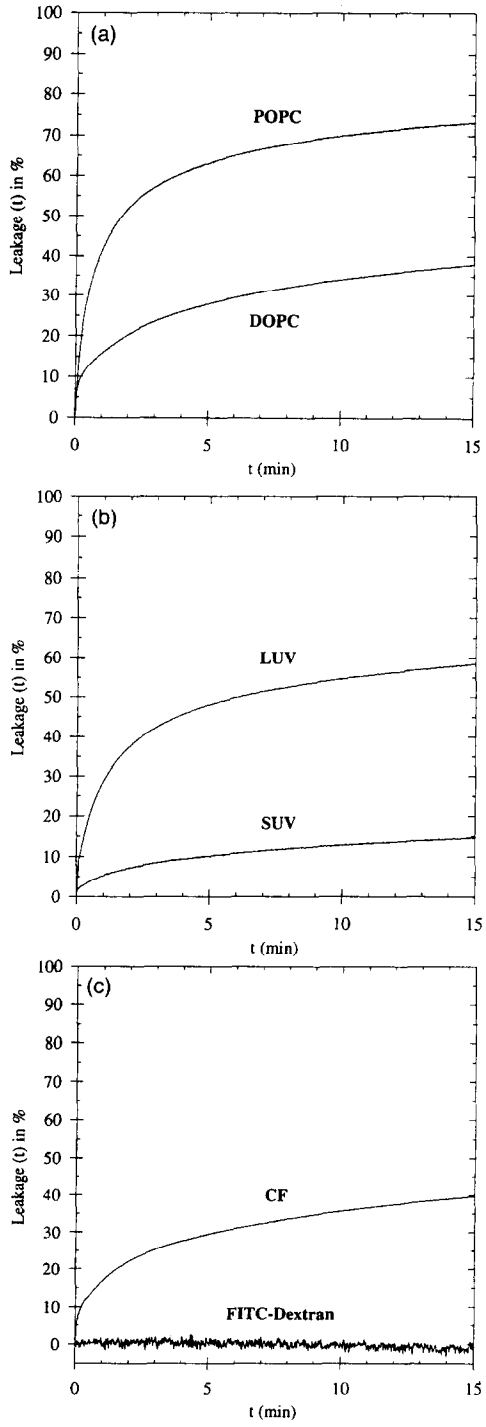

Fig. 3. (a) Influence of the lipid composition on the melittin induced leakage. POPC and DOPC are compared. The vesicle size and fluorescent dye are constant. Here the upper curve represents POPC and the lower one DOPC. The sample contained LUV filled with CF. Typically each vesicle is associated with $x = 61$ melittin molecules on average (this is equivalent to $r = 6.2 \cdot 10^{-4}$). (b) Influence of the vesicle size on the melittin induced leakage. SUV and LUV are compared. The lipid composition and fluorescent dye are constant. Here the upper curve represents LUV and the lower one SUV. The sample contained vesicles made of DOPC and filled with CF. Typically each vesicle had the same surface concentration $s = 5.3 \cdot 10^{-5}$ nm^{-2}. That means the SUV are associated with 9 melittin molecules and the LUV with 186 molecules on average. (c) Influence of the size of fluorescent dyes on the melittin induced leakage. CF and FITC-dextran are compared. The vesicle size and lipid composition are the same. The upper curve represents CF and the lower one FITC-dextran. The sample contained LUV made of DOPC. Typically each vesicle is associated with $x = 45$ melittin molecules on average (this is equivalent to $r = 4.6 \cdot 10^{-4}$).

melittin molecules x is associated to a vesicle. In our experiment was $x = 61$ on average. The same leakage behaviour is qualitatively obtained for SUV filled with CF and for LUV filled with FITC-dextran indicating that this difference in efflux is independent of vesicle size and encapsulated dye.

4.3.2. Vesicle size

A comparison of the melittin induced leakage between SUV and LUV is presented in Fig. 3b. The lipid composition and the size of the encapsulated dye were constant. For this measurement we have taken vesicles of DOPC filled with carboxyfluorescein. The data were compared on the basis of an equal surface concentration s. The calculation of s which has been explained above in detail was based on the assumption of a same partition coefficient $K_p = 3 \cdot 10^{-4}$ M^{-1} for SUV and LUV. However, Fig. 3b. shows that there is a difference in the leakage for the two vesicle sizes of DOPC. Using vesicles consisting of POPC and loaded also with CF we found qualitatively the same result.

4.3.3. Size of the encapsulated fluorescent dye

As one can see in Fig. 3c. there exists a remarkable difference in the leakage if we use two dyes of different molecular weights (CF: 376, FITC-dextran: 9400) and different molecular sizes (CF = 0.5 nm [34], FITC-dextran = 2–3 nm radius). The average number of associated melittin molecules per vesicle, x, was the same ($x = 45$). For the measurement we used LUV made of DOPC. The same result is qualitatively obtained for LUV consisting of POPC indicating that this difference is not influenced by the lipid composition.

To transport a FITC-dextran molecule with a hydrodynamic radius of 2–3 nm through a pore of melittin about 20 peptide molecules are necessary. This was theoretically calculated if a melittin molecule is assumed to be roughly a cylinder of 4 nm length and 1 nm diameter, and if it is oriented perpendicular to the membrane (barrel stave model). From the above efflux experiment with 45 melittin molecules we concluded that obviously the formed pores have to be smaller then 2–3 nm in radius. Consequently, less then 20 melittin molecules are needed to build up a pore. If the average number of melittin molecules per vesicle is increased up to 300 or 400 we see a slight leakage of FITC-dextran (data not shown). This effect could be an indication for the formation of larger pores consisting of more than 20 molecules at high peptide concentrations.

If the same calculations (barrel stave model) are performed with carboxyfluorescein (radius of 0.5 nm) the melittin pore should have a radius of approx. 1 nm and build up of about 6 single peptide molecules which is in good agreement with our efflux measurements (data not shown).

5. Discussion

5.1. Dynamic light scattering and fusion experiments

We found that fusion only takes place for SUV and not for LUV. The second result was that the melittin induced fusion of the SUV is not the cause for the efflux of the encapsulated dye. The efflux takes place for both types of vesicles. From our findings we concluded that melittin does not promote fusion significantly. Only the structure of vesicles seems to be the reason for fusion. The small unilamellar vesicles have a high curvature of the bilayer membrane and defects in the membrane structure are possible. Therefore the SUV can be considered as being in a metastable state. In contrast, the large unilamellar vesicles are not so highly curved. The inner and outer lipid monolayer are – but in the opposite way – bent almost equal in magnitude in the case of LUV. This results in much smaller bending moments than for SUV. And according to this feature we did not detect any fusion for LUV in the investigated time and concentration range.

In contrast to our results Morgan et al. [32] found melittin induced fusion for large unilamellar vesicles with a radius of about 40 nm. They worked below and above the phase transition temperature of the applied lipids. About the fusion mechanism not much is known in detail up to now. Bradrick and Georghiou [33] established a simple model for short times which was checked only for one concentration ratio of lipid to peptide.

Melittin induced fusion is obviously dependent on the kind of lipids which are used.

5.2. Efflux measurements

5.2.1. Lipid composition

The difference in the leakage data shown in Fig. 3a is quite remarkable because both lipids are very similar. The headgroups are identical. A structural difference exists only in one of the carbon chains of each lipid. POPC has a palmitoyl acid chain (16:0) instead of an oleoyl acid chain (18:1) as in DOPC. One double bond and two C-atoms cause such a change in the efflux behaviour. Another astonishing point is that the leakage for DOPC is less than for POPC. We can argue that the DOPC bilayer should have a higher fluidity due to the two double bonds. Therefore the associated melittin molecules should show a faster diffusion in a DOPC membrane than in a POPC membrane. From this fact it would follow that the aggregation process and the pore formation in DOPC should be faster than in POPC bilayers. That is what we expected. But we observed the opposite. The only explanation we have up to now for this behaviour is the shape of the lipids. DOPC has a bit larger area per fatty acid chain than POPC due to the second double bond. Therefore the insertion of melittin is more difficult in the case of DOPC than in the case of POPC.

5.2.2. Vesicle size

The reasons why we have got different results in leakage for small and large unilamellar vesicles are numerous. At first we have to emphasize that the same partition coefficient for SUV and LUV was assumed for the calculation of the variable s. This assumption has to be checked in further binding measurements.

Apart from the binding of melittin to the vesicles there are other possible reasons for the mentioned difference. The membrane curvature of SUV and LUV is not the same. Due to this the mobility of melittin molecules can vary between SUV and LUV. Therefore the diffusion coefficient may differ and the rate of pore formation is influenced. When a pore of melittin is formed in the membrane it can have a certain life time or duration of opening which may be different for SUV and LUV. The time to completely unload a vesicle depends on this duration of opening and the amount of dye which has to flow out. Although the included volume of LUV is much larger then for SUV the leakage is higher for LUV than for SUV. It has to be checked in detail which one of the listed reasons is the most probable.

5.2.3. Size of the encapsulated fluorescent dye

The method of encapsulating dyes of different molecule sizes is a well established method. It is often used in the literature in order to get an idea about pore sizes of various peptides or proteins [21,35,36].

Our data suggested pore radii between 0.5 to 3 nm which requires about 6–20 melittin molecules. This gives a first idea about the possible structure of a melittin pore. It will be useful to apply other, smaller FITC-dextrans (e.g. with MW = 4000) in the efflux measurements to come closer to the true radius of such a pore.

These three comparisons were done with the assumptions that the partition coefficients estimated for POPC- and DOPC-SUV can be used for all investigated systems, and second that the dye release is based on an all-or-none release so that the efflux function $E(t)$ can be used without correction. However, the shown results give an first indication for the influence of vesicle size and lipid composition on the melittin induced efflux and the size of a melittin pore.

Acknowledgements

I am very grateful to Prof. Gerhard Schwarz for stimulating discussion and useful advice as well as Dr. Mathias Winterhalter for critically reading the manuscript. I am also grateful to Dr. Frank Stieber (Institute of Physical Chemistry of this university) for enabling the DLS measurements and discussing the results. This study was supported by grant No. 31-32188.91 from the Swiss National Science Foundation.

References

[1] E. Habermann, Science, 177 (1972) 314–322.
[2] G. Schwarz and G. Beschiaschvili, Biochim. Biophys. Acta, 979 (1989) 82–90.
[3] C.E. Dempsey, Biochim. Biophys. Acta, 1031 (1990) 143–161.

[4] T.C. Terwilliger and D. Eisenberg, J. Biol. Chem., 257 (1982) 6016–6022.
[5] L.R. Brown, W. Braun, A. Kumar and K. Wütherich, Biophys. J., 37 (1982) 319–328.
[6] A.F. Drake and R.C. Hider, Biochim. Biophys. Acta, 555 (1979) 371–373.
[7] J.C. Talbot, J. Dufourcq, J. DeBony, J.F. Faucon and C. Lussan, FEBS, 102 (1979) 191–193.
[8] S.C. Quay and C.C. Condie, Biochemistry, 22 (1983) 695–700.
[9] H. Vogel and F. Jähnig, Biophys. J., 50 (1986) 573–582.
[10] A. Okada, K. Wakamatsu, T. Miyazawa and T. Higashijima, Biochemistry, 33 (1994) 9438–9446.
[11] W.F. DeGrado, G.F. Musso, M. Lieber, E.T. Kaiser and F.J. Kezdy, Biophys. J., 37 (1982) 329–338.
[12] G. Schwarz, R. Zong and T. Popescu, Biochim. Biophys. Acta, 1110 (1992) 97–104.
[13] J. Dufourcq, J.F. Faucon, G. Fourche, J.L. Dasseux, M. LeMaire and T. Gulik-Krzywicki, Biochim. Biophys. Acta, 859 (1986) 33–48.
[14] G.D. Eytan and T. Almary, FEBS, 156 (1983) 29–32.
[15] M.T. Tosteson and D.C. Tosteson, Biophys. J., 36 (1981) 109–116.
[16] M.S.P. Sansom, Prog. Biophys. Mol. Biol., 55 (1991) 139–235.
[17] G. Boheim, J. Membrane Biol., 19 (1974) 277–303.
[18] G. Schwarz, S. Stankowski and V. Rizzo, Biochim. Biophys. Acta, 861 (1986) 141–151.
[19] J. Barbet, P. Machy, A. Truneh and L.D. Leserman, Biochim. Biophys. Acta, 772 (1984) 347–356.
[20] E. Poitevin and P. Wahl, Biophys. J., 31 (1988) 247–258.
[21] I. Lang, H. Scholz and R. Peters, J. Cell Biol., 102 (1986) 1183–1190.
[22] C.F.J. Böttcher, C.M. van Gent and C. Fries, Anal. Chim. Acta, 24 (1961) 203–204.
[23] L.D. Mayer, M.J. Hope and P.R. Cullis, Biochim. Biophys. Acta, 858 (1986) 161–168.
[24] P. Schurtenberger and H. Hauser, Biochim. Biophys. Acta, 778 (1984) 470–480.
[25] S. Kölchens, V. Ramaswami, J. Birgenheier, L. Nett and D.F. O'Brien, Chem. Phys. Lipids, 65 (1993) 1–10.
[26] G. Schwarz and C. Robert, Biophys. J., 58 (1990) 577–583.
[27] D.K. Struck, D. Hockstra and R.E. Pagano, Biochemistry, 20 (1981) 4093–4099.
[28] G. Schwarz and C. Robert, Biophys. Chem., 42 (1992) 291–296.
[29] G. Beschiaschvili and H.D. Baeuerle, Biochim. Biophys. Acta, 1068 (1991) 195–200.
[30] C. Huang and J.T. Mason, Proc. Natl. Acad. Sci. USA, 75 (1978) 308–310.
[31] S. Nir, T. Stegmann and J. Wilschut, Biochemistry, 25 (1986) 257–266.
[32] C.G. Morgan, H. Williamson, S. Fuller and B. Hudson, Biochim. Biophys. Acta, 732 (1983) 668–674.
[33] T.D. Bradrick and S. Georghiou, Biochim. Biophys. Acta, 905 (1987) 494–498.
[34] J. Zimmerberg, R. Blumenthal, D.P. Sarkar, M. Curran and S.J. Morris, J. Cell Biol., 127 (1994) 1885–1894.
[35] R. Peters, EMBO J., 3 (1984) 1831–1836.
[36] H. Tournois, Ph.D. thesis, Utrecht, Netherlands, 1990.