Biochimica et Biophysica Acta, 1189 (1994) 101–107

BBAMEM 76173

Lipid unsaturation influences melittin-induced leakage of vesicles

Nanda K. Subbarao * and Robert C. MacDonald

Department of Biochemistry, Molecular Biology and Cell Biology, Northwestern University, 2153 Sheridan Road, Evanston, IL 60208 (USA)

(Received 22 December 1992)
(Revised manuscript received 7 September 1993)

Key words: Melittin; Calcein; Liposome; Unsaturated lipid; Lipid–peptide interaction; Lipid–protein interaction

Investigation of vesicles composed of different phosphatidylcholines revealed that the extent of leakage of internal contents induced by the lytic agent melittin can range from practically none to essentially complete, depending upon the fatty acyl chain composition of the phospholipid. The extent of leakage increases with the number of double bonds in the series dioleoylphosphatidylcholine < dilinoleoylphosphatidylcholine < dilinolenoylphosphatidylcholine. It depends on the length of the saturated chain with 1-myristoyl-2-arachidonoylphosphatidylcholine vesicles being more sensitive to melittin induced leakage than 1-palmitoyl-2-arachidonoylphosphatidylcholine, 1-stearoyl-2-arachidonoylphosphatidylcholine or 1-palmitoyl-2-docosahexaenoylphosphatidylcholine vesicles. The extent of leakage induced by melittin from vesicles composed of 1-palmitoyl-2-oleoylphosphatidylcholine, dioleoylphosphatidylcholine, 1-palmitoyl-2-arachidonoylphosphatidylcholine and 1-palmitoyl-2-docosahexaenoylphosphatidylcholine increases with the free volume parameter of these lipids for 1,6-diphenylhexatriene (Straume, M. and Litman, B.J. (1987) Biochemistry 26, 5113–5120). Among the lipids examined here, diphytanoylphosphatidylcholine vesicles were least susceptible to melittin induced leakage. The results indicate that lipid fatty acyl structure may be important in lipid–protein interactions of the kind simulated by melittin.

Introduction

Studies of lipid–protein interactions tend to emphasize the role of protein structure. Examples include the determination of the structural requirements for a peptide to mimic the serum apolipoprotein A1 [1], the role of the different regions of melittin in its function as a hemolytic agent [2], and the structural requirements for signal sequences [3]. It has been observed that interactions of these peptides depend on the fatty acyl composition of the lipid. Peptides which are models for ApoA1, for instance, mimic this protein to different extents depending on the fatty acyl composition of the lipid [4]. However, in contrast to the attention given to protein structure, there have been few systematic investigations of the requirements for lipids in interactions with peptides or proteins, with respect to either chemical structure or physical properties.

The structure of the lipid in the fatty acyl region may be expected to have an important role in lipid–protein interactions since the latter present the hydrophobic component needed for interaction with intrinsic membrane proteins [5]. The penetration of a peptide or a segment of a protein into a bilayer is likely to depend on its fluidity, compressibility or accessibility of its hydrophobic regions. Depth of penetration of a peptide into the phospholipid may well depend on the density of packing of the fatty acyl chains at different distances from the headgroup region. The extent of bilayer disruption due to peptide penetration may depend on the extent to which the bilayer distorts to accommodate the peptide and how well the lipid packing can withstand such distortion. Finally, the fatty acyl composition could alter the organization in the headgroup region and thereby affect the interaction of the lipid with the peptide in an indirect way.

Melittin, in conjunction with lipid bilayers is a popular model system for the study of protein–lipid interac-

* Corresponding author. Fax: +1 (708) 4671380.

Abbreviations: 18:2-PC, dilinoleoylphosphatidylcholine; 18:3-PC, dilinolenoylphosphatidylcholine; DOPC, dioleoylphosphatidylcholine; DPH, 1,6-diphenyl-1,3,5-hexatriene; DPhyPC, diphytanoylphosphatidylcholine; DTPA, diethylenetriaminepentaacetic acid; fv, free volume available for tumbling of 1,6-diphenyl-1,3,5-hexatriene, see Ref. 21; MAPC, 1-myristoyl-2-arachidonoylphosphatidylcholine; POPC, 1-palmitoyl-2-oleoylphosphatidylcholine; PAPC, 1-palmitoyl-2-arachidonoylphosphatidylcholine; PDPC, 1-palmitoyl-2-docosahexaenoylphosphatidylcholine; R_i, lipid/melittin molar ratio; SAPC, 1-stearoyl-2-arachidonoylphosphatidylcholine; TMA-DPH, 1-[4-(trimethylammonio)phenyl]-6-phenyl-1,3,5-hexatriene.

SSDI 0005-2736(93)E0283-G

tions because of minimal complexity in structure, commercial availability and its short length which allows easy synthesis of analogs [6,2]. Melittin rapidly penetrates the fatty acyl chain region of liposomes composed of many common lipids. The rate of penetration, as measured by the blue shift of the fluorescence maximum by stop-flow techniques, is of the order of 50 s^{-1} for dimyristoylphosphatidylcholine and dimyristoleoylphosphatidylcholine vesicles [7]. The binding of melittin to phospholipid is primarily through hydrophobic forces [8] and the interaction of the peptide with liposomes results in leakage of their internal contents [9–12]. An indication of the potentially important role of unsaturation in melittin-induced leakage comes from earlier experiments on phospholipid-cholesterol-dicetyl phosphate liposomes which showed that vesicles containing palmitoyloleoylphosphatidylcholine (POPC) are more susceptible to melittin-induced leakage than vesicles containing dioleoylphosphatidylcholine (DOPC) [9].

We have used melittin as a model to investigate the effect of varying the lipid fatty acyl structure on lipid-peptide interactions by studying the extent to which melittin-induced leakage of liposomes depends upon the fatty acyl composition of the phospholipid. We chose phosphatidylcholine as the phospholipid in order to minimize electrostatic effects with the cationic peptide and restricted ourselves to the study of lipids in the fluid phase. We have systematically varied the number of double bonds as well as the difference in chain lengths between the fatty acids in the *sn*-1 and *sn*-2 positions. We have also examined the effect of branching of the fatty acid chains by including diphytanoylphosphatidylcholine (DPhyPC) in the investigation.

Methods

Melittin

Melittin, 99.9% pure by reverse phase HPLC, was obtained from Serva Biochemicals (Paramus, NJ) and stored at −20°C, in the dark. A stock solution, about 0.5 mg/ml in deionized water, was stored at 4°C in a brown bottle and used within 10 days. The peptide is monomeric under these conditions as verified by the tryptophan emission spectrum [13]. The concentration of this solution was determined by A_{280}, assuming an absorption coefficient of 5600 [11]. Incubation of extruded vesicles of POPC with melittin at the lipid/melittin molar ratio (R_i) = 1 at room temperature for 3 h in 5 mM Tris, 100 mM NaCl, 0.1 mM EDTA, 60 μM diethylenetriaminepentaacetic acid (DTPA), pH 7.4 (leakage assay buffer) resulted in no visible lysophosphatidylcholine spot on silica gel thin-layer chromatography by iodine stain when 0.5 mg lipid was spotted on the TLC plate.

Preparation of vesicles

Phospholipids were obtained from Avanti Polar Lipids (Alabaster, AL). They were stored at −20°C under argon. Chloroform solutions with 1 mg of the phospholipids were dried under vacuum and hydrated with 200 μl 60 mM calcein, 60 μM DTPA at pH 7.4 by freeze thawing 10 times. The vesicles were extruded through two 0.1 μm diameter pore polycarbonate filters (Nuclepore, Pleasanton, CA) 19 times, by means of a hand held extrusion unit [14]. In this apparatus, vesicles are transferred from one syringe to another through extrusion filters. Multiple extrusions can be made with no exposure of the lipids to air and this feature is invaluable in handling the oxygen sensitive lipids studied here. The vesicles were chromatographed over a short Sephadex G-75 column equilibrated with degassed leakage assay buffer to separate the vesicles from the free calcein. The vesicles were stored at room temperature, for not more than 5 h, until used. The phospholipid was assayed by a modification of the Bartlett method [15]. To determine the extent to which the vesicles composed of the different lipids varied in size, small aliquots of vesicles and calcein stock solution were added to 2.5 ml leakage assay buffer with 30 μl Triton X-100 (10% w/v in water), to obtain final calcein concentrations where self quenching and inner filter effect were negligible and the resulting increase in fluorescence intensities (Ex: 490 nm and Em: 520 nm) were determined. The ratio of the calculated increase in fluorescence intensity due to lysis of liposomes composed of 1 mg phospholipid was divided by the calculated increase due to addition of 200 μl of the calcein stock solution to the leakage buffer and expressed as a percentage. The % calcein entrapped ranged from 1.7 to 2.3% indicating little variation in the total internal volume of the vesicles and therefore, little variation in the size of the vesicles. Incubation of the vesicles without melittin in the leakage buffer resulted in no change in fluorescence for 4–5 h indicating that the vesicles were stable over the time period of the experiments. Care was taken at every step to minimize oxidation of the lipids. All buffers were deoxygenated by bubbling argon through them. The extrusion apparatus was flushed with degassed buffer before use. Failure to degas buffers resulted in a decreased susceptibility of the vesicles to lysis. The A_{233}/A_{217} of the vesicles showed that the extent of oxidation was less than 2% [16]. Inclusion of butylated hydroxytoluene, a common lipid antioxidant, resulted in increased susceptibility of DOPC vesicles to leakage induced by melittin and therefore was not used.

Leakage assay

Leakage assays were based on fluorescence measurements done on a PTI Alphascan spectrofluori-

meter equipped with a xenon lamp, a magnetic stirrer and an aperture in the roof of the sample chamber that allowed injection of solutions into the cuvette while monitoring the fluorescence.

R_i was chosen to be between 1000 and 250 to minimize the formation of rouleaux type complexes [17]. A diluted melittin stock solution (5 μM) was prepared in the leakage assay buffer and stored in a brown bottle; the peptide remains monomeric under this condition as verified by the tryptophan fluorescence emission spectrum [13]. For all leakage experiments, the lipid was suspended in 2.5 ml leakage assay buffer at a concentration of 10 μM, stirred at room temperature and its fluorescence monitored (excitation at 490 nm and emission at 520 nm) by collecting data at the rate of three points per second. A 5–20 μl aliquot of the diluted melittin stock solution was injected (final melittin concentrations of 10–40 nM) into the magnetically stirred cuvette and recording of the fluorescence signal from the cuvette was continued. Triton X-100 (30 μl, 10% w/v in water) was injected after about 3 min to completely lyse the vesicles. Single deviant time points, approximately once in 20 leakage measurements, appeared immediately after the addition of melittin, possibly due to injected air bubbles. These were ignored for the curve fitting. Injection of a small volume of peptide into the large volume in the cuvette is known to often result in irreproducible time-courses in vesicle lysis/leakage studies. However, under the conditions chosen for these experiments, at these relatively low concentrations, speed of cuvette stirring, etc., we obtained reproducible time-courses, except for deviant points immediately after addition of melittin as mentioned above. The measurements were made in triplicate. For each lipid, the leakage experiment was performed on two different liposome preparations. The data presented here thus represent the means of values from six leakage time-courses for each lipid at each R_i. Standard deviations were calculated for each group. After addition of melittin, the cuvette walls became hydrophobic, as was evident from the pattern of water streaming down its sides during washing. Addition of Triton X-100 for complete lysis of the vesicles resulted in the removal of this hydrophobic layer. Leakage time-courses were determined under identical conditions at the beginning and the end of each experiment session to confirm that the vesicles were not becoming gradually damaged by lipid oxidation or any other process. There were no occasions on which a change in the leakage patterns was seen during the course of the day.

An equation in the form

$$\Delta F = A \cdot (1 - \exp[-Bt]) + C \cdot t$$

was the simplest expression that fitted (Inplot graphical software, GraphPAD, San Diego, CA) the experimentally obtained increase in fluorescence intensity due to release of calcein self quenching (ΔF) with time (t), for the time range studied here (250 time points after injection of melittin; 82 s). Such an equation represents a biphasic process; an initial fast leakage concomitant with a slow one and has been used in the past to describe melittin-induced leakage of vesicles and hemolysis of erythrocytes [6,12]. The amplitude of leakage due to the fast step was calculated using the expression $L = 100 \cdot A/$ (Fluorescence intensity increase due to complete lysis of the vesicles). L represents the percentage of the total amount of entrapped dye which is released due to the fast leakage step. B and C, the time constants for release of dye per min due to the fast phase and slow phase respectively, are expressed as percentages of the fluorescence intensity released due to complete lysis of the vesicles.

Results

Injection of melittin into the suspension of liposomes resulted in a sharp increase in the fluorescence intensity of the solution. The leakage time-course was similar to that reported for melittin-induced hemolysis but was somewhat faster [6,19,20]. The lag time before onset of leakage was 0.5 s or less and the fast phase was nearly complete in 15 s. The leakage curve was fitted by non-linear regression to an equation in the form given in the experimental section.

We studied the effect of the number of double bonds in the phosphatidylcholine on the susceptibility of the liposomes to melittin-induced leakage. For this purpose we chose three phospholipids, dioleoylphosphatidylcholine, dilinoleoylphosphatidylcholine (18:2-PC) and dilinolenoylphosphatidylcholine (18:3-PC). For these lipids, at a given R_i, the extent of leakage due to the fast leakage step (L) was highest for 18:3-PC and lowest for DOPC (Fig. 1). In each case, L decreased sharply as R_i increased up to about $R_i = 500$, beyond which the decrease was more gradual (Fig. 1). The value of B was $0.0026 \pm 0.0005\%$ per min for DOPC at $R_i = 1000$ and increased to $0.0067 \pm 0.0005\%$ per min at R_i of 250. 18:2- and 18:3-PC also exhibited similar values for B. The rate of the slow leakage phase (C) was close to zero for these three lipids at all the R_i values examined and the leakage did not reach completion even 24 h after injection of melittin.

The second set of phosphatidylcholines all have arachidonic acid (C_{20}, Δ 5,8,11,14) at the *sn-1* position and the saturated myristic (C_{14}), palmitic (C_{16}) or stearic (C_{18}) acids at *sn-2*. At any R_i, L decreased in the series 1-myristoyl-2-arachidonoylphosphatidylcholine (MAPC) > 1-stearoyl-2-arachidonoylphosphatidylcholine (SAPC) > 1-palmitoyl-2-arachidonoylphosphatidylcholine (PAPC). As in the case of the lipids used in the experiments of Fig. 1, the L decreased as

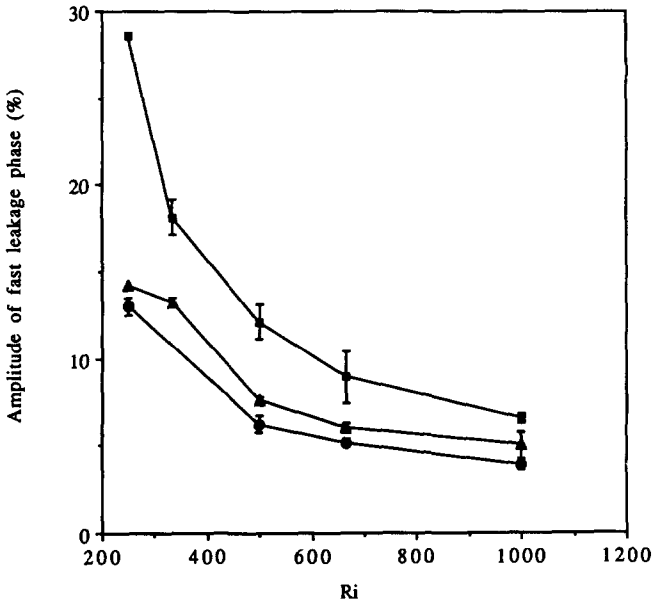

Fig. 1. Percent leakage of internal contents due to the fast leakage phase induced by melittin, from extruded vesicles composed of DOPC (●), 18:2-PC (▲) and 18:3-PC (■) induced at different values of R_i, with a constant lipid concentration of 10 μM in 5 mM Tris, 100 mM NaCl, 0.1 mM EDTA, 60 μM DTPA (pH 7.4). Melittin was injected into the stirred cuvette as a stock solution in the monomeric form and the induced leakage was followed by the release of self quenching of calcein trapped inside the vesicles. The vesicles were lysed with Triton X-100 to obtain the fluorescence increase after complete lysis.

R_i increased (Fig. 2). However, there were other marked differences in the leakage behavior. First, C was substantial and decreased as R_i increased except

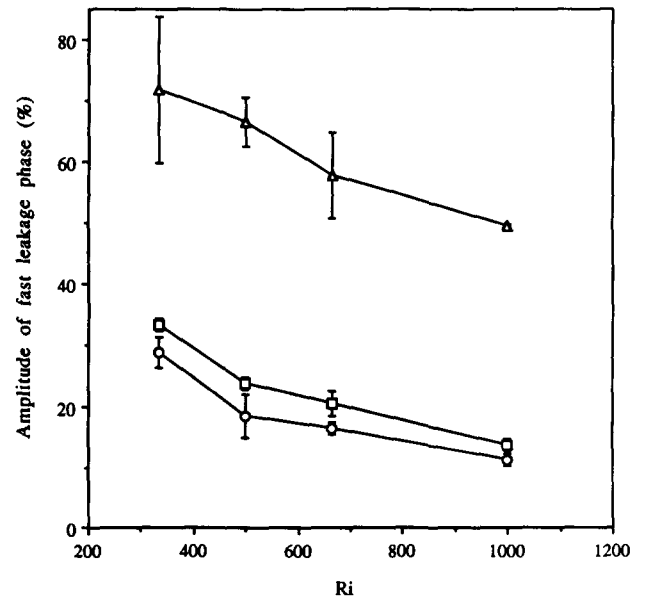

Fig. 2. Percent leakage of internal contents due to the fast leakage phase induced by melittin, from extruded vesicles composed of PAPC (○), SAPC (□) and MAPC (△) at varying values of R_i and constant lipid concentration of 10 μM. The assay procedure is described in the legend to Fig. 1.

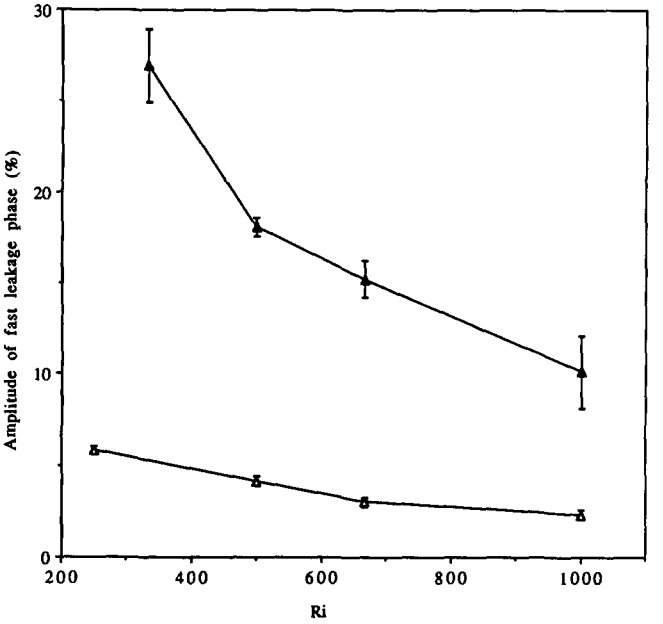

Fig. 3. Percent leakage of internal contents due to the fast leakge phase induced by melittin, from extruded vesicles composed of PDPC (▲) and POPC (△) at varying values of R_i and constant lipid concentration of 10 μM. The assay procedure is described in the legend to Fig. 1.

for MAPC in which case it reached a maximum at $R_i = 600$ (At $R_i = 1000$, $C = 11.7 \pm 0.5$, 3.5 ± 1 and 4.5 ± 1.4% per min for MAPC, PAPC and SAPC, respectively). Moreover, the total extent of leakage reached 100% within a few hours. B values for MAPC ranged from 0.0015 ± 0.0005% per min at $R_i = 1000$ to 0.0023 ± 0.0007% per min at $R_i = 250$ and were in the same range for SAPC and PAPC.

We also studied the susceptibility of DPhyPC vesicles to leakage induced by melittin, since this lipid may form an unusual bilayer due to the bulky substituents at positions 3, 7, 11 and 15 on the fatty-acyl chains. These vesicles proved to resist attack by melittin, with virtually no fast leakage phase component in the leakage pattern. The values of C were 1 ± 0.1, 1.1 ± 0.1 and 1.5 ± 0.2% per min for $R_i = 1000$, 500 and 333, respectively.

Melittin-induced leakage of vesicles could depend on the availability of 'empty spaces' in the bilayer needed to accommodate the peptide. Such 'empty spaces' have been quantified in terms of a parameter denoted as free volume (fv), which is derived from the rate of tumbling of the fluorescent probes DPH (1,6-diphenyl-1,3,5-hexatriene) and TMA-DPH (1-[4-(trimethylammonio)phenyl]-6-phenyl-1,3,5-hexatriene) in the bilayer [21]. We studied the relationship between fv of lipids and melittin induced leakage of liposomes composed of them by examining liposomes of 1-palmitoyl-2-docosahexaenoylphosphatidylcholine (PDPC), 1-palmitoyl-2-oleoylphosphatidylcholine (POPC), DOPC

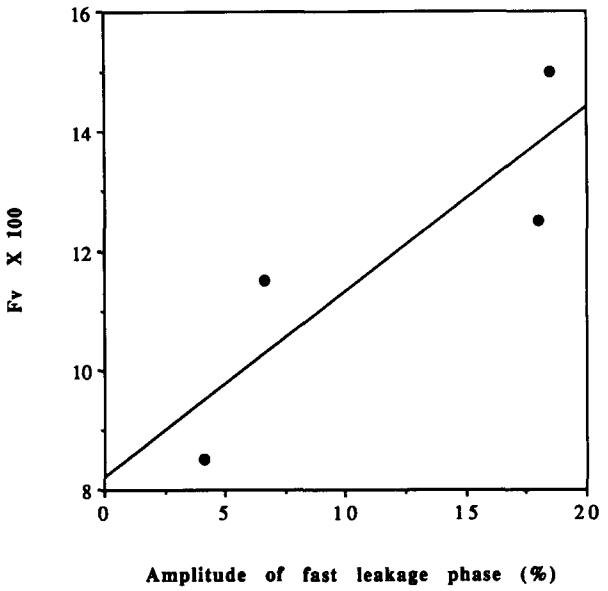

Fig. 4. Variation of the extent of leakage due to the fast leakage phase with fv of the lipids composing the liposomes at $R_i = 500$ and 10 μM lipid concentration. The experimental details are provided in the legend to Fig. 1 and in the text.

and PAPC for which fv values have been reported. Fig. 3 depicts the variation of L with R_i for PDPC and POPC and Fig. 4 shows that the fv of the four lipids does indeed parallel L. C was negligibly small for POPC and varied between $4 \pm 2.5\%$ per min at $R_i =$ 1000 to $6.7 \pm 2.5\%$ per min at $R_i = 333$ for PDPC.

The fv for TMA-DPH for these lipids [21] shows no relationship to leakage similar to the one observed for DPH. We also observed that elevating the temperature decreases L but increases fv (DPH) for each lipid. For example, L of PAPC vesicles ($R_i = 500$) decreases from 24 at room temperature to 15 at 37°C, while the fv increased 2–3-fold when the temperature increases from 5°C to 37°C [21].

Discussion

Our results indicate that melittin-induced leakage of internal contents from vesicles is strongly influenced by the nature of the fatty-acyl chains of the phospholipid, indicating that the latter are important for lipid–protein interactions of the kind simulated by this peptide.

Leakage of vesicle contents follows a time-course that is described by a fast leakage process in parallel with a slow one. The amplitude of the fast process increased steeply with the number of double bonds when both chains were unsaturated, whereas the rate constant for the slow process was small and relatively unaffected by change in unsaturation. The amplitude of the fast leakage phase from vesicles of lipids with 6 double bonds in one chain and none in the other was similar to that from vesicles of lipids with 3 double bonds in both chains. However, the rate of the slow phase was larger in the former case. When one chain was unsaturated (arachidonoyl) and the other saturated, the extent and rate of leakage increased with decreasing chain length of the saturated chain. In these cases, the slow leakage rate was significant. A highly fluid and relatively expanded lipid with saturated but branched chains, DPhyPC, was remarkably resistant to perturbation by melittin.

In these experiments, we are measuring release of a dye caused by melittin-induced discontinuities in the bilayer. There is a linear relationship between the amout of dye released and ΔF (no inner filter effect or self quenching at these low concentrations). However, since we are examining a multi-step phenomenon, we do not attribute either the exponential or the linear leakage kinetics to any particular process or stage thereof. The kinetics of the melittin-induced leakage process suggests that there is a relatively rapid process which leads to an initial abrupt loss of contents from at least some vesicles. Since the binding of melittin to lipid vesicles is known to be very fast [7], this phase should be essentially over before we took our first time point and it seems unlikely that the fast process can be identified with the initial melittin-vesicle interaction. Rather, it more possibly reflects the perturbing action, per se, of melittin such as association of several melittin molecules to form a functional channel [12]. The slow process suggests that the membranes of the vesicles with bound peptide are compromised such that they lose contents slowly and at a fairly constant rate. These two phases have been observed by others [6,12].

Two fairly detailed models for the lytic activity of melittin have been proposed [12,22]. Although these models differ with respect to the actual mechanism by which the integrity of the membrane is violated, they are not sufficiently detailed with respect to lipid-peptide interaction that they have predictive value with respect to effects of fatty acyl chain variation. In the following discussion we therefore concentrate on the physical properties of the core of bilayers that could depend upon lipid chain composition and how these properties could influence the action of melittin.

Among the properties of bilayers affected by the number of double bonds, microviscosity is mentioned most frequently. Clearly, whenever movement is involved in membrane phenomena, the viscosity of the relevant medium – the bilayer – must play a role. In the present examples, which do not appear atypical, large effects are seen even though the viscosity changes are relatively small [23]; the influence of fluidity may thus be relatively minor. Somewhat correlated with microviscosity is free volume, a parameter which, according to Straume and Litman [21], is related to the freedom of a fluorescent probe to tumble within a bilayer. We see a general parallel between melittin-in-

duced vesicle leakage and the magnitude of this parameter which probably reflects a composite of membrane properties including compressibility and dilational viscosity, as well as microviscosity.

The extent to which melittin partitions into the bilayer could be an important factor which controls the extent of leakage (see Note added in proof). There are several bilayer associated physical properties which could affect the peptide–lipid interaction. One such is the configurational entropy of the fatty acyl chains. The less fluid the membrane, the more a molecule embedded within it is restricted. A modest entropy barrier is thus expected in the penetration of molecules into membranes from the aqueous phase. Another property that increases systematically with the number of double bonds is the area per molecule occupied by the lipid at the air/water interface [24]. This would suggest that the more double bonds, the larger the fraction of the hydrophobic core exposed to the aqueous phase, an effect which may influence the ease with which the melittin molecules could congregate into a membrane-perturbing array. Another parameter which would increase with numbers of double bonds is polarizibility. As a consequence, dispersion forces should increase in parallel. This would mean that a relatively polarizable molecule such as melittin would reside at a lower energy, the more double bonds in its environment, an effect which could lead to deeper or more frequent penetration of the bilayer, the more unsaturated the lipid. This phenomenon might explain why DPhyPC is relatively resistant to the action of melittin, although the steric influences of the methyl group on the chains of this lipid could have effects on this lipid that we are unable to anticipate. Finally, we note that bilayer thickness is dependent on acyl chain composition. Influences of lipids on enzyme activities earlier ascribed to fluidity effects have now been attributed to thickness differences [25]. If leakage induced by melittin is primarily due to a pore, then bilayer thickness could well be an important factor in both rate and extent of transport.

Whatever the explanation for the lipid-chain dependent effects presented here, it is clear that the hydrophobic interior of bilayers plays a major role in their interaction with melittin. It is likely that these effects will extend to many other proteins that penetrate or reside within membranes. Well-designed investigations of lipid unsaturation on lipid–protein interactions may thus yield information with significant import for many areas of cell biology.

Note added in proof: (received 23 November 1993)

More recent experiments by Naoto Oku and Robert MacDonald have indicated that based on the blue shift of the melittin fluorescence emission maximum from 357 nm to 332 nm when lipid (6.25 μM) is titrated with melittin (0.35 μM to 7 μM), the partition coefficient between the aqueous phase and the lipid phase for melittin is basically the same for the different phospholipids, and is very similar to that reported in literature [12]. This suggests that the differences in leakage are not due to differences in lipid-peptide association but to differences in perturbation of the lipid by melittin.

Acknowledgments

This study was supported by NIH grant GM38244 and a Junior Research Fellowship from the American Heart Association of Metropolitan Chicago to N.K.S. The authors are grateful to one of the reviewers for several helpful comments and corrections.

References

1 Ananthramiah, G.M. (1986) Methods Enzymol. 128, 627–647.
2 Kaiser, E.T. and Kezdy, F.J. (1987) Annu. Rev. Biophys. Biophys. Chem. 16,561–81.
3 Briggs, M.S. and Gierasch, L.M. (1986) Adv. Protein Chem. 38, 109–180.
4 Subbarao, N.K., Fielding, C.J., Hamilton, R. and Szoka, F.C. (1988) Proteins Struct. Funct. Genet. 3, 187–198.
5 Jähnig, F. (1983) Proc. Natl. Acad. Sci. USA 80, 3691–3695.
6 Dempsey, C.E. (1990) Biochim. Biophys. Acta 1031, 143–161.
7 Sekharam, K.M., Bradrick, T.D. and Georghiou, S. (1991) Biochim. Biophys. Acta 1063, 171–174.
8 Dufourcq, J. and Faucon, J.F. (1977) Biochim. Biophys. Acta 467, 1–11.
9 Bhakoo, M., Birkbeck, T.H. and Freer, J.H. (1985) Can. J. Biochem. Cell Biol. 63, 1–6.
10 Yianni, Y.P., Fitton, J.E. and Morgan, C.G. (1986) Biochim. Biophys. Acta 856, 91–100.
11 Portlock, S.H., Clague, M.J. and Cherry, R.J. (1990) Biochim. Biophys. Acta 1030, 1–10.
12 Schwarz, G., Zong, R.-T. and Popescu, T. (1992) Biochim. Biophys. Acta 1110, 97–104.
13 Talbott, J.C., Dufourcq, J., DeBony, J., Faucon, J.F. and Lussan, C. (1979) FEBS Lett. 102, 191–193.
14 MacDonald, R.C., MacDonald, R.I., Menco, B.P.M., Takeshita, K., Subbarao, N.K. and Hu, L.-R. (1991) Biochim. Biophys. Acta 1061, 297–303.
15 Bartlett, G.R. (1959) J. Biol. Chem. 234, 466–468.
16 Thompson, S. and Smith, M.T. (1985) Chem. Biol. Interactions 55, 357–366.
17 Dufourcq, E.J., Faucon, J.D., Fourche,, G. Dufourcq, J. Gulik-Krzywicki, T. and LeMaire, M. (1986) FEBS Lett. 201, 205–209.
18 Kaszycki, P. and Wasylewski, Z. (1990) Biochim. Biophys. Acta 1040, 337–345.
19 Degrado, W.F., Kezdy, F.J. and Kaiser, E.T. (1981) J. Am. Chem. Soc. 103, 679–681.

20 Tosteson, M.T., Holmes, S.J., Razin, M. and Tosteson, D.C. (1985) J. Membr. Biol. 87, 35–44.
21 Straume, M. and Litman, B.J. (1987) Biochemistry 26, 5113–5120.
22 Katsu, T., Kuroko, M., Morikawa, T., Sanchika, K., Fujita,Y., Yamamura, H. and Uda, M. (1989) Biochim. Biophys. Acta 983, 135–141.
23 Stubbs, C.D. and Smith, A.D. (1984) Biochim. Biophys. Acta 779, 89–137.
24 Evans, R.W. and Tinoco, J. (1978) Chem. Phys. Lipids 22, 207–220.
25 Caffrey, M. and Fiegelson, G.W. (1981) Biochemistry 20, 1949–1961.