Biochimica et Biophysica Acta, 1151 (1993) 43–50

BBAMEM 76060

Interaction of hydrophobic peptides with model membranes: slow binding to membranes and not subtle variations in pore structure is responsible for the gradual release of entrapped solutes

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(Received 8 March 1993)

Key words: Membrane perturbation; Hydrophobic peptide; Pardaxin; Fluorescence spectroscopy; Membrane association; Binding; Peptide toxin; Model membrane

Investigation of the mechanism of action of membrane-perturbatory peptides often involves monitoring the release of entrapped solutes from small unilamellar vesicles. Complete release of vesicular contents can take 15 min or more. Theoretical calculations suggest that the process should be of the order of seconds and not minutes. We have investigated the membrane-perturbatory abilities of hydrophobic peptides corresponding to regions of pardaxin that are important for toxin action. Peptide-induced release of entrapped carboxyfluorescein (CF) from lipid vesicles under various conditions was monitored by fluorescence spectroscopy. Several minutes were required for the complete release of CF. This has been shown to be due to lack of instantaneous and complete association by all the added peptide with the lipid vesicles. In addition, for a given peptide/lipid molar ratio, an increase in lipid concentration causes an increase in the rate of CF-release. It is likely that increased binding following a greater number of collisions between peptide and vesicles is responsible for this observation. A Fast Protein Liquid Chromatography assay confirms that a significant amount of peptide remains unbound from the vesicles. Other investigators have reported the requirement for a similar time span for the complete release of vesicular contents by pardaxin and several other membrane-perturbatory peptide toxins. The proposed reason for the delay in lysis may therefore be applicable to a large variety of membrane-perturbants. Thus, the assay of peptide-induced release of vesicular contents is likely to predominantly reflect only the rate of association of peptide with the membrane, and not more subtle variations in the nature of the pore formed.

Introduction

A large number of antibacterial and hemolytic peptides are known whose mode of action involves membrane-perturbation [1]. These peptides have varying lengths, charge distributions, primary and secondary structures and conformations (e.g., alamethicin, δ-endotoxin, gramicidin A, gramicidin S, magainin, melittin, and pardaxin). Efforts to understand their mechanism(s) of action have included conformational studies of the peptide, in whole [1] or in part [2] and assays monitoring membrane-perturbation for the entire toxin [3], its fragments [4] or its variants [5]. Assays used to ascertain the degree of membrane-perturbatory activity on model membranes include changes in the scatter profile of the liposomes reflecting aggregation or fusion [6], leakage of fluorescent markers [7], degree of dissipation of a diffusion potential and the maximal rate of dissipation of diffusion potential [3]. It has been proposed that the mechanism of action of several peptides involves the formation of an aggregate surrounding a central pore. The nature of the pores formed has been sought to be established at better resolution using changes in membrane conductivity [3,8,9].

We have been interested in the membrane-perturbatory activities of fragments of pardaxin (GFFALIPKIISSPLFKTLLSAVGSALSSSGEQE), an ichthyotoxic neurotoxic peptide produced by the sole *Pardachirus pavoninus* [10]. We have shown that the 15-re-

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Abbreviations: Am, amide; Boc, *tert*-butyloxycarbonyl; Bzl, benzyl; CF, 5(and 6)-carboxyfluorescein; FPLC, fast protein liquid chromatography, Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; LUV, large unilamellar vesicle; MLV, multilamellar vesicle; OMe, methyl ester; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PS, phosphatidylserine; RBC, red blood cell; SUV, small unilamellar vesicle; Z, benzyloxycarbonyl. The standard one letter code has been used for (L-) amino acids.

sidue peptide corresponding to the hydrophobic N-terminal region of pardaxin has the ability to cause the release of carboxyfluorescein (CF) from small unilamellar vesicles (SUVs) like pardaxin although at somewhat lower lipid/peptide ratios [11]. The release of hydrophilic solutes trapped in small unilamellar vesicles has been extensively used to quantitate the membrane-perturbatory activities of several peptides. Thus, the activities of closely related peptides has been compared for peptides related to alamethicin [12] and the apolipoproteins [13]. The maximal rate of pore formation by peptides related to pardaxin has been defined by the plateau in the profile of percentage dissipation of diffusion potential over time and the differences in leakage profiles have been explained as the differences in the perturbatory activities of the peptides in question [5]. The delay in complete release of entrapped dye has been interpreted as different time-dependent changes in peptide arrangement, conformation or modes of lysis [3,13]. In general, these assays have been taken to reflect the channel forming properties of the peptides under study. Theoretical calculations have suggested that the leakage of vesicular contents should take seconds and not minutes [14]. In this report we demonstrate that it is the binding of peptide to the vesicles that is responsible for this time delay of several minutes. It is likely that this cause for delay is common to many membrane perturbants.

Experimental procedures

Synthesis of peptides

Peptides related to the N-terminal and central segments of pardaxin shown in Table I were synthesized by solid-phase and solution-phase methods by procedures commonly used for hydrophobic peptides [15–17]. Alamethicin was obtained from Sigma. Purification of the synthetic peptides was carried out by Fast Performance Liquid Chromatography (FPLC) using reverse phase columns (Pep RPC 5/5, Pharmacia). The peptides were characterized by quantitative amino acid analysis, using an LKB 4151 Alpha Plus Amino Acid Analyser and sequence analysis on an Applied Biosystems 473A sequencer.

Preparation of lipid vesicles

Multilamellar vesicles (MLVs) were prepared as follows: a chloroform solution of lipid was dried using the rotavaporator, buffer (120 mM NaCl, 5 mM 4-(2-hydroxyethyl)-1-piperazine ethanesulphonic acid (Hepes), pH 7.4) were added and this was then vortexed to generate the vesicles. The MLVs were then sonicated using a titanium tip sonifier (Branson) for 30 min, to generate small unilamellar vesicles (SUVs). Large unilamellar vesicles (LUVs) were prepared by passing the MLVs through the 0.1 μm filters of an Extruder (Lipex, Canada) 10 times. For the carboxyfluorescein release assay, the entrapped buffer was 100 mM 5(and 6)-carboxyfluorescein (CF), 50 mM NaCl or KCl and 5 mM Hepes at pH 7.4. The liposomes were separated from unentrapped CF by gel filtration and quantitated by a colorimetric assay for phospholipids [18]. For the CF release experiment, SUVs were then diluted 30-fold into a 120 mM NaCl, 5 mM Hepes (pH 7.4) buffer. Egg phosphatidylcholine (PC) was obtained from Sigma. Microsomal lipid was obtained from rat liver microsomes by standard procedures [19].

Lysis experiments

A Hitachi 650-10S fluorimeter was used for the scatter and fluorimetric studies. For the CF release assays, $\lambda_{ex} = 490$, $\lambda_{em} = 520$ nm and slit widths were 3 nm each for excitation and emission. The vesicles were stirred continuously and peptide was added in 3–7-μl aliquots using a Hamilton syringe. The lysis profile was recorded continuously, using chart speeds varying from 30–120 mm/min. The maximal rate of lysis is defined as the maximal slope, as lysis commences, over 5–10 s. It was calculated manually from the profiles and was obtained at the highest chart speed.

The lysis profile of B16P at different peptide concentrations was examined and it was found that different rates of release of entrapped dye resulted. In order to determine whether even low concentrations of pep-

TABLE I

Primary structures of pardaxin and synthetic peptides used in the studies of peptide–lipid interaction

Pardaxin:	G	F	F	A	L	I	P	K	I	I	S	S	P	L	F	K	T	L	L	S	A	V	G	S	A	L	S	S	S	G	E	Q	E
					5					10					15					20					25					30			
15P-OMe:	G	F	F	A	L	I	P	K	I	I	S	S	P	L	F-OMe																		
16P-OMe:	G	F	F	A	L	I	P	K	I	I	S	S	P	L	F	K-OMe																	
B16P:	Boc-G	F	F	A	L	I	P	K	I	I	S	S	P	L	F	K-OMe																	
14P-Am:									I	I	S	S	P	L	F	K	T	L	L	S	A	V-$CONH_2$											
Alamethicin [a]:	Ac-X	P	X	A	X	A	Q	X	V	X	G	L	X	X	P	V	X	E	Q	F-CH_2OH													

[a] X, α-amino isobutyric acid.

tide could permeabilize the SUVs completely, a continuous recording of lysis was made over 15 min. The rate of leakage of CF from liposomes was also investigated after separation of unbound peptide from the vesicles using a gel-filtration column. A 10 ml column was filled with 6.5 ml lipid saturated Sephadex G-50. SUVs of varying lipid composition (with 100 mM CF entrapped) were prepared as above. In order to determine the void volume of the column, 1 ml of 100 μM liposomes was loaded on the column and found to elute in the 3rd–4th ml of the eluate (as judged by CF fluorescence after treating 1-ml fractions of the eluate with Triton X-100). Peptide was added to 1 ml of 100 μM SUVs and the initial rise in fluorescence (reflecting ongoing leakage of vesicular contents) recorded. The conditions for recording were exactly the same as for the other CF release experiments described above. After 30 s the same sample was loaded on the Sephadex gel filtration column and 1 ml fractions collected. The fluorescence ($\lambda_{ex} = 490$ nm, $\lambda_{em} = 520$) of successive fractions of eluate was then recorded before and after the addition of Triton (0.1%).

The variation in the rates of lysis by B16P for a given peptide/lipid ratio was also determined at different lipid concentrations. Lipid concentration was varied from 50 μM to 100 μM, and the peptide/lipid ratio ranged from 1:60 to 1:100.

The scatter profile of the liposomes in the presence of B16P was also investigated. For the scatter studies, the PC SUVs were made in 120 mM NaCl, 5 mM Hepes (pH 7.4). 100 μM lipid was used for each experiment. Fluorimeter settings were 400 nm for both excitation and emission monochromators with a slit width of 2 nm each.

Quantitation of bound peptide

A semi-quantitative assay to determine the degree of peptide bound was established using the FPLC. A known concentration of peptide (14P-Am or alamethicin) was injected onto a C-18 PepRPC 5/5 (Pharmacia) column and a single peak obtained. Peptide was added to 1 ml buffer, stirred for half an hour and spun down at $14000 \times g$ for 8 min. The supernatant was injected into the FPLC, to establish the percentage recovery. The same amount of peptide was then added to PC MLVs (prepared as above). It was assumed that the MLVs have 10% of their lipid content in the outermost layer [20] and the lipid content used for calculating the peptide/lipid ratios (1:7 or 1:35 for 14P-Am and 1:15 for alamethicin) refer to 10% of the total lipid in the suspension. The peptide-lipid system was stirred for 0.5 h or 1.5 h and pelleted down (as above). An assay for phospholipid [18] showed no phospholipid in the supernatant after these conditions for centrifugation. The supernatant was then injected into the FPLC to detect the fraction of peptide unbound.

All experiments were performed at least twice and reproducibility was very similar. Peptide/lipid ratio indicated are molar ratios.

Results

Studies on several pardaxin analogs [3,21,22] indicate that the amino terminal region is important for activity. It has also been proposed that the central region is the channel lining segment [3]. We have shown that the peptide corresponding to the 1–15, N-terminal region of the toxin has the ability to cause release of CF from lipid vesicles [11]. Hence, the interaction of peptides corresponding to the N-terminal and central regions of the toxin with model membranes has been investigated.

Release of CF when B16P was added to SUV with entrapped dye at self quench concentration is shown in Fig. 1. Increasing concentrations of peptide results in increased rate of CF release (estimated by calculating steepest slope of each efflux curve). Peptides 15P-OMe, 16P-OMe and 14P-Am showed release profiles similar to that shown in Fig. 1. However, the percentage CF release in the initial 1 min was less than B16P indicating that they are less effective in permeabilizing the lipid vesicles. We observed that even low concentrations of B16P can cause a high percentage of CF release, if left sufficiently long. Thus, at the end of 15 min, 95% release is observed as shown in Fig. 2. Even at this time point, a plateau is not reached and it is likely that 100% release would be observed were even more time allowed. Release of CF in the absence of peptide was less than 10% over 40 min. These results

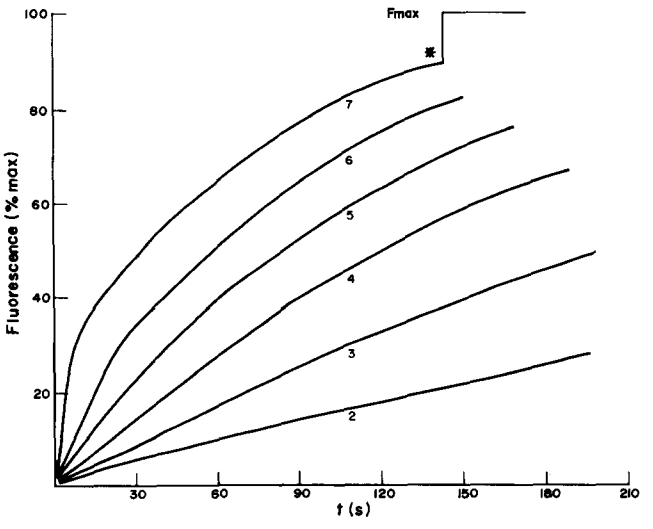

Fig. 1. Release of CF from PC SUVs in the presence of varying concentrations of B16P. The numbers in the figure correspond to concentration of peptide in μM. Lipid = 200 μM. F^{max} corresponds to the fluorescence value when the lipid vesicles were disrupted with Triton X-100 (0.1% v/v). Asterisk, addition of Triton.

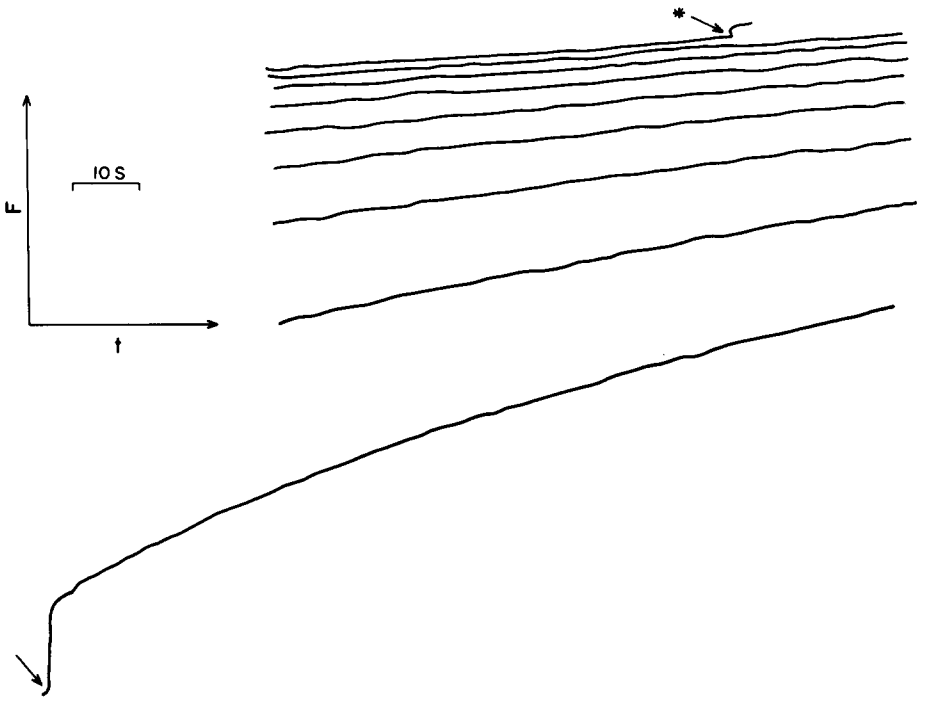

Fig. 2. Release of CF from PC SUVs in the presence of B16P. The total curve is shown in successive traces, presented from bottom to top. Lipid/peptide molar ratio, 300:1. *Y*-axis, fluorescence, (*F*), arbitrary units; *X*-axis, time. Arrow, addition of peptide; asterisk, addition of Triton (0.1% v/v).

indicate that even at a peptide/lipid ratio of 1:300, complete release of CF from all the vesicles is possible given sufficient time. Similar results were obtained with 16P-OMe, 15P-OMe and 14P-Am.

In an attempt to distinguish between the possible rate-limiting steps, the vesicles were passed through a gel filtration column after CF release was initiated. The experiment is outlined in Fig. 3 and the results are summarized in Table II. The following conditions were varied in these experiments: the peptide investigated (B16P, 16P-OMe or 14P-Am), lipid composition (microsomal lipid or PC), size of the liposomes (SUV or LUV), peptide/lipid ratio (1:5 or 1:25) and the time after initiation of lysis at which the peptide vesicle mixture was loaded onto the column (almost immediately, or after ≈ 50% lysis had occurred). After gel filtration, the recovered vesicles do not exhibit ongoing CF release as seen in Fig. 3. However, when these vesicles were treated with Triton, significant release is observed. Similar results were obtained for the different peptide and lipid systems investigated. Attempts to quantitate the unbound peptide from the column eluate were not successful. Since the columns were presaturated with lipid, it is possible that the unbound peptide becomes associated with this lipid. The recovery of vesicles (based on the total CF released by Triton from all the vesicles) was complete.

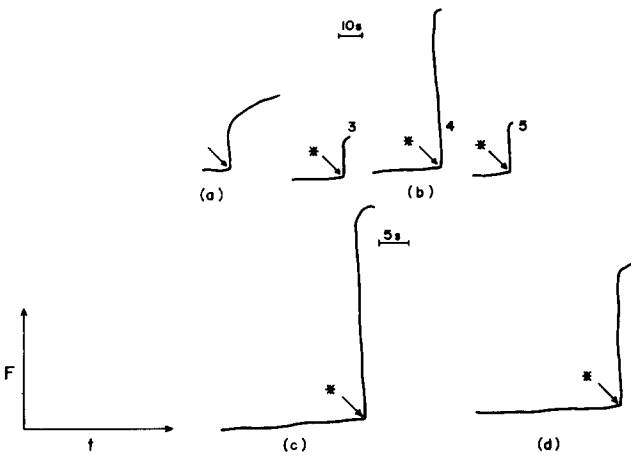

Fig. 3. Lysis profiles of lipid vesicles after gel filtration of vesicles plus peptide. (a) B16P was added to SUV composed total microsomal lipids at a 1:25 peptide/lipid molar ratio to initiate lysis (arrow, addition of peptide). (b) After the vesicles were loaded onto a gel filtration column, successive fractions of column eluate were checked for CF fluorescence before and after the addition of Triton. Fractions 3, 4 and 5 are shown sequentially. (c) 14P-Am was added to PC-LUVs at a 1:5 peptide/lipid ratio. The tube with maximal release of CF after Triton treatment is shown. (d) 16P-OMe was added to PC LUVs at a 1:25 peptide/lipid ratio and separated from unbound peptide after 45% CF was released. The tube with maximal release of CF after Triton treatment is shown. Lipid concentration = 100 μM for all experiments. Asterisk, addition of Triton. *Y*-axis, fluorescence (*F*), arbitrary units; *X*-axis, time.

TABLE II

Release of CF from the vesicles after treatment with Triton, subsequent to passage of the peptide-vesicles through a gel-filtration column [a,b]

Eluate tube No.	Fluorescence (B16P) [c]	Fluorescence (14P-Am) [d]	Fluorescence (16P-OMe) [e]
1	none	1	none
2	none	1	none
3	10	25	17
4	38	36	24
5	12	9	3
6		2	

[a] No increase in fluorescence, reflective of ongoing lysis, is observed before treatment of any of the peptide-vesicle systems with Triton.
[b] The numbers are fluorescence intensities (arbitrary units).
[c] B16P was added to 100 μM microsomal SUVs at a 1:25 peptide/lipid ratio and loaded onto the gel-filtration column almost immediately after lysis was initiated.
[d] 14P-Am was added to 100 μM PC LUVs at a 1:5 peptide/lipid ratio and loaded onto the column almost immediately after lysis was initiated.
[e] 16P-OMe was added to 100 μM PC LUVs at a 1:25 peptide/lipid ratio and loaded onto the column after 45% of the CF had leaked from the vesicles.

In order to quantitate better the amount of peptide left unbound from the vesicles, an FPLC assay was used. 7 nmol of 14P-Am eluted as a single peak on the FPLC, as seen in Fig. 4a. The same amount of peptide was stirred for 0.5 h with 250 μM MLVs at a peptide/lipid ratio of 1:5, the lipid spun down and the supernatant injected into the FPLC column. The profile of peptide recovered is as shown in Fig. 4b. Based on the area under the curve, recovery was 83%. Recovery was 109% after stirring for 1.5 h with MLVs as shown in Fig. 4c. 93% peptide was recovered after incubation with 5 × lipid concentration (peptide/lipid ratio 1:35) as seen in Fig. 4d. Alamethicin, a 20-residue peptide whose channel forming properties as well as interaction with model membranes has been well characterized [2,23–25] has the ability to cause the

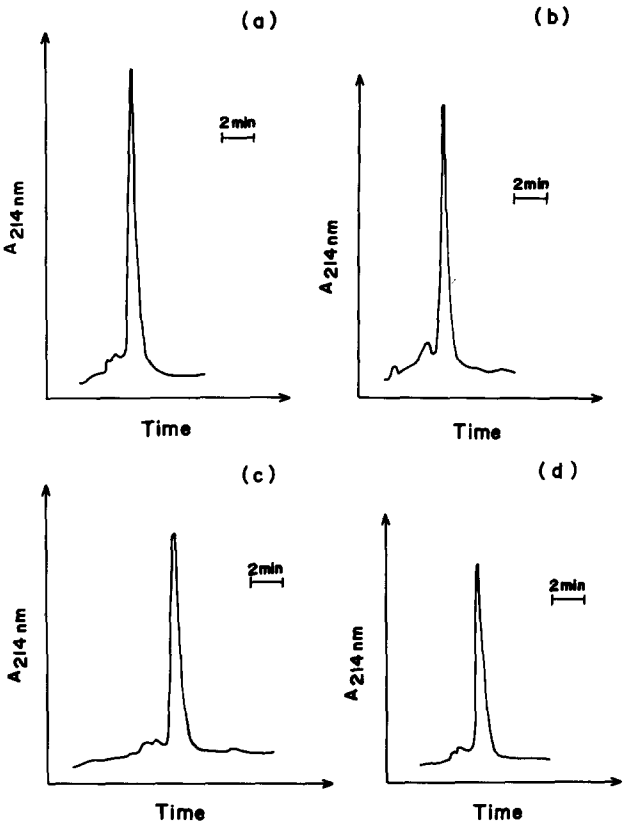

Fig. 4. Estimation of peptide 14P-Am unbound to MLV by FPLC. (a) 7 nmol 14P-Am eluted as a single peak on a PrepRPC column. (b) 7 nmol 14P-Am stirred for 0.5 h with 250 μM PC MLVs, the lipid spun down and the supernatant injected into the FPLC column. (c) 7 nmol 14P-Am stirred for 1.5 h with 250 μM PC MLVs, the lipid spun down and the supernatant injected into the FPLC column. (d) 7 nmol 14P-Am stirred with 1250 μM PC MLVs, the lipid spun down and the supernatant injected into the FPLC column. Solvent A: 0.1% TFA/water, Solvent B: 0.1% TFA/acetonitrile. Gradient: 25% to 70% solvent B in 25 min.

release of CF from SUV [24]. In order to examine whether alamethicin also binds slowly to lipid vesicles, analysis similar to that described in the previous paragraph was carried out. Alamethicin (18 nmoles) eluted

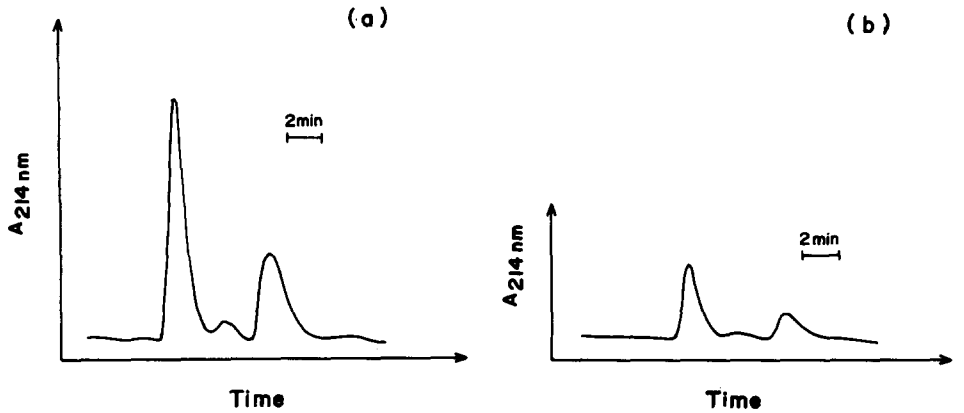

Fig. 5. Estimation of alamethicin unbound to MLV by FPLC. (a) Alamethicin (18 nmol) injected onto the pepRPC FPLC column eluted as two peaks. (b) Alamethicin (18 nmol) incubated with PC MLVs at a 1:15 peptide/lipid ratio, the lipid spun down, and the supernatant injected into the FPLC column. Gradient as in Fig. 4.

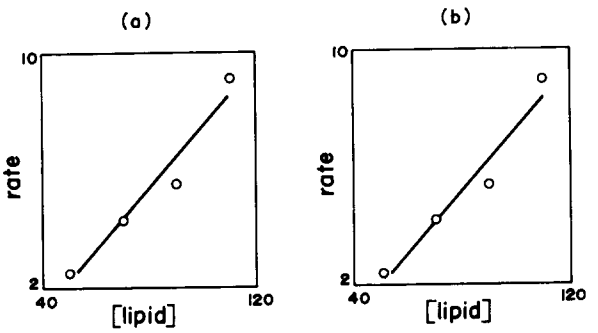

Fig. 6. Plot of maximal rate of CF release from PC vesicles by B16P as a function of lipid concentration at a fixed peptide/lipid molar ratio. (a) 1:80, (b) 1:100. Lipid concentration varied between 50 and 100 μM.

as two peaks – a major peak (alamethicin I [26] followed by a less intense peak (alamethicin II [26] as seen in Fig. 5a. Peptide was stirred with lipid at a 1:15 peptide/lipid ratio. Recovery of peptide in the supernatant was only 33% as is evident in Fig. 5b.

Using the CF release assay, the rate of leakage (initial, maximal slope, after initiation of lysis) was also investigated at a fixed peptide/lipid ratio over different lipid concentrations. The results are presented in Fig. 6. A straight line with a positive slope is obtained, thus indicating an increase in lysis with an increase in lipid concentration.

Inspection of the scatter profiles for liposomes incubated with B16P reveals that although nonlytic concentrations of peptide do not bring about any change in the scatter profile, lytic concentrations had two effects. An immediate increase in the scatter was followed by no further change (over minutes) as shown in Fig. 7.

Discussion

Release of CF from SUVs by B16P, 16P-OMe and 14P-Am is a slow process with complete release occuring only after 15 min. This behaviour is observed even at high lipid-peptide molar ratios where all the peptide would be expected to be bound. It has been shown that $\approx$ 30 s is need to permeabilize a 100 nm diameter SUV with a pore of 10 pS conductance and a permeating ion

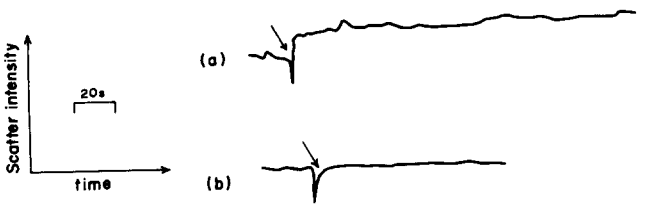

Fig. 7. The scatter profile on the addition of B16P to 100 μM PC SUVs. (a) An immediate increase, followed by no change, on the addition of 2.0 μM peptide. (b) No change in scatter on the addition of 0.2 μM peptide. *Y*-axis, arbitrary units of scatter; *X*-axis, time in min. Arrow, addition of peptide. Lipid = 100 μM.

concentration of 150 mM [14]. 10 pS is a very low figure with melittin forming 20–2000 pS channels [27] and δ-endotoxin, 30–420 pS channels [8] and hence 30 s (or less) should suffice to permeabilize an SUV. The rate-limiting process in the permeabilization of an SUV could be any one or more of the following steps: (i) adsorption of peptide to the bilayer, (ii) insertion into the hydrophobic core of the membrane, (iii) aggregation of monomers into a functional multimeric aggregate and any conformational change necessary for the formation of this functional pore-forming unit [28,29]. In order to distinguish between some of these steps, peptide loaded vesicles were separated from the unbound peptide by gel filtration soon after lysis was initiated. No further CF release was observed. The lack of CF release after passage of the vesicles and peptides through a gel filtration column indicates that the association of unbound peptide with the vesicles is an important rate limiting step in the ongoing lytic process. There is a possibility that peptide is adsorbed to the vesicle surface, and that incorporation of peptide into the lipid bilayer is the cause of delayed lysis. The adsorbed peptide could be 'stripped' from the vesicles during passage through the column. However, the results of the semi-quantitative assay used to detect the fraction of unbound peptide support the gel filtration experiment in suggesting that the presence of a large fraction of unbound peptide that is responsible for the slow rates of leakage of entrapped dye. The small discrepancies in percentage recovery (such as 109%) arise from the difficulty in establishing the baseline of the peak due to the small contaminating shoulder. Even in the case of alamethicin, an effective membrane-perturbatory peptide which should exhibit a greater degree of binding, $\approx$ 33% of peptide was recovered in the supernatant.

The gel filtration experiment therefore suggests that (a) the extent of association of unbound peptide with the vesicles is critically rate limiting to the ongoing lytic process, (b) during lysis, dissociation of vesicle-bound peptide occurs to an insignificant degree. If such dissociation were significant, the associated peptide would have caused continued release of entrapped dye after the liposomes had eluted from the column, (c) steps subsequent to the binding by peptide, such as reorientation of the membrane-bound peptide from a nonlytic to lytic configuration or aggregation is not responsible for the delay in lysis of several minutes. Were this to be critical to peptide action, continued release of CF from the liposomes would have been observed after their passage through the column. It is conceivable that mixed peptide/lipid micelles may be formed at the fairly high peptide/lipid ratios used for the lysis experiments, as is observed for melittin [30] and peptides related to apolipoprotein [31]. These micelles could release peptide which would cause the continued lysis

in a non-fractionated peptide-lipid system. Although this possibility has not been tested, it seems unlikely for the following reasons: (a) in view of the need for peptide in the buffer for ongoing release of vesicular contents, the ratio of bound peptide to lipid is likely to be much lower than that of free peptide/lipid, and (b) a decrease in scatter is not seen during lysis. This is in contrast to the strong decrease in scatter for Triton micellization of SUVs.

For a given peptide/lipid ratio an increased rate of lysis is observed with an increase in lipid concentration. This may happen for two reasons. If the formation of one lytic unit is sufficient to empty vesicular contents several 'lytic units' of peptide may be formed per vesicle with only the first one being reported by the CF-release assay. With an increase in lipid concentration, the lytic units may be distributed over more vesicles, and the total amount of CF released is more. Alternatively, there may be greater collision frequency between peptide and vesicles due to the increase in absolute concentration of each component. We have not distinguished between these possibilities, but the second alternative supports the concept that low degrees of release of vesicular contents are due to incomplete binding of peptide.

The tendency of hydrophobic peptides to take several minutes for the complete release of SUV contents has been described for very many peptides that interact with membranes. Examples include apolipoprotein analogues [13], δ-endotoxin [32], dodecylamine [6], gramicidin S [6,33], magainin 1 [34], melittin [32], pardaxin and its analogues [5], a peptide fragment of the MS2 lysis protein [35] and tachyplesin [36]. None of the lysis profiles plateau after several minutes of lysis and degrees of lysis are reported with respect to a given time point [5]. This result therefore holds for perturbants of very different structures with dodecylamine not being a peptide, gramicidin S and tachyplesin forming cyclic and non-cyclic β-sheet structures, respectively, and δ-endotoxin, magainin and melittin adopting helical structures, though very different in their primary sequences. In a study of forming pardaxin binding to LUVs, toxin labelled with ^{125}I was added to LUVs and passed through a Sepharose 4B column in order to separate free from bound peptide [37]. Only 15% of the added radioactivity was associated with the lipid vesicles. In studies on gramicidin A, although channel activity was observed after the addition of peptide to the aqueous phase bathing the two sides of the bilayer, breaking and remaking the membrane drastically enhanced channel formation [38]. It was also found that the numbers of gramicidin A channels formed per minute increases steadily over 20 min which must therefore reflect increasing incorporation of peptide over time. The association equilibrium of several apolipoproteins with phospholipid has been shown to take from milliseconds to minutes [39] and the inactivity of some cecropin analogues has been shown to be due to their inability to bind to planar bilayers [40].

Thus, membrane-perturbatory peptides take an order of magnitude more time than required to completely permeabilize small unilamellar vesicles. There is an immediate cessation of the lytic reaction after separation of unbound peptide from the vesicles. Hence we propose that in the permeabilization of small unilamellar vesicles by peptides it is the binding by peptide to vesicles that is responsible for the large delay in the completion of lysis. Although several assays are routinely used for assaying a peptide's membrane-perturbatory activity like the rate of dissipation of a diffusion potential, the maximal extent of dissipation of a diffusion potential or the extent of release of calcein or carboxyfluorescein from a vesicle, all of these reflect the rate of association of the peptide with the membrane. Thus, these assays do not provide information on the mechanism of membrane perturbation after association of the perturbant with the membrane such as subtle variations in the structure of the pore formed, as is often assumed and implied.

Acknowledgements

We thank V.M. Dhople for amino acid analysis and S. Thennarasu for synthesizing one of the peptides by semi-automated methods and help in some experiments. We are also grateful to M.V. Jagannadham for sequence analysis of peptides.

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