Biochimica et Biophysica Acta 937 (1988) 387–397
Elsevier

BBA 73859

Gramicidin S and dodecylamine induce leakage and fusion of membranes at micromolar concentrations

Gera D. Eytan, Rachel Broza and Yechiel Shalitin

Department of Biology, Technion-Israel Institute of Technology, Haifa (Israel)

(Received 12 August 1987)

Key words: Gramicidin S; Liposome; Membrane fusion; Dodecylamine; Cationic amphipath

The effect of the antibiotic gramicidin S and the synthetic cationic amphipath dodecylamine on membranes was studied with large unilamellar vesicles containing phosphatidylcholine and varying concentrations of cardiolipin. Fusion of vesicles composed of equal amounts of the two phospholipids occurred with both drugs at concentrations lower than 10 μM. Fusion was accompanied by leakage of the contents, while higher drug concentrations caused complete loss of vesicle contents. Drug concentrations at least one order of magnitude lower were needed to induce leakage from vesicles containing only phosphatidylcholine. Under these conditions, contents leakage occurred with no measurable aggregation or membrane intermixing. On the other hand, much higher concentrations of both drugs were required to induce leakage from vesicles containing predominantly cardiolipin. Release of contents occurred upon aggregation of the vesicles and collapse of the vesicular organization, as well as formation of paracrystalline structure when dodecylamine was employed or amorphous material when gramicidin A was used. In contradistinction to other model systems, phosphatidylcholine was needed for fusion induced by the cationic amphipaths, and its presence reduced the threshold concentration of the drugs needed to induce leakage of the contents. The similar effects of the two drugs on membranes imply that, at least in these model membranes, the relevant feature of both drugs is only their amphiphatic nature.

Introduction

Many fundamental biological processes involve membrane fusion. Despite the huge amount of research data collected, the molecular mechanisms of membrane fusion are still largely unknown. Certain proteins that play a role in fusion have been identified, such as the glycoproteins involved in the entry of membrane-coated viruses into cells [1,21], synexin, which plays a role in exocytosis [3], and lysin, which seems to have a function in the fusion of mollusk spermatozoa and eggs [4]. Fusion has been studied extensively using lipid vesicles composed of defined phospholipids. Fusion was induced by divalent ions, mainly calcium ions [5,6], and proteins, i.e., synexin [7] and lysin [4] and by other agents: polylysine [8], spermine [9], polymyxin B [10,11], melittin [12,13] and myelin basic protein [14].

Recently, it has been demonstrated that a variety of cationic amphipaths, such as dodecylamine, induce fusion of small unilamellar

Abbreviations: Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; N-NBD-PE, *N*-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)phosphatidylethanolamine; N-Rh-PE, *N*-(lissamine Rhodamine B sulfonyl)phosphatidylethanolamine; DPA, dipicolinic acid; CL, cardiolipin; PC, phosphatidylcholine.

Correspondence: G.D. Eytan, Department of Biology, Technion-Israel Institute of Technology, Haifa 32000, Israel.

vesicles containing cardiolipin [14]. It was suggested that the minimum characteristics for such a fusogen are a hydrophobic moiety, that allows incorporation of the fusogen in the liposome, and a positive charge, which promotes the tight appregation of the liposomes and thus triggers the fusion reaction itself.

In the present research we investigated the effect of dodecylamine on fusion of large vesicles, which better approximate biological membranes, to test whether a drug such as gramicidin S, which is a cationic amphipath, will indeed promote fusion.

The antibiotic gramicidin S is a cyclic decapeptide (-Val-Orn-Leu-D-Phe-Pro-Val-Orn-Leu-D-Phe-Pro-), produced by certain strains of *Bacillus brevis* [15,16]. Structural studies have indicated that the two positive charges of the ornithines are located only 0.8–1.0 nm apart, forming a cationic hydrophilic pole, while the other amino acids constitute a hydrophobic moiety [17]. It possesses bacteriostatic and bactericidal effects on numerous Gram-positive bactertia, but is less active against Gram-negative ones [18]. Its interactions with various membranes, such as bacteria, mitochondria, erythrocytes and model membranes, have been documented (see Ref. 17 for a review, and Refs. 19–21). It has been postulated that the positive charges are required both for the antimicrobial activity and for its binding to membranes. At a high drug to lipid ratio, gramicidin S solubilizes the membrane structure and transforms it into small dense micelles consisting of gramicidin S/lipid (2 : 1).

Based on structure-function studies of many gramicidin S analogues, Kato and Izumiya [22] proposed the 'sidedness' hypothesis, which proposes that the organization of the drug with hydrophobic and hydrophilic moieties is essential for its antibacterial function. Katsu et al. [23,24] extended this type of study on the effect of the drug on permeability of biological membranes and liposomes. The present study demonstrates that the effects of gramicidin S and the synthetic amphipath dodecylamine on neutral and acidic liposomes are strikingly similar, and this implies that the essential feature of gramicidin S for induction of fusion and leakage is its amphipathic nature.

Materials and Methods

Materials

Gramicidin S, dodecylamine, phosphatidylcholine, cardiolipin, cholic acid and Hepes were purchased from Sigma. N-NBD-PE and N-Rh-PE were purchased from Avanti Polar Lipids. 5(6)-Carboxyfluorescein was purchased from Eastman and further purified according to the method of Ralston et al. [25]. Pigments were extracted from spinach leaves [26] and chlorophylls *a* and *b* were purified by paper chromatography with 10% acetone in petroleum ether (boiling point, 60–80 °C) as eluant. All phospholipids were tested for purity by thin-layer chromatography, and proved to be at least 99% pure. Phospholipid concentrations were determined by assaying the lipid phosphorus content according to the method of Bartlett [27] and are expressed in terms of phosphorus concentration.

Liposome preparation

Membrane intermixing was monitored with the fusion assay based on resonance energy transfer from chlorophyll *b* to *a* [28]. Unilamellar vesicles were prepared in 100 mM NaCl/0.1 mM EDTA/5 mM Hepes (pH 7.4) by the reverse-phase evaporation technique [29], followed by extrusion through 0.2-μm polycarbonate membranes [30]. The Tb-DPA fusion assay was used for monitoring intermixing of vesicle contents [31]. Large unilamellar vesicles were prepared as above in (a) 5 mM $TbCl_3$/50 mM sodium citrate/5 mM Hepes (pH 7.4), (b) 50 mM DPA/20 mM NaCl/5 mM Hepes (pH 7.4), (c) 2.5 mM $TbCl_3$/25 mM DPA/10 mM NaCl/25 mM sodium citrate/5 mM Hepes (pH 7.4). After extrusion through the polycarbonate membrane, the external medium was exchanged by passage of the vesicles through a Sephadex G-75 column equilibrated with 100 mM NaCl/1 mM EDTA/5 mM Hepes (pH 7.4). Vesicles prepared for experiments designed to monitor membrane intermixing were prepared in 100 mM NaCl/0.1 mM EDTA/5 mM Hepes (pH 7.4).

Vesicles with trapped 5(6)-carboxyfluorescein were prepared in a medium similar to the last one, except that it contained also 80 mM 5(6)-carboxyfluorescein. External dye was removed by pas-

sage of the vesicles through a Sephadex column equilibrated with dye-free medium.

Assays

The Tb-DPA assay for vesicle contents intermixing was performed as described by Wilschut et al. [31] in a MPF-44B Perkin Elmer Spectrofluorometer. A typical reaction mixture consisted of Tb and DPA vesicles at a 1:1 ratio together with 0.1 mM EDTA/100 mM NaCl/5 mM Hepes (pH 7.4). The vesicles, amounting to a final concentration of 100 μM, were added last. The maximal fluorescence intensity was determined with the Tb-DPA vesicles.

Gramicidin S and dodecylamine quenched the fluorescence intensity of N-Rh-PE to such an extent that the fusion assay based on this probe [32] could not be used for monitoring membrane intermixing. A similar observation has been described by Parente and Lentz [33]. On the other hand, the assay utilizing chlorophylls *b* and *a* as donor and acceptor, respectively, of resonance energy proved useful even in the presence of the cationic amphipaths. Both dodecylamine and gramicidin S, in the concentration range used in the present work, had no effect on the resonance energy transfer efficiency in chlorophylls-containing vesicles in the absence of non-pigmented ones. Presumably, the chlorophylls were protected by their intra-membraneous location, in contrast to the external location of the fluorescent groups of the other probes. Vesicles containing 0.6 and 1.2% purified chlorophylls *b* and *a*, respectively, were mixed with a 5-fold amount of non-pigmented vesicles and added to the reaction mixture containing 100 mM NaCl/0.1 mM EDTA/5 mM Hepes (pH 7.4) and, wherever appropriate, a cationic amphipath. The total concentration of vesicles in the reaction mixture amounted to 100 μM. The efficiency of resonance energy transfer and the membrane-intermixing extent were calculated as described [28].

Leakage of liposome contents was monitored as dequenching of 5(6)-carboxyfluorescein fluorescence upon leakage and dilution of dye trapped in vesicles [34]. Assay conditions were similar to those described above for the fusion assays.

Turbidity measurements, as an assay for vesicle aggregation and size increase, were performed in a Beckman DU-8 spectrophotometer. Absorbance at 500 nm was monitored under conditions similar to those used for monitoring memrbane intermixing.

For determination of gramicidin S binding to vesicles, various concentrations of the drug were

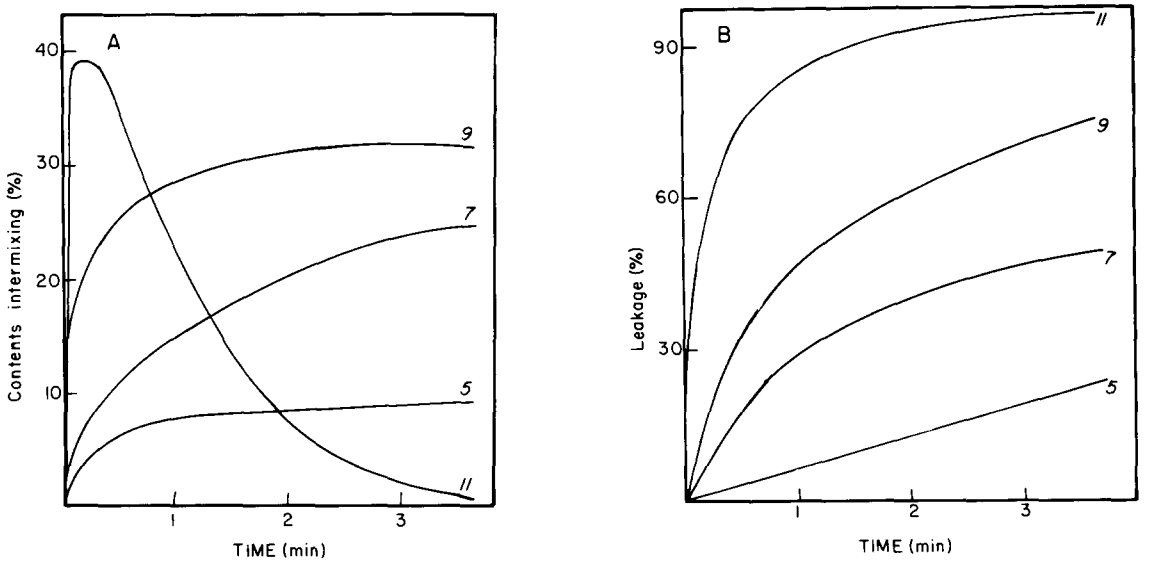


Fig. 1. The effect of gramicidin S on PC/CL liposomes. Large unilamellar vesicles (100 μM final concentration) composed of CL and PC (1:1 ratio) were added to a reaction mixture containing various concentrations of gramicidin S. Aqueous contents intermixing (A) and leakage (B) were determined as described in Materials and Methods. The numbers in the figure represent the gramicidin S concentrations in μM.

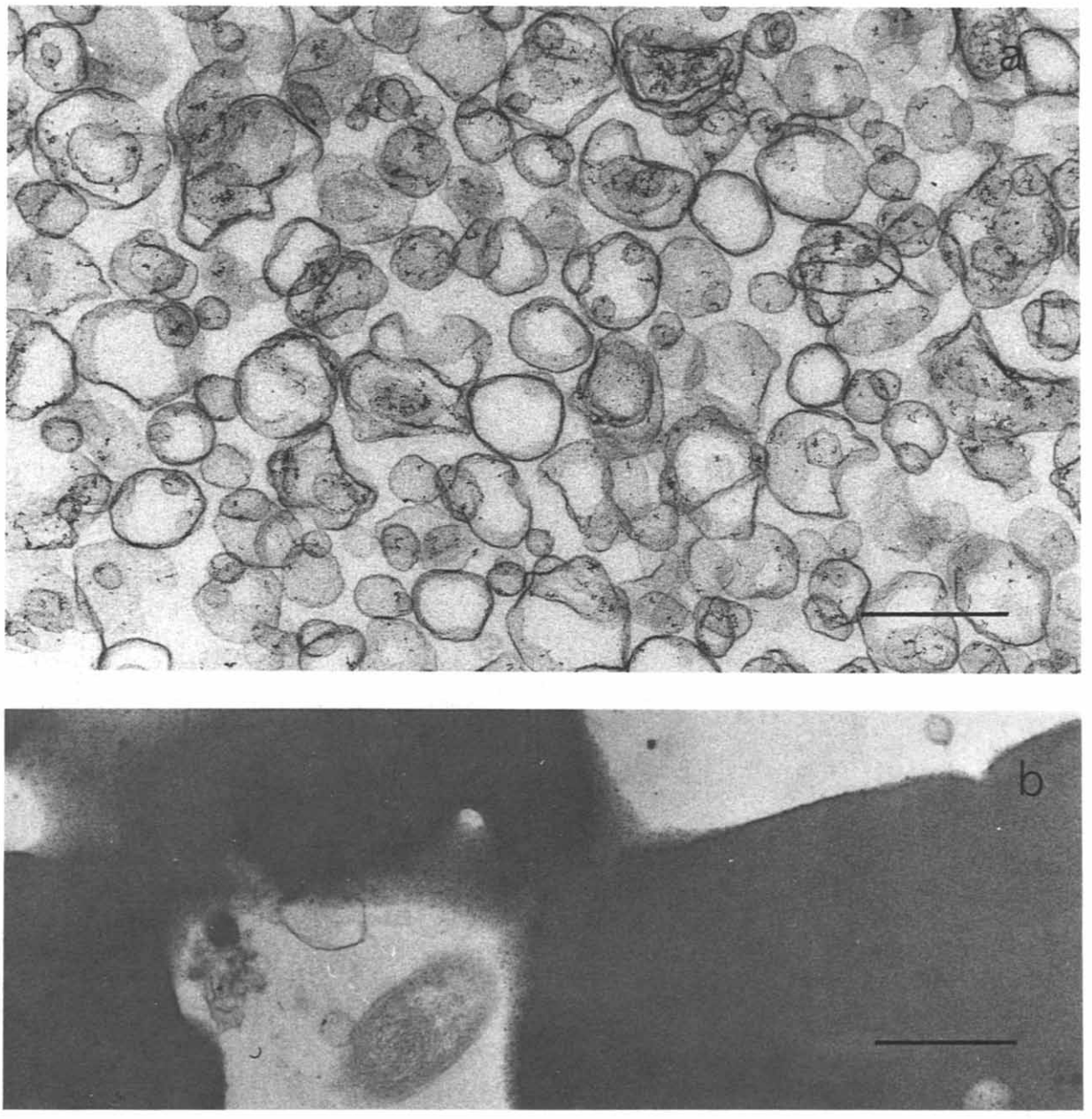
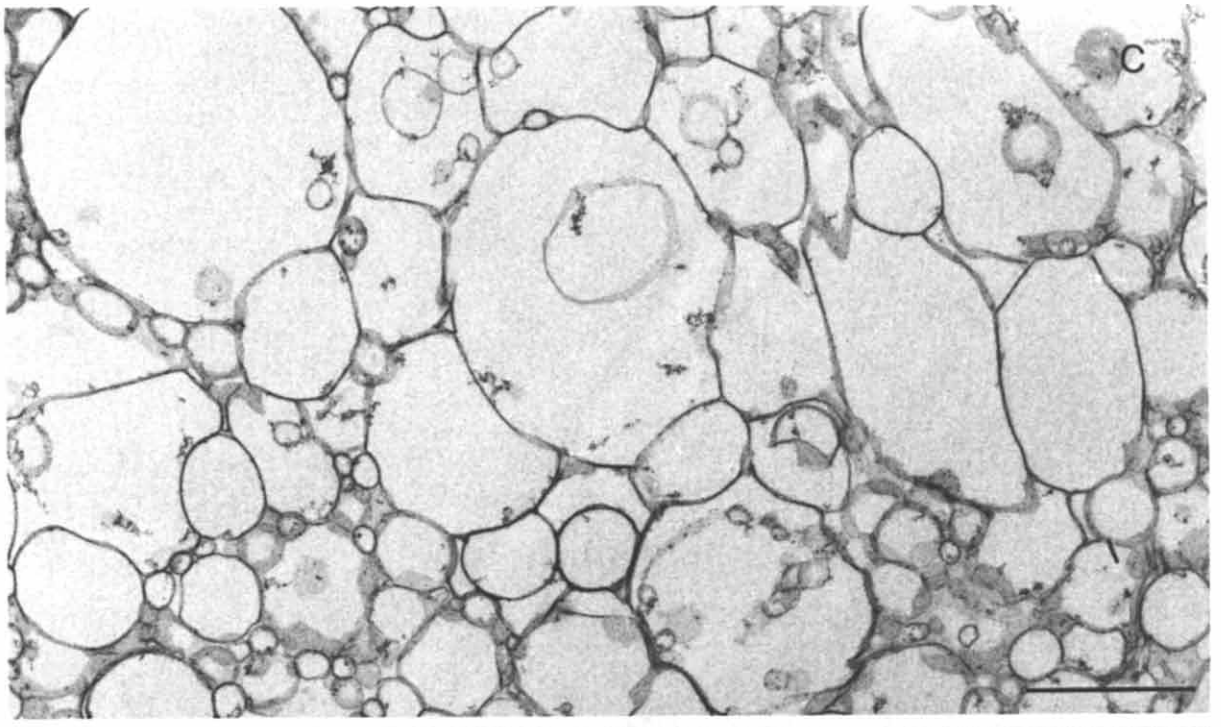


Fig. 2. Electron micrographs of liposomes fused in the presence of gramicidin S and dodecylamine. Large unilamellar vesicles composed of PC/CL (1:1) were incubated for 15 min with either gramicidin S (a) or dodecylamine (c) at a drug to CL ratio of 0.25 and processed for electron microscopy as described in Materials and Methods. Large unilamellar vesicles composed of CL were incubated with either gramicidin S (b) or dodecylamine (d) at a drug to CL ratio of 1.5. The length of the bar in the figures is (a) 1.0, (b) 0.5, (c) 0.75 and (d) 0.2 μm.

incubated for 10 min with 100 μM Tb vesicles as described above. The vesicles were removed by centrifugation at 150000 $\times$ g for 60 min and the gramicidin S remaining in the supernatant fluid was measured by the fluorescamine technique [35]. Since Hepes interfered with this assay, it was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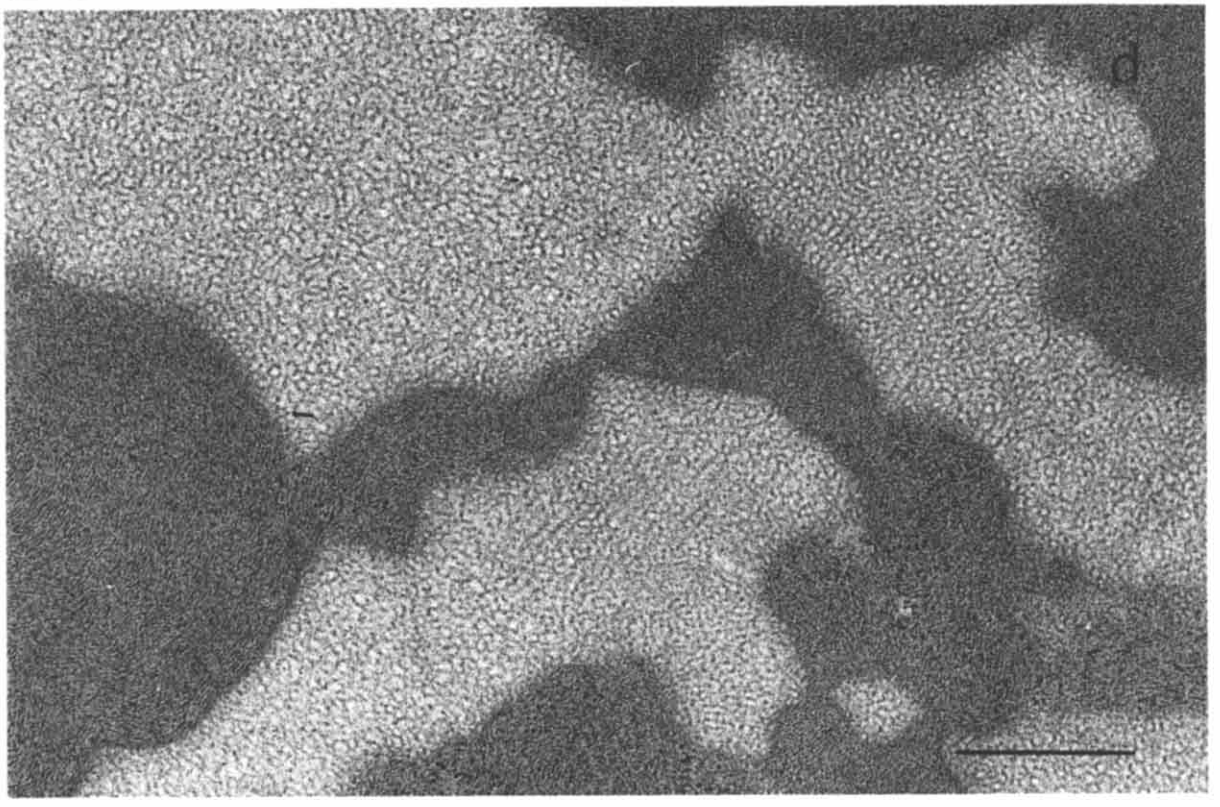
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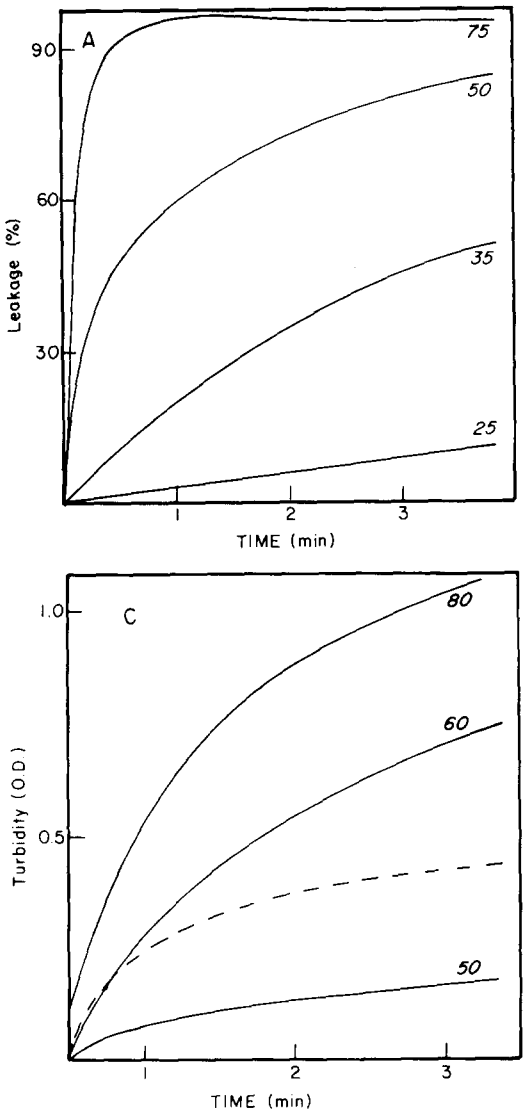
substituted in all media by sodium phosphate buffer.

Samples for electron microscopy were prepared as previously described [8].

Results

Gramicidin S-induced fusion of PC / CL liposomes

As demonstrated in Fig. 1, gramicidin S induced leakage and fusion of large unilamellar vesicles composed of PC/CL (1:1). The threshold concentration for fusion-induction was 4 μM, corresponding to a charge ratio of gramicidin S to CL of about 0.15. Already at this concentration, leakage of vesicle contents was evident. At drug concentrations higher than 10 μM, intermixing of liposome membranes was complete (data not shown), but the leakage proved massive and interfered with the intermixing of vesicle contents. Vesicles prepared for electron microscopy appeared as round vesicles of 0.1 μm diameter (data not shown). After addition of gramicidin S, the vesicles underwent a few rounds of fusion, and most of the population had a diameter of 0.3–0.6 μm (Fig. 2a). Addition of defatted bovine serum albumin before the addition of vesicles to the drug



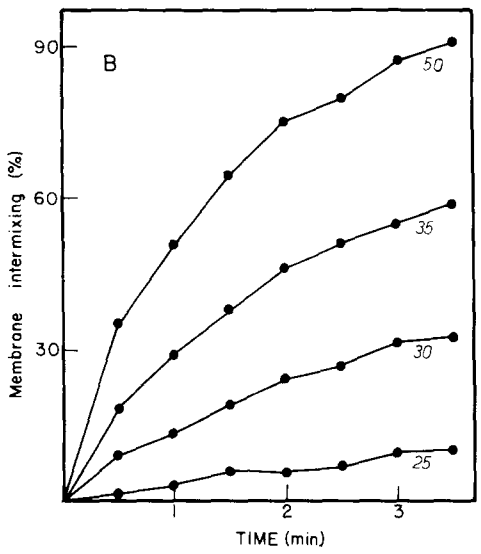


Fig. 3. The effect of gramicidin S on CL liposomes. Large unilamellar vesicles (100 μM final concentration) composed of CL were added to a reaction mixture containing various concentrations of gramicidin S. Leakage of aqueous contents (A), membrane intermixing (B) and turbidity (C) were determined as described in Materials and Methods. The increase in turbidity of a vesicle sample added to a reaction mixture containing 20 mM $CaCl_2$ is presented in C by the broken line. The numbers in the figure represent the gramicidin S concentrations in μM.

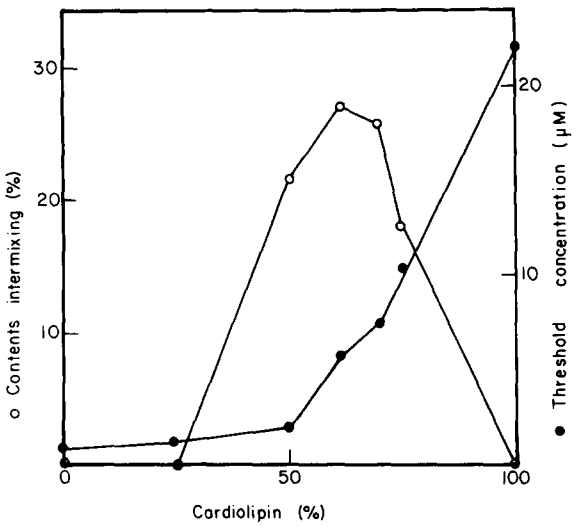


Fig. 4. The effect of liposome composition on gramicidin S-induced fusion. Large unilamellar vesicles (100 μM final concentration) composed of various proportions of PC and CL were added to various concentrations of gramicidin S and leakage of contents was determined. The minimal concentration allowing observable leakage, namely, the threshold concentration is presented in the figure (filled symbols). The extent of contents intermixing 1 min after addition of the vesicles to a medium containing double the threshold concentration is presented in the figure (empty symbols).

prevented the gramicidin S-induced fusion, presumably by binding the drug. Addition of the bovine serum albumin after the addition of the vesicles to the drug caused at least partial disaggregation, resulting in reduction in turbidity, but had no effect either on the extent of vesicle contents coalescence or on the the extent of membrane intermixing.

No intermixing of vesicle contents was observed when gramicidin S was added to liposomes composed only of CL. The concentrations of the drug needed to cause leakage from CL liposomes were higher compared to PC/CL vesicles. The leakage near the threshold concentration was accompanied by membrane intermixing, while at higher concentrations the leakage was much faster than membrane intermixing (Fig. 3). Gramicidin S induced a massive increase in turbidity of the vesicle suspesion (Fig. 3c) and electron microscopy revealed formation of large dense amorphous structures (Fig. 2b).

Fig. 4 shows that there is an optimal ratio of CL to PC in the liposomes for gramicidin S-induced intermixing of contents. At both high and low CL concentrations, leakage of vesicles occurred without contents intermixing. The threshold concentration for leakage induction was 20-times lower for pure PC vesicles compared to CL vesicles. The leakage from pure PC liposomes was unaccompanied by either membrane intermixing or aggregation assayed as turbidity (data not shown).

A possible explanation for the greater sensitivity of PC liposomes could be tighter binding of gramicidin S to these vesicles. However, as shown in Fig. 5, at the concentration range relevant to the present work, practically all the added drug was bound to CL liposomes, whereas the binding to PC liposomes was somewhat lower.

There was a synergistic effect of calcium ions and gramicidin S on liposome fusion. In the presence of subthreshold concentrations of calcium ions, lower concentrations of gramicidin S were required for promotion of fusion. This was most

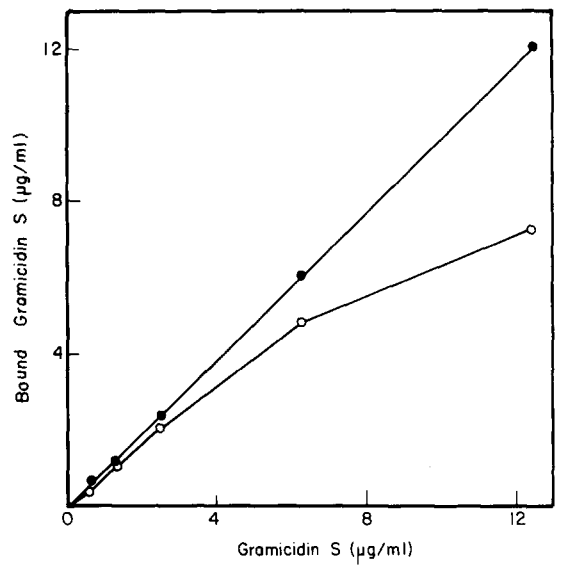


Fig. 5. Binding of gramicidin S to liposomes. Large unilamellar vesicles (100 μM final concentration) containing either PC (empty symbols) or CL (filled symbols) were added to reaction mixtures containing various concentrations of gramicidin S. After 15 min the vesicles were removed by centrifugation and the amount of gramicidin S bound to them was determined as described in Materials and Methods.

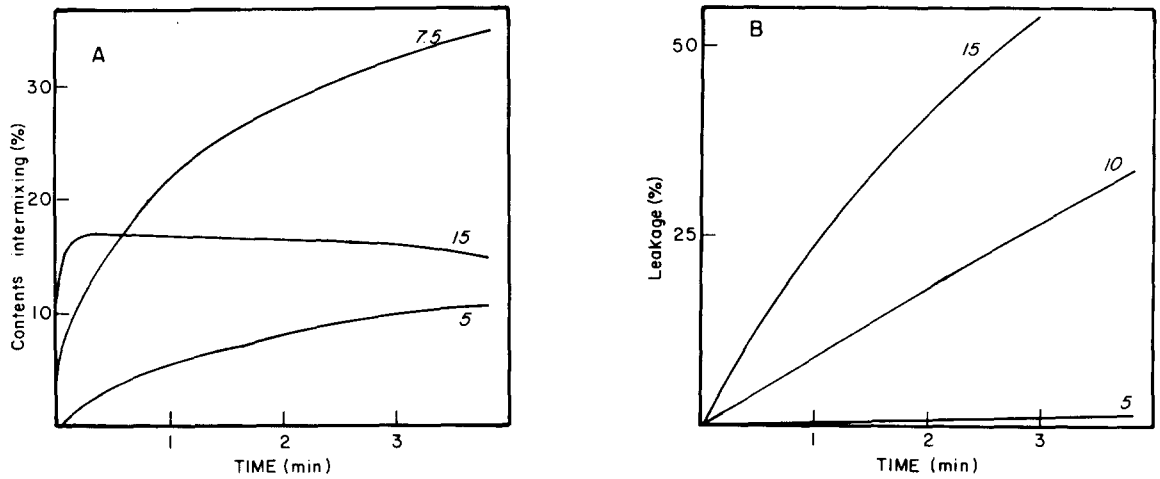


Fig. 6. The effect of gramicidin S on CL liposomes in the presence of subthreshold concentrations of calcium ions. Large unilamellar vesicles (100 μM final concentration) composed of CL were added to a medium containing $CaCl_2$ (4 mM) and various concentrations of gramicidin S. Aqueous contents intermixing (A) and leakage (B) were determined as described in Materials and Methods. The numbers in the figure represent the gramicidin S concentrations in μM.

evident with liposomes containing pure CL, where in the presence of subthreshold concentrations of calcium ions, vesicle fusion occurred at relatively low gramicidin S concentrations and resulted in intermixing of vesicle contents (Fig. 6).

In order to investigate whether the effects described above were specific for gramicidin S, the effects of another cationic amphipath, dodecylamine, have been tested. As the PC contents of the liposomes were increased, the concentration of dodecylamine required to induce leakage also decreased (Figs. 7–9). Liposomes composed of PC/CL (1:1), were fused in the presence of dodecylamine to cardiolipin ratios higher than about

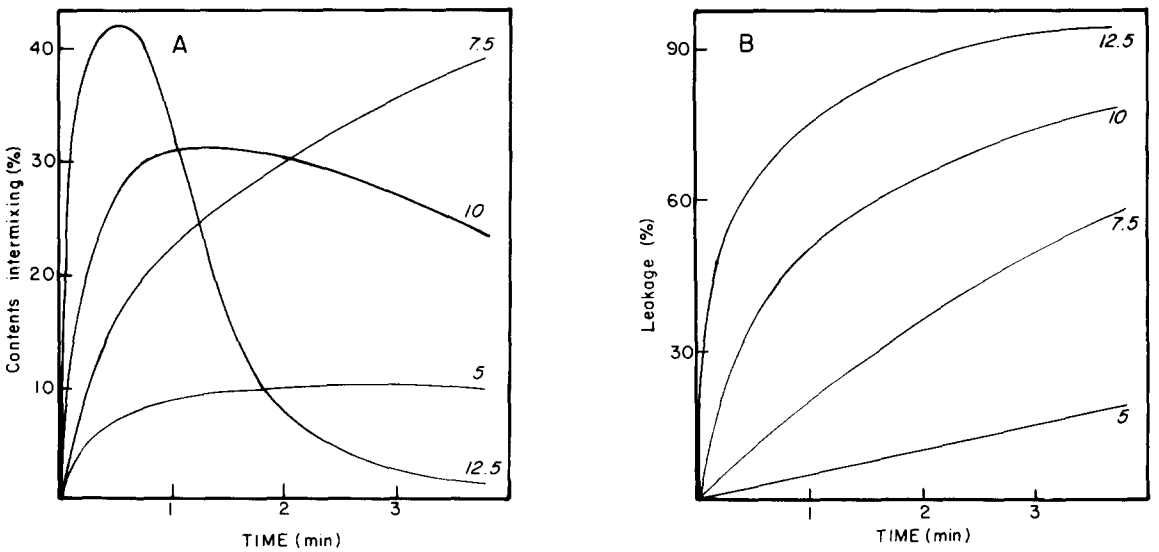


Fig. 7. The effect of dodecylamine on PC/CL liposomes. Large unilamellar vesicles (100 μM final concentration) composed of CL and PC (1:1 ratio) were added to a reaction mixture containing various concentrations of dodecylamine. Aqueous contents intermixing (A) and leakage (B) were determined as described in Materials and Methods. The numbers in the figure represent the dodecylamine concentrations in μM.

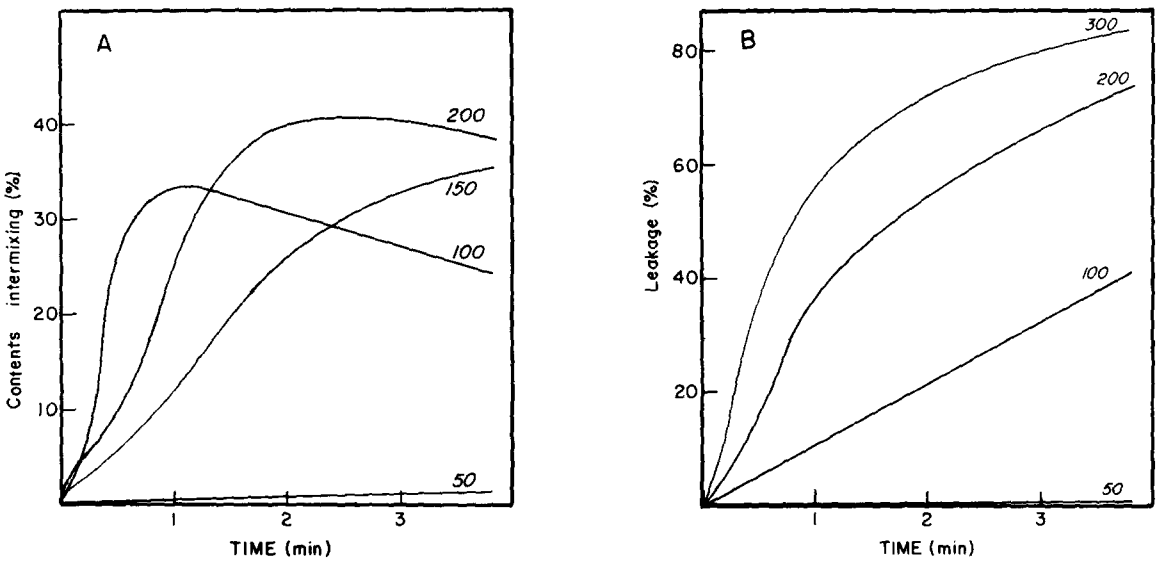


Fig. 8. The effect of dodecylamine on CL liposomes. Large unilamellar vesicles (100 μM final concentration) composed of CL were added to a reaction mixture containing various concentrations of dodecylamine. Intermixing (A) and leakage (B) of aqueous contents were determined as described in Materials and Methods.

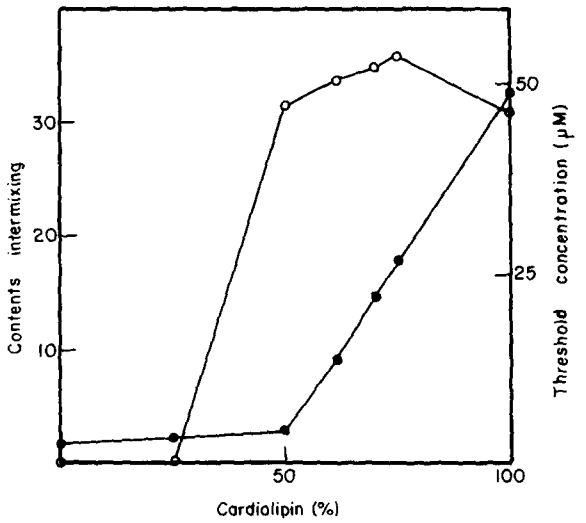


Fig. 9. The effect of liposome composition on dodecylamine-induced fusion. Large unilamellar vesicles (100 μM final concentration) composed of various proportions of PC and CL were added to various concentrations of dodecylamine and leakage of contents was determined. The minimal concentration allowing observable leakage, namely, the threshold concentration is presented in the figure (filled symbols). The extent of contents intermixing 1 min after addition of the vesicles to a medium containing double the threshold concentration is presented in the figure (empty symbols).

0.1. Electron microscopy revealed that the product of dodecylamine-induced fusion was vesicles with diameters ranging from 0.2 μm to several μm (Fig. 2c). In contradistinction to gramicidin S, dodecylamine did induce fusion of CL vesicles, resulting in intermixing of contents but only limited leakage of contents. The threshold concentrations for both fusion and leakage of contents was a ratio of dodecylamine to CL of about 0.5. At this concentration range the fusion product appeared as round vesicles with diameters of about 0.2–0.5 μm (data not shown). At higher dodecylamine concentrations, the vesicular structure seemed to collapse and instead two paracrystalline structures were formed, one with a basic cubic unit of 20 nm dimension and the other composed of densely packed membranes (fig. 2d).

Discussion

Despite the apparent structural difference between gramicidin S, a circular peptide, and synthetic amphipath, dodecylamine, there is a striking similarity in their effect on leakage and fusion of vesicles containing PC and CL. Gramicidin S has

for many years been one of the most popular objects of conformational studies in the peptide field. Its relatively simple primary structure and considerable biological potency presented possibilities for elucidating its structure-function relationship (for an extensive review, see Ref. 17). Various physico-chemical and theoretical methods suggested a conformational model which resembles, in general terms, the amphipathic structure of dodecylamine. Gramicidin S assumes a 'pleated sheet' structure, with the two positively charged ornithines located in spatial proximity and forming a hydrophilic moiety, while the rest of the molecule, which is essentially non-polar, forms a hydrophobic moiety [36]. Study of the anti-microbial activity of many gramicidin S analogues revealed that changes leading either to neutralization of its positive charge or to introduction of positive charges into its hydrophobic moiety result in its inactivation [17,22]. Moreover, changes in the peptide ring, leading to separation of the ornithines and interference with the sample amphipathic nature of the molecule, abolish its activity. It should be borne in mind that these structure-function studies have been performed for the antimicrobial activity of gramicidin S, and thus their relevance to fusion and leakage from liposomes is only suggestive. Recently, it has been suggested that the amphipathic nature of the drug is also important in promoting leakage from bacteria, erythrocytes and mitochondria. The similarity in effect of gramicidin S and dodecylamine indicates that their crucial structural feature is their amphipathic nature and that the two positive charges of gramicidin S do not constitute an advantage in promoting fusion.

The generally accepted concept of the fusion process is that initially the membranes have to aggregate and, subsequently, upon destabilization of the bilayer structure fusion occurs [37,38]. A possible explanation for the efficacy of cationic amphipaths, such as dodecylamine and other amines [39], gramicidin S, polymyxin B [9,10] and melittin [12], is that they bind tightly to the membranes and their positive charges attract negative charges located on other vesicles. Formation of intermembrane dehydrated complexes could by itself lead to destabilization of the membranes, as proposed by Papahadjopoulos et al. [38] for the calcium-induced fusion of acidic liposomes. On the other hand, these amphipaths possess the capacity to destabilize membranes, and at high enough concentrations serve as detergents and transform the lipid bilayer into small mixed micelles of lipid/amphipath (for an example, see Refs. 19 and 20). Physico-chemical studies have suggested that gramicidin S, in contradistinction to polymyxin B interacts only with the headgroups of PC liposomes and is not inserted into the hydrophobic core of the membrane [17,19,20]. If this is also the case for liposomes containing CL, aggregation of fusing membranes could be explained by hydrophobic interaction between bound gramicidin S molecules. However, recent studies have indicated that gramicidin S is inserted into the hydrophobic core of dipalmitoylphosphatidylcholine liposomes and immobilizes boundary lipids [22,23].

Two features of the fusion induced by gramicidin S and dodecylamine distinguish it from the fusion induced by calcium ions. The leakage induced by the amphipaths is only partially caused by liposome fusion or even aggregation. In the case of PC vesicles, leakage occurs with no apparent aggregation or fusion, and with all liposomes containing mainly PC, the leakage rate was faster than the rate of membrane intermixing (data not shown).

For this reason the fluorescence assayed in the Tb-DPA assay was not corrected as suggested by Nir and coworkers [10,41] and the fluorescence curves are actually underestimations of the actual fusion rates.

Whereas PC inhibits calcium-induced fusion and leakage of acidic liposomes, its presence in PC/CL liposomes actually increased the potency of the amphipaths in causing leakage. Since at the relevant concentration range the amphipaths bind tightly to CL liposomes, the presence of CL seems to interfere with the capacity of the amphipaths to induce lysis. The phenomenon is not explained by simple charge neutralization, since the increase in threshold concentrations needed to induce leakage in CL liposomes is 10-fold higher compared to PC/CL liposomes. The greater sensitivity of PC liposomes to lysis by gramicidin S has been described in the past [21], and it has been suggested that since liposomes composed of PC and phos-

phatidylinositol are sensitive to the effect of the drug, while liposomes composed of phosphatidylserine and phosphatidylethanolamine are immune, that the protecting element is the presence of amino group and not the charge. Clearly, the protection afforded by cardiolipin shows this explanation to be an oversimplification. The similarity between the behavior of dodecylamine and gramicidin S suggests that the lipid specificity of the latter could be explained by its amphipathic nature and cannot be due to specific interactions of certain amino acids with the liposomes.

Acknowledgments

This research was supported by the Israel Academy of Sciences and Humanities-Basic Research Foundation and by the Fund for the Promotion of Research at the Technion. We are grateful to Professor David Glick for his help with the manuscript and to David Berkovich for the drawing of the figures.

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