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Gramicidin A Induced Fusion of Large Unilamellar Dioleoylphosphatidylcholine Vesicles and Its Relation to the Induction of Type II Nonbilayer Structures†

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ABSTRACT: The fusogenic properties of gramicidin were investigated by using large unilamellar dioleoylphosphatidylcholine vesicles. It is shown that gramicidin induces aggregation and fusion of these vesicles at peptide to lipid molar ratios exceeding 1/100. Both intervesicle lipid mixing and mixing of aqueous contents were demonstrated. Furthermore, increased static and dynamic light scattering and a broadening of ^{31}P NMR signals occurred concomitant with lipid mixing. Freeze–fracture electron microscopy revealed a moderate vesicle size increase. Lipid mixing is paralleled by changes in membrane permeability: small solutes like carboxyfluorescein and smaller dextrans, FD-4(M_r ~4000), rapidly (1–2 min) leak out of the vesicles. However, larger molecules like FD-10 and FD-17 (M_r ~9400 and 17200) are retained in the vesicles for >10 min after addition of gramicidin, thereby making detection of contents mixing during lipid mixing possible. At low lipid concentrations (5 μM), lipid mixing and leakage are time resolved: leakage of CF shows a lag phase of 1–3 min, whereas lipid mixing is immediate and almost reaches completion during this lag phase. It is therefore concluded that leakage, just as contents mixing, occurs subsequent to aggregation and lipid mixing. Although addition of gramicidin at a peptide/lipid molar ratio exceeding 1/50 eventually leads to hexagonal H_{II} phase formation and a loss of vesicle contents, it is concluded that leakage during fusion (1–2 min) is not the result of H_{II} phase formation but is due to local changes in lipid structure caused by precursors of this phase. By making use of gramicidin derivatives and different solvent conformations, it is shown that there is a close parallel between the ability of the peptide to induce the H_{II} phase and its ability to induce intervesicle lipid mixing and leakage. It is suggested that gramicidin-induced fusion and H_{II} phase formation share common intermediates.

Membrane fusion is an essential event in many processes in cellular physiology and in viral infectivity. Studies on enveloped viruses in particular have provided strong evidence for direct involvement of proteins and (poly)peptides in the initiation, specificity, and control of biological fusion processes. Despite enormous progress in this field, the molecular mechanism of the dynamic structural reorganization during this process remains elusive.

Basic concepts concerning lipid structure during fusion have been derived from extensive explorations of model membrane systems. For pure lipid membranes, which in close resemblance to biomembranes are rich in type II lipids (preferring inverted nonbilayer structures, e.g., hexagonal H_{II} phase), consensus is reached that inverted micellar intermediates which have been proposed a decade ago (Verkleij et al., 1979a,b) play an important role in the fusion process (Ellens et al., 1989; Gruner et al., 1988).

Model membrane studies have also documented the lipid vesicle fusion[1] modulating activity of a variety of proteins and (poly)peptides [see for reviews Hong et al. (1987), Blumenthal (1987), and Bentz and Ellens (1988)]. A common denominator for (poly)peptide fusogens seems to be the ability of the molecule to destabilize the bilayer. This can be accomplished by either predominantly electrostatic [e.g., polylysines (Walter et al., 1986; Gad et al., 1985)] or hydrophobic [e.g., alamethicin (Lau & Chan, 1975)] interactions, but most often by a combination of both [e.g., melittin (Morgan et al., 1983; Batenburg et al., 1987a)]. Interestingly, for a number of cases, positive correlations have been found between the ability of a polypeptide to modulate vesicle fusion and the formation of type II nonbilayer lipid structures. Examples are glycophorin (Taraschi et al., 1982a,b), myelin basic protein (Smith & Cornell, 1985; Lampe & Nelstuen, 1982), a cardiotoxin (Batenburg et al., 1985), and melittin (Batenburg et al., 1987a,b). However, a satisfactory molecular picture connecting these observations has not been obtained because of

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[1] In the references quoted in the introduction, the term fusion is not always well-defined and is often used if, e.g., only lipid mixing is observed. Throughout the present paper, fusion refers to the process leading to both mixing of lipids and mixing of contents of vesicles.

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insufficient data on fusion and lipid structure in the same experimental system or because of a lack of information on the structure of the polypeptide in the membrane. Nevertheless, suggestions on the role of type II nonbilayer lipid structural intermediates in the mechanism of protein-induced biomembrane fusion are frequently made (Yamada & Ohnishi, 1986; Scheule, 1987).

In this study, we address the question on the relation between polypeptide-induced vesicle fusion and formation of type II nonbilayer lipid structures using gramicidin as a model peptide. The peptide antibiotic gramicidin A′ is a hydrophobic linear pentadecapeptide produced by *Bacillus brevis* prior to sporulation (Katz & Demain, 1977; Pschorn et al., 1982).

The primary structure of naturally occurring gramicidins is (Sarges & Witkop, 1965a,b) HCO-L-Val[1]-Gly[2]-L-Ala[3]-D-Leu[4]-L-Ala[5]-D-Val[6]-L-Val[7]-D-Val[8]-L-Trp[9]-D-Leu[10]-L-Y[11]-D-Leu[12]-L-Trp[13]-D-Leu[14]-L-Trp[15]-$NHCH_2CH_2OH$ in which Y is tryptophan, phenylalanine, or tyrosine in gramicidin A, B, and C, respectively.

Gramicidin is a strong modulator of lipid polymorphism [for a review, see Killian and de Kruijff (1986)] and can induce H_{II} phase formation both in model bilayer systems made of a variety of phospholipids and in bilogical membranes such as the erythrocyte (Tournois et al., 1987b). This effect is very specific; formylation of the four tryptophan residues as in tryptophan N-formylated gramicidin A′ (NFGRA′)[2] totally blocks the peptide-induced H_{II} phase formation (Killian et al., 1985; Aranda et al., 1987), and even a subtle change such as the replacement of Trp[11] by Phe was shown to drastically reduce the H_{II} phase promoting effect (Killian et al., 1987). In addition, recently it was reported that the solvent history of gramicidin, which can determine the folding motif of the polypeptide upon incorporation in a DOPC membrane, influences its lipid structure-modulating abilities (Tournois et al., 1987a). These authors suggested that for the induction of the H_{II} phase the $\beta^{6.3}$ conformation, which is predominant upon insertion from dimethyl sulfoxide or trifluoroethanol, is a prerequisite. Gramicidin incorporates into the membrane out of an ethanolic solution most likely as a relatively stable antiparallel dimer, without the induction of the H_{II} phase (Killian et al., 1988; Tournois et al., 1987a).

These properties make gramicidin an excellent model to establish the relationship between peptide structure and induction of nonbilayer structures on one hand and the involvement of these structures in the fusion process on the other. In view of the extensive structural characterization of the interaction of gramicidin with dioleoylphosphatidylcholine (Killian & de Kruijff, 1985; Killian et al., 1987; Chupin et al., 1987; Tournois et al., 1987a), large unilamellar vesicles of this lipid were used as an experimental system in fusion experiments.

It will be shown that gramicidin induces both vesicle aggregation and mixing of lipids of two separate populations of vesicles, which is paralleled by leakage of small solutes like carboxyfluorescein and smaller dextrans. Larger dextran molecules, however, are retained in the vesicles for a longer time, and make detection of contents mixing during lipid mixing possible. A striking correlation is observed between the peptides ability to cause vesicle fusion and H_{II} phase formation. The results suggest common structural intermediates in membrane fusion and H_{II} phase formation and are discussed in light of mechanisms proposed for fusion and H_{II} phase formation in pure lipid systems.

[2] Abbreviations: ANTS, 8-aminonaphthalene-1,3,6-trisulfonic acid; CF, 6-carboxyfluorescein; DMSO, dimethyl sulfoxide; DOPC, 1,2-dioleoyl-*sn*-glycero-3-phosphocholine; *E*, excimer fluorescence intensity; *M*, monomer fluorescence intensity; FD-4, fluorescein isothiocyanate dextran-4000; FITC, fluorescein isothiocyanate; GR, gramicidin; GR-(EtOH), gramicidin added from ethanolic solution; NFGRA′, tryptophan N-formylated gramicidin A′; pyrene-PC, 1-palmitoyl-2-(1-pyrenyldecanoyl)-*sn*-glycero-3-phosphocholine; TFE, trifluoroethanol.

Materials and Methods

Gramicidin A′, natural mixture, was obtained from Sigma (St. Louis, MO) and was used without further purification, unless otherwise stated. Gramicidins A, B, and C were obtained from the natural mixture as described by Killian et al. (1987). Tryptophan N-formylated gramicidin A′ (NFGRA′) was derived from gramicidin according to the method of Killian et al. (1985). 6-Carboxyfluorescein (Eastman Kodak Co., Rochester, NY) was purified by active carbon treatment, recrystallization from water/ethanol (2/1 v/v), and Sephadex LH20 (Pharmacia) column chromatography (Ralston et al., 1981).

1,2-Dioleoyl-*sn*-glycero-3-phosphocholine (DOPC) was synthesized and purified as described before (van Deenen & de Haas, 19694; Dekker et al., 1983). 1-Palmitoyl-2-(1-pyrenyldecanoyl)-*sn*-glycero-3-phosphocholine (pyrene-PC) was obtained from Molecular Probes Inc. (Eugene, OR). Fluorescein isothiocyanate dextrans (MW_{av} 3900, 9400, and 17 200; 0.003–0.01 mol of FITC/mol of glucose) were purchased from Sigma (St. Louis, MO).

All phospholipids and lipid probes were tested for purity by HPTLC and proven to be at least 99% pure. All other reagents were analytically pure, and solvents were of analytical grade.

Vesicle Preparation. Large unilamellar vesicles (diameter determined by dynamic light scattering to be on the average 150 nm) were prepared from DOPC (15 mM) by using the extrusion technique (0.4-μm polycarbonate filters, Nuclepore) described by Mayer et al. (1986). The unilamellarity of the vesicles was determined by means of quenching of the ^{31}P NMR signal of the outer monolayer with Mn^{2+} (Mayer et al., 1986). From these measurements, it could be inferred that 80–90% of the lipids reside in the outermost bilayer. The buffers used are specified below. Fluorescent lipids were incorporated by mixing them with DOPC prior to mixed film hydration. Unless otherwise indicated, all experiments were performed at 25 °C, and gramicidin and its analogues were added to the preformed unilamellar vesicles from 0.5 mM stock solutions in dimethyl sulfoxide by means of a Hamilton micro syringe. Phospholipids were quantified after perchloric acid destruction, by the method of Fiske and Subbarrow (1925).

Fusion Assays. In order to monitor intermixing of vesicle contents, we made use of the Tb/dipicolinic acid assay according to Wilschut et al. (1980), the 8-aminonaphthalene-1,3,6-trisulfonic acid/*p*-xylylenebis(pyridinium bromide) assay as described by Ellens et al. (1985), and also an assay using FITC–dextrans adapted from Stutzin (1986). In the FITC–dextran assay, aqueous contents mixing is monitored as dequenching of the FITC fluorescence due to dilution of vesicle contents in acceptor vesicles. FITC–dextrans of various average molecular weights 3900 (FD-4), 9400 (FD-10), and 17 200 (FD-17), were enclosed in self-quenching concentrations (FD-4, 20 mM; FD-10, 20 mM; FD-17, 4 mM) in 40 mM Tris-HCl and 40 mM NaCl at pH 7.0 in DOPC vesicles. Nonentrapped material was removed by gel filtration on a Sephacryl S-300 HR column (1 × 40 cm) in 65 mM NaCl and 40 mM Tris-HCl, pH 7.0. Contents mixing between these vesicles and a 20-fold excess of acceptor vesicles (final lipid concentration 150 μM) was monitored as fluorescence dequenching. Acceptor vesicles contained 65 mM NaCl/40 mM Tris-HCl at pH 7.0 or 40 mM NaCl, 20 mM dextran 4, and

40 mM Tris-HCl at pH 7.0. The 100% dequenching values were determined by addition of 20 μL of 10% (v/v) Triton X-100. All data points were corrected for small changes in scattering (1–10% of the total signal) upon peptide-induced aggregation or Triton X-100 addition by subtraction of the signal obtained after addition of the same amount of gramicidin to acceptor vesicles only, at the same final lipid concentration. The diameters of the FITC–dextrans (2.4, 3.2, and 4.0 nm for respectively FD-4, FD-10, and FD-17) were calculated from the radius of raffinose using the cube root ratio of their molecular weights. Fluorescence was measured on an SLM 500C spectrofluorometer (SLM instruments Inc., Urbana, IL). Excitation was at 465 nm and emission at 543 nm through a 530-nm cutoff filter.

Lipid Mixing Assay. Coalescence of membranes was monitored by fluorescence using pyrene-PC-containing vesicles. This assay is based on the characteristic ability of pyrene groups to form excimers, a process that is strongly concentration dependent (Birks, 1970; Galla & Sackman, 1974; Doody et al., 1980). To monitor lipid mixing, large unilamellar DOPC vesicles containing 10 mol % pyrene-PC were prepared in 150 mM NaCl, 0.2 mM EDTA, 0.5 mM NaN_3, and 10 mM Tris-HCl, pH 7.0. In the assay, these vesicles were mixed (1/20 mol/mol on basis of phosphorus) with unlabeled vesicles prepared in the same buffer. Excitation was at 343 nm, and emission was measured from 360 to 550 nm. The ratio of excimer (E) and monomer (M) fluorescence intensities serves as a measure for the surface concentration of pyrene-PC. Since E is the difference in fluorescence intensity at 480 nm before and after addition of TX-100, E/M is expressed as

$$E/M = (F_{480} - F_{T480})/F_{377}$$

F_{480} is the fluorescence intensity at 480 nm before TX-100 addition, F_{T480} is the intensity at 480 nm after addition of 20 μL of 10% (v/v) Triton X-100. F_{377} is the monomer intensity at 377 nm.

CF Leakage Assay. 6-Carboxyfluorescein (CF) and the FITC–dextrans were used to detect changes in barrier properties of DOPC vesicles. CF was entrapped by preparing vesicles in 50 mM CF, 40 mM NaCl, and 40 mM Tris–acetate, pH 7.0. The unencapsulated material was removed by gel filtration through a Sephadex G-75 column (1 × 30 cm; Pharmacia), eluting with 100 mM NaCl/40 mM Tris–acetate, pH 7.0. Excitation was at 430 nm, and the carboxyfluorescein release was monitored as an increase in emission intensity at 513 nm, due to a dequenching of CF fluorescence (Weinstein et al., 1977). The total amount of CF entrapped was determined by lysing the vesicles by the addition of 50 μL of 10% (v/v) Triton X-100. The CF release is expressed as

$$\% \text{ leakage} = (F_t - F_0)100/(F_T - F_0)$$

F_0 is the initial fluorescence, F_t is the fluorescence after an incubation period of t minutes, and F_T is the fluorescence after TX-100 addition. The amount CF released from vesicles in the absence of peptide (<1%/10 min) was subtracted from the peptide-induced leakage.

A comparable assay was performed using the FITC–dextrans FD-4, FD-10, and FD-17 both for control purposes in the assay for intermixing of vesicle contents and also for obtaining insight in the size of the gramicidin-induced defects in the vesicle bilayer. For prolonged incubations to determine slow leakage, vesicles with dextrans were kept in the dark.

Na^+ Leakage Assay. Vesicles were prepared in 77.5 mM sodium acetate/acetic acid, pH 5.5, buffer, and nonenclosed material was exchanged for isotonic magnesium acetate/acetic acid, pH 5.5, by means of a Sephadex G75 column. The amount of Na^+ released was measured 5 min after addition of peptide with a Na^+-specific electrode (Philips G15-Na)–reference electrode (Philips R44/2-SD/1) combination connected to a millivolt meter (Radiometer, TTT2).

Light Scattering. Static light scattering was measured on an SLM 500C spectrofluorometer under 90° at 500 nm. Vesicles were prepared in 100 mM NaCl/40 mM Tris-HCl at pH 7.0 and measured in the same buffer at a 5 or 150 μM lipid concentration. Dynamic light scattering on the same vesicles at 25 μM was measured at 632.8 nm under 90° with a Malvern 4700 (Malvern Ltd., Malvern, U.K.) equipped with a 25-mW helium/neon laser (NEC Corp., Tokyo, Japan) and using Malvern PCS software. The mean diameter and the polydispersity of the dispersions were determined with the cumulant expansion method (Brown et al., 1975).

^{31}P NMR. ^{31}P NMR was used to study the macroscopic structure of the gramicidin–DOPC system. ^{31}P NMR spectra were recorded on a Bruker MSL-300 spectrometer as described earlier (Chupin et al., 1987) using an interpulse time of 1.0 s. An aliquot of gramicidin solution (10 mM) was dropwise added to 8 mL of a vigorously stirred dispersion of large unilamellar vesicles (1.9 mM DOPC) in buffer (100 mM NaCl, 0.2 mM EDTA, and 10 mM Tris-HCl, pH 7.5) and either transferred to a 20-mm NMR tube and measured in a 20-mm dedicated ^{31}P probe (sensitive volume 8 mL) or centrifugated (17500g, 10 min at 4 °C), after which the pellet was transferred to a 10-mm NMR tube and measured in a 10-mm VSP broad-band probe. Pure H_{II} phase structures could be pelleted quantitatively, and the percentage of H_{II} phase could be determined by quantifying the amount of phosphorus in the pellet relative to the total phosphorus content; 0 ppm corresponds to the chemical shift position of the ^{31}P NMR resonance position of lysophosphatidylcholine micelles in water.

Small-Angle X-ray Diffraction. Small-angle X-ray diffraction was performed as described earlier by Killian and de Kruijff (1985).

Freeze–Fracture Electron Microscopy. For freeze–fracturing, vesicles were prepared in 100 mM NaCl, 0.2 mM EDTA, and 10 mM Tris-HCl, pH 7.0, at a lipid concentration of 15 mM. Prior to addition of gramicidin (from 10 mM stock solutions), the vesicle dispersions were diluted to 1.9 mM in order to prevent direct effects of the solvent (TFE or DMSO) used to add gramicidin. The final solvent concentration was maximally 2% v/v. After addition of the peptide, samples were centrifuged (17500g, 2 min) and subsequently resuspended in as little as possible buffer and either fast-frozen by rapid plunging into liquid propane using the plunge freezing device (KF80) manufactured by Reichert Jung (Wien, Austria) or supplemented by 30% v/v glycerol as cryoprotectant and quenched in solid–liquid N_2. The samples were subsequently fractured in a Balzers freeze–etch machine according to standard procedures. Replicas were examined in a Philips CM-10 electron microscope.

Results

Lipid Mixing. It was first established whether gramicidin induces mixing of lipids of two separate vesicle populations by means of an assay based on pyrene-PC whose fluorescence is concentration dependent. After excitation at 343 nm, the pyrenyl monomer has a fluorescence spectrum dominated by two sharp monomer emission bands with maxima at 377 and 395 nm. An excited monomer is able to dimerize with a ground-state pyrenyl group, thus forming an excimer which has a broad fluorescence band with a maximum at approxi-

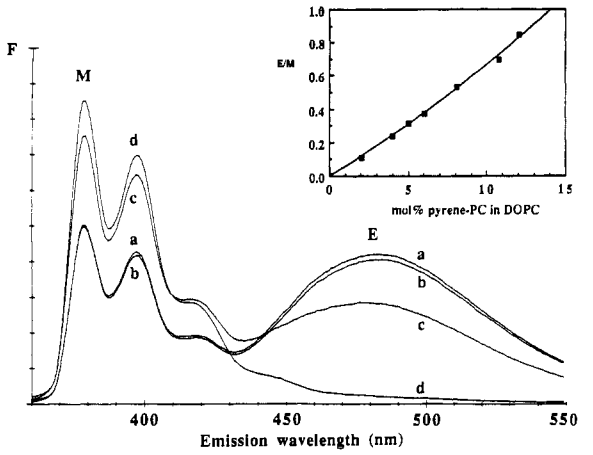

FIGURE 1: Fluorescence emission spectra of pyrene-PC (0.1 mol/mol of DOPC) (a) in large unilamellar vesicles, (b) after addition of a 20-fold excess of unlabeled vesicles (final concentration 150 μM), (c) 1 min after addition of gramicidin A′ from DMSO (0.07 mol/mol of lipid), and (d) after addition of 50 μL of 10% (v/v) Triton X-100. The latter spectrum was recorded at a 2× less sensitive scale. The insert depicts the relation between the percentage pyrene-PC in DOPC vesicles (150 μM) and the recorded E/M value.

mately 480 nm. A typical spectrum obtained from DOPC vesicles containing 10 mol % pyrene-PC is shown in Figure 1 (spectrum a). For the ratio of the intensities of the excimer (E) and monomer (M) fluorescence signals of pyrene, the following equation is valid (Birks, 1970; Galla & Sackman, 1974; Doody et al., 1980): $E/M \sim \tau T/\eta$[pyrene], where τ is the fluorescence decay time, T the absolute temperature, and η the viscosity. This indicates that at constant temperature the excimer to monomer ratio is proportional to the local probe concentration. By preparation of vesicles of varying mole percent pyrene-PC, the validity of this relation was confirmed for the range of pyrene-PC concentrations used (Figure 1, insert). Upon addition of a 20-fold molar excess of unlabeled vesicles, there is a slight decrease in intensity of both excimer and monomer emissions due to the dilution of the fluorescently labeled vesicles (curve b), but the ratio of E/M remains constant (0.715). Subsequent addition of gramicidin leads to a large decrease in excimer and an increase in monomer intensity (curve c), resulting in a decreased E/M ratio. Addition of Triton X-100 leads to lysis of the vesicles and a disappearance of the excimer band (curve d). Addition of dimethyl sulfoxide did not show a significant effect on the E/M ratio in the concentration range used to add gramicidin (data not shown). Because the E/M ratio is also dependent on the fluidity (η^{-1}) of the environment and on the fluorescence decay time of the excimer, which both can be influenced by protein incorporation of phase changes, as a control, the E/M ratio was measured upon addition of gramicidin to 10% pyrene-PC-labeled vesicles only, with an identical final lipid concentration (i.e., 150 μM). This results in a slight decrease in the E/M ratio which can be entirely explained by a reduction of the surface concentration of pyrene-PC as a result of the gramicidin incorporation. Therefore, the large reduction in E/M ratio induced by gramicidin in Figure 1 is mainly the result of lipid mixing.

Figure 2 shows the time course of the effect of gramicidin on the E/M ratio at 5 and 150 μM DOPC concentrations. Clearly, incorporation of gramicidin immediately leads to a *drastic* decrease in the E/M ratio during the first minute after addition. Ninety degree static light scattering demonstrated that the decrease in E/M is paralleled by a considerable increase in average particle size (data not shown). Dynamic light

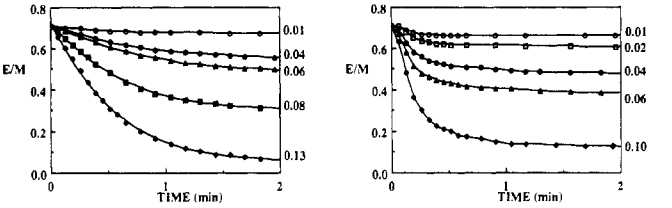

FIGURE 2: Effect of gramicidin A′ on the E/M ratio of DOPC vesicles containing 10% (mol/mol) pyrene-PC, in the presence of a 20-fold excess of unlabeled vesicles. The final lipid concentrations were (left) 5 μM and (right) 150 μM. Gramicidin to DOPC molar ratios are indicated in the figure.

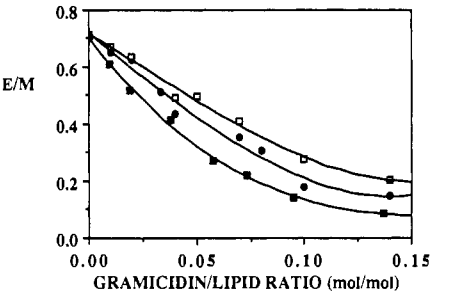

FIGURE 3: Gramicidin dependence of lipid mixing 1 min after addition of gramicidin A′ added from DMSO to vesicles of DOPC containing 10 mol % pyrene-PC, in the presence of a 20-fold excess of unlabeled vesicles at final lipid concentrations of 5 (□), 75 (●), and 150 μM (■).

scattering was used to quantify the average size of particles before and after addition of gramicidin and showed an increase from approximately 150 to 340 nm (at a 0.04 molar ratio of gramicidin to lipid, lipid concentration 25 μM). These data clearly demonstrate gramicidin-induced mixing of lipids in unilamellar vesicles, accompanied by aggregation and/or an increase in vesicle size.

In both the lipid mixing and light-scattering experiments, a plateau is reached 1 min after gramicidin addition. The extent of lipid mixing after 1 min as a function of the gramicidin concentration is shown in Figure 3. A significant decrease in E/M is already observed at a gramicidin to lipid ratio as low as 0.01. The largest decrease of E/M at a particular gramicidin/lipid ratio is observed for the highest lipid concentration. Comparable results were obtained if gramicidin was added from TFE instead of DMSO (data not shown).

Lipid mixing was also determined by monitoring changes in fluorescence energy transfer between the headgroup-labeled *N*-(7-nitro-2,1,3-benzoxadiazol-4-yl)phosphatidylethanolamine (*N*-NBD-PE) and *N*-(lissamine rhodamine B sulfonyl)-dioleoylphosphatidylethanolamine (*N*-Rh-PE) as described by Struck et al. (1981). However, gramicidin was found to quench the fluorescence of both these probes to such an extent that use of an assay based on these probes to quantify the extent of lipid mixing was unreliable. Comparable complications of probe quenching have been described earlier (Parente & Lentz, 1986; Eytan et al., 1988). Results obtained with this assay (data not shown) were nevertheless found to be qualitatively in agreement with those obtained by using pyrene-PC to monitor lipid mixing.

In experiments on aqueous contents mixing by means of the Tb/dipicolinic acid (Wilschut et al., 1980) and the ANTS/*p*-xylylenebis(pyridinium bromide) assay (Ellens et al., 1985), we noticed a pronounced gramicidin-induced leak of Tb^{3+} or ANTS under conditions of lipid mixing. Before presenting an alternative approach toward measuring coalescence of aqueous contents in this system, let us first consider the gramicidin-induced loss of barrier function of the vesicle

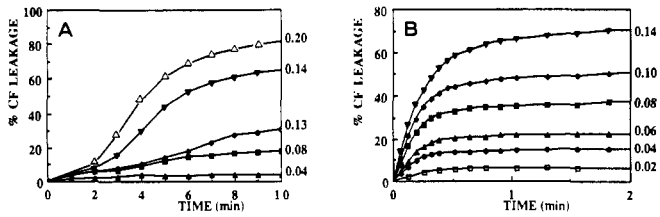

FIGURE 4: Leakage of CF from DOPC vesicles induced by gramicidin A' at a total lipid concentration of (A) 5 μM and (B) 150 μM. Molar ratios of gramicidin to DOPC are indicated in the figure.

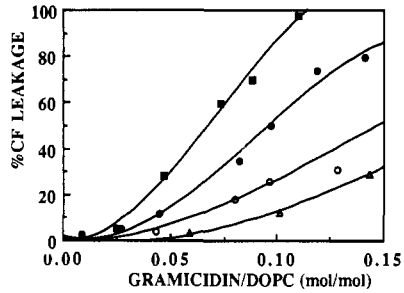

FIGURE 5: Gramicidin concentration dependence of CF leakage, measured 10 min after addition at final lipid concentrations of 1.2 (△), 5 (○), 10 (●), and 150 μM (■).

membranes as established in studies using the carboxyfluorescein assay.

Leakage of Contents. Gramicidin is well-known for its ability to form channels for small monovalent cations (Hladky & Haydon, 1970; Urry, 1972). Indeed, leakage of Na^+ from vesicles (200 μM) was already detectable at a molar ratio of 10^{-6} gramicidin/DOPC (this study). At a 10-fold higher molar ratio, complete loss of enclosed Na^+ occurs within 1.5 min (data not shown). Only at much higher molar ratios, gramicidin causes rapid release of CF from vesicles (Figure 4), possibly as a consequence of its effect on the lipid organization. In contrast to lipid mixing (compare with Figure 2), at a lipid concentration of 5 μM, the gramicidin-induced CF leakage shows a lag time of approximately 1–2 min, followed by a steep increase that levels off after another 4 min (Figure 4A). The lag time is strongly temperature dependent: at 10 °C, a lag of 2–3 min was observed whereas at temperatures above 30 °C no lag phase could be detected. Whereas rates of gramicidin-induced lipid mixing appeared to be only slightly dependent on lipid concentration (Figure 3), a strong vesicle concentration dependence was noticed for the CF release. At 150 μM DOPC, the CF leakage has lag times no longer than 15 s, and in all cases, a plateau is reached after 1 min (Figure 4B). Furthermore, at this higher lipid concentration, gramicidin-induced leakage of CF is much faster and more extensive then at 5 μM (compare Figure 4A). These data reveal different kinetics for CF leakage and lipid mixing. From the gramicidin dependence of the induced CF leakage (Figure 5), it can be concluded that leakage is only observed above a threshold molar ratio of 0.025 or 0.04 for lipid concentrations of 150 and 1.2 μM, respectively. Above this threshold value, the extent of CF release increases roughly linearly with the peptide/lipid ratio. Addition of gramicidin from TFE instead of DMSO leads to virtually the same results [data not shown; see also Tournois et al. (1987a)].

By comparing the time scales of lipid mixing and CF leakage [Figures 2 (left panel) and 4A], it can be noticed that at a lipid concentration of 5 μM, lipid mixing precedes CF leakage and is already considerable within the lag time of leakage. Also the gramicidin dependence of both processes is different in that lipid mixing does not show a threshold ratio and even at high (e.g., 0.13) gramicidin/DOPC ratios, which give rise

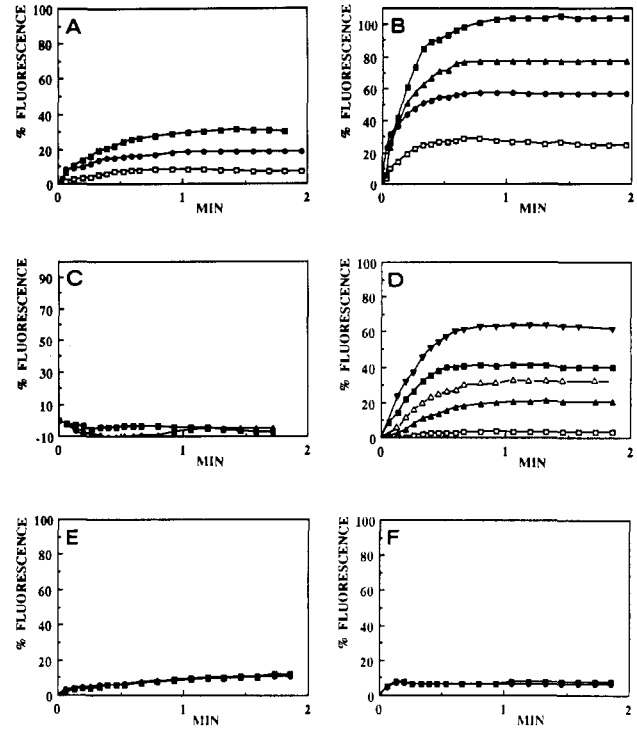

FIGURE 6: Dequenching of FTIC-fluorescence induced by addition of gramicidin to FITC–dextran containing large unilamellar vesicles either in the absence (A, C, and E) or in the presence of a 20-fold excess of acceptor vesicles (B, D, and F). FITC–dextrans used were FD-4 (A, B), FD-10 (C, D), and FD-17 (E, F). The total lipid concentration was 150 μM. Gramicidin was added in molar ratios of 0.02 (□), 0.04 (●), 0.05 (▲), 0.06 (△), 0.07 (■), and 0.10 (▼). Enclosed FD concentrations were FD-4, 20 mM; FD-10, 20 mM; and FD-17, 4 mM.

to a decrease in the E/M ratio of approximately 90% (Figure 2, left), only approximately 30% leakage of CF is observed (Figure 4A). The data indicate that, depending on the vesicle concentration, membrane fusion and leakage can be resolved in time.

Mixing of Aqueous Contents. We made use of FITC–dextrans in order to determine the size of the defect in the permeability barrier induced by gramicidin and to establish if, as a consequence of membrane fusion, mixing of aqueous contents occurs. The strategy in the experiments is to enclose FITC–dextrans of various sizes in DOPC vesicles and to measure in one set of experiments their possible release (increase in fluorescence due to dilution in the medium) and in another set of experiments their possible dilution into vesicles that do not contain FITC–dextran, as a result of gramicidin-induced fusion. Figure 6 shows leakage and contents mixing induced by gramicidin and monitored by dextrans of various sizes. Gramicidin-induced leakage of FD-4 (Figure 6A) is almost as efficient as CF leakage under comparable conditions (compare with Figure 4B). Upon increasing the molecular weight of the FITC–dextrans from 3900 to 9400 or 17 200, virtually no leakage is observed on the time scale of the experiment (Figure 6C,E), demonstrating a defined size or size limit of the gramicidin-induced defect in the bilayer. Interestingly, significant gramicidin-induced leakage of larger dextrans could be detected during prolonged incubation. Addition of gramicidin (0.10 mol/mol of lipid) to vesicles (150 μM) containing FD-10 (20 mM) or FD-17 (4 mM) and prolonged incubation lead to a leakage of the dextrans which amounts to 25% of the total fluorescence after 20 min (data not shown). This result suggests a time-dependent general loss in barrier function.

Whereas in the case of CF the extent and rate of dequenching are unaffected by the presence of acceptor vesicles (data not shown), the dequenching of the FITC fluorescence of FD-4 and FD-10 occurs more readily and to a larger extent in the presence of acceptor vesicles (Figure 6B,D). In the case of FD-4, all fluorescence is regained within 1 min after addition of gramicidin (0.07 mol/mol; Figure 6A). The most obvious explanation of these results is a preferential dilution of the FITC–dextrans into the lumen of acceptor vesicles as a result of gramicidin-induced vesicle fusion. Such aqueous contents mixing is most obvious in the case of FD-10, where virtually no leakage is observed (compare Figure 6C,D). The largest dextran tested (FD-17) reveals neither leakage (Figure 6E) nor mixing of contents (Figure 6F) in the time range studied. DMSO up to 3% (v/v), the maximal concentration introduced in the system as a solvent for gramicidin, does not show any effect on the fluorescence (data not shown). Because relatively high concentrations of FD's are used in this assay (4–20 mM), several controls were performed to exclude the possibility that the dextrans themselves affect the fusion or leakage properties of the vesicles. First, dextrans do not seem to affect the extent or rate of fusion since enclosure of 20 mM nonfluorescent dextran-4 does not affect gramicidin-induced lipid mixing as monitored with pyrene-PC (data not shown). Second, when CF (20 mM) is enclosed together with dextran-4 (20 mM), the gramicidin-induced CF release is comparable to that found in vesicles without dextran-4 (data not shown). Furthermore, in the presence of acceptor vesicles, CF dequenching is not enhanced as was found in the case of FD-4. Third, it might be anticipated that FITC–dextrans bind to the membranes depending on their solubility and molecular weight which might cause them to act as lipid mixing probes. This would also prevent FD-17 and FD-10 from being diluted. Stable binding (>10 min) of FD-17 (4 mM) to DOPC membranes, however, could not be detected by gel filtration experiments (data not shown). We cannot exclude weak or reversible binding of FD-17 to the lipid bilayer, which could complicate the interpretation of the data. Finally, gramicidin-induced increase in light scattering of vesicles is virtually the same in the absence or presence of incorporated dextran-4 (20 mM). It cannot be excluded that the larger FITC–dextrans become entrapped in H_{II} phase structures which could prevent them from leaking out. This could result in an absence of dequenching and an apparent fusion signal, when in fact H_{II} phase formation occurs. This would, however, not explain the lack of fluorescence increase of FD-17 in the presence of acceptor vesicles.

Effect of Different Gramicidin Analogues and Conformations. In order to get insight into the mechanism of gramicidin-induced vesicle fusion, some gramicidin analogues and different solvent-induced molecular conformations of gramicidin were investigated for their ability to induce lipid mixing (Figure 7A) and CF leakage (Figure 7B). Replacement of Trp^{11} in gramicidin A by Phe (yielding gramicidin B) drastically reduces the H_{II} phase inducing ability as compared to gramicidin A or A', whereas replacement by Tyr (gramicidin C) only leads to a small change in this respect (Killian et al., 1987). Interestingly, the same order in activity is found for induction of lipid mixing GR A > GR C ≫ GR B (Figure 7A). Table I gives the final membrane pyrene-PC concentrations which are obtained 1 min after addition of various peptides to a mixture of labeled and unlabeled vesicles at a molar ratio of 0.07 and starting at 10 mol % pyrene-PC. Tryptophan N-formylated gramicidin (NFGRA') and gramicidin added from ethanolic solution [GR(EtOH)], which have

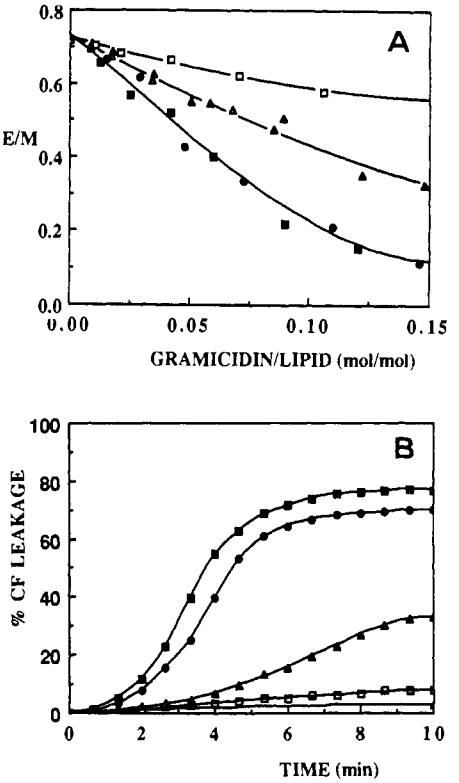

FIGURE 7: Lipid mixing after 1 min (A) and leakage of carboxyfluorescein (B) induced by gramicidins A (■), B (▲), and C (●) and NFGRA' (□) added from DMSO and by gramicidin A added from ethanol (△). The final lipid concentration was 5 μM (A) and 150 μM (B). In (B), the peptide/lipid ratio was 0.07.

Table I: Membrane Concentration of Pyrene-PC in Large Unilamellar DOPC Vesicles and Percentage of CF Release after Addition of Peptide at a Molar Ratio to Lipid of 0.07[a]

compound	mol % pyrene-PC[b]	% CF-release[c]
none	10.0	<1
gramicidin A'	1.7	80
gramicidin A	1.7	80
gramicidin B	5.6	34
gramicidin C	1.8	70
NFGRA'	6.8	8
GR(EtOH)	5.3	4

[a] The concentration of pyrene-PC prior to addition was 10 mol %. [b] Measured 1 min after addition of peptide at a lipid concentration of 150 μM. [c] Measured 10 min after addition of peptide at a lipid concentration of 5 μM.

both lost the ability to induce H_{II} phase formation in DOPC (Killian et al., 1985; Tournois et al., 1987a), have strongly reduced lipid mixing capabilities as compared with gramicidin A added from DMSO (Figure 7A) or TFE (not shown). A striking correlation is also found between the ability to induce leakage of CF in DOPC vesicles (Figure 7B and Table I) and the ability to induce H_{II} phase formation. Whereas gramicidins A and A' (not shown) are nearly equally efficient and gramicidin C is only slightly reduced in its ability, gramicidin B has partially and NFGRA' and GR(EtOH) have almost totally lost their capacity to cause release of CF from vesicles (Table I).

Interestingly, these data show that NFGRA' and GR(EtOH) are able to induce a limited degree of lipid mixing without inducing CF leakage. In order to see if these peptides are able to induce mixing of vesicle contents, they were tested in the contents mixing assay, using FD-4. NFGRA' (molar ratio to lipid 0.1) induces a comparably small (5–10%) increase in the fluorescence of FD-4 in the presence or absence of

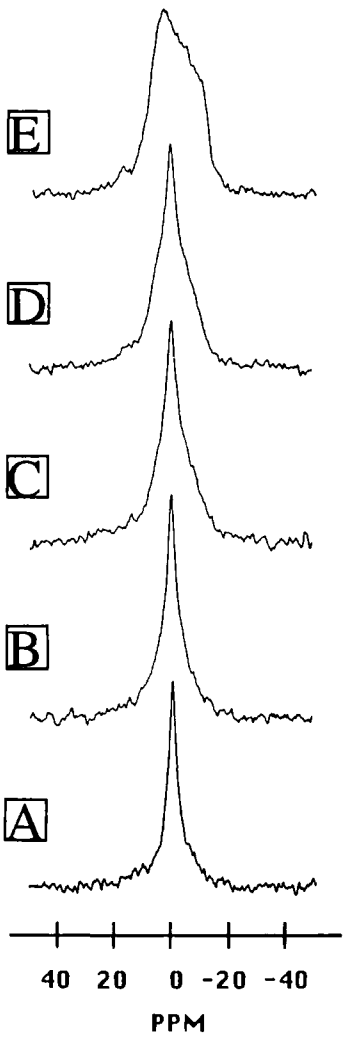

FIGURE 8: Effect of gramicidin A′ on 121.5-MHz proton-decoupled ^{31}P NMR spectra of large unilamellar DOPC vesicles (1.9 mM). Gramicidin to DOPC molar ratios are (A) 0, (B) 0.04, (C) 0.07, and (D) 0.10. (E) was the same as (D) except that the spectrum was recorded after pelleting the sample by centrifugation. Spectra A–D originally had the same integral intensity but are scaled to the same height. Spectrum E results from 30% of the total lipid. Gramicidin was added from DMSO such that in all samples, including (A), the final DMSO concentration was 2% v/v.

unlabeled vesicles, demonstrating that the peptide is unable to induce large-scale leakage or mixing of contents. Upon addition of gramicidin A′ from ethanol (molar ratio to lipid 0.1), the FD-4 fluorescence increase is slightly but significantly larger in the presence (20–30%) than in the absence (10%) of unlabeled vesicles, indicating limited mixing of contents (data not shown).

H_{II} Phase Formation. ^{31}P NMR was used to determine whether gramicidin addition results in H_{II} phase formation for the large unilamellar vesicles used in the fusion experiments. This technique can conveniently detect this phase (Cullis et al., 1985). Large unilamellar DOPC vesicles of the size used give rise to a sharp, nearly isotropic ^{31}P NMR signal due to motional averaging of the chemical shift anisotropy arising from the phospholipid lateral diffusion and vesicle tumbling (Burnell et al., 1980). The spectrum does not change upon addition of 2% DMSO (Figure 8A). Addition of gramicidin in DMSO leads to a broadening of the ^{31}P NMR signal as is shown in Figure 8B–D for various gramicidin/lipid ratios. Although such line shapes cannot be satisfactory interpreted in terms of lipid structure, the line broadening indicates the formation of larger structures in which only partial averaging of the chemical shift anisotropy occurs (Burnell et al., 1980). Addition of gramicidin leads to a precipitation of some of the lipid as a result of vesicle aggregation and/or fusion. The precipitate can be readily collected either by centrifugation (17500*g*, 10 min at 4 °C) or simply by allowing it to settle to the bottom of the tube which occurs in approximately 30 min. The resuspended precipitate (gramicidin/DOPC ~ 0.1), collected by centrifugation (Figure 8E) or by spontaneous sedimentation (not shown), shows a ^{31}P NMR signal characteristic of a hexagonal H_{II} lipid organization (Cullis et al., 1985), with a low-field peak and a high-field shoulder and a chemical shift anisotropy of approximately 20 ppm. In the suspension before centrifugation (Figure 8D), no defined signal originating from the H_{II} phase can be detected. This might be due to a combination of the presence of a large superimposed isotropic signal masking an underlying H_{II} phase signal and the fact that H_{II} phase structures are small and highly curved. Already at a molar ratio of 0.02 gramicidin to DOPC, small amounts of H_{II} phase could be spun down and the amount of H_{II} phase quantified by determination of the amount of lipid in the pellet as specified above, increased in a gramicidin-dependent way (not shown). Using this method, it could be determined that at a molar ratio of 0.10 and after 30 min the H_{II} phase is the final configuration for approximately 30% of the lipid. Small-angle X-ray diffraction experiments performed on precipitated or centrifuged samples with gramicidin added from DMSO or TFE (gramicidin/DOPC ~ 0.1) revealed, in conjunction with the ^{31}P NMR data and freeze–fracture data (see below), H_{II} phase formation by the appearance of reflections at 56.8 and 33.6 Å with a H_{II} phase characteristic relation of $1:(1/\sqrt{3})$ (Luzzati, 1980) (data not shown).

We have examined the products of fusion by freeze–fracture electron microscopy (Figure 9). The control vesicles show a reasonable homogeneity in size measuring diameters of 50–200 nm (Figure 9A) which is in good agreement with the dynamic light-scattering data. In centrifuged samples that were quenched 5 min after addition of gramicidin (0.10 mol/mol of DOPC) from TFE, highly curved H_{II} phase structures are observed (Figure 9C). Next to these structures are numerous vesicles that are seemingly unaffected by the peptide as their size falls within the range of the control vesicles; however, some vesicles are considerably larger than the largest vesicles found in the controls (~400–500 nm), revealing a moderate general size increase. In samples resuspended in buffer containing glycerol (30% v/v) as a cryoprotectant, and quenched as 5 min after gramicidin addition, comparable morphology was observed with the exception that in rare occasions vesicle aggregates could be visualized with numerous lipidic particle-like structures located at zones where membranes are closely apposed (data not shown). Such structures have not been observed in the absence of glycerol. Because concentrations of both lipid and peptide necessary for freeze–fracture electron microscopy experiments are roughly 10-fold higher then those used in fusion assays, in view of the fast kinetics (at 150 μM, fusion is complete within 1 min), early steps in fusion are not expected to be visualized with this technique.

Discussion

The aim of this study was to investigate the possible relationship between peptide-induced fusion of large unilamellar DOPC vesicles and the induction of nonbilayer lipid structures, using gramicidin A′ as a model peptide. The data demonstrate that gramicidin induces fusion of DOPC large unilamellar vesicles. Gramicidin-induced intervesicle lipid mixing could

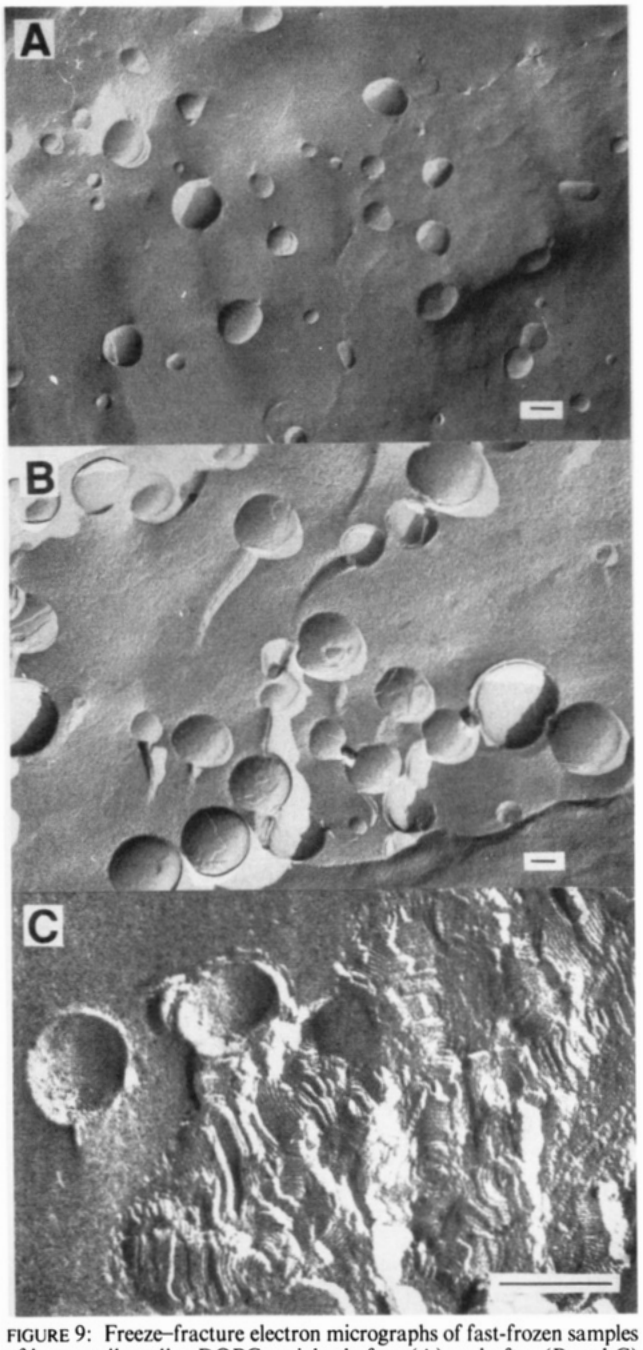

FIGURE 9: Freeze–fracture electron micrographs of fast-frozen samples of large unilamellar DOPC vesicles before (A) and after (B and C) addition of gramicidin (0.1 mol/mol of DOPC from TFE). 15 mM vesicle dispersions (A) were diluted to 1.9 mM prior to addition of gramicidin. After addition of the peptide, samples were centrifuged (17500*g*, 2 min) and subsequently resuspeneded in 50 μL of buffer and fast-frozen by rapid plunging into liquid propane (B and C) 5 min after addition of the peptide. Bars indicate 150 nm.

be demonstrated by means of a fluorometric assay based on the lipid analogue pyrene-PC. Mixing of aqueous contents could be shown to occur by use of FITC–dextrans. Furthermore, it is shown that gramicidin causes (i) an increase in (static and dynamic) light scattering concomitant with the lipid mixing and contents mixing, (ii) a broadening of the ^{31}P NMR signal of the large unilamellar vesicles corresponding to the formation of larger structures, and (iii) a moderate vesicle size increase revealed by freeze–fracture electron microscopy.

Let us consider the various effects of the peptide on the lipid system in more detail, first at a lipid concentration of 150 μM. At a molar ratio exceeding 0.01, intervesicle lipid mixing can be detected, indicating coalescence of donor membranes and unlabeled acceptor membranes. Both the rate and the extent of lipid mixing increase with the molar ratio of gramicidin. This lipid mixing is paralleled by an increase in light scattering. Also at low molar ratios (0.01) mixing of aqueous contents can be demonstrated by using FD-4 and FD-10-based assays. At gramicidin/DOPC molar ratios exceeding 0.025, the gramicidin-induced vesicle fusion is accompanied by a rapid leakage of enclosed solutes like CF, Tb^{3+}, and ANTS. This leakage is in agreement with recently published data (Tournois et al., 1987a) and is not due to the channel function of gramicidin, since CF, Tb^{3+}, and ANTS cannot pass through the gramicidin channel. Interestingly, the observed leakage is dependent on the size of the enclosed solute. Whereas CF and FD-4 show a considerable leakage, FD-10 and FD-17 are retained in the vesicles for longer times, indicating that gramicidin induces membrane defects of specific size. On the basis of the FD leakage experiments, the maximal diameter of the putative defects can be estimaed to fall between 2.4 and 4 nm. Similar defects have also been observed as a consequence of gramicidin incorporation in erythrocytes (Classen et al., 1987) and were estimated to have slightly smaller diameters of 1–3.4 nm. It has been suggested that this gramicidin-induced leakage is mechanistically related to H_{II} phase formation (Tournois et al., 1987a,b). Because H_{II} phase formation is expected to result in a loss of vesicle integrity and thus a release of the enclosed contents, the relatively slow leakage of larger dextrans implies that limited H_{II} phase formation or contact-mediated lysis may occur in these unilamellar systems on the time scale of the experiments (2 min) and supports the suggestion that precursor forms of H_{II} phase formation rather than this phase itself are involved in the leakage process (Tournois et al., 1987b). Centrifugation of the vesicles leads to a substantial (30–40%) release of vesicle contents (also FD-17) within 2 min. This indicates that centrifugation increases leakage possibly by enhancing H_{II} phase formation.

For a given peptide concentration, fusion (lipid and contents mixing but also light scattering and leakage) does not proceed to completion but rather appears to approach a limit which increases with peptide concentration, suggesting either that the peptide incorporation is inhomogeneous and only a limited amount of vesicles contain sufficient gramicidin to be affected or that gramicidin is only active for a short period. It could be argued that fusion is an aspecific process occurring as a result of the incorporation of the hydrophobic peptide; however, the peptide analogues NFGRA′ and GR(EtOH), which have comparable incorporation efficiencies, do not cause fusion. Alternatively, the peptide may undergo additional rearrangements, e.g., self-aggregation in the membrane during which fusion can occur.

At the relatively high lipid concentration of 150 μM, discussed so far, gramicidin-induced CF release, lipid mixing, and contents mixing run closely parallel, and lipid mixing is always accompanied by leakage. However, at a 30-fold lower concentration (5 μM), the leakage of CF shows a temperature-dependent lag phase of 1–3 min. In contrast, lipid mixing under comparable conditions has an immediate onset, independent of temperature. These data suggest that lipid mixing at low lipid concentration can occur without loss of contents and that processes occur in the following order: (1) aggregation and lipid mixing; (2) changes in membrane permeability and contents mixing; (3) H_{II} phase formation. This suggestion is consistent with contemporary theory for H_{II} formation in lipid vesicles (Siegel, 1984, 1986a–c, 1987; Verkleij, 1984).

The observation that NFGRA′ and GR(EtOH) to some extent induce lipid mixing but not contents mixing or leakage moreover suggests that these processes are separate and subsequent steps.

From data in Table I, it can be calculated that gramicidin A′ (0.1 mol/mol of DOPC) causes vesicles to fuse with on average four or more other vesicles, which would lead to an approximately 2-fold radius increase. Dynamic light-scattering data indicate a much larger increases in particle size. A 2-fold increase is already observed at much lower molar ratios of gramicidin (0.04 mol/mol of DOPC). This is most likely due to additional vesicle aggregation.

The present data show that induction of fusion is highly specific and reveal an excellent correlation between the ability of a peptide to induce fusion and its ability to induce H_{II} phase formation. Upon modification of gramicidin by formylation of the tryptophan indole rings, resulting in NFGRA′, its capability to induce lipid mixing and contents mixing has diminished. Even more subtle changes in the primary structure, such as replacement of the tryptophan at position 11 for phenylalanine (GR B), lead to a strongly reduced ability to bring about intervesicle lipid mixing. On the other hand, a $Trp^{11} \rightarrow Tyr$ substitution (GR C) did not result in any detectable changes in this respect. Whereas addition of gramicidin from TFE or DMSO leads to comparable fusion, the extent of both lipid mixing and contents mixing is drastically reduced upon addition from an ethanolic solution, suggesting that the $\beta^{6.3}$-helical conformation is a prerequisite for the capacity to induce fusion. The sequential order established for the fusogenic activity (Figure 7A), GR A = GR A′ = GR C $\gg$ GR B > NFGRA′ and GR(TFE) = GR(DMSO) $\gg$ GR(EtOH), closely matches that found for induction of CF release (Figure 7B) and is the same as that reported for the efficiency of H_{II} phase induction by these derivatives (Killian et al., 1987) and solvent conformations (Tournois et al., 1987a). The close correlation between the ability to induce H_{II} phase and to bring about fusion of large unilamellar DOPC vesicles strongly suggests that type II intermediates are involved in the fusion mechanism and that the specific gramicidin–lipid interactions which eventually lead to H_{II} phase formation are the same as those involved in vesicle fusion. A detailed model for the gramicidin-induced H_{II} phase formation has been proposed by Killian and de Kruijff (1988). In this model, the tendency of gramicidin to aggregate in curved structures by a combination of strong intermolecular attractive forces and the pronounced cone shape of the $\beta^{6.3}$-helices, both involving the tryptophans, is the driving force for H_{II} phase formation. The peptide is believed to form the backbone of the inverted H_{II} tubes. Recent data reported by Scarlata et al. (1988) indicate aggregation of gramicidin in DOPC membranes occurring already at a peptide/lipid molar ratio of 1/50–1/123. Such aggregate formation might be relevant for the mechanism of fusion.

The molecular mechanism of the fusion process is considered to involve at least three distinct steps: vesicle aggregation, membrane destabilization, and merging of membranes and (aqueous) contents (Lucy, 1982; Blumenthal, 1987). In considering possible mechanisms of gramicidin-induced membrane fusion, it should be noted that large unilamellar DOPC vesicles do not fuse spontaneously. Approach of membranes to less than approximately 30 Å results in strong hydration repulsion, which counteracts closer approach. Forces between interacting bilayers have been evaluated extensively (Gruen et al., 1984; Parsegian & Rand, 1983; Rand, 1981). Gramicidin (and its derivatives) can be expected to influence these forces in several ways. First, when it adsorbs to the outer leaflet of the membrane, it reduces hydration barriers. Second, upon insertion, it separates the lipid headgroups and thereby reduces the electrostatic repulsive energy between vesicles. Third, in the membrane, the hydrophobic molecules will contribute to the surface hydrophobicity. Together this will lead to a reduction of the interaction potential between vesicles. For the scope of this paper, it would lead too far to discuss all these factors, but clearly gramicidin (derivatives) can be expected to influence these forces such as to enhance aggregation of vesicles.

The next step would be a destabilization of the membrane. The observed sequence of events that occurs during gramicidin-induced fusion is in close agreement with contemporary theory for lipid vesicle fusion and H_{II} phase formation via inverted micellar intermediates (Siegel, 1984, 1986a–c, 1987; Verkleij, 1984) and also with other observations in model membranes (Ellens et al., 1986, 1989). Gramicidin might be able to induce formation of such IMI's between pairs of apposed bilayers by imposing a smaller "intrinsic radius of curvature" (Kirk et al., 1984; Gruner, 1985; Gruner et al., 1988) or increased "headgroup-area ratio" (Siegel, 1986c) on the lipid. It might do so because of the conical shape of the peptide or small peptide aggregates, or by enlarging the hydrophobic volume of the system. These putative transient type II nonbilayer intermediates, in analogy with the model proposed by Siegel (1984, 1986a–c, 1987) for pure lipid systems, could either initiate H_{II} phase formation (if sufficient gramicidin is present) or result in interlamellar attachment sites, which are transient points of fusion between vesicles that connect membranes and aqueous contents. In this context, it is tempting to speculate that the inability of FD-17 to reveal contents mixing (Figure 6E,F) reflects the inability of the molecules to pass through the interlamellar attachment sites. We should keep in mind, however, that it is difficult to compare data obtained with FD's of different sizes and quenching efficiencies quantitatively.

The reduced ability of the gramicidin derivatives and the ethanolic conformation of gramicidin to induce type II nonbilayer intermediates might then reflect a change in efficiency to affect the hydrophobic volume or intrinsic curvature of the lipid layer either due to a different shape of the gramicidin/DOPC complex or due to the aggregation state of the peptide molecules. Although the exact conformations of NFGRA′ and GR B are unknown, it has been shown by CD measurements (Killian et al., 1988) and by monolayer experiments (Tournois et al., 1989) that the conformation of these derivatives is quite distinct from that adopted by GR A. Furthermore, sucrose density gradient centrifugation experiments revealed a stronger tendency to self-aggregate for NFGRA′ and GR B than for GR A (Killian et al., 1987).

The present data demonstrate a direct correlation between the ability to induce H_{II} phase formation and the ability to induce fusion by the antibiotic peptide gramicidin. Furthermore, they show a high degree of peptide structure specificity in these processes. It is interesting to compare these findings with virus membrane fusion events. It has been shown that the pH-induced fusion of Sindbis virus with model membranes is dependent on the acyl chain composition of the target membrane and is greatly enhanced by the presence of type II nonbilayer lipids such as PE or cholesterol in these membranes (Scheule, 1987). These observations were explained in terms of a tentative model in which the target membrane must have sufficiently sized domains containing poorly hydrated lipids which are capable of adopting a nonbilayer ar-

rangement. A comparable observation is the preference of the vesicular stomatitis virus G protein for cis-unsaturated lipids (Yamada & Ohnishi, 1986). This indicates the involvement of inverted nonbilayer intermediates in virus–membrane fusion. The obvious suggestion is that small hydrophobic peptides in viral fusion proteins, which become exposed, e.g., as a response to low pH, trigger the actual fusion step by locally and transiently destabilizing the bilayers involved in the fusion reaction.

ACKNOWLEDGMENTS

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Registry No. DOPC, 4235-95-4; NFGRA′, 109050-07-9; gramicidin A, 11029-61-1; gramicidin A′, 9066-06-2; gramicidin B, 9062-60-6; gramicidin C, 9062-61-7.

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Effects of Phospholipids on Binding of Calcium to (Ca^{2+}–Mg^{2+})-ATPase†

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ABSTRACT: The (Ca^{2+}–Mg^{2+})-ATPase purified from skeletal muscle sarcoplasmic reticulum binds two Ca^{2+} ions per ATPase molecule. On reconstitution into bilayers of dioleoylphosphatidylcholine ((C18:1)PC) or dinervonylphosphatidylcholine ((C24:1)PC) the stoichiometry of binding remains unchanged, but when the ATPase is reconstituted into bilayers of dimyristoleoylphosphatidylcholine ((C14:1)PC) the stoichiometry changes to one Ca^{2+} ion per ATPase molecule. Nevertheless, the level of phosphorylation is the same for the ATPase reconstituted with (C18:1)PC or (C14:1)PC. The effect of (C14:1)PC on the stoichiometry of Ca^{2+} binding is prevented by androstenol at a 1:1 molar ratio with the phospholipid.

The activity of the (Ca^{2+}–Mg^{2+})-ATPase purified from the sarcoplasmic reticulum of skeletal muscle is known to be sensitive to the structure of the phospholipid molecules surrounding it in phospholipid bilayers. The phospholipid supporting highest ATPase activity is dioleoylphosphatidylcholine ((C18:1)PC),[1] and phospholipids with either shorter or longer fatty acyl chains or different headgroups support lower activities (Warren et al., 1974; Johannsson et al., 1981; Caffrey & Feigenson, 1981; East & Lee, 1982; Froud et al., 1986a). The low ATPase activity supported by phosphatidylcholines with short fatty acyl chains can be increased very considerably by addition of a wide variety of hydrophobic molecules, including sterols, fatty acids, and alcohols, alkanes, and pyrethroids (Johannsson et al., 1981; Simmonds et al., 1982, 1984; Jones & Lee, 1985; Jones et al., 1985, 1986; Froud et al., 1986b; Michelangeli et al., 1989). The low ATPase activities supported by phosphatidylcholines with long fatty acyl chains, however, are unaltered by addition of such hydrophobic molecules (Froud et al., 1986b). This argues that the changes to the ATPase that follow from reconstitution with a short-chain phosphatidylcholine are very different to those that follow from reconstitution with a long-chain phosphatidylcholine. We have been attempting to define the steps in the reaction sequence for the ATPases that are sensitive to phospholipid structure (Froud et al., 1986b; Michelangeli et al., manuscript in preparation). Here we focus on the steps in which Ca^{2+} binds to the ATPase on the cytoplasmic side of the membrane.

The binding of two Ca^{2+} ions to the native ATPase is a critical step since only in the Ca^{2+}-bound form can the ATPase by phosphorylated by MgATP; in the Ca^{2+}-free conformation the ATPase can instead be phosphorylated by P_i (de Meis & Vianna, 1979). Two Ca^{2+} ions bind per ATPase molecule in a cooperative manner, involving at least one slow conformational change. Rates of binding and dissociation of $^{45}Ca^{2+}$ and of changes in the tryptophan fluorescence of the ATPase that

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[1] Abbreviations: (C14:1)PC, dimyristoleoylphosphatidylcholine; (C18:1)PC, dioleoylphosphatidylcholine; (C24:1)PC, dinervonylphosphatidylcholine; SR, sarcoplasmic reticulum.

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