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Fusion of phospholipid vesicles produced by the anti-tumour protein α-sarcin

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The anti-tumour protein α-sarcin causes fusion of bilayers of phospholipid vesicles at neutral pH. This is demonstrated by measuring the decrease in the efficiency of the fluorescence energy transfer between *N*-(7-nitro-2-1,3-benzoxadiazol-4-yl)-dimyristoylphosphatidylethanolamine (NDB-PE) (donor) and *N*-(lissamine rhodamine B sulphonyl)-diacylphosphatidylethanolamine (Rh-PE) (acceptor) incorporated in dimyristoylphosphatidylcholine (DMPG) vesicles. The effect of α-sarcin is a maximum at 0.15 M ionic strength and is abolished at basic pH. α-Sarcin promotes fusion between 1,6-diphenylhexa-1,3,5-triene (DPH)-labelled DMPG and dipalmitoyl-PG (DPPG) vesicles, resulting in a single thermotropic transition for the population of fused phospholipid vesicles. Bilayers composed of DMPC and DMPG, at different molar ratios in the range 1:1 to 1:10 PC/PG, are also fused by α-sarcin. Freeze-fracture electron micrographs corroborate the occurrence of fusion induced by the protein. α-Sarcin also modifies the permeability of the bilayers, causing the leakage of calcein in dye-trapped PG vesicles. All of the observed effects reach saturation at a 50:1 phospholipid/protein molar ratio, which is coincident with the binding stoichiometry previously described.

INTRODUCTION

The process of fusion between membranes is an important step in many cellular events such as secretion, endocytosis and translocation of macromolecules. From a molecular point of view, this process has mainly been studied in lipid model systems. It is known that bivalent and multivalent cations can induce fusion between vesicles containing negatively charged phospholipids (Duzgunes *et al.*, 1981, 1987; Ohki, 1982; Papahadjopoulos, 1987). Some large cationic molecules, such as polylysin and polymyxin, have also been reported to aggregate and fuse vesicles containing acid phospholipids (Gad, 1983; Walter *et al.*, 1986; Oku *et al.*, 1986). Proteins can also induce bilayer fusion, involving both electrostatic and hydrophobic interactions between the protein and the participating membranes (Walter *et al.*, 1986). Notwithstanding, the molecular mechanism inherent to this process has not yet been elucidated due to, among other reasons, the large variety of biological functions exhibited by fusion-promoting proteins, e.g. viral spike proteins (White *et al.*, 1983; Eidelman *et al.*, 1984), synexin (Hong *et al.*, 1981*b*), clathrin (Steer *et al.*, 1982), cardiotoxin (Batenburg *et al.*, 1985), and sperm proteins such as bindin and lysin (Glabe, 1985; see Hong *et al.*, 1981*a*, for a review). However, the possibility that bilayer fusion phenomena may participate in the transport of proteins across membranes has been reported for diphtheria toxin (Blewitt *et al.*, 1985; Panini *et al.*, 1987) and tetanus toxin (Cabiaux *et al.*, 1985). α-Sarcin, a cytotoxic protein that shares with other toxins the characteristic of having an intracellular target, specifically crosses tumour cell membranes (Olson *et al.*, 1965; Roga *et al.*, 1971). We have previously reported that α-sarcin binds specifically to negatively charged lipids and induces their aggregation (Gasset *et al.*, 1989). In this paper, we demonstrate that α-sarcin induces the fusion of acid phospholipid vesicles, this process being dependent on temperature, pH and ionic strength, resulting in vesicle permeability changes.

EXPERIMENTAL PROCEDURES

Lipid preparation

Synthetic phospholipids, dimyristoylphosphatidylcholine (DMPC), dimyristoylphosphatidylglycerol (DMPG), *N*-(7-nitro-2-1,3-benzoxadiazol-4-yl)-dimyristoylphosphatidylethanolamine (NBD-PE) and *N*-(lissamine rhodamine B sulphonyl)-diacylphosphatidylethanolamine (Rh-PE) were purchased from Avanti Polar Lipids (Birmingham, AL, U.S.A.). Egg phosphatidylglycerol (PG) was purchased from Lipids Products (South Nutfield, Surrey, U.K.). Calcein was obtained from Sigma, and was purified on a Sephadex LH-20 column before use (Ralston *et al.*, 1981).

The different vesicles were prepared at a phospholipid concentration of 1 mg/ml in 30 mM-Tris/HCl buffer, pH 7.0, containing 0.1 M-NaCl, in a water-bath sonifier (Martínez del Pozo *et al.*, 1988*a*; Gasset *et al.*, 1989). The temperature was maintained 5 °C above the phase-transition value of the corresponding phospholipid. The presence of lysophospholipids after the preparation of the vesicles was not observed by chromatographic analysis of the lipid component (Gavilanes *et al.*, 1981).

Lipid-vesicles–protein complexes were obtained by adding α-sarcin to recently prepared vesicles at different lipid/protein molar ratios.

Abbreviations used: PG, phosphatidylglycerol; DMPG, dimyristoyl-PG; DPPG, dipalmitoyl-PG; PC, phosphatidylcholine; DMPC, dimyristoyl-PC; DPH, 1,6-diphenylhexa-1,3,5-triene; NDB-PE, *N*-(7-nitro-2-1,3-benzoxadiazol-4-yl)-dimyristoylphosphatidylethanolamine; Rh-PE, *N*-(lissamine rhodamine B sulphonyl)-diacylphosphatidylethanolamine; RET, resonance energy transfer.

Fusion assays

Intermixing of membrane lipids was measured by resonance energy transfer (RET) assays (Struck *et al.*, 1981). A vesicle population (11.7 μM), containing a 1% molar ratio of each fluorescent lipid analogue (Rh-PE and NBD-PE), was mixed with unlabelled phospholipid vesicles at a 1:9 molar ratio. As shown by Struck *et al.* (1981), when the energy donor and the energy acceptor are in lipid vesicles at appropriate surface densities, considered as the ratio of fluorescent lipid/total lipid, an efficient energy transfer process is observed. The decreasing fluorophore density resulting from fusion is reflected in a less efficient RET. Steady-state emission spectra were recorded by using a Perkin–Elmer MPF-44E spectrofluorimeter. The excitation and emission slit-widths were set at 7 nm and 5 nm respectively. Cells of 0.2 cm optical path were used in order to reduce the effect of the light scattering of some samples; the temperature was maintained at 37 °C. Spectra of vesicle preparations containing both NBD-PE and Rh-PE show emission maxima around 520 nm and 585 nm for excitation at 450 nm (see Fig. 1). The main part of the fluorescence emission at 520 nm arises from NBD-PE, whereas most of the fluorescence intensity at 585 nm is due to fluorescence energy transfer between the donor (NBD-PE) and the acceptor (Rh-PE). The efficiency of the energy transfer (ET) (%) in such samples is defined by the equation:

$$\text{ET}\ (\%) = [(1-F)/F_0] \times 100 \qquad (1)$$

where F and F_0 are the fluorescence emission intensities at 520 nm in the presence and in the absence respectively of Rh-PE.

Fusion induced by α-sarcin was estimated from the increase of the NDB-PE fluorescence (emission band at 520 nm) when fluorescent lipids were diluted into unlabelled vesicles in the presence of the protein. This is due to a decrease in the efficiency of RET between NBD-PE and Rh-PE. After each set of measurements, vesicles were disrupted with Triton X-100 (1% final concentration) and the emission bands of NBD-PE and Rh-PE were recorded by using direct excitation. This treatment allows the determination and normalization of the donor/acceptor amounts present for each particular sample, since it eliminates the energy transfer (Struck *et al.*, 1981).

Absorbance measurements

Absorbance measurements were carried out using either a Cary 118 or a Beckman DU-8 spectrophotometer equipped with a thermostated cell holder. The u.v. spectrum of the protein and a specific absorption coefficient ($A^{0.1\%}_{1\,\text{cm},280}$) of 1.34 litre·g^{-1}·cm^{-1} (Gavilanes *et al.*, 1983) were used to calculate the concentration of α-sarcin solutions. The protein used throughout the described experiments was kindly supplied by Dr. E. Méndez and Dr. F. P. Conde (Hospital Ramón y Cajal, Madrid, Spain), and it corresponds to a highly purified sample of α-sarcin (used for protein sequence studies; Rodríguez *et al.*, 1982; Gavilanes *et al.*, 1983). The protein was employed without any previous denaturing treatment.

Fluorescence polarization

The labelling of the phospholipid vesicles with 1,6-diphenylhexa-1,3,5-triene (DPH) was performed as previously described (Gavilanes *et al.*, 1985). Polarization of the fluorescence emission of DPH was measured with the corresponding accessory on the MPF-44E spectrofluorimeter in thermostated cells. Emission was measured at 425 nm for excitation at 365 nm. The contribution of α-sarcin to the degree of polarization of DPH was negligible, as shown by the results of separate experiments (not shown). The contribution of sample turbidity to the polarization of DPH fluorescence emission was also negligible at the lipid concentrations, protein/lipid molar ratios and optical path length (0.2 cm) employed, as deduced from the values corresponding to successive dilution of the samples.

Freeze-fracture electron microscopy

Fusion of DMPG induced by α-sarcin was also examined by freeze-fracture electron microscopy. DMPG vesicles were incubated in the absence or in the presence of α-sarcin at 37 °C for 1 h, using a lipid concentration of 0.67 mg/ml and lipid/protein molar ratios of 1000:1, 500:1 and 50:1. Samples were then taken without centrifugation for examination by freeze-fracture electron microscopy. The samples were fast-frozen without cryoprotectant from 37 °C in liquid propane using a Reichert–Jung KF80 freezing device, and were freeze-fracture platinum/carbon-replicated using a Balzers' 400F freeze-etch apparatus. Replicas were cleaned using commercial bleach and examined using a Philips CM10 electron microscope.

Calcein release experiments

Calcein-trapped vesicles were prepared by a modification of the freeze–thaw technique (Kasahara & Hinkle, 1977). A dried film (5 mg) of egg PG was hydrated in 30 mM-Tris/HCl, pH 7.0, containing 25 mM-calcein, up to a final lipid concentration of 10 mg/ml. The egg PG dispersion was sonicated in a probe-sonicator, frozen in liquid N_2 and thawed at room temperature. This cycle was repeated three times in order to obtain an adequate yield of trapped calcein. Non-trapped calcein was removed by centrifugation on a Beckman L8 centrifuge for 60 min at 100000 *g*, rotor SW-65. The pellet was washed twice and resuspended in 30 mM-Tris/HCl, pH 7.0, containing 0.1 M-NaCl, at a final concentration of 0.6 mg/ml. Different α-sarcin concentrations were added to phospholipid vesicles solutions at a lipid concentration of 8 μM, based on their phosphorus content determined according to Fiske & SubbaRow (1925). The mixture was maintained for 1 h at 37 °C and then centrifuged for 1 h at 100000 *g* (rotor R-40.3). Steady-state emission spectra of the obtained supernatants were recorded for excitation at 490 nm. Fluorescence intensity was measured at 515 nm.

RESULTS AND DISCUSSION

α-Sarcin is a cytotoxic protein which specifically degrades the larger ribosomal RNA of some tumour cells (for a review, see Wool, 1984), thus exhibiting an effective anti-tumour activity. In recent studies, we have examined the changes in the state of aggregation of phospholipid vesicles induced by α-sarcin (Gasset *et al.*, 1989). The results obtained indicated that the α-sarcin–phospholipid-vesicle interaction leads to the formation of large structures. However, from those data, we were not able to

distinguish between aggregation and fusion of the lipid bilayers induced by α-sarcin. We therefore performed a series of experiments, based on the measurement of the RET between two fluorescence probes incorporated into the same vesicle, to detect the existence of fusion induced by α-sarcin. The freeze fracture patterns of α-sarcin–vesicle systems have also been studied. We also analysed the different thermotropic behaviour of protein–phospholipid vesicles complexes to study the potential lipid intermixing in the presence of α-sarcin. In addition, the release of calcein was determined by measurement of the alterations in membrane permeability induced by the protein.

The intermixing of membrane lipids resulting from membrane fusion can be monitored by using RET measurements. The concept relies upon the ability of a fluorescent energy donor to excite an energy acceptor if both are free to diffuse in the same membrane at an appropriate surface density (Struck *et al.*, 1981).

Fig. 1 shows the emission spectra of phospholipid vesicles containing both NBD-PE (donor) and Rh-PE (acceptor) (DMPG/NBD-PE/Rh-PE, 98:1:1 molar ratio; fluorescence-labelled vesicles) for excitation at 450 nm. Two emission maxima at 520 nm and 585 nm, corresponding to NBD-PE (donor) and Rh-PE (acceptor) respectively, are observed in the absence of α-sarcin (spectrum c, Fig. 1). Addition of α-sarcin to a mixture of both fluorescence-labelled and unlabelled vesicles caused an increase in the intensity of the emission peak at 520 nm, as well as a concomitant reduction in the emission peak at 585 nm. The fluorescence emission variation reaches equilibrium after a very short period of time, of the order of seconds. Fig. 2 shows the time course of the increase in fluorescence intensity at 520 nm caused by α-sarcin. Increasing amounts of the protein result in a progressive reduction in the emission peak at 585 nm and in an increase in the fluorescence quantum yield of NBD-PE at 520 nm (Fig. 1, spectra d–g; Fig. 2). These fluorescence variations occur with increases in the size of the particles present in the interaction medium. This variation was analysed by measuring the absorbance at 360 nm of the different lipid/protein mixtures which was caused by the light scattering of the α-sarcin–phospholipid complexes. The time course of the absorbance increase is given in Fig. 2 for different amounts of α-sarcin. The final value of the absorbance at 360 nm requires different lengths of time for free stabilization, depending on the concentrations of protein and phospholipid. Nevertheless, the main increase in absorbance occurs within a few seconds. Thus the spectra in Fig. 1 were recorded after 1 h (the longest period of time required for the stabilization of the absorbance at 360 nm is about 40 min) in order to consider only the energy transfer variations of the final α-sarcin–phospholipid complexes formed.

The fluorescence changes described above indicate a

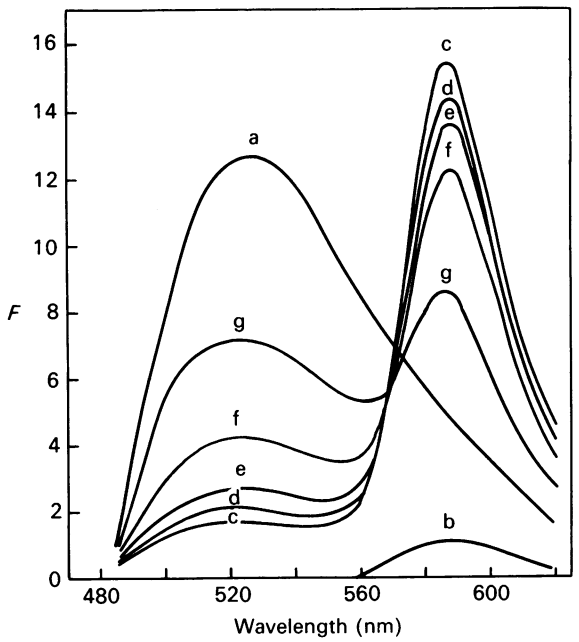

Fig. 1. Effect of α-sarcin on energy transfer between NDB-PE and Rh-PE incorporated into the same DMPG vesicles

Labelled vesicles (11.7 μM total lipid) were mixed with unlabelled DMPG vesicles (105.3 μM total lipid) in 30 mM-Tris/HCl buffer, pH 7.0, containing 0.1 M-NaCl (DMPG/NBD-PE/Rh-PE molar ratio, 98:1:1). α-Sarcin was added to the mixture at different lipid/protein molar ratios. Spectra were recorded after 1 h of incubation at 37 °C. The excitation wavelength was 450 nm. Spectrum c was obtained in the absence of α-sarcin. Spectra d–g were the result of the incubation of lipid with increasing amounts of α-sarcin (lipid/protein molar ratios of 1000:1, 500:1, 250:1 and 50:1 respectively). Spectrum a was the result of mixing the DMPG/NBD-PE (99:1 molar ratio) vesicles with unlabelled DMPG vesicles in a 1:9 proportion. Spectrum b was the result of mixing DMPG/Rh-PE (99:1 molar ratio) vesicles with unlabelled vesicles in a 1:9 proportion. Fluorescence intensity is expressed in arbitrary units.

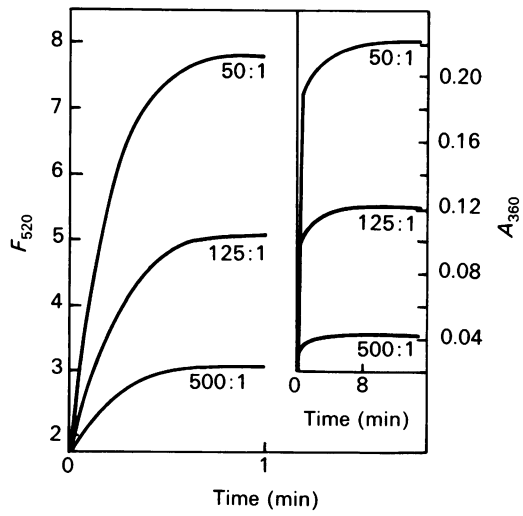

Fig. 2. Time course of the α-sarcin-induced fusion of DMPG vesicles

The study was performed as in Fig. 1 (the labelled/unlabelled vesicles molar ratio was 1:9). The fluorescence intensity at 520 nm (*F*, expressed in arbitrary units, as in Fig. 1) was continuously recorded for excitation at 450 nm. Different lipid/protein molar ratios, as indicated in the Figure, were used. The total phospholipid concentration was 117 μM. Inset: Time course of the increase in absorbance at 360 nm produced by α-sarcin at different unlabelled DMPG/protein molar ratios. The total phospholipid concentration was 300 μM.

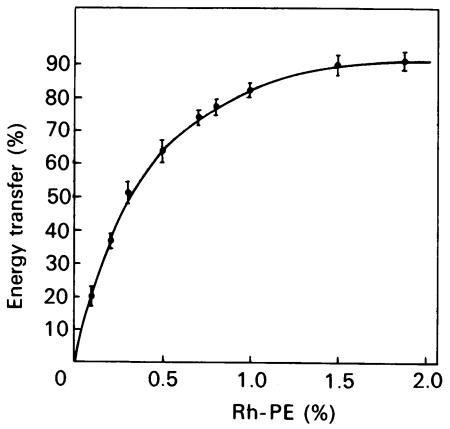

Fig. 3. Efficiency of the energy transfer process between NBD-PE (donor) and Rh-PE (acceptor) as a function of the percentage of energy acceptors/phospholipid molecule in the vesicles (surface density)

The experiments were performed at a donor concentration of 1% of the phospholipid concentration (11.7 μM); the labelled/unlabelled vesicle molar ratio was 1:9. The energy transfer efficiency (mean values ± S.D. corresponding to three different experiments) was calculated according to eqn. (1) (see the text). Fluorescence at 520 nm (excitation at 450 nm) was used for these calculations.

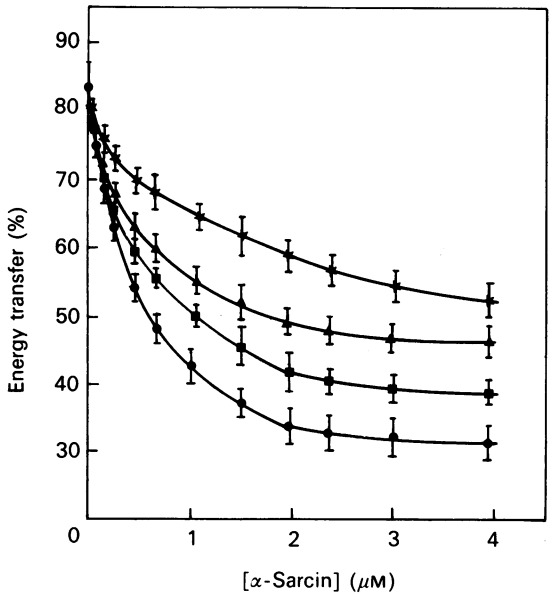

Fig. 4. Effect of α-sarcin concentration on energy transfer efficiency

The total phospholipid concentration was 117 μM and energy transfer was measured as described in the legend to Fig. 1. Energy transfer efficiency was calculated as for Fig. 3. Vesicles: ●, DMPG; ■, DMPG/DMPC (10:1); ▲, DMPG/DMPC (5:1); ★, DMPG/DMPC (1:1) (molar ratios). Values are expressed as means ± S.D. for three different experiments.

reduction in the efficiency of the energy transfer between NBD-E and Rh-PE. We have studied the efficiency of the energy transfer process as a function of the number of energy acceptors (Rh-PE) per phospholipid molecule (surface density) for the lipid system considered herein. The results obtained are given in Fig. 3. According to these data, the maximum decrease in the efficiency of energy transfer produced by α-sarcin in these experiments (from about 80% to about 30%; Fig. 4) represents a decrease in the acceptor surface density of (8.4 ± 1.6)-fold. Considering that the proportion of fluorescence-labelled vesicles/unlabelled vesicles in our assay system is 1:9, the above decrease in the surface density of the energy acceptor is consistent with an α-sarcin-induced fusion between the labelled and unlabelled DMPG vesicles, followed by lateral diffusion of the fluorescent probes in the plane of the newly enlarged membranes. Simple aggregation of the vesicles would not result in such a change in energy transfer (Blumenthal *et al.*, 1983).

The energy transfer efficiency calculated according to eqn. (1) (see the Experimental procedures section) versus the α-sarcin concentration for DMPG vesicles is shown in Fig. 4. A decrease in the energy transfer efficiency is observed with increasing α-sarcin concentration, resulting in a saturation plot. As indicated above, an (8.4 ± 1.6)-fold decrease in acceptor surface density is obtained at a saturating lipid/protein molar ratio of about 50:1 (Fig. 4). This saturating value is the same as that obtained from the aggregation studies (Gasset *et al.*, 1989); binding experiments also revealed a similar stoichiometry for the lipid–α-sarcin complex (Gasset *et al.*, 1989).

When the fluorescent vesicles were incubated with α-sarcin in the absence of unlabelled vesicles, there was no change in the energy transfer (results not shown). This indicates that the effect observed above was not simply due to a change in the shape or in the radius of curvature of the vesicles. If the mixture of fluorescence-labelled and unlabelled vesicles is incubated in the absence of α-sarcin, no change in the efficiency of energy transfer is observed. Thus the vesicles used are stable for at least 24 h, even at 0 °C, as also deduced from electron microscopy examination (results not shown). Therefore an apparent fusion resulting from vesicle instability must be discarded.

The effect of α-sarcin on the fusion of phospholipid vesicles has also been studied for DMPG/DMPC mixtures. The results obtained are also given in Fig. 4 for vesicles of different DMPG/DMPC molar ratios. The decrease in the energy transfer efficiency (and consequently the extent of the fusion process) caused by α-sarcin diminishes as the proportion of PC increases. When DMPC vesicles were used, no change in the emission spectrum of the mixture of labelled and unlabelled vesicles was observed after α-sarcin addition. The lack of an effect of α-sarcin on the fusion of PC vesicles agrees with previous results which showed that the protein does not induced aggregation of such vesicles (Gasset *et al.*, 1989).

The membrane fusion induced by α-sarcin is dependent on ionic strength, pH and temperature. The effects of both pH and ionic strength on the efficiency of energy transfer are given in Fig. 5. When the ionic strength increases, the degree of fusion decreases (maximum at 0.15 M ionic strength), being effectively abolished at 0.35 M (Fig. 5*a*). The ionic strength does not produce any conformational change in the protein, at least up to 0.5 M-salt (Martínez del Pozo *et al.*, 1988*b*); thus the influence of the salt in the fusion process cannot be

related simply to direct effects upon the protein itself. An increase in the ionic strength does not produce aggregation of α-sarcin, as deduced from gel filtration chromatography in columns equilibrated at different NaCl concentrations. Therefore the effect of the ionic strength should be interpreted in terms of the electrostatic component which induced the formation of the acid-phospholipid–α-sarcin complex (Gasset *et al.*, 1989). When this is abolished, fusion does not take place, which is what would occur at higher ionic strength. On the other hand, if the ionic strength increases after complex formation, the degree of fusion is not modified (results not shown). Thus fusion induced by α-sarcin is not reversed by increasing ionic strength. This indicates the involvement of hydrophobic interaction in the overall process.

There are at least three essential requirements for fusion of vesicles: (1) aggregation of the vesicles, (2) a close apposition of membranes, and (3) a transitional destabilization of the bilayers (Hong & Vacquier, 1986). Previous results indicated that α-sarcin causes aggregation of the acid phospholipid vesicles even at 1.0 M ionic strength (Gasset *et al.*, 1989). There are cases in which aggregated vesicles do not fuse, mainly because there is not a sufficiently close apposition of membranes due to the hydration shell of the bilayer surface, which is thought to prevent fusion (Hong *et al.*, 1981*a*). This occurs for α-sarcin-induced fusion when the ionic strength is increased. Thus from the results on the effect of the ionic strength, the existence of both an electrostatic and a hydrophobic component may be deduced for α-sarcin-induced fusion.

The results of the RET assays at different pH values are presented in Fig. 5(*b*). A pH-induced transition is found at pH 7–8, the intermixing of the phospholipids between labelled and unlabelled vesicles being abolished at basic pH. The phospholipid vesicles would not be expected to be modified at this pH value. However, α-sarcin does exhibit a conformational transition affecting tryptophan residues at pH 8 (Martínez del Pozo *et al.*, 1988*b*), which has been attributed to the deprotonation of the α-NH_2 group of the protein. Thus the pH-induced transition of the fusion of acid phospholipids produced by α-sarcin may be related to the behaviour of the protein itself, as has been shown previously for the pH dependence of α-sarcin-induced vesicle aggregation (Gasset *et al.*, 1989).

α-Sarcin-induced DMPG vesicle fusion is also temperature-dependent (Fig. 6). This dependence can be ex-

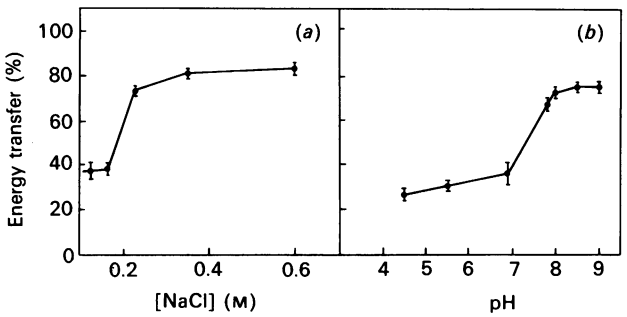

Fig. 5. Effect of (*a*) ionic strength and (*b*) pH on α-sarcin-induced fusion of DMPG vesicles

The lipid/protein molar ratio was 50:1 and the total phospholipid concentration 117 μM. Ionic strength was increased by adding NaCl to 30 mM-Tris/HCl, pH 7.0. The experiments in (*b*) were performed in 30 mM-citrate buffers at the different pH values. Values are means ± S.D. for three different experiments. Adequate controls without protein were carried out, and no significant changes in energy transfer were observed.

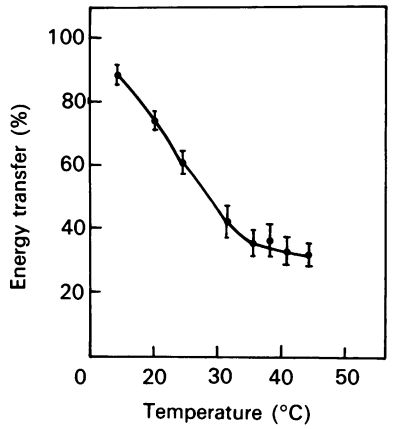

Fig. 6. Effect of temperature on the fusion of fluorescent and unlabelled DMPG vesicles induced by α-sarcin

Percentages of the RET (means ± S.D. for three experiments) were calculated as described in the legend to Fig. 3. The phospholipid concentration in all of these experiments was 117 μM in 30 mM-Tris/HCl, pH 7.0, containing 0.1 M-NaCl. The lipid/protein molar ratio was 50:1. Adequate controls without protein were performed and no significant changes in energy transfer were observed.

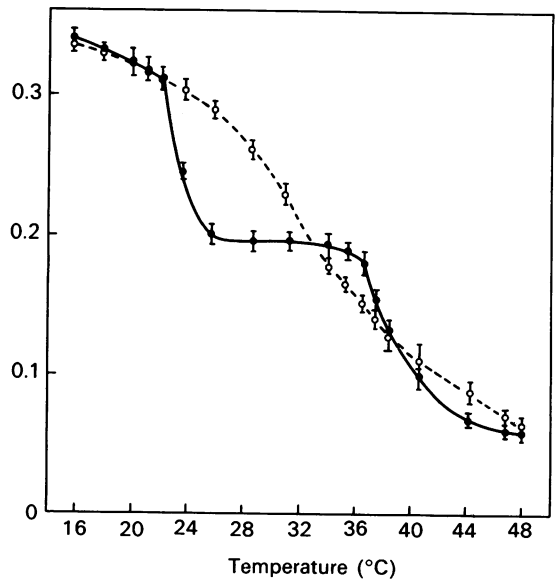

Fig. 7. Phase-transition temperature profiles of mixtures of DPH-labelled DMPG and DPPG vesicles

Anisotropy (*r*) values are means ± S.D. of two different determinations. ●, Mixture of DMPG and DPPG vesicles; ○, mixture of DMPG and DPPG vesicles in the presence of α-sarcin at a 50:1 lipid/protein molar ratio. The analyses were performed in 30 mM-Tris/HCl buffer, pH 7.0, containing 0.1 M-NaCl. Anisotropy was determined after equilibration of the sample for 10 min at each temperature.

plained in terms of phospholipid phase transition. Fusion is negligible at temperatures lower than 20 °C, whereas above 20 °C the extent of DMPG vesicle fusion increases with temperature (Fig. 6). This suggests that the lipid phase-transition temperature is a critical factor in the fusion process. Thus fusion occurs preferentially above the phase-transition temperature of the phospholipid (23 °C for DMPG). α-Sarcin also induces, to the same extent, fusion of egg PG at room temperature (results not shown).

Fusion therefore seems to be dependent on the extent of hydrophobic penetration of α-sarcin, which in turn is largely dependent on the bilayer packing. A large hydrophobic perturbation produced by the protein would facilitate a transitional intermembrane destabilization in aggregated vesicles and consequently lead to fusion (Hong *et al.*, 1981*a*).

The fluorescence depolarization of DPH-containing vesicles also demonstrates that extensive fusion occurs during aggregation of acidic vesicles with α-sarcin. The thermotropic behaviour of a mixture of DMPG vesicles and DPPG vesicles is modified in presence of α-sarcin (Fig. 7). The vesicles without the protein show two different transition temperatures corresponding to each phospholipid (23 °C for DMPG and 42 °C for DPPG). After incubation with α-sarcin, the thermotropic behaviour changes and the anisotropy of the mixture loses its previous biphasic character, corresponding to only one set of mixed DMPG/DPPG vesicles. Membrane destabilization is required for fusion to take place in the

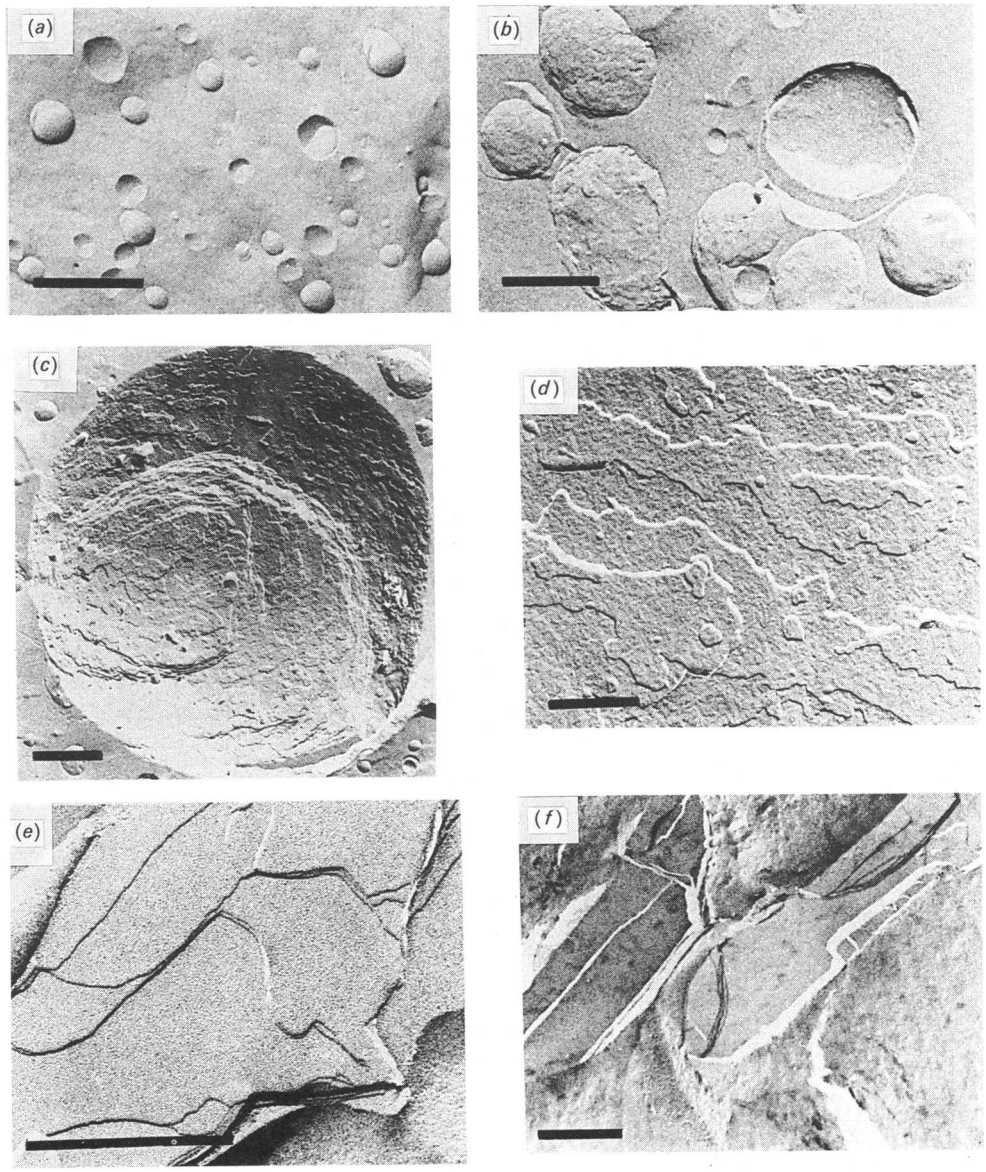

Fig. 8. Electron micrographs of PG vesicles incubated in the absence and in the presence of α-sarcin for 1 h at 37 °C

See the Experimental procedures section for further details. The Figure shows DMPG vesicles (*a*) and PG vesicles incubated in the presence of α-sarcin at lipid/protein molar ratios of 1000:1 (*b*), 500:1 (*c*, *d*) and 50:1 (*e*, *f*). Bars represent 500 nm for micrographs (*a*)–(*e*) and 5000 nm for (*f*).

aggregated system. Thus a major role of proteins in vesicle fusion may arise from their capability to destabilize the membranes via hydrophobic insertion into lipid bilayers. In this regard, the existence of electrostatic and hydrophobic components in the acid-phospholipid–α-sarcin interaction has previously been reported (Gasset *et al.*, 1989).

The freeze-fracture electron micrographs in Fig. 8 illustrate dramatically the fusion capabilities of α-sarcin. Fig. 8(*a*) shows that the DMPG suspension is mostly composed of small vesicular structures. The main part of the sample is apparently present as unilamellar vesicles of approx. 150–200 nm in diameter. Incubation with α-sarcin for 1 h at 37 °C at molar ratios as low as 1000:1 (lipid/protein) has resulted in the marked fusion of the small vesicles into large multilamellar vesicular structures. Small vesicles are still clearly present in this sample, which is in agreement with the lower degree of fusion observed using RET fusion assays. At lipid/α-sarcin molar ratios of 500:1 (Fig. 8*c*), very large multilamellar fusion structures have become apparent, with far fewer of the small vesicular structures being present. Interestingly, at this molar ratio of α-sarcin, the lipid bilayer faces are no longer flat and smooth but are very uneven. The fracture face frequently jumps from one bilayer to another, indicative of close membrane juxtaposition, as is clearly illustrated in Fig. 8(*d*). At the highest molar ratio of α-sarcin investigated (50:1, lipid/protein), there is a complete absence of the small vesicular structures. The lipid is exclusively organized in planar sheets (Figs. 8*e* and 8*f*), indicating that the fusion processes have probably gone to completion. In this study, there has been no evidence for the existence of fusion structures, namely inter- or intramembrane particles. This is perhaps to be expected, as such fusion structures are very rarely seen in studies with protein-induced membrane fusion, with the exception of those studies with peptides such as gramicidin which induce fusion by promoting the hexagonal II phase in phospholipids (Killian *et al.*, 1985).

We also investigated the ability of α-sarcin to modify the permeability of egg-PG vesicles by measuring the leaking out of trapped calcein from the vesicles upon α-sarcin addition. High concentrations of the dye were trapped inside the vesicles; under these conditions, the fluorescence of the dye was almost completely quenched. Upon leakage, the dye was diluted and became highly fluorescent (Weinstein *et al.*, 1976). The rate of increase in calcein fluorescence intensity was taken as an index of the changes in vesicle permeability. The release of calcein was complete in a very short period of time (of the order of seconds). Nevertheless, the calcein fluorescence intensity was measured after 1 h to consider final structures of the protein–lipid complexes, as indicated above for the energy transfer study. The results at different α-sarcin/egg-PG molar ratios are shown in Fig. 9. Egg-PG vesicles were used instead of DMPG due to the higher efficiency of dye encapsulation shown by this lipid (results not shown).

There was release of calcein upon addition of α-sarcin. Thus α-sarcin induces fusion with concomitant alteration of the bilayer permeability. The greatest leakage was observed at a lipid/protein molar ratio of 50:1 (Fig. 9), which is coincident with the binding stoichiometry (Gasset *et al.*, 1989). Therefore the calcein release induced by α-sarcin can be related to the dramatic change observed by freeze-fracture analysis of the lipid vesicles.

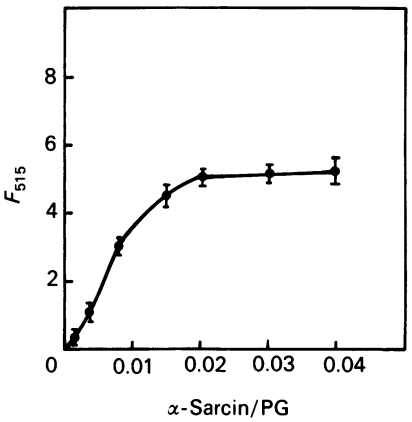

Fig. 9. Variation of the fluorescence of released calcein from PG vesicles upon addition of α-sarcin at various lipid (egg-PG)/protein molar ratios

The PG concentration was 8 μM and the α-sarcin/PG mixture was incubated for 1 h at 37 °C in 30 mM-Tris/HCl, pH 7.0, containing 0.1 M-NaCl. The lipid–protein complex was removed by centrifugation at 100 000 *g* for 60 min and the fluorescence intensity of the supernatant at 515 nm (*F*, expressed in arbitrary units; mean values ± S.D. of three different experiments) was measured by excitation at 490 nm.

Kimelberg & Papahadjopoulos (1971) have proposed that an asymmetric charge distribution produced when a basic polypeptide binds to one side of the bilayer could promote the flip-flop of this patch. Partial loss of internal content could occur during this inversion. A second possibility, the more probable, is the spillage of internal content that could occur during α-sarcin-induced fusion of acid vesicles. Thus the experiments with large unilamellar vesicles trapping calcein shed some light on the interaction of α-sarcin with egg-PG bilayers.

In conclusion, α-sarcin is able to promote the fusion of phospholipids, causing modification of both the permeability of the bilayers and the lipid structures. Bilayer fusion would reflect the processes occurring during the passage of α-sarcin across membranes, and it gives an insight into the molecular basis of the cytotoxicity of this protein.

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