Fusion of Liposomes Induced by a Cationic Protein from the Acrosome Granule of Abalone Spermatozoa†

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Received December 17, 1984; Revised Manuscript Received August 28, 1985

ABSTRACT: Lysin, a protein of M_r 16 000 from the acrosome granule of the abalone, is responsible for the dissolution of the egg vitelline layer. The primary structure of this cationic protein projects some hydrophobic domains in the secondary structure. Lysin was found to associate nonselectively with phospholipid bilayers and cause a spontaneous release of encapsulated carboxyfluorescein in liposomes. The association of lysin with phosphatidylcholine liposomes suggests that there is a hydrophobic interaction between lysin and lipid bilayers. Binding of lysin to phospholipid resulted in the aggregation of phosphatidylserine-containing liposomes, but aggregation was not observed in neutral phosphatidylcholine liposomes. Resonance energy transfer and dequenching of fluorescent 1-palmitoyl-2-*cis*-parinaroylphosphatidylcholine were both used to determine the fusogenic activity of lysin in aggregated liposomes. Results from both assays are consistent. Lysin-induced fusion was observed in all the phosphatidylserine-containing liposomes, and the general trend of fusion susceptibility was phosphatidylserine/phosphatidylcholine (1:2) $\simeq$ phosphatidylserine/phosphatidylcholine/phosphatidylethanolamine (1:1:1) > phosphatidylserine/phosphatidylethanolamine (1:2). Cholesterol up to 30% did not affect the intrinsic fusion susceptibility. A hydrophobic penetration by protein molecules and the packing of phospholipid bilayers are used to interpret the fusion susceptibility. Lysin-induced liposome aggregation was highly independent of the state of self-association of lysin in ionic medium. However, the fusogenic activity of self-associated lysin was found to be much less than the monodispersed one. Liposomes preincubated with Ca^{2+} did not fuse initially as readily as those without Ca^{2+} treatment. This early inhibition was subsequently reduced while lysin-induced fusion was taking place, and the same level of lipid mixing was reached. Our observations suggest that positively charged lysin may utilize its hydrophobic domain to interact with the hydrophobic region of lipid bilayers and facilitate the fusion of negatively charged membranes, which are in close apposition to each other, because of the polycationic nature of lysin. We propose that lysin may play two roles in fertilization: dissolution of the egg vitelline layer and promotion of fusion between sperm and egg plasma membrane.

Membrane fusion is a fundamental part of various cellular processes, such as exocytosis, endocytosis, neurotransmitter release, fertilization, and formation of multinucleated fibers in skeletal muscle (Poste & Allison, 1973). The fusion of sperm and egg plasma membranes during fertilization is one of the few natural examples of intercellular membrane fusion accessible to experimental study (Poste & Nicolson, 1978). The gametes of marine invertebrates are ideal for such studies because they can be obtained in vast quantities, and the fusion event occurs in seawater external to the adult body.

Spermatozoa of mollusks of the order Archeogastropoda possess a very large acrosome granule at the anterior apex of the cell (Haino-Fukushima, 1974; Lewis et al., 1980; Shiroya & Sakai, 1984). The eggs are surrounded by a protective, elevated vitelline layer about 1 μm in thickness composed of glycoproteins (Lewis et al., 1982). During fertilization, the anterior apex of the sperm binds to the vitelline layer, the acrosome granule opens, and proteins are released, dissolving a hole in the egg vitelline layer (Lewis et al., 1982). The sperm passes through the hole and fuses with the egg. The acrosomal proteins have been termed "lysins" because of their ability to dissolve egg investments (Dan, 1967). Present evidence suggests that the mechanism by which the abalone sperm lysin dissolves the vitelline layer is nonenzymatic; it seems to be based on the hydrophobic competition of the lysin for intermolecular hydrophobic bonds among the glycoprotein molecules comprising the vitelline layer (Lewis et al., 1982). The fact that the vitelline layer is readily dissolved in a mixture of 7 parts seawater and 3 parts 2-propanol (V. D. Vacquier, unpublished results) indicates that the vitelline layer is held together by hydrophobic bonding.

An acrosomal lysin from the red abalone *Haliotis rufescens* has been purified in milligram quantities (Lewis et al., 1982). The amino acid sequence of this protein has been determined by automated Edman degradation. The molecule is composed of 134 amino acids, has a calculated molecular weight of 16 070, and hereafter will be termed 16K lysin (Fridberger et al., 1985). The 16K lysin has a p*I* of about 9, is soluble in aqueous low-salt solutions, and must have exposed hydrophobic domains, since it binds to paraffin-coated glass (Lewis et al., 1982). Observing that in situ the lysin surrounds the area where membrane fusion between sperm and egg occurs, we wondered if it might be also involved in membrane fusion. Due to technical difficulties in isolating sperm and egg membrane and the complex nature of these membranes, we have not as yet attempted to answer this question by directly working on sperm and egg. However, phospholipid model

† This work was supported by National Institutes of Health Grants GM-28117 (K.H.) and HD-12986 (V.D.V.).

0006-2960/86/0425-0543$01.50/0

membranes have been considered a convenient experimental system, because phospholipid bilayers are the fundamental structure of biological membranes and they can be well characterized and manipulated. Thus, the factors affecting membrane fusion can be investigated in considerable detail. In this report, we have examined the interaction of 16K lysin with phospholipid membranes and have found that 16K lysin is a very potent promoter of liposome fusion.

Materials and Methods

Purification of Lysin. The 16K lysin was isolated from *Haliotis rufescens* spermatozoa as previously described (Lewis et al., 1982). When stored at −70 °C, the lysin retains activity for at least 2 years. For fusion experiments, the protein was dialyzed at 4 °C against 5 mM *N*-(2-hydroxyethyl)-piperazine-*N*′-2-ethanesulfonic acid (HEPES)[1]/100 mM NaCl/0.1 mM EDTA (pH 7.4). All the experiments were carried out at room temperature unless otherwise specified.

Other Proteins. Bovine serum albumin (fatty acid free), ribonuclease A, and melittin were obtained from Sigma (St. Louis, MO).

Lipids. Phosphatidylserine (PS) and PE (transphosphatidylated from egg PC) were purchased from Avanti Polar Lipids (Birmingham, AL). Egg PC was purified as described by Papahadjopoulos & Miller (1967). Cholesterol from Sigma was recrystallized twice before use. The lipids were stored as a chloroform solution in sealed ampules under argon at −40 °C. Labeled phospholipids, NBD-PE, Rh-PE, and PnAPC were obtained from Avanti and used without further purification.

Chemicals. Carboxyfluorescein (Eastman Kodak) was purified by chromatography on Sephadex LH-20 (Pharmacia, Piscataway, NJ). Carboxyfluorescein was loaded on the column as a sodium salt solution and eluted with water at neutral pH. All other chemicals were purchased in the highest purity available.

Preparation of Liposomes. Large unilamellar vesicles were prepared in 5 mM HEPES/100 mM NaCl/0.1 mM EDTA (pH 7.4) by the reverse-phase evaporation technique (Szoka & Papahadjopoulos, 1978) and then extruded through polycarbonate membranes (Nucleopore, Pleasanton, CA) with a pore size of 0.1 μm (Olson et al., 1979). Carboxyfluorescein vesicles were prepared in 50 mM sodium carboxyfluorescein, pH 7.4. The nonencapsulated carboxyfluorescein was removed by gel filtration on Sephadex G-75 (elution buffer, 5 mM HEPES/100 mM NaCl/0.1 mM EDTA, pH 7.4). The liposome concentration was determined by measuring lipid phosphorus according to the procedure of Bartlett (1959).

Liposome Aggregation. Aggregation of liposomes was followed by a change of absorbance at either 360 or 400 nm in a Beckman Model 34 spectrophotometer. The change in absorbance over time was recorded. The total volume of each sample was 1 mL containing 50 nmol of phospholipids.

Release of Liposomal Content. An SLM-4000 fluorometer was used for continuous monitoring of fluorescence intensity. Carboxyfluorescein, entrapped initially at a self-quenched concentration in liposomes, increased in fluorescence after being released from liposomes. The excitation wavelength was 430 nm, and emission above 520 nm was measured by using a Corning 3-68 cutoff filter. The 100% carboxyfluorescein fluorescence was determined by lysing the vesicles with 0.1% (v/v) Triton X-100. Fluorescence measurements were carried out at 25 °C, unless otherwise specified.

Fusion Assays. In the resonance energy transfer (RET) assay (Struck et al., 1981; Hoekstra, 1982), NBD-PE and Rh-PE were incorporated into one set of liposomes ("labeled liposomes") at 1 mol % each. Under this condition, there was NBD quenching by Rh in labeled liposomes. The labeled liposomes were mixed with liposomes containing no fluorescent phospholipids ("unlabeled liposomes") at a ratio of 1:9 for the fusion measurement. The residual fluorescence of mixed liposomes (50 nmol of total phospholipid of 1 part labeled and 9 parts unlabeled liposomes in 1 mL) before fusion was taken as 0% fluorescence. A complete intermixing of all the bilayers upon fusion would be expected to result in a membrane containing 0.1 mol % of each fluorescent phospholipid. This theoretical maximal fluorescence was considered to be 100%. For the calibration of 100% fluorescence, a third set of liposomes containing 0.1 mol % of each fluorescent phospholipid was prepared. The measurements of NBD fluorescence were recorded continuously at an excitation wavelength of 455 nm and an emission wavelength of 530 nm. Fusion was indicated by the increase of NBD fluorescence resulting from the dilution of the fluorescent lipids into unlabeled liposomes which in turn is due to a decrease in the efficiency of resonance energy transfer between NBD and Rh.

A second fusion assay was developed by measuring fluorescence dequenching of PnAPC in liposomes (Somerharju et al., 1981). Labeled liposomes were prepared by incorporating a self-quenched level of PnAPC into bilayers. In general, 1 mol % of butylated hydroxytoluene was added as an antioxidant in all the experiments involving PnAPC. The labeled liposomes were mixed with unlabeled liposomes (containing egg PC instead of PnAPC) at a ratio of 1:5 for the fusion measurement. Measurements were carried out as described for the RET assay with excitation at 324 nm (0.5-nm slit) and emission at 410 nm (16-nm slit). For calibration of the fluorescence scale and determination of the extent of lipid mixing, the residual self-quenched fluorescence of the labeled liposomes was set as the 0% fluorescence level and the 6-fold dilution of PnAPC was set as 100%. By keeping the absolute concentration of PnAPC in liposomes constant and changing the ratio of egg [PC] vs. [PnAPC], a dequenching curve was obtained. The extent of fusion determined experimentally could then be correlated to the average round of fusion.

Results

Association of Lysin with Liposomes. It is known that the interaction of a protein with lipid bilayers can alter the structure and change the permeability of lipid bilayers (Papahadjopoulos et al., 1975; Steer et al., 1982). The leakage of encapsulated carboxyfluorescein from the vesicles can be easily used for examining lipid–protein interactions. There was instantaneous release of carboxyfluorescein upon addition of the purified lysin at a lipid to protein mole ratio of 160:1 (Figure 1). The ability of lysin to induce leakage was not dependent on the composition of phospholipid vesicles. However, a higher rate of release was observed in PS-containing vesicles compared to neutral PC vesicles under the same conditions. The crude lysin preparation which was released from the acrosome granule had a similar effect on the release of carboxyfluorescein, indicating that other proteins associated with lysin do not interfere with the lysin–liposome interaction. Ca^{2+} or Mg^{2+} inhibited the lysin-induced release of carboxy-

[1] Abbreviations: HEPES, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; NBD-PE, *N*-(7-nitro-2,1,3-benzoxadiazol-4-yl)-phosphatidylethanolamine; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PnAPC, 1-palmitoyl-2-*cis*-parinaroylphosphatidylcholine; PS, phosphatidylserine; RET, resonance energy transfer; Rh-PE, *N*-(lissamine rhodamine B sulfonyl)phosphatidylethanolamine; EDTA, ethylenediaminetetraacetic acid.

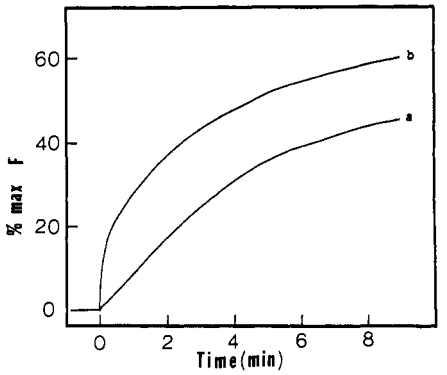

FIGURE 1: Time course of lysin-induced release of carboxyfluorescein encapsulated in (a) PC and (b) PS/PE (1:2) liposomes. Lysin (5 μg) was added to vesicles of 50 μM phospholipid in 1 mL at time = 0.

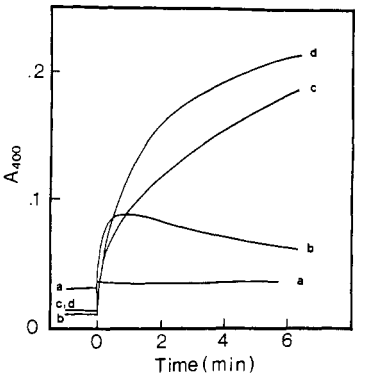

FIGURE 2: Time course of the lysin-induced turbidity change of liposomes composed of (a) egg PC, (b) PS/PC (2:1), (c) PS/PE (1:2), and (d) PS/PC/cholesterol (1:2:1). All samples contained 50 μM phospholipid. Lysin (18 μg) was added at time = 0 at room temperature in each experiment. Buffer (5 mM HEPES/100 mM NaCl/0.1 mM EDTA, pH 7.4) was used to set the zero absorbance.

fluorescein, indicating there was a charge-dependent interaction of lysin with PS-containing vesicles.

We attempted to determine the kinetics of lysin binding to liposomes but found it impossible because of the high nonspecific adsorption of lysin to glass or plastic tubes in the absence of liposomes. What we found in those experiments was that in the presence of PS/PC (1:2) or PS/PE (1:2) vesicles, more than 95% of the lysin would bind to vesicles instead of to the tube surface.

Lysin-Induced Aggregation of Liposomes. Lysin-induced aggregation of liposomes composed of different phospholipids is shown in Figure 2. The extent of aggregation as a function of the amount of protein added is shown in Figures 3 and 4. Aggregation of vesicles induced by 16K lysin was observed in all PS-containing vesicles but not in neutral PC vesicles. A similar aggregation pattern was also seen on negative-stained electron micrographs. The kinetics of aggregation was markedly dependent on the ratio of negative charged phospholipid to lysin. A clear difference was observed between PS/PC (2:1) and PS/PC/cholesterol (1:2:1). The molar ratio of phospholipid to lysin in these experiments was 45:1. For PS/PC (2:1) liposomes, there were 15 PS molecules available on the liposomal surface for lysin. This high ratio of PS to lysin may explain the biphasic aggregation profile of PS/PC (2:1) liposomes. Further detailed investigations are needed to establish the relationship between the structural change and the charge ratio of lipid to protein. The aggregation profiles of PS/PE (1:2), PS/PC (1:2), or PS/PE/PC (1:1:1) liposomes were similar.

Lysin-Induced Liposome Fusion. In an attempt to determine whether fusion occurred during the aggregation of vesicles induced by lysin, two assays of lipid mixing were performed. Figure 5 shows a representative time course of an RET fusion assay. For this particular assay, an increase of fluorescence over 26% represents that each labeled liposome has gone through at least one round of complete lipid mixing. No fluorescence increase was induced by lysin in nonaggre-

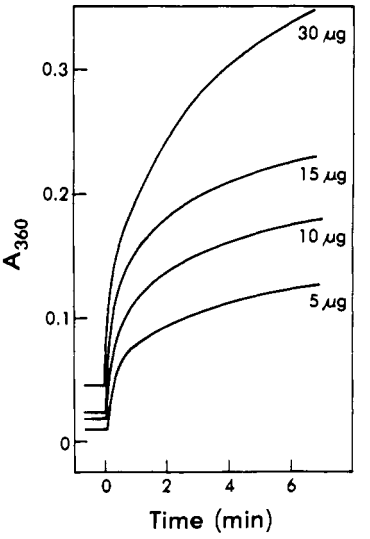

FIGURE 3: Time course of turbidity change of PS/PE/PC (1:1:1) liposomes induced by lysin. Liposome suspension (50 nmol of lipid in 100 μL) was added to 0.9 mL of buffer containing various quantities of lysin (5, 10, 15, and 30 μg) at time = 0. Absorbance at 360 nm was recorded at room temperature.

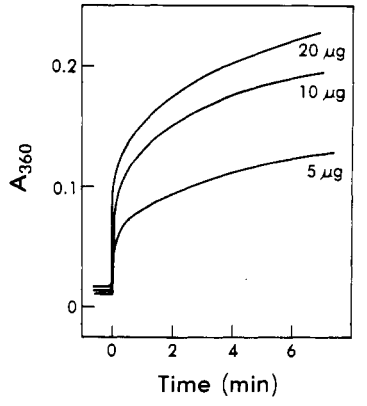

FIGURE 4: Time course of turbidity change of PS/PE (1:2) liposomes induced by lysin (5, 10, and 20 μg). Experimental conditions were given in the Figure 3 legend.

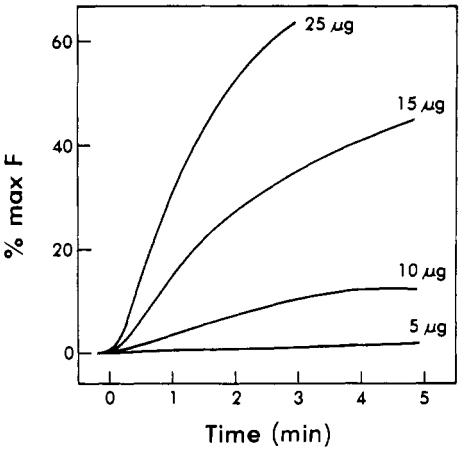

FIGURE 5: Fusion of PS/PE/PC (1:1:1) liposomes induced by lysin as measured by resonance energy transfer assay. Lysin (5, 10, 15, and 25 μg) was mixed with 50 μM liposomes at time = 0.

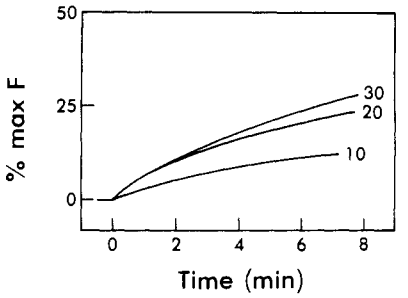

FIGURE 6: Fusion of PS/PE (1:2) liposomes induced by lysin as measured by resonance energy transfer assay. Lysin (10, 20, and 30 μg) was mixed with liposomes at time = 0.

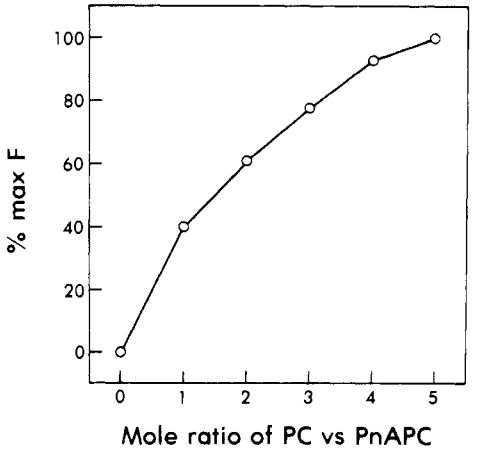

FIGURE 7: Fluorescence dequenching curve of PS/PE/PC (1:1:1) liposomes containing 1-palmitoyl-2-*cis*-parinaroylphosphatidylcholine. The residual fluorescence of PS/PE/PnAPC (1:1:1) liposomes was set as 0% maximum fluorescence. Dequenching was achieved by diluting PnAPC with egg PC but maintaining the same ratio of phospholipids, i.e., mole ratio of PS:PE:(PC + PnAPC) = 1:1:1. The fluorescence of PS/PE/PC/PnAPC (6:6:5:1) liposomes was set as maximum fluorescence. Each sample has an equal concentration of PnAPC (4.125 μM).

gated PC liposomes. Furthermore, no lipid mixing was observed upon the addition of lysin to the mixture of labeled PS/PC (1:2) vesicles and unlabeled PC vesicles, indicating that lysin does not promote the exchange of lipid probes between nonaggregated liposomes. In marked contrast to PC liposomes, all PS-containing liposomes scored in the RET fusion assay. With higher amounts of lysin added, the extent of lipid mixing was estimated to correspond to slightly more than two rounds of liposome fusion. In principle, there should be a higher fluorescence increase corresponding to higher lipid mixing; however, the extensive aggregation of liposomes promoted by lysin probably led to underestimating it. This was observed clearly in PS/PE (1:2) liposomes (Figure 6) where lysin-induced fusion was not as rapid as other liposomes, even though there was no substantial difference in the rate or extent of aggregation (Figure 4). Among PS-containing liposomes, a higher specific fusion rate was generally observed in bilayers containing PC. A low-fusing system like PS/PE (1:2) liposomes could be improved by including PC as seen in PS/PE/PC (1:1:1) liposomes. Cholesterol up to 30% did not affect the fusion pattern.

Even though NBD-PE and Rh-PE have been shown to be nonexchangeable (Struck et al., 1981; Kumar et al., 1982), the possibility that lysin may promote exchange of probes between aggregated lipid bilayers in an RET fusion assay cannot be ruled out. An alternative fusion assay was developed by measuring the fluorescence dequenching of PnAPC during its mixing with unlabeled phospholipid. Figure 7 shows a

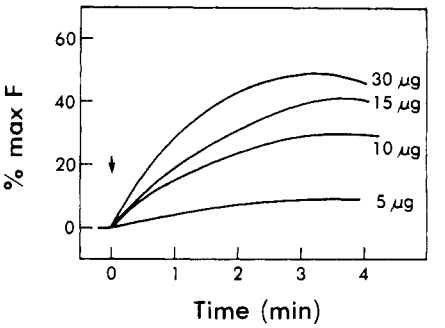

FIGURE 8: Fusion of PS/PE/PC (1:1:1) liposomes induced by lysin (5, 10, 15, and 30 μg) as measured by fluorescence dequenching of PnAPC. Lysin was added to the liposome suspension (50 μM) at time = 0. Lysin concentrations higher than 30 μg/mL did not increase the rate and extent of fusion.

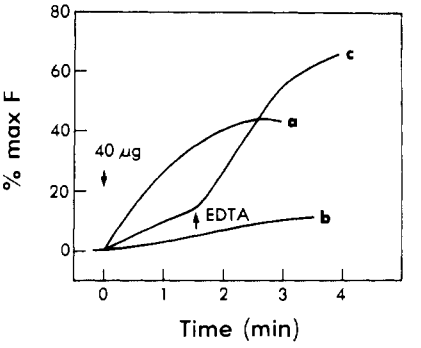

FIGURE 9: Effect of Ca^{2+} on lysin-induced fusion of PS/PE/PC (1:1:1) liposomes. Fusion based on the fluorescence dequenching of PnAPC was monitored as described under Materials and Methods. Ca^{2+} was added to the liposome suspension prior to the addition of lysin (40 μg). Curve a, fusion induced by lysin in the absence of Ca^{2+}; curve b, fusion induced by lysin in the presence of 2 mM Ca^{2+}; curve c, inhibition of fusion by Ca^{2+} (1 mM) eliminated by EDTA (2 mM).

Table I: Effect of Ca^{2+} Preincubation on Lysin-Induced Fusion of PS/PC (1:2) Vesicles Measured by RET Assay[a]

$[Ca^{2+}]$ (mM)	[lysin] (μg mL^{-1})	fusion rate (%F/min)
0	9	6.4
0	10	6.8
0	18	28.0
0.1	18	28.0
0.25	18	16.0
0.5	18	8.0
1.0	18	2.4
2.0	18	0.0

[a] Liposomes were preincubated with Ca^{2+} for 1 min before the addition of lysin.

dequenching curve of PnAPC in PS/PE/PC (1:1:1) liposomes as a function of the mole ratio of unlabeled PC to PnAPC. The time course of dequenching measurement of PnAPC in PS/PE/PC (1:1:1) liposomes induced by lysin is illustrated in Figure 8. The extent of lipid mixing obtained in this dequenching experiment corresponds to less than two rounds of liposomal fusion.

Effect of Divalent Cations. When liposomes were preincubated with Ca^{2+} (1–2 mM), fusion induced by lysin was inhibited (Figure 9, Table I). However, if Ca^{2+} was added to lysin-induced fusing liposomes, only a slight inhibition was detected. The inhibition was slowly reduced as fusing took place, and the extent of lipid mixing was the same as in the case with no Ca^{2+} (Figure 10A). This inhibition could be reversed rapidly by adding EDTA to the slowing fusing mixture (Figure 9). Magnesium at higher concentration (3–4 mM) had a similar effect as Ca^{2+}. It should be noted that addition of Ca^{2+} to a slow-fusing lysin–liposome mixture at

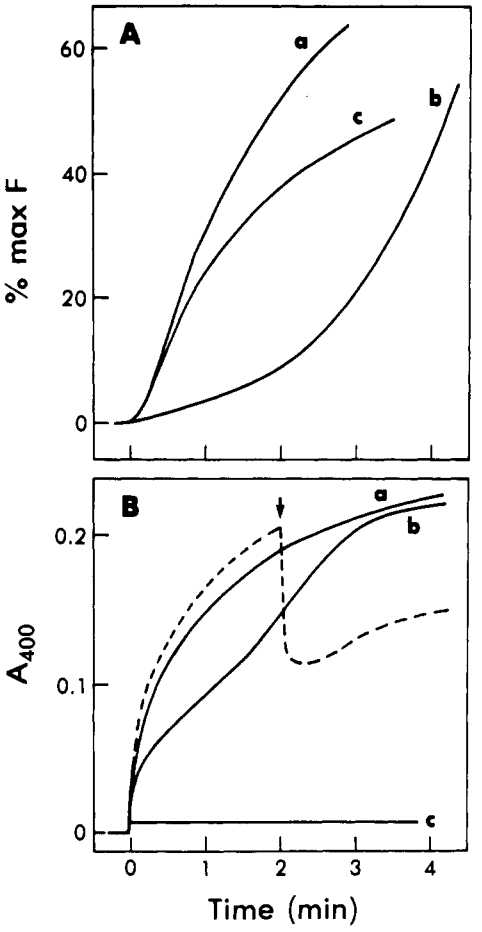

FIGURE 10: Fluorescence development in RET assay (A) vs. corresponding turbidity change at 400 nm (B) of PS/PE/PC (1:1:1) liposomes. Lysin (25 μg) was added to liposomes (50 μM) preincubated with 0 mM Ca^{2+} (a) or 2 mM Ca^{2+} (b). Lysin was added at time = 0. Melittin (5 μg) was added to liposomes (50 μM) preincubated with 2 mM Ca^{2+} (c). Dashed line in (B): Melittin (15 μg) was mixed with liposomes in the absence of Ca^{2+} initially; Ca^{2+} (2 mM) was added to the aggregated liposomes at the arrow.

a later stage (>10 min) initiated further fusion and doubled the final extent of lipid mixing. In this respect, lysin behaves as an aggregation agent like other physiological polycations, polyamines (Hong et al., 1983; Schuber et al., 1983).

Other Proteins. The fusogenic nature of other proteins, bovine serum albumin and cationic proteins, such as ribonuclease and melittin, was also tested. As expected, bovine serum albumin and ribonuclease showed no effect on liposome aggregation or fusion. Lysin-induced fusion was not affected even in the presence of large amounts of bovine serum albumin or ribonuclease. As for melittin, there was a very rapid development of fluorescence when melittin was injected into the liposome suspension in both fusion assays. The turbidity change upon the addition of melittin to liposomes appeared to correlate only to the fluorescence increase within an optimal range of the ratio of protein to lipid. For 50 μM of PS/PE/PC (1:1:1) liposomes, 10–15 μg/mL melittin was found to be the optimal range for correlating turbidity change to fluorescence increase. When the amount of melittin used was outside this range, an instant time-independent increase in fluorescence was observed along with no change in turbidity. In the presence of Ca^{2+}, melittin induced a time-dependent increase in fluorescence along with no change in turbidity, as seen in Figure 10. This further complicates the nature of the interaction of melittin with liposomes. Despite the complexity, it is clear that any liposome fusion induced by melittin has to be among those cases in which turbidity and time-dependent fluorescence increase are correlated. Since the efficiency of both fluorescence dequenching and energy transfer is highly dependent on the surface density of probes in the bilayer, any significant insertion of protein molecules into the lipid bilayer will certainly increase the fluorescence intensity. Such an increase in fluorescence intensity was observed in melittin–liposome systems and could be quantified by mixing melittin with labeled liposomes. The increase was time independent, and Ca^{2+} was found to promote further increase. This observation fits with the established capability of melittin readily to insert the apolar region of lipid bilayers (Mollay & Kreil, 1973). In contrast, measurements of lysin–liposome interactions reveal no significant time-independent fluorescence increase under the same experimental conditions as those of melittin.

Discussion

The interaction of 16K lysin of abalone sperm with liposomes has been described in this paper. To characterize interactions of lysin with the phospholipid bilayer, understanding the structure of the protein is essential. The complete amino acid sequence of 16K lysin has been resolved recently (Fridberger et al., 1985). Lysin, a cationic protein, contains a segment (residues 29–72) which has 10 positively charged but no negatively charged residues. An obvious hydrophobic stretch of amino acids is seen from residues 62–70. A model representing secondary structures of lysin reveals that four α-helices display hydrophobic and neutral amino acid residues on half of the circumference and charged residues on the other half. In view of these separate domains lysin displays, it is not surprising that lysin is able to interact nonspecifically with liposomes of various composition. The spontaneous release of entrapped carboxyfluorescein in liposomes suggests a penetration of the lysin molecule into bilayers or other structural changes (e.g., phase separation) due to the binding of lysin to lipid bilayers. The interaction of lysin with neutral PC liposomes must come from the hydrophobic domain. This is consistent with the fact that lysin binds avidly to nonpolar surfaces through its hydrophobic domains. Even though lysin tends to self-associate in a high-salt medium at high protein concentration, this protein–protein bonding is too weak to bridge adjacent PC liposomes as evidenced in the fact that no aggregation of PC liposomes was observed in the presence of lysin. It is clear that the binding of positively charged lysin to neutral vesicles enhances the repulsion between adjacent vesicles and thus merely the perturbation of bilayer integrity by lysin is seen as the release of fluorescent dye.

There are at least three essential requirements for fusion of liposomes: (1) aggregation of the liposomes; (2) a close apposition of membranes; and (3) a transitional destabilization of the bilayers. Results of the present study indicate that lysin can meet some or all of these requirements, depending upon the lipid composition of liposomes.

Surface electrostatics of liposomes containing acidic phospholipid are modified by the presence of multivalent cations, both by specific binding and by electrostatic screening of the double layer (Nir et al., 1983). Lysin, a natural polycation with some distinctive positively charge domains (Fridberger et al., 1985), is expected to decrease the surface charge density and surface potential. The consequent reduction of the electrostatic repulsion by lysin seems to induce liposomes to aggregate, as seen in PS-containing liposomes (Table II). Aggregation can also occur when protein molecules bridge adjacent liposomes through their multibinding sites. According to the predicted secondary structure of lysin, there are several

Table II: Interaction of Lysin with Liposomes

phospholipid compn	carboxy-fluorescein release	aggregation	fusion RET	fusion PnAPC
PC	+	-	-	-
PS/PE (1:2)	++	++	+	
PS/cholesterol (2:1)	++	++	++	
PS/PC (1:2)	++	++	++	++
PS/PC/PE (1:1:1)	++	++	++	++

separate positively charged and hydrophobic domains on α-helices. The positively charged sites of lysin, which are associated with PC liposomes via hydrophobic insertion, may bind to PS-containing liposomes and lead to aggregation and fusion between PC and PS-containing liposomes. In contrast to this prediction, no fusion between PC and PS/PC (1:2) liposomes was detected. It is concluded that PS is required for lysin-induced fusion. On close examination of lysin-induced aggregation of PS-containing liposomes, we found that the kinetics seems not to depend on what neutral phospholipid is used in PS-containing liposomes as long as they have the same charge density, as evidenced in PS/PE (1:2), PS/PC (1:2), and PS/PE/PC (1:1:1) liposomes (Figures 2–4). In addition, the aggregation kinetics is markedly dependent on the charge density of the bilayer surface as seen in PS/PE (1:2) vs. PS/PC (2:1) liposomes (Figure 2).

There are cases in which aggregated liposomes do not fuse, mainly because there is not a close apposition of membranes: a hydration shell of the bilayer surface is thought to prevent fusion (Papahadjopoulos et al., 1977; Düzgünes et al., 1981; Hong et al., 1981). Membrane destabilization is required for fusion to take place in the aggregated system. A major role for lysin in liposome fusion may arise from its capability to destabilize the membrane via hydrophobic insertion into lipid bilayers. It appears that lysin detects the structural difference between PS/PE (1:2) and PS/PC (1:2) liposomes as reflected in the difference in the rate of fusion. A small head group and the capability of PE to form intermolecular hydrogen bonding permit PE to pack more tightly than PC in lipid bilayers. Thus, it is more difficult for lysin molecules to penetrate PE-containing bilayers than PC-containing ones. As pointed out above, any significant insertion of protein into lipid bilayer can be quantified by measuring either the dequenching of PnAPC liposomes or the decrease of energy transfer in labeled liposomes. This correlation is currently under investigation. Our preliminary observation shows a fair correlation between the bilayer packing and the extent of protein penetration. This may also explain why the PS/PE/PC (1:1:1) liposomes fuse more readily than PS/PE (1:2) liposomes despite both liposome systems having similar aggregation kinetics (Figures 3 and 4). For PS/PC (1:2) or PS/PC/PE (1:1:1) liposomes, preincubation with Ca^{2+} or Mg^{2+} inhibited lysin-induced fusion but not aggregation (Figure 10). When the order of the addition of Ca^{2+} and lysin was reversed (i.e., lysin first followed by Ca^{2+}), fusion was only slightly inhibited. Ca^{2+} or Mg^{2+} only slows down the initial fusion: the same extent of fusion is eventually reached (Figure 10). Since aggregation is not sensitive to the order of Ca^{2+} and lysin addition, the difference in fusion rate is most likely due to the degree of the hydrophobic interaction between lysin and the bilayer. Since divalent cations are known to condense bilayer packing, the binding of divalent cations to the bilayer surface prior to the addition of lysin may reduce lysin's hydrophobic interaction with the bilayer.

It has been speculated that the inhibitory action of PC in Ca^{2+}-induced fusion of PS/PC liposomes may be related to the hydration shells remaining around the PC head groups even in the presence of Ca^{2+} (Jendrasiak & Hasty, 1974; Düzgüneş et al., 1981). Unlike with Ca^{2+}, the fusogenic activity of lysin is not reduced by PC in PS-containing liposomes. A similar observation is described in clathrin-induced pH-dependent fusion of liposomes (Hong et al., 1985).

Other cationic proteins, ribonuclease and melittin, have been tested for fusogenic activity in liposomes. Liposomes, which are susceptible to lysin-induced fusion, do not interact with ribonuclease. Even at the very high concentration of ribonuclease present in the liposome suspension, lysin-induced fusion is not affected. Melittin, with 13 of the first 20 residues being hydrophobic and 4 of the last 6 being positively charged (Habermann & Jentsch, 1967), somewhat resembles lysin. However, there are some distinctly different features in aggregation and lipid mixing between these two proteins. Melittin integrates readily into phospholipid bilayers and shows a narrow concentration range for liposome aggregation. Only in this range does there exist a possibility that aggregated liposomes will fuse (Figure 10). The incorporation of melittin into the lipid bilayer is rapid, and there is no saturation point. A large amount of melittin makes PS-containing vesicles positively charged, and no aggregation of liposomes occurs. A time-independent fluorescence increase represents a fast initial incorporation and is followed by a slow redistribution of melittin to both half-leaflets of bilayer. The magnitude of this fluorescence increase due to the physical insertion of melittin is less than the total fluorescence increase of the liposomes aggregated by an optimal amount of melittin. The net fluorescence increase seems to represent the extent of lipid mixing among aggregated liposomes. In contrast, all the time-dependent lysin-induced fluorescence increases we have detected are correlated well with an increase in turbidity. The rate of both turbidity and fluorescence increase reaches a plateau when one continues to increase the amount of lysin in liposome suspension. (A note of caution: A time-dependent fluorescence increase caused by a combination of melittin and Ca^{2+} in an RET assay cannot be interpreted as fusion since it correlates with absolutely no change in turbidity; see curve c of Figure 10.)

There is a tendency that lysin self-associated at higher protein concentrations in salt solution. In view of lysin's predicted secondary structure (Fridberger et al., 1985), in ionic media lysin may form a molecular complex with most charged residues located on the surface via hydrophobic protein–protein bonding. Thus, the site for hydrophobic bilayer penetration is not available. This may explain our preliminary results that self-associated lysin promotes a rapid aggregation of liposomes but its fusogenic activity is largely reduced.

The fusogenic activity of viral protein can be considered to come from a conformational change by lowering the pH (White & Helenius, 1980; Skehel, et al., 1982; Florkiewicz & Rose, 1984). The conformational change leads to an increase of the hydrophobic surface which, in turn, facilitates the membrane fusion. In this respect, lysin may utilize its existing hydrophobic domain to interact with the hydrophobic region of phospholipid bilayers and facilitate the fusion of membranes, which are in close apposition engendered by the polycationic feature of lysin.

We have shown in two fusion assays that PS, the most abundant negatively charged phospholipid in biological membranes, is required for lysin-induced fusion. Recently, Glabe (1985) has shown that the sperm adhesive protein, bindin, also induces fusion of mixed-phase liposomes. For bindin-induced fusion, PS is required. It will be interesting to examine the

fusogenic activity of lysin in other negatively charged lipids such as phosphatidate, phosphatidylinositol, and phosphate derivatives of phosphatidylinositol. If this fusion model can be related to the fusion of sperm and egg plasma membrane, this major protein released by abalone sperm after acrosome reaction may play two roles in fertilization: dissolution of the vitelline layer of the egg surface by breaking the hydrophobic bonding of the glycoprotein in the vitelline gel and further promotion of the close apposition and fusion of the sperm and egg plasma membrane.

ACKNOWLEDGMENTS

We gratefully acknowledge Dr. Demetrios Papahadjopoulos for his support and interest in this project. We also thank Drs. Patricia Baldwin and Stephen Murray for valuable comments.

Registry No. Ca, 7440-70-2; Mg, 7439-95-4; melittin, 37231-28-0.

REFERENCES

Bartlett, G. R. (1959) *J. Biol. Chem. 234*, 466–468.

Dan, J. C. (1967) in *Fertilization* (Metz, C. B., & Monroy, A., Eds.) pp 237–293, Academic Press, New York.

Düzgüneş, N., Wilschut, J., Fraley, R., & Papahadjopoulos, D. (1981) *Biochim. Biophys. Acta 642*, 182–195.

Florkiewicz, R. Z., & Rose, J. K. (1984) *Science (Washington, D.C.) 225*, 721–723.

Fridberger, A., Sundelin, J., Vacquier, V. D., & Peterson, P. A. (1985) *J. Biol. Chem. 260*, 9092–9099.

Glabe, C. G. (1985) *J. Cell Biol. 100*, 800–806.

Habermann, E., & Jentsch, J. (1967) *Hoppe-Seyler's Z. Physiol. Chem. 348*, 37–50.

Haino-Fukushima, K. (1974) *Biochim. Biophys. Acta 352*, 179–191.

Hoekstra, D. (1982) *Biochemistry 21*, 2833–2840.

Hong, K., Düzgüneş, N., & Papahadjopoulos, D. (1981) *J. Biol. Chem. 256*, 3641–3644.

Hong, K., Schuber, F., & Papahadjopoulos, D. (1983) *Biochim. Biophys. Acta 732*, 469–472.

Hong, K., Yoshimura, T., & Papahadjopoulos, D. (1985) *FEBS Lett. 191*, 17–23.

Jendrasiak, G. L., & Hasty, J. H. (1974) *Biochim. Biophys. Acta 337*, 79–91.

Kumar, N., Blumenthal, R., Henkart, M., Weinstein, J. N., & Klausner, R. D. (1982) *J. Biol. Chem. 257*, 15137–15144.

Lewis, C. A., Leighton, D. L., & Vacquier, V. D. (1980) *J. Ultrastruct. Res. 72*, 39–46.

Lewis, C. A., Talbot, C. F., & Vacquier, V. D. (1982) *Dev. Biol. 92*, 227–239.

Mollay, C., & Kreil, G. (1973) *Biochim. Biophys. Acta 316*, 196–302.

Olson, F., Hunt, C. A., Szoka, F. C., Vail, W. J., & Papahadjopoulos, D. (1979) *Biochim. Biophys. Acta 557*, 9–23.

Papahadjopoulos, D., & Miller, N. (1967) *Biochim. Biophys. Acta 135*, 624–638.

Papahadjopoulos, D., Moscarello, M., Eylar, E. H., & Isac, T. (1975) *Biochim. Biophys. Acta 401*, 317–335.

Papahadjopoulos, D., Vail, W. J., Newton, C., Nir, S., Jacobson, K., Poste, G., & Lazo, R. (1977) *Biochim. Biophys. Acta 465*, 579–598.

Poste, G., & Allison, A. C. (1973) *Biochim. Biophys. Acta 300*, 421–465.

Poste, G., & Nicolson, G. L. (1978) in *Cell Surf. Rev. 5*, 1–111.

Schuber, F., Hong, K., Düzgüneş, N., & Papahadjopoulos, D. (1983) *Biochemistry 22*, 6134–6140.

Shiroya, Y., & Sakai, Y. T. (1984) *Dev. Growth Differ. 26*, 25–32.

Skehel, J. J., Bayley, P. M., Brown, E. B., Martin, S. R., Waterfield, M. D., White, J. M., Wilson, I. A., & Wiley, D. C. (1982) *Proc. Natl. Acad. Sci. U.S.A. 79*, 968–972.

Somerharju, P., Brockerhoff, H., & Wirtz, K. W. A. (1981) *Biochim. Biophys. Acta 649*, 521–528.

Steer, C. J., Klausner, R. D., & Blumenthal, R. (1982) *J. Biol. Chem. 257*, 8533–8540.

Struck, D., Hoekstra, D., & Pagano, R. G. (1981) *Biochemistry 20*, 4093–4099.

Szoka, F. C., & Papahadjopoulos, D. (1978) *Proc. Natl. Acad. Sci. U.S.A. 75*, 4194–4198.

White, J., & Helenius, A. (1980) *Proc. Natl. Acad. Sci. U.S.A. 77*, 3273–3277.