Promotion and Inhibition of Vesicle Fusion by Polylysine†

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ABSTRACT: Polylysine induced rapid aggregation of large unilamellar vesicles composed of phosphatidylcholine–cardiolipin (1:1 molar ratio) but not their fusion. Application of the terbium–dipicolinic acid fusion assay showed that addition of polylysine at nanomolar concentrations enabled a significant lowering of the Ca^{2+} threshold concentration for vesicle fusion from 9 to 1 mM. Analysis of the kinetics of fusion with a mass-action kinetic model showed that polylysine enhanced significantly the rate of aggregation but affected only slightly the rate of fusion per se. Maximal enhancement of overall fusion rates occurred at a charge ratio (polylysine/cardiolipin) of about 0.5. At larger polylysine concentrations, e.g., at charge ratios greater than 3, polylysine inhibited vesicle fusion.

Vesicle fusion induced by polycations has been demonstrated for polylysine (Vail & Stollery, 1979; Gad et al., 1982; Gad & Eytan, 1983; Gad, 1983; Uster & Deamer, 1985), polymyxin B (Gad & Eytan, 1983; Gad, 1983), melittin (Eytan & Almary, 1983), cytochrome *c* (Gad et al., 1982), and polyamines (Schuber et al., 1983). Polylysine, polymyxin B, melittin, and cytochrome *c* induced fusion of negatively charged small unilamellar vesicles. When compared with Ca^{2+} or Mg^{2+}, the potency of these polycations was remarkably greater in terms of the amounts needed to induce fusion (Gad & Eytan, 1983; Gad, 1983; Eytan & Almary, 1983). Polyamines were shown to promote Ca^{2+}-induced fusion of LUV[1] by lowering the cation's threshold concentration and also to induce fusion of certain binary and ternary vesicles, which included phosphatidylethanolamine (Schuber et al., 1983). Polycations such as polylysine may be of great importance as inducers of membrane fusion, since they may promote vesicle–cell interactions, e.g., for the introduction of macromolecules into cells. So far no induction of fusion by polylysine has been recorded in vesicle–cell systems.

Our preliminary examination showed that polylysine alone could not induce fusion of LUV, which did not contain phosphatidylethanolamine. In the present work, we demonstrate the use of polylysine to increase the potential of Ca^{2+} in inducing fusion of negatively charged vesicles. However, the enhancing effect is reversed with excess polylysine. With the mass-action kinetic model (Nir et al., 1980, 1982, 1983a; Bentz et al., 1983a,b), the role of polylysine is elucidated. The importance of competitive binding and other mechanisms are considered.

Materials and Methods

Materials. Phosphatidylcholine (PC), cardiolipin (CL), cholic acid, polylysine (average M_r 35000), poly(aspartic acid), and poly(glutamic acid) were purchased from Sigma Chemicals Co. The poly amino acids were suspended in buffer and kept in small aliquots at −18 °C. Each aliquot was never refrozen since degradation occurred during repetitive thawing. Cholic acid was further purified by twice recrystallizing it from ethanol (Kagawa & Racker, 1971). All phospholipids were assayed for purity on thin-layer chromatography and proved to be at least 99% pure. Phospholipid concentration was determined by the concentration of phosphate as described (Bartlett, 1959); thereafter, vesicle concentration was given as lipid phosphorus content. In binding experiments, polylysine concentration was determined by both ninhydrin (Yemm & Cocking, 1955) and protein (Bradford, 1976) assays.

Vesicle Preparation. The Tb–DPA fusion assay (Wilschut et al., 1980) was used for monitoring fusion. Large unilamellar vesicles (LUV) were prepared by the reverse-phase evaporation technique (Wilschut et al., 1981) followed by extrusion through 0.2- and 0.1-μm polycarbonate membranes (Olson et al., 1979). Vesicle contents were as follows: (a) 5 mM $TbCl_3$, 50 mM sodium citrate, and 5 mM Hepes, pH 7.4; (b) 50 mM DPA, 20 mM NaCl, and 5 mM Hepes, pH 7.4; (c) 2.5 mM $TbCl_3$, 25 mM DPA, 10 mM NaCl, 25 mM sodium citrate, and 5 mM Hepes, pH 7.4. After extrusion through the polycarbonate membrane, the vesicles were passed through a Sephadex G-75 column equilibrated and eluted with 100 mM NaCl, 1 mM EDTA, and 5 mM Hepes, pH 7.4. To remove EDTA, aliquots of vesicles a and b were passed through a second Sephadex G-75 column, the elution buffer not containing EDTA. After the second column Tb-containing and DPA-containing vesicles were used to determine entrapped volume. These vesicles had an internal volume of 3–4 μL/μmol of P_i, and their intrinsic leakage rate was less than 1% per day at 4 °C.

Fusion Assay. All measurements were carried out as described (Wilschut et al., 1980) in a SLM 4800 spectrofluorometer. The Tb–DPA complex was excited at 276 nm, and the fluorescence was measured at 545 nm after passing through a Corning 3-68 cutoff filter (>530 nm). All measurements were carried out at 25 °C. Measurements were carried out at several vesicle concentrations of 5–100 μM lipid phosphorus. A typical reaction mixture consisted of Tb and DPA vesicles at a 1:1 ratio, 0.1 mM EDTA, 100 mM NaCl, and 5 mM Hepes, pH 7.4. Vesicles were always added last, unless oth-

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[1] Abbreviations: CL, cardiolipin (bovine heart); DPA, dipicolinic acid; EDTA, ethylenediaminetetraacetic acid; Hepes, *N*-(2-hydroxyethyl)-piperazine-*N*′-2-ethanesulfonic acid; LUV, large unilamellar vesicles; PC, phosphatidylcholine (egg yolk); PL, polylysine; PS, phosphatidylserine.

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erwise mentioned, into the vigorously stirred suspension inside the fluorometer. Maximal fluorescence intensity was calibrated with Tb-containing vesicles after passage through the EDTA-free column and the addition of 0.5% sodium cholate in the presence of 20 μM DPA. The reduction in the fluorescence of preformed Tb–DPA complex entrapped in vesicles (type c) was determined in a medium containing 0.1 mM EDTA. This quantity is denoted D (Bentz et al., 1983b) and represents the leakage as well as the entry of EDTA and Ca^{2+} into the vesicles. The corrected fusion curves in a typical vesicle mixture were given initially by $F_c = F + 0.5D$ (Nir et al., 1980; Bentz et al., 1983b), F denoting the percent of maximal fluorescence of the Tb–DPA complex. Chelation of polylysine was achieved by the addition of 5-fold excess of poly(aspartic acid) or poly(glutamic acid) in 100 mM NaCl and 5 mM Hepes (pH 7.4).

Preliminary fusion experiments were conducted by employing the resonance energy transfer method with chlorophylls *a* and *b*, according to Gad & Eytan (1983) with the exception of having the pigments separated by paper chromatography (10% acetone in light petrol ether) from the carotenoids, which interfered with the preparation of the LUV. The results obtained with this method of mixing of membranes agreed with the data obtained with the TB–DPA method when corrected for leakage.

Analysis of Fusion Kinetics. The analysis followed the mass-action kinetic model Nir et al., 1980, 1982, 1983a; Bentz et al., 1983a), which views the overall fusion reaction as a sequence of two gross steps: (1) aggregation whose rate constant $C_{\|}$, has the units M^{-1} s^{-1}; (2) fusion, i.e., membrane destabilization and merging, whose rate constant, $f_{\|}$, has the unit s^{-1}. Dissociation of aggregates with a rate constant, D (s^{-1}), was explicitly considered.

The values of F_c (% Tb–DPA fluorescence corrected for leakage) were first simulated by the equation (Bentz et al., 1983a)

$$F_c = 100A(t)\mathcal{F}(t) \quad (1)$$

in which t is the time

$$A(t) = (1 + 4C_{\|}V_0t)^{1/4} - 1 \quad (2)$$

V_0 is the molar concentration of the vesicles, and

$$\mathcal{F}(t) = 1 + [\exp(-f_{\|}t) - 1]/(f_{\|}t) \quad (3)$$

The fusion of preaggregated vesicles, e.g, preincubated with polylysine, was simulated by the equation (Bentz et al., 1983b)

$$F_c(t) = 50[1 - \exp(-f_{\|}t)] \quad (4)$$

At the second stage more accurate numerical calculations were carried out, which enabled an extension up to $F_c = 40\%$ (S. Nir, unpublished data). In these calculations, deaggregation is explicitly accounted for. We utilized the approximate relations $\hat{f}_{\|} = f_{\|} + D_{\|}$ and $\hat{C}_{\|} = C_{\|}/(1 + D_{\|}/f_{\|})$, in which $C_{\|}$ and $f_{\|}$ are the true values, whereas $\hat{C}_{\|}$ and $\hat{f}_{\|}$ are the effective values that coincide with the true values when $D_{\|}/f_{\|} \ll 1$ [see Bentz et al. (1983a, 1985) for more details].

Turbidity Measurements. Continuous turbidity measurements for determination of aggregation were carried out at 500 nm in gilford 2535 spectrophotometer. Vesicles and incubation conditions were the same as in the fusion assay.

Polylysine Binding Measurements. Vesicles, labeled with 1 mol % of the fluorescent probe *N*-NBD-PE [*N*-(7-nitro-2,1,3-benzoxadiazol-4-yl)phosphatidylethanolamine] were incubated at varying concentrations (50–400 μM) for 10 min with polylysine at varying concentrations (2–16 μg/mL), corresponding to charge ratios of 0.25–1 (polylysine:CL), in

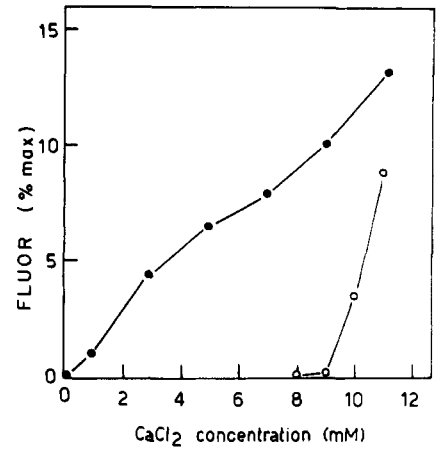

FIGURE 1: Effect of polylysine on calcium-induced fusion. The 50 μM LUV consisting of PC:CL (1:1 molar ratio) were injected into suspensions containing varying concentrations of $CaCl_2$ with (●) or without (○) 35 nM polylysine (charge ratio of 0.5 PL:CL). Fusion was monitored by the Tb–DPA assay. The corrected extent of fusion (see Materials and Methods) was calculated 3 s after LUV injection.

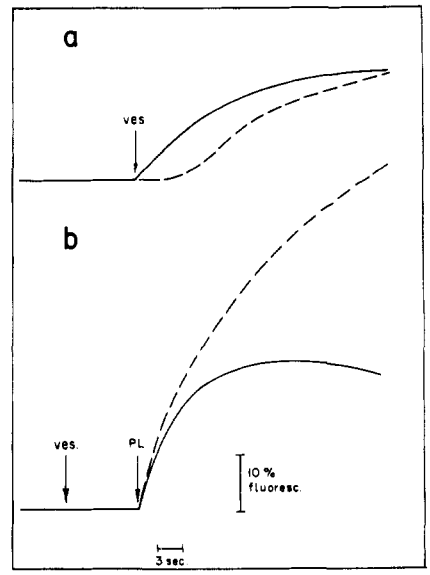

FIGURE 2: Enhancement of fusion by polylysine. The 50 μM LUV (ves.) consisting of PC:CL (1:1 molar ratio) were injected into suspensions containing either 10 mM $CaCl_2$ (a) or 8 mM $CaCl_2$ (b). Polylysine (35 nM, equivalent to a charge ratio of 0.5) was added (b). Tb fluorescence (solid lines) and leakage (broken lines) were monitored.

100 mM NaCl and 5 mM Hepes (pH 7.4). The suspension was spun for 90 min at 100000*g*. Aliquots of the supernatant were assayed for remaining polylysine and traces of liposomes. In parallel experiments, 8 mM $CaCl_2$ was included in the reaction mixture, and polylysine binding was assayed in a similar fashion. The amount of polylysine in the supernatant was given as the fraction of the polylysine in the supernatant of samples that did not include vesicles.

RESULTS

Effect of Polylysine on Calcium-Induced Fusion. A significant rate of fusion was observed when PC:CL (1:1 molar ratio) LUV were mixed in suspension with Ca^{2+} concentrations higher than 9 mM (Figure 1). When polylysine was added to a suspension of vesicles and subthreshold Ca^{2+} concentrations, e.g., 8 mM, the rate of fusion increased dramatically (Figures 1 and 2). The same effect was observed when subthreshold concentrations of Ca^{2+} were added to a suspension of LUV and polylysine. While neither Ca^{2+} nor polylysine

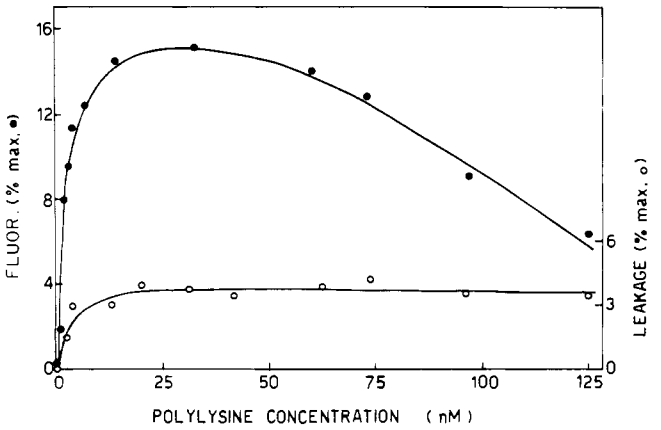

FIGURE 3: Effect of polylysine concentration on the extent of fusion. The 50 μM LUV consisting of PC:CL (1:1 molar ratio) were injected into suspensions containing 8 mM $CaCl_2$ and varying concentrations of polylysine. The corrected Tb fluorescence (●) and the leakage of contents (○) were calculated after 3 s.

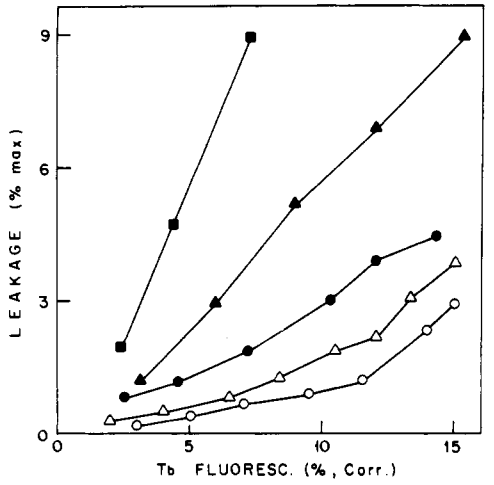

FIGURE 4: Effect of polylysine and vesicle concentration on fusion and leakage. LUV consisting of PC:CL (1:1 molar ratio) were incubated at concentrations of 100 (○, ●), 20 (△, ▲), and 5 μM (■) lipid phosphorus with 10 mM $CaCl_2$ (empty symbols) and 8 mM $CaCl_2$ with polylysine (42, 8.4, and 2.1 μg/mL, respectively, full symbols). In each sample, the Tb fluorescence and the leakage were recorded. Corrected Tb fluorescence was plotted vs. leakage.

could induce fusion, their combination did. When polylysine was also present in the incubation mixture, at concentrations as low as 35 nM (with 50 μM vesicles, corresponding to a charge ratio of 0.5 PL:CL), extensive rates of fusion were observed even with a Ca^{2+} concentration as low as 1 mM (Figure 1). With 11 mM Ca^{2+}, polylysine still had an enhancing effect on the fusion process, the initial fusion rates being 3 and 5% maximal fluorescence/s without and with polylysine, respectively.

When poly(glutamic acid), a chelator of polylysine (Gad et al., 1982), was added before the polycation, no fusion occurred. Addition of EDTA after initiation of fusion resulted in termination of the fusion process while the addition of poly(glutamic acid), under similar conditions, changed the fusion rate only slightly. Poly(glutamic acid) affected fusion only when lower concentrations of $CaCl_2$ were used, e.g., 6 mM and less.

When Ca^{2+} was kept at a subthreshold concentration (8 mM) and polylysine was added at varying concentrations, i.e., at varying PL:CL charge ratios, the following phenomenon was observed (Figure 3). Polylysine promoted fusion only up to a certain concentration. At higher concentrations the effect diminished by more than 50%, whereas the extent of

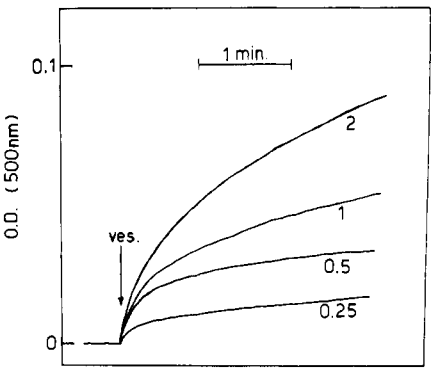

FIGURE 5: Effect of polylysine on vesicle aggregation. The 50 μM LUV consisting of PC:CL (1:1 molar ratio) were added to a suspension containing varying amounts of polylysine, corresponding to charge ratios of 0.25, 0.5, 1, and 2. The time course of the change in the turbidity (500 nm) was recorded.

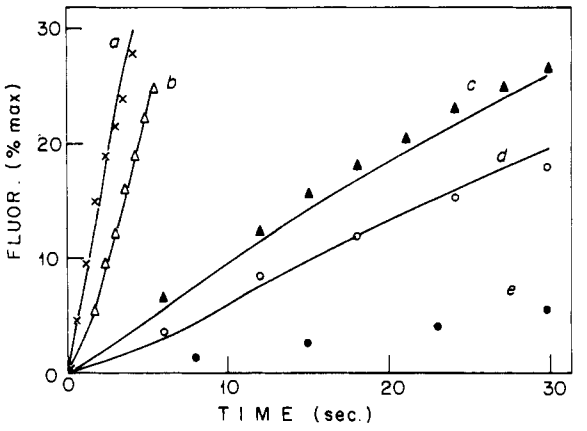

FIGURE 6: Effect of polylysine on the time course of Ca^{2+}-induced vesicle fusion. LUV consisting of PC:CL (1:1 molar ratio) at final concentrations of 100 (a, ×; b, △) and 5 μM (c, ▲; d, ○; e, ●) were added to suspensions containing 25 (a, ×; e, ●) and 1.25 nM (c, ▲) polylysine. In all cases, the reaction media contained 10 mM $CaCl_2$. Tb fluorescence was monitored and corrected for leakage as described under Materials and Methods. The experimental data are reported by the symbols, and the corresponding simulations are represented by the lines. The case of excess polylysine with 5 μM LUV is not accompanied by a simulation.

leakage of the vesicles' contents over this concentration range was rather uniform. Similar results were obtained with all vesicle concentrations used (5–100 μM P_i). Inclusion of polylysine (in addition to Ca^{2+}) in the reaction mixture resulted in increased leakage as compared to its absence (Figure 2). Plotting Tb fluorescence vs. leakage allowed assessment of the relative yield of fusion with or without polylysine and at various vesicle concentrations (Figure 4). Without polylysine the fusion to leakage ratio was consistently larger than that with polylysine present in the incubation mixture. Furthermore, the use of a larger vesicle concentration resulted in larger fusion to leakage ratio; i.e., there was less leakage per fusion event.

Effect of Polylysine on Aggregation. In order to assess the role of polylysine in this system, the extent of aggregation was measured by increased turbidity of the reaction mixture. A Ca^{2+} concentration of 8 mM was not sufficient to induce aggregation. Polylysine induced vesicle aggregation at a rate and extent that increased with its amount (Figure 5). The rate and extent of polylysine-induced aggregation were somewhat enhanced by the presence of Ca^{2+}. Aggregation could be reversed by the addition of poly(glutamic acid) in the absence of Ca^{2+}.

Enhancement and Inhibition of Fusion by Polylysine. The results in Figure 6 and in Table I demonstrate the mode of

Table I: Rate Constants of Aggregation, $C_{\|}$, Fusion, $F_{\|}$, and Deaggregation, $D_{\|}$

Ca^{2+} (mM)	PL:CL P_i	charge ratio (P1:CL)	$C_{\|}$ (M^{-1} s^{-1})	$f_{\|}$ (s^{-1})	$D_{\|}$ (s^{-1})
10[a]	0	0	$(1.4–1.8) \times 10^8$	0.3 (0.2–0.4)[b]	
10[a]	1:2800	0.2	$(5–6.5) \times 10^8$	0.35 (0.3–0.5)	0.7 (0.5–0.75)
10	1:1400	0.4	$(8–9) \times 10^8$	0.29 (0.24–0.34)	0.35 (0.3–0.5)
8	1:2800	0.2	$(5–6) \times 10^8$	0.25 (0.2–0.3)	0.8 (0.5–0.9)
8[c]	1:1400	0.4	$(6.5–7) \times 10^8$	0.27 (0.23–0.3)	0.4 (0.3–0.5)
10[d]	1:420	1.5		0.3 (0.28–0.35)	
8[d]	1:420	1.5		0.35 (0.3–0.38)	

[a] In these cases the experimental and the calculated results of $F_c(t)$ are given in part in Figure 5 (for 5 and 100 μM LUV). The determination of parameters was based on four LUV concentrations from 5 to 100 μM. [b] Values of $C_{\|}$ and $f_{\|}$ were recently determined for this case at 25 and 37 °C (Wilschut et el., 1985). Our value of $f_{\|}$ is somewhat larger. This difference is mainly attributed to the different PC's used, dioleyl-PC as compared to egg PC in the present work. In this case, reversibility of aggregation could be ignored. [c] The measurements included four LUV concentrations of 1:1 Tb:DPA and 1:9 Tb:DPA vesicles at 50 μM. [d] Vesicles were incubated with polylysine and $f_{\|}$ values were obtained by a simulation that employed eq 4.

enhancement and inhibition of vesicle fusion with polylysine. The results in Figure 6 include experimental as well as calculated F_c values in the presence of 10 mM $CaCl_2$. It illustrates that the calculations with the mass-action kinetic model simulate well the experimental data. It also illustrates the increase in overall fusion rate with phospholipid concentration. A comparison of curves a and b (100 μM lipid) and c and d (5 μM lipid) reveals an enhancement in the overall fusion rate upon the addition of polylysine at a concentration of 25 and 1.25 nM, respectively, which corresponds, in both cases, to a charge ratio of 0.2 (PL:CL). On the other hand, a comparison of curves d and e (5 μM lipid) illustrates that the addition of polylysine at the same initial concentration (25 nm) causes a reduction in the overall fusion rate. In this case (curve e), the charge ratio is 4.

Kinetic Analysis of Fusion. Table I summarizes the effect of Ca^{2+} and polylysine concentrations on the rate of aggregation, $C_{\|}$, and the rate of fusion, $f_{\|}$. No aggregation or fusion can occur with 8 mM $CaCl_2$ without polylysine; i.e., in this case the relative increase in the rate constants is very large. In the presence of 10 mM $CaCl_2$ the rate of aggregation increases appreciably when polylysine is included. The effect of polylysine, when added at suboptimal concentrations (see Figure 3), is proportional to its concentration.

Effect of Preincubation with Polylysine on the Rate of Fusion. Preincubation of the vesicles, for 15 s with polylysine (90 nM), resulted in faster overall fusion rates (Figure 7). Similar results were obtained with 8 and 10 mM $CaCl_2$. Preincubation enhanced the initial rates 2–3-fold. Preincubation of the vesicles with Ca^{2+} at subthreshold concentration (8 mM) did not change the rate of fusion. These results were simulated by means of eq 4 and yielded $f_{\|}$ values similar to those obtained by simultaneous determination of $C_{\|}$ and $f_{\|}$ (table I).

Binding of Polylysine to Vesicles. Incubation of polylysine with vesicles resulted in removal of more than 90% of the polylysine after pelleting of the vesicles regardless of the concentration of polylysine employed. This was found for polylysine concentrations corresponding to charge ratios of 0.25–1, irrespective of vesicle concentrations. In the presence of 8 mM $CaCl_2$ more than 70% of the polylysine was bound to the vesicle fraction.

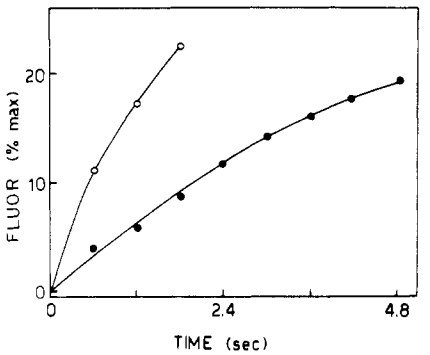

FIGURE 7: Effect of preincubation with polylysine on fusion. The 50 μM LUV consisting of PC:CL (1:1 molar ratio) were added to a medium containing 8 mM $CaCl_2$ and 90 nM polylysine (●) or were incubated for 15 with 90 nM polylysine prior to the addition of 8 mM $CaCl_2$ (○). To fluorescence was monitored, and its extent was corrected for leakage as described under Materials and Methods.

Discussion

Vesicle fusion consists of two gross steps: (1) vesicle aggregation or close apposition of surfaces; (2) local bilayer destabilization followed by the merging of membranes (Nir, 1977; Papahadjoupoulos et al., 1977; Nir et al., 1983a; Bentz et al., 1983a). Small unilamellar vesicles composed of PC–CL (5:1 molar ratio) underwent aggregation and fusion after addition of polylysine (Gad et al., 1982; Gad & Eytan, 1983). In contradistinction, LUV with larger ratio of CL (1:1 with PC) were only induced to aggregate under similar conditions. Their aggregation was rapid and could be reversed only by addition of polyanions. The smaller fusion capacity of LUV has been thoroughly described for several vesicle systems (Liao & Prestegard, 1979; Sundler & Papahadjopoulos, 1981; Düzgünes et al., 1981a; Lichtenberg et al., 1981; Wilschut et al., 1981; Nir et al., 1982). Huang & Mason (1978) have pointed out the existence of molecular-packing constraints and asymmetry in packing between the two monolayers of the membrane in small unilamellar vesicles. Thus, such vesicles are more susceptible to structural changes, e.g., fusion, because the forces holding the molecules in an ordered bilayer are less optimized in them than in LUV. Enhancing effects of added chemical such as Mg^{2+} (Sundler et al., 1981) or polyamines (Schuber et al., 1983) on fusion induced by Ca^{2+} have also been documented.

The application of the mass-action kinetic model to the analysis of kinetics of vesicle fusion yielded the rate constant of aggregation, $C_{\|}$ and the rate constant of fusion, $f_{\|}$ (see Table I), and enabled a more detailed characterization of the action of polylysine. Both the Tb–DPA fluorescence, or fusion results, and the turbidity or aggregation measurements indicate that polylysine promoted vesicle vesicle aggregation, and thus it reduced the threshold concentrations of Ca^{2+} for inducing vesicle fusion. The results in Figure 6 demonstrate that the kinetics of vesicle aggregation and fusion, a process that is promoted by the combined effect of Ca^{2+} and polylysine, can be well simulated and predicted by the model. Furthermore, when preaggregation of vesicles is achieved by means of preincubation of vesicles with polylysine, the analysis yields $f_{\|}$ values that are similar to the values deduced from experi-

ments which yield both $C_{\parallel}$ and $f_{\parallel}$ values. Enhancement of the overall rate of vesicle fusion by preaggregation was noted before in a study on the effect of polyamines (Schuber et al., 1983).

The action of polylysine in aggregating negatively charged vesicles at nanomolar concentrations is certainly related to the charge neutralization that it produces, which results in reduced electrostatic repulsion between vesicles. Studies on vesicles of various compositions (Schuber et al., 1983) showed that threshold concentrations of spermine (a tetravalent cation) for induction of vesicle aggregation vary from 15 to 110 μM and those of a trivalent polyamine, spermidine, are at least 1 order of magnitude larger. In comparison, the threshold concentrations of divalent cations are in the millimolar range (Papahadjopulos et al., 1977; Düzgünes et al., 1981a,b; Wilschut et al., 1980; Nir et al., 1980a; Ohki et al., 1982), whereas the concentrations of monovalent cations required to induce the aggregation of PS vesicles are several hundred millimolar (Nir et al., 1980b; Day et al., 1980; Ohki et al., 1982). It was pointed out that in the analysis of fusion kinetics we considered a wide range of lipid concentrations. The model could simulate all these results with the same rate constants only when the ratio PL:CL remained constant, e.g., 25 nM polylysine with 100 μM phosphate and 1.25 nM polylysine with 5 μM phosphate. This means that most of the polylysine in solution was bound to the vesicles (see Table I) as was indeed confirmed by the polylysine binding studies. Hence, the important factor is charge ratio rather than absolute amount or concentration. Polylysine was shown to compete efficiently with Ca^{2+} and Mn^{2+} for binding to negatively charged vesicles (Gad, 1983). The fact that polylysine was strongly bound to the vesicles even in the presence of 8 mM $CaCl_2$ emphasizes its potential to compete with Ca^{2+} for binding. At the optimal charge ratio of 0.5 (PL:CL) as a consequence of polylysine binding, less sites are available for Ca^{2+}, yet the overall fusion rate is at least as fast as that obtained with 11 mM $CaCl_2$ alone. This demonstrates that the actual Ca^{2+} concentration needed for fusion is lower than that observed without polylysine because the limiting factor is the ability to induce aggregation. Furthermore, chelation of polylysine is effective in stopping the fusion process only at lower $CaCl_2$ concentrations (less than 6 mM). At subthreshold (8 mM) $CaCl_2$ concentrations, once the aggregates form it seems that the amount of bound Ca^{2+} is sufficient to allow the fusion to proceed.

The results in Figure 6 and Table I indicate that polylysine has little effect, if any, on the rate constant of fusion per se, $f_{\parallel}$. In this sense the action of polylysine is similar to that of lectins (Düzgünes et al., 1984) and synexin (Creutz et al., 1979; Hong et al., 1981, 1982), which was shown to enhance the overall rate of vesicle fusion and to reduce the Ca^{2+} threshold concentration merely by promoting vesicle aggregation, i.e., increasing only $C_{\parallel}$ values (Hong et al., 1983). Another confirmation to our conclusion is the fact that removal of polylysine after initiation of fusion does not alter significantly the rate of fusion, while the addition of EDTA terminates the process. This phenomenon takes place even with 8 mM $CaCl_2$, a concentration at which Ca^{2+} alone cannot induce aggregation and fusion. This also brings forth the possibility, confirmed by the preincubation experiments, that once the vesicles are aggregated the threshold concentration of the fusion inducer can be significantly reduced. Regarding the action of polyamines in promoting fusion, it was shown (Schuber et al., 1983) that spermine can induce the fusion of vesicles of several compositions without Ca^{2+}. However, we encounter here an additional peculiar result that the overall fusion rate is reduced in the presence of larger concentrations of polylysine.

The possibility that excess polylysine inhibits fusion because of reduced rates of aggregation was ruled out on the basis of the observation that the extent of aggregation increased with increasing charge ratios up to 10 (results not shown). The inhibition of fusion in the presence of large charge ratios is expressed in terms of a significant reduction (by at least 1 order of magnitude) of the rate of fusion. The optimal charge ratio PL:CL is about 0.5 and significant fusion inhibition is observed when the charge ratio PL:CL equals 4 (polylysine per outer layer charge; we make an assumption that the phospholipid distribution in LUV is symmetrical). The above reduction in the fusion rate could be attributed to a reduction in the binding of Ca^{2+} to the vesicles. Portis et al. (1979) suggested that fusion depended on the formation of cation–phospholipid complexes. Since polylysine alone could not induce fusion and the fusion reaction could be arrested by the addition of EDTA but not poly(glutamic acid) at subthreshold Ca^{2+} concentration, we concluded that Ca^{2+} was the inducer of fusion, as in the above-mentioned paper (Portis et al., 1979). Thus, when polylysine is present in excess (about 80% are bound to the vesicles at a charge ratio of 1), it prevents Ca^{2+} binding and consequently inhibits fusion. Recent fusion and binding studies on PS vesicles, in which large concentrations of monovalent cations were used, demonstrated that a steep reduction in $f_{\parallel}$ values was well correlated with a reduction in Ca^{2+} binding to the membranes (Nir et al., 1983b; Bentz et al., 1983:), whereas Düzgünes et al. (1981b) showed a similar effect on initial fusion rates of PS–PC vesicles. However, it appears that at certain combinations of Ca^{2+} and polylysine the fusogenicity of PC–CL is optimal and greater than in the absence of polylysine.

In conclusion, we propose that polylysine amplifies Ca^{2+}-induced fusion by enhancing aggregation of the vesicles. At subthreshold concentrations of Ca^{2+}, the limiting factor for vesicle fusion is the capacity to cause aggregation, and the enhancement of fusion by polylysine is dramatic. In view of these findings, polylysine may be useful in significantly reducing Ca^{2+} concentrations needed for membrane fusion, e.g., in vesicle–cell systems that are sensitive to excess Ca^{2+}.

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Two Types of Hydrocarbon Chain Interdigitation in Sphingomyelin Bilayers†

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ABSTRACT: Vibrational Raman spectroscopic experiments have been performed as a function of temperature on aqueous dispersions of synthetic DL-*erythro*-*N*-lignoceroylsphingosylphosphocholine [C(24):SPM], a racemic mixture of two highly asymmetric hydrocarbon chain length sphingomyelins. Raman spectral peak-height intensity ratios of vibrational transitions in the C–H stretching-mode region show that the C(24):SPM–H_2O system undergoes two thermal phase transitions centered at 48.5 and 54.5 °C. Vibrational data for fully hydrated C(24):SPM are compared to those of highly asymmetric phosphatidylcholine dispersions. The Raman data are consistent with the plausible model that the lower temperature transition can be ascribed to the conversion of a mixed interdigitated gel state (gel II) to a partially interdigitated gel state (gel I) and that the higher temperature transition corresponds to a gel I → liquid-crystalline phase transition. The observation of a mixed interdigitated gel state (gel II) at temperatures below 48.5 °C implies that biological membranes may have lipid domains in which some of the lipid hydrocarbon chains penetrate completely across the entire hydrocarbon width of the lipid bilayer.

Among the various choline-containing phospholipids present in eukaryotic biological membranes, sphingomyelin or *N*-acyl-sphingosylphosphocholine is a major component of most plasma membranes. The sphingomyelin isolated from plasma membranes is, in general, a mixutre of many molecular species with various fatty acyl chain moieties. Stearic, nervonic, lignoceric, and behenic acids constitute the major fatty acyl

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