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Polyamines as Modulators of Membrane Fusion: Aggregation and Fusion of Liposomes†

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ABSTRACT: We have studied the effect of the polyamines (spermine, spermidine, and putrescine) on the aggregation and fusion of large (approximately 100 nm in diameter) unilamellar liposomes in the presence of 100 mM NaCl, pH 7.4. Liposome fusion was monitored by the Tb/dipicolinic acid fluorescence assay for the intermixing of internal aqueous contents, and the release of contents was followed by carboxyfluorescein fluorescence. Spermine and spermidine at physiological concentrations aggregated liposomes composed of pure phosphatidylserine (PS) or phosphatidate (PA) and mixtures of PA with phosphatidylcholine (PC) but did not induce any fusion. However, liposomes composed of mixtures of acidic phospholipids, cholesterol, and a high mole fraction of phosphatidylethanolamine could be induced to fuse by spermine and spermidine in the absence of divalent cations. Putrescine alone in the physiological concentration range was ineffective for both aggregation and fusion of these liposomes. Liposomes made of pure PC did not aggregate in the presence of polyamines. Addition of aggregating concentrations of spermine caused a drastic increase in the rate of Ca^{2+}-induced fusion of PA liposomes and a large decrease in the threshold Ca^{2+} concentration required for fusion. This effect was less pronounced in the case of PS or PA/PC vesicles. Preincubation of PA vesicles with spermine before the addition of Ca^{2+} resulted in a 30-fold increase in the initial rate of fusion. We propose that polyamines may be involved in the regulation of membrane fusion phenomena accompanying cell growth, cell division, exocytosis, and fertilization.

The naturally occurring polyamines (putrescine, spermidine, and spermine) are ubiquitous components of living material (Cohen, 1971; Bachrach, 1973). Their biosynthesis is exquisitely controlled, and major changes in intracellular polyamine concentrations occur after stimulation of cell growth and division and at certain phases of the cell cycle (Jänne et al., 1978; Heby & Jänne, 1981). The requirement of polyamines for cellular events such as division was demonstrated by the use of polyamine biosynthesis inhibitors (Mamont et al., 1976, 1978; Heby & Jänne, 1981).

At physiological pH, polyamines are polycations, and an important part of their function in the biosynthesis of nucleic acids and proteins has been ascribed to their properties as counterions of polynucleotides [reviewed in Cohen (1971, 1978), Caldarera et al. (1976), and Algranati & Goldemberg (1977)]. Because of their cationic properties, polyamines are also expected to interact with acidic phospholipids in biomembranes. Some effects of polyamines on membrane properties have been described, including membrane stabilization against osmotic stress (Tabor, 1962; Harold, 1964), changes in membrane fluidity (Spisni et al., 1976), and effects on electrokinetic properties of red blood cells (Chun et al., 1976). However, few studies have tried to delineate the possible involvement of polyamines in physiological functions of biomembranes. Among such functions, membrane fusion is particularly important since it is a prerequisite in exocytosis (secretion, neurotransmission, cell growth), endocytosis, formation of secondary lysosomes, and cell division (Poste & Allison, 1973).

We have studied the effect of polyamines on the aggregation and fusion of phospholipid vesicles (liposomes) of different composition in order to understand the interaction of polyamines with different components of biological membranes. The fusion of liposomes containing acidic phospholipids has been extensively investigated (Papahadjopoulos et al., 1979;

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Düzgüneş et al., 1980; Hong et al., 1982) and shows characteristics similar to those of biological membrane fusion. In this report, we show that spermidine and spermine at physiological concentrations induce aggregation of liposomes containing acidic phospholipids and are able to decrease the threshold levels of divalent cations required for fusion. Moreover, polyamines promote fusion of certain vesicle types in the absence of any divalent cation. The results obtained in this study suggest that polyamines, especially spermidine and spermine, could be intracellular modulators of membrane fusion.

Experimental Procedures

Materials. Bovine brain phosphatidylserine (PS)[1] and PE and PA (both transphosphatidylated from egg PC) were purchased from Avanti Polar Lipids (Birmingham, AL). Egg PC was purified as described by Papahadjopoulos & Miller (1967). Spermidine and spermine, as hydrochlorides, were obtained from Sigma (St. Louis, MO). Cholesterol (Sigma) was recrystallized twice before use. The lipids were stored as a chloroform solution in sealed ampules under argon at −40 °C. $TbCl_3 \cdot 6H_2O$ was obtained from Alfa (Danvers, MA); DPA and nitrilotriacetic acid were from Sigma. CF (Eastman Kodak) was purified by chromatography on Sephadex LH-20 (Pharmacia, Piscataway, NJ). All other chemicals used were of the highest purity available.

Vesicle Preparation. Large unilamellar vesicles encapsulating the desired aqueous contents were prepared by the reverse-phase evaporation method (Szoka & Papahadjopoulos, 1978) with minor modifications described by Wilschut et al. (1980). The vesicles for aggregation studies were prepared in 5 mM Tes or 5 mM Hepes containing 100 mM NaCl, pH 7.4. The vesicles for fusion experiments were made in either (a) 2.5 mM $TbCl_3$ and 40 mM sodium nitrilotriacetate, (b) 50 mM DPA (sodium salt) and 20 mM NaCl, or (c) 50 mM CF (sodium salt). In addition, the media contained 5 mM Tes, pH 7.4. The vesicles were sized by extrusion through polycarbonate membranes (Unipore; Bio-Rad, Richmond, CA) with a pore size of 0.1 μm (Olson et al., 1979). The resulting vesicles were separated from nonencapsulated material by gel filtration on Sephadex G-75 (elution buffer, 100 mM NaCl, 5 mM Tes, and 1 mM EDTA, pH 7.4; in the case of CF-containing vesicles, the EDTA concentration was 0.1 mM). Lipid phosphorus was determined according to Bartlett (1959).

Fusion Assay. The assay is based on the formation of the highly fluorescent $Tb(DPA)_3^{3-}$ chelation complex resulting from intermixing of the aqueous contents inside the fusing vesicles (Wilschut et al., 1980). The assay mixture consists of Tb and DPA-containing vesicles, at a 1:1 ratio (25 μM phospholipid for each), in 1 mL (final volume) of 100 mM NaCl, 5 mM Tes, and 0.1 mM EDTA (pH 7.4) at 25 °C. The fluorescence was measured with an SLM-4000 fluorometer. The Tb–DPA complex was excited at 276 nm and the fluorescence (>530 nm) measured through a Corning 3-68 cutoff filter. The fluorescence intensity was calibrated, i.e., set at 100%, with Tb-containing vesicles (25 μM phospholipid, free of EDTA) and lysed with 0.5% sodium cholate (Calbiochem-Behring, San Diego, CA; recrystallized twice) in the presence of 20 μM DPA. Light scattering at 276 nm was measured simultaneously by using the second emission channel connected to a monochromator. Leakage of vesicle contents was assessed with CF-containing vesicles (50 μM). CF was excited at 430 nm and the fluorescence measured through the 3-68 cutoff filter. The 100% fluorescence setting was obtained with a vesicle preparation containing 0.1% (v/v) Triton X-100.

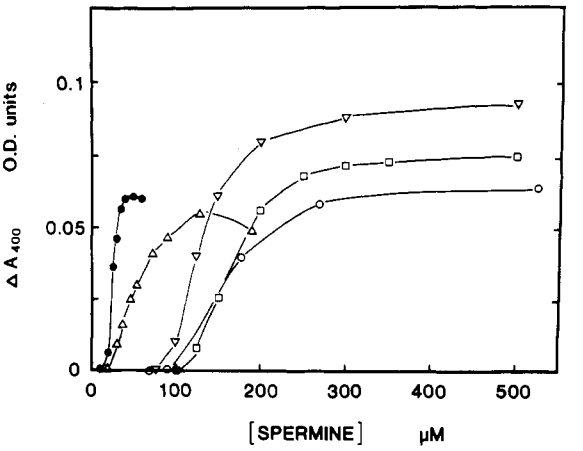

FIGURE 1: Turbidity changes (ΔA_{400}) of vesicles of different phospholipid composition vs. spermine concentration. PS (●), PS/PE/cholesterol (6:14:10) (□), and PA/PS/PE/cholesterol (1:5:14:10) (▽) were 50 μM phospholipid; PA/PE (1:3) (△) and PS/PE (1:3) (○) were 75 μM phospholipid in 0.1 M NaCl–5 mM Tes, pH 7.4. Turbidity changes at 2 min (1 min for PA/PE) were plotted against the final spermine concentration.

Vesicle Aggregation. The aggregation was monitored by the turbidity changes of the vesicle suspension, at 400 nm, by use of a Beckman Model 34 spectrophotometer. The concentration range of phospholipid was 50–80 μM, in 5 mM Tes and 100 mM NaCl (pH 7.4) at room temperature (final volume 0.5 mL). The change of absorbance, after addition of polyamine, was recorded. It was found convenient to plot the absorbance change obtained 2 min after polyamine addition as a function of the polyamine concentration. In order to compare the aggregation induced by polyamines of vesicles of different phospholipid composition, a "threshold concentration" was defined as the concentration of polyamine obtained by extrapolating the curve of turbidity change to zero ΔA_{400} (see Figure 1). Subthreshold concentrations of polyamines will correspond in the text to concentrations which did not induce any measurable aggregation or fusion, on the time scale of our experiments (up to 30 min).

Results

Kinetics of Aggregation of Vesicles Induced by Polyamines. Spermidine and spermine induced the aggregation of large unilamellar vesicles containing acidic phospholipids. The increase in absorbance measured at equilibrium after the addition of the polyamine suggested that aggregation was extensive: e.g., in the case of PS vesicles, the absorbance increased by a factor of 10 in the presence of spermine or spermidine. The kinetics of aggregation were markedly dependent on the polyamine concentration and exhibited a threshold (Figure 1). The threshold concentration was dependent on the phospholipid composition of the vesicles (Table I); e.g., vesicles composed of a mixture of acidic and zwitterionic phospholipids, such as PC or PE, resulted in an increased threshold value. In contrast, polyamines did not induce the aggregation of vesicles made of PC.

The presence of polyamines also facilitated the effect of Ca^{2+} or Mg^{2+} in aggregating phospholipid vesicles (Table II). For example, in the case of PS vesicles, spermine at just below its threshold concentration decreased by 40% the threshold con-

[1] Abbreviations: CF, carboxyfluorescein; DPA, dipicolinic acid; EDTA, ethylenediaminetetraacetic acid; Hepes, *N*-(2-hydroxyethyl)-piperazine-*N*′-2-ethanesulfonic acid; PA, phosphatidate; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PI, phosphatidylinositol; PS, phosphatidylserine; Tes, *N*-[tris(hydroxymethyl)methyl]-2-aminoethanesulfonic acid; Chol, cholesterol.

Table I: Threshold Concentrations of Polyamines for Aggregation of Large Unilamellar Vesicles of Different Phospholipid Compositions[a]

phospholipid	threshold value [spermine] (μM)	[spermidine] (μM)
PS	20	270
PA	15	130
PS/PE (1:3)	95	1600
PA/PE (1:3)	20	460
PA/PC (1:1)	70	ND[b]
PA/PS/PE/Chol (1:2:3:2)	30	640
PA/PS/PE/Chol (1:5:14:10)	90	ND
PS/PE/Chol (6:14:10)	110	ND

[a] The threshold concentrations were determined as described under Experimental Procedures. The mixed vesicles contained molar ratios of phospholipids as indicated. [b] ND, not determined.

Table II: Influence of Spermine and Magnesium on the Threshold of Calcium-Induced Aggregation of Vesicles Made of Different Phospholipids

phospholipid	threshold of Ca^{2+} (mM)	additions
PS	1.7	
	1.0	spermine (20 μM)
PA/PS/PE/Chol (1:2:3:2)	3.5	
	2.5	spermine (20 μM)
	1.5	magnesium (1.9 mM)
	1.0	spermine (20 μM) magnesium (1.9 mM)

centration of Ca^{2+} required to induce aggregation. The effects of the addition of spermine at concentrations just below threshold (20 μM) or Mg^{2+} (1.9 mM) on the threshold of Ca^{2+} required to induce aggregation of liposomes composed of PA/PS/PE/cholesterol (1:2:3:2) are also shown in Table II.

The polyamine-induced aggregation of PS vesicles was found to be reversible. At given times, a vesicle suspension incubated in the presence of 30 μM spermine was diluted 50% by addition of the same volume of the vesicle suspension. After dilution, the spermine concentration became subthreshold, and the absorbance returned to the original level, indicating that the aggregation was essentially reversible and that the dissociation of aggregates was very fast (within seconds).

Polyamines as Modulators of Membrane Fusion. The close apposition of vesicle membranes during aggregation is considered to be an initial step and a necessary condition for the membranes to fuse. It was of interest, therefore, to determine whether polyamines could also trigger membrane fusion or modulate the divalent cation requirement for fusion. In the absence of divalent cations, polyamines were unable to induce the fusion of vesicles composed of pure PA or PS and mixtures of PA with PC, as judged by the assay monitoring the intermixing of aqueous contents of vesicles (Wilschut et al., 1980). Lack of apparent intermixing of aqueous contents in these cases is not due to leakage of contents, since addition of polyamines even at high concentrations did not promote the release of CF. In spite of the lack of fusion and the complete retention of vesicle contents, these vesicles aggregated rapidly after addition of polyamines to threshold concentrations. Nevertheless, polyamines did modify quite drastically the effects of divalent cations on these vesicles. The following three subsections will describe in detail the characteristic behavior of these vesicles.

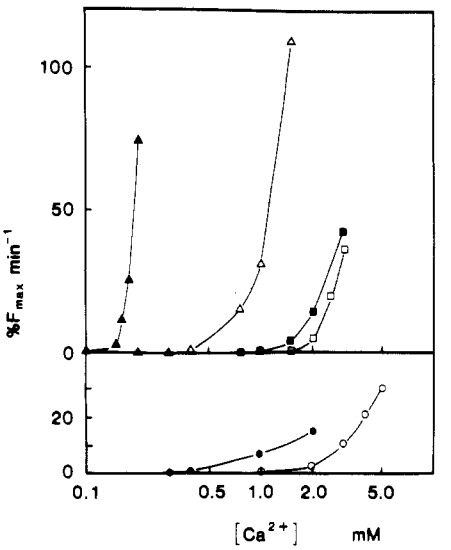

FIGURE 2: Comparative kinetics of Ca^{2+}-induced fusion of vesicles in the absence and presence of spermine. Large unilamellar vesicles of PA (△, ▲), PA/PC (1:1) (○, ●), and PS (□, ■) (50 μM phospholipid) were in 0.1 M NaCl, 5 mM Tes, and 0.1 M EDTA (pH 7.4) at 25 °C. The fusion was induced by addition of Ca^{2+} in the absence (open symbols) or in the presence (closed symbols) of 20, 100, and 30 μM spermine at time zero. The initial rates of fusion, the percent of maximal Tb–DPA fluorescence per minute, are given as a function of the Ca^{2+} concentration.

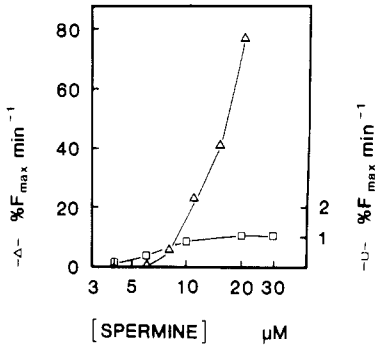

FIGURE 3: Influence of spermine concentration on the kinetics of fusion of vesicles in the presence of subthreshold concentrations of Ca^{2+}. PA (△) and PS (□) vesicles (50 μM phospholipid) were incubated in the presence of subthreshold concentrations of Ca^{2+}, 0.2 and 1.0 mM, respectively. Spermine was given at time zero. The initial rates of fusion are given as a function of the spermine concentration.

(*i*) *Vesicles Composed of Phosphatidate.* Addition of fixed concentrations of spermine (20 μM), which induce PA vesicle aggregation, resulted in dramatic increases in the observed rates of Ca^{2+}-induced fusion and in a large shift of the threshold concentration of Ca^{2+} required for fusion (Figure 2). Because of the presence of 0.1 mM EDTA in the medium, the threshold value for Ca^{2+} may be in the range of 10^{-5}–10^{-4} M. The addition of small concentrations of spermine together with a fixed subthreshold concentration of Ca^{2+} (0.2 mM) resulted in a drastic increase in the rate of fusion (Figure 3). Spectacular effects of spermine on the rates of Ca^{2+}-induced fusion were observed when the vesicles were allowed to preaggregate in the the presence of the polyamine alone. Thus, a preincubation of 0.5 min with 15 μM spermine, before the addition of a subthreshold concentration of Ca^{2+} (0.16 mM), was sufficient to obtain an increase in the observed fusion rate by a factor of 30 (Figure 4). In a control experiment, the preincubation of the vesicles with Ca^{2+}, followed by the addition of spermine, did not alter the observed fusion rate compared to the case where both cations were added together. These results indicate that low concentrations of Ca^{2+}, together

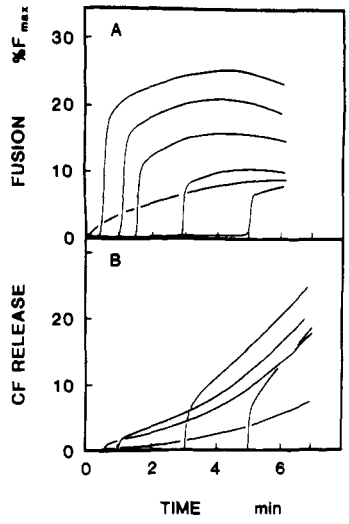

FIGURE 4: Effect of the preincubation of PA vesicles with spermine on the kinetics of Ca^{2+}-induced fusion. Vesicles (50 μM phospholipid) were incubated with a spermine concentration (15 μM) which causes aggregation. Ca^{2+} (0.16 mM) was added at 0, 0.5, 1.0, 1.5, 3.0, and 5 min, respectively (A). For comparison, the kinetics of the release of vesicle contents (CF) under the same experimental conditions (pH 7.4, 25 °C) are shown (B). The sharp change of the rate of fusion at a later stage in (A) is due to the fast release of vesicle contents as shown in (B).

Table III: Influence of Spermine and Preincubation with Spermine on the Kinetics of Calcium-Induced Fusion of Phosphatidate/Phosphatidylcholine Vesicles[a]

vesicles	[spermine] (μM)	time (min)	[calcium] (mM)	initial fusion rate (% F_{max} min^{-1})
PA/PC		0	1	0.1
(1:1)	100	0	1	6.2
	100	1	1	37
PA/PC		0	3	0.0
(1:3)	700	0	3	2.9
	700	1	3	4.3
	700	5	3	18.7

[a] Spermine, at the concentrations indicated, was added to vesicle suspensions (50 μM phospholipid) at time = 0 min, and calcium was added at the times indicated. The initial rate of fusion is given as the percent of the maximal fluorescence per minute.

with spermine, can lead to very fast rates of vesicle fusion. Moreover, they point to the paramount influence of the rate of vesicle aggregation on the fusion rate.

(*ii*) *Vesicles Composed of Phosphatidate Mixed with Phosphatidylcholine.* Inclusion of phosphatidylcholine in acidic phospholipid vesicles is inhibitory to Ca^{2+}-induced fusion (Papahadjopoulos et al., 1974; Düzgüneş et al., 1981a; Sundler et al., 1981). When 50 and 75 mol % PC was included in PA vesicles, the threshold Ca^{2+} concentration required for fusion increased severalfold, and the extent of fusion was markedly reduced [see Figure 2 and Sundler et al. (1981)]. Aggregating concentrations of spermine decreased substantially the threshold concentration of the divalent cation induced fusion of such vesicles and increased the rate (Figure 2) and extent of fusion (not shown). This effect was particularly striking after a preincubation with the polyamine (Table III). It appears, therefore, that spermine is able to counteract the inhibitory effect of PC on fusion.

(*iii*) *Vesicles Composed of Phosphatidylserine.* In contrast to the effects observed with PA vesicles, concentrations of

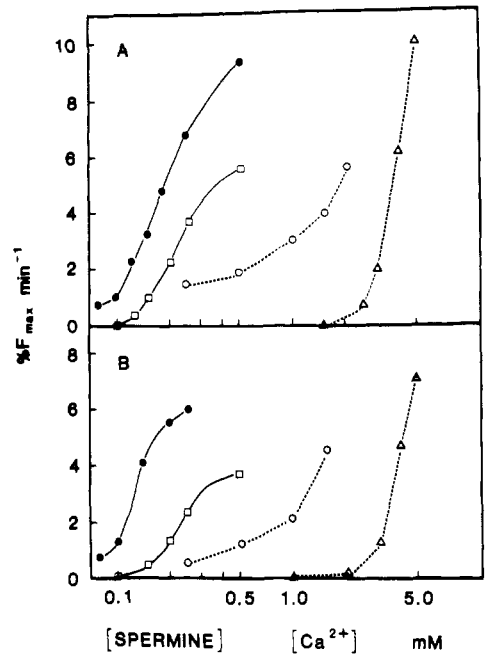

FIGURE 5: Kinetics of the fusion of mixed vesicles induced by spermine and Ca^{2+}. PA/PS/PE/cholesterol (1:5:14:10) (A) and PS/PE/cholesterol (6:14:10) (B) vesicles, both at a concentration of 50 μM phospholipid, were incubated at 25 °C, pH 7.4, with spermine in the absence (□) or presence (●) of 1 mM Ca^{2+}, or with Ca^{2+} in the absence (△) or presence (○) of 150 μM spermine. The initial rates of fusion are given as a function of spermine (solid lines) or Ca^{2+} (dotted lines) concentrations.

spermine which cause vesicle aggregation (30 μM) had a relatively modest influence on the threshold and fusion rates of Ca^{2+}-induced fusion of PS vesicles (Figure 2). Addition of increasing concentrations of spermine or spermidine to vesicles in the presence of subthreshold concentrations of Ca^{2+} (1 mM) resulted in increased initial rates of fusion, but again, the results were not as pronounced as with PA vesicles. The results with spermine are shown in Figure 3. Parallel experiments with CF-containing vesicles indicated that leakage of contents would not account for the lower apparent initial rates of fusion in the case of PS vesicles. Preincubation of vesicles with spermine (30 μM) 4.5 min before addition of Ca^{2+} (1 mM) led to an increase of the initial rate of fusion, by a factor of 9, compared to the case where both ions were given at time zero (data not shown).

Vesicle Fusion Induced by Polyamines. The fusion of phospholipid vesicles composed of PA/PS/PE/cholesterol (1:2:3:2) can be induced by millimolar concentrations of Ca^{2+} and Mg^{2+} (Hong et al., 1983). Polyamines by themselves did not induce the fusion of such vesicles, but by allowing their aggregation, they changed dramatically the rate, extent, and threshold of divalent cation induced fusion. By using such multicomponent vesicles, containing higher proportions of PE, i.e., PA/PS/PE/cholesterol (1:5:14:10) and PS/PE/cholesterol (6:14:10), we could demonstrate for the first time that spermidine and spermine, in the absence of any divalent cation, were able to promote membrane fusion. In the case of spermine, the concentration needed for fusion was much lower than that of Ca^{2+} (Figure 5), whereas millimolar spermidine concentrations were needed in order to obtain the same observed fusion rates (not shown). In addition, polyamines and Ca^{2+} showed a synergistic effect on fusion (Figure 5). For example, subthreshold concentrations of Ca^{2+} (1 mM) and spermine (100 μM), when given together, yielded substantial fusion rates. Similarly, addition of 1 mM spermidine to the PA-containing mixed vesicles incubated in the presence of 1.0 mM

Ca^{2+} increased the initial fusion rate from nondetectable to 2% F_{max} min^{-1}. The concentrations of polyamines which were effective in inducing fusion were close to the ones which induced vesicle aggregation (Figures 1 and 5). Finally, it is interesting to compare the kinetics of fusion of the two vesicle systems (Figure 5A,B). The replacement of one-sixth of the PS content in the vesicles by PA is enough to affect significantly the rate of polyamine- and Ca^{2+}-induced fusion, indicating the high fusogenic capacity of PA.

Contrary to the cases studied before, the time course of the fusion of the vesicles studied in this section exhibited biphasic behavior, i.e., an initial slow lag phase of fusion followed by a faster phase, the duration of the slow phase being shortened at higher concentrations of spermine or Ca^{2+}. A similar trend was found before by Düzgüneş et al. (1981a) in the case of Ca^{2+}- or Mg^{2+}-induced fusion of PS/PE vesicles and in Ca^{2+}- and Ca^{2+}/polyamine-induced fusion of PA/PS/PE/Chol (1:2:3:2) vesicles. Interestingly, in the latter example, the lag phases disappeared when the vesicles were preincubated with the polyamines (Hong et al., 1983). The rates reported in Figure 5 are the initial rates and do not represent the maximum fusion rates obtained. Such kinetic behavior is expected when the fusion process departs significantly from a purely aggregation rate-limiting case (Bentz et al., 1983a; Nir et al., 1983). However, at present, one cannot exclude the occurrence during the aggregation of a relatively slow lateral phase separation creating zones of enhanced fusogenic capacity.

Discussion

Enhancement of Liposome Aggregation and Fusion. Fusion of phospholipid vesicles consists basically of two distinct steps: first, vesicle aggregation, resulting in a close apposition of their surface; second, fusion of the membranes involving local bilayer destabilization (Nir, 1977; Papahadjopoulos et al., 1977; Düzgüneş et al., 1981b; Nir et al., 1982). The results of the present study indicate that polyamines can influence both steps.

Surface electrostatics of acidic phospholipid vesicles are modified by the presence of multivalent cations, both by specific binding and by electrostatic screening of the double layer (Nir et al., 1983). Polyamines are polycations and, as counterions to acidic phospholipids, are expected to greatly decrease the surface charge density and surface potential. The consequent reduction of the mutual electrostatic repulsion between vesicles could induce the vesicles to aggregate, as a result of the increase in the contribution of the van der Waals attractive forces to the total free energy of interaction. The interaction of polyamines, as counterions, with vesicles containing acidic phospholipids results in an intense aggregation. The "threshold" concentrations of spermidine and spermine which induce vesicle aggregation are well within their known physiological range; this is in contrast to putrescine which showed virtually no effect under similar conditions (for this reason, putrescine was omitted in most of our studies). For a given vesicle composition, spermine is more efficient in inducing aggregation as compared to spermidine (Table I). The polyamines act at lower concentrations than divalent cations. This is not unexpected since the surface potential is related to the cation concentration near the vesicle surface and is thus dependent on the valency of the ion (Ohki, 1976; McLaughlin, 1977). In this context, it was noted previously that polyamines are also able to aggregate biomembranes such as rat liver microsomes (Tabor, 1960; Jamdar, 1979).

Fusion of liposomes induced by divalent cations has been studied extensively as a model for membrane fusion (Papahadjopoulos et al., 1979; Düzgüneş et al., 1980; Düzgüneş & Papahadjopoulos, 1983). In the case of large vesicles, Ca^{2+} was shown to be highly effective in inducing fusion of vesicles containing acidic phospholipids, which is in accordance with the known physiological role of this divalent cation in exocytic events such as secretion or neurotransmitter release. However, the threshold Ca^{2+} concentrations required for fusion in these model systems vary from 10^{-4} to 10^{-2} M, which is above the physiological intracellular Ca^{2+} concentration range. As a consequence, the possibility exists that intracellular changes in spermidine and spermine levels during the cell cycle, or when cells are stimulated to divide, could be accompanied by large effects on the rate of membrane aggregation and membrane fusion. Moreover, changes in polyamine levels could differentially affect membranes, depending, for example, on the proportion of acidic phospholipid or phosphatidylethanolamine in membrane domains, and the synergism with Ca^{2+} and Mg^{2+} could result in an amplification and modulation, by the polyamines, of membrane aggregation and fusion induced by these divalent ions.

There are other nonphysiological polyvalent cations, such as La^{3+} (Hammoudah et al., 1981) and polylysine (Gad et al., 1982), which can induce aggregation of acidic phospholipid vesicles and destabilize small PS vesicles to form large vesicular structures. The aggregation and fusion induced by polylysine are dependent on the size of the polycation: i.e., the longer the molecule, the less that is needed to induce similar aggregation. In this regard, polyamines behave like polylysine even though the detailed molecular basis of the interaction of the polyamines with phospholipid vesicles is not known. The observed effects of polyamines could be due to binding to individual phosphate anionic sites and/or delocalized binding in the double layer according to the counterion condensation theory of Manning (1969). This latter interaction has been proposed to be prevalent in the interaction between polyamines and deoxyribonucleic acids (Bloomfield & Wilson, 1981). Comparison of the distances separating the ammonium groups in polyamines with the distances between neighboring anionic head groups in phospholipid membranes shows that multiple ionic interactions could be possible between the polyamines and the vesicles, resulting in neutral complexes. In addition, the great conformational flexibility of the polyamines should permit the formation of such multiple complexes which are favored for entropic reasons. The distal ammonium groups of spermidine and spermine are separated by 11 and 16 Å, respectively (Liquori et al., 1967), while in PS bilayers, the phosphate head groups are separated by approximately 8 Å. Since the binding of divalent cations to PS appears to be an important determinant of fusion in pure PS or PS/PC vesicles (Portis et al., 1979; Düzgüneş et al., 1981b; Bentz et al., 1983b), it would be of interest to determine the bound polyamine per acidic phospholipid at the threshold of aggregation and fusion.

Phospholipid Specificity. Studies on the divalent cation induced aggregation and fusion of liposomes have shown that although aggregation is a prerequisite for fusion, it may or may not lead to membrane fusion, depending on the phospholipid composition. Mg^{2+} will only aggregate large unilamellar PS vesicles, whereas Ca^{2+} is able to both aggregate and fuse these membranes (Wilschut et al., 1981). In contrast, both Ca^{2+} and Mg^{2+} will induce aggregation and fusion of vesicles composed, for example, of PA, PA/PE, or PS/PE (Sundler & Papahadjopoulos, 1981; Düzgüneş et al., 1981a; Sundler et al., 1981). A similar behavior was found in this study in the case of spermidine and spermine. Polyamines affect profoundly the kinetics of the fusion of PA vesicles, induced by Ca^{2+}: they decrease the Ca^{2+} threshold, increase the fusion

rates, and interfere with the fusion-inhibitory action of PC in mixtures with PA. This interference by polyamines suggests that the hydrated PC head group, which accounts for the inhibition of fusion, may be affected by the polyamines bound to the membrane surface, since it is known that the hydrocarbon segments of bound polyamines perturb and release structured water (Loftfield et al., 1981). Dramatic changes in overall fusion rates were observed especially when the vesicles were preincubated with the polyamines before addition of the fusogenic agent. In contrast to the PA vesicles, the Ca^{2+}-induced fusion of PS vesicles is less affected by the polyamines.

At this point, it is interesting to compare the effects of polyamines and Mg^{2+} on the fusion of large unilamellar vesicles. In the case of PS vesicles, spermine and spermidine exert a synergistic effect on Ca^{2+}-induced fusion, while Mg^{2+} is inhibitory (Wilschut et al., 1981). The situation is quite different with PA vesicles where Mg^{2+}, in contrast to the polyamines, is able to induce the fusion of the vesicles. This property is lost on inclusion of PC in the vesicle composition (Sundler et al., 1981). The quantitative difference between the Ca^{2+}-induced fusion of PA and PS vesicles with regard to the modulation by polyamines is striking. It would be of interest to elucidate the mechanism for this difference in relation to the close approach of the vesicles induced by the polyamines and their effects on Ca^{2+} binding and bilayer destabilization.

It has been demonstrated before that aggregation of PS vesicles, induced by Ca^{2+} or Mg^{2+}, results in the emergence of a higher affinity for Ca^{2+} (Portis et al., 1979; Ekerdt & Papahadjopoulos, 1982). It is possible that such effects might be even more amplified in the PA vesicles. In the present case, it is possible that the aggregation of the vesicles induced by the polyamine allows the appearance of intermembrane structures which present a high affinity for Ca^{2+}. This would explain the decreased threshold for divalent cations in the presence of aggregating concentrations of polyamines. The difference observed between the PA and PS vesicles may arise from the closeness of their approach and thus in the creation of structures favorable to Ca^{2+} binding. Because of the bulk and hydration of the serine moiety, aggregated PS vesicles might establish larger intermembrane distances than the PA vesicles.

Although liposomes composed of pure PS or PA do not undergo membrane destabilization and fusion in the presence of polyamines, liposomes containing a large mole fraction of PE in addition to the acidic phospholipids can be induced to fuse by spermine (Figure 5). This observation is reminiscent of the action of Mg^{2+} on PS/PE liposomes (Düzgüneş et al., 1981a) and of Ca^{2+} on PI/PE liposomes (Sundler et al., 1981), since these divalent cations do not induce the fusion of liposomes made of the pure acidic phospholipid component (Wilschut et al., 1981; Sundler & Papahadjopoulos, 1981). These results indicate that PE, contrary to PC, has an intrinsic capacity to promote membrane fusion.

This capacity may be related to the smaller repulsive hydration forces between PE bilayers (Lis et al., 1982) and the tendency of PE bilayers to transform into the hexagonal phase (Reis-Husson, 1962; Cullis & de Kruijff, 1979; Hauser et al., 1981) as suggested earlier (Düzgüneş et al., 1981a). The interaction of the PA or PS molecules with the polyamines may induce a local phase separation of the acidic lipids from the PE and produce molecular packing defects at the domain boundaries. These regions would then be favorable for close intermembrane contact and molecular mixing between apposed vesicles. Differential scanning calorimetry experiments have indicated that polyamines do not induce large phase separations in such types of vesicles, at least under experimental conditions which apply to fusion studies (F. Schuber and D. Papahadjopoulos, unpublished results). Therefore, massive lateral phase separation does not seem to be required for the fusion process, in agreement with the recent work of Hoekstra (1982) and Düzgüneş et al. (1983).

Possible Physiological Significance. The molecular factors which control cellular events involving membrane fusion are largely unknown. Liposome fusion has been used extensively as a model system to elucidate the involvement of various phospholipids, proteins, and divalent cations in membrane fusion (Papahadjopoulos et al., 1979; Hong et al., 1982; Düzgüneş et al., 1980; Nir et al., 1983). The results of the present study indicate that polyamines such as spermine and spermidine are significant modulators of divalent cation induced membrane fusion, as well as inducers of fusion in certain types of phospholipid vesicles. These phospholipid-specific effects of polyamines could be involved in processes such as cell growth, cell division, and exocytosis. The regulation of polyamine levels in the cytoplasm could thus exert its influence on the fusion of specific membranes.

The increase of the cell surface during cell growth has been explained by the membrane flow theory (Morrê, 1977), which implies the fusion of the plasma membrane with vesicles derived from the Golgi apparatus. Cell growth is accompanied by increased levels of polyamines, and one can speculate that normal membrane fusion rates might need optimal polyamine concentrations. In this respect, an interesting observation was made by Harada et al. (1981) that CHO cells which have been limited of their polyamines by incubation with an ornithine decarboxylase inhibitor stop dividing and accumulate a great number of "vacuoles". Upon addition of polyamines to the culture medium, these vacuoles disappear, and the cells resume dividing. A link between membrane fusion and normal polyamine levels is one of the possibilities which might account for this observation.

Polyamines could also act as modulators of exocytic events triggered by Ca^{2+} because of their metabolically regulated intracellular concentrations. The effect of polyamines could be especially important in membrane domains enriched in PA, whose production increases at the expense of PI during stimulation of secretion in a process termed the "phospholipid effect" (Hokin & Hokin, 1953; Michell, 1975; Lapetina & Cuatrecasas, 1979). It should also be noted that polyamines in the presence of physiological concentrations of Mg^{2+} have a significant effect on the threshold concentration of Ca^{2+} required for membrane fusion (Hong et al., 1983; this study). The observation that polyamines themselves can trigger fusion of membranes containing significant amounts of PE may also be of physiological significance especially since this phospholipid appears to be preferentially located in the inner monolayer of plasma membranes (Op den Kamp, 1979).

The role of the high concentrations of spermidine and spermine in the seminal plasma of several animal species is still open to speculation (Bachrach, 1973). Bearer & Friend (1982) have recently shown that the fusogenic region of the guinea pig sperm membrane has anionic lipid domains. It is therefore possible that the polyamines present in the seminal fluid participate in the membrane fusion events occurring during fertilization.

In this study, using liposomes as model systems, we have demonstrated that polyamines such as spermidine and spermine are involved in membrane fusion. This finding adds a

new dimension to the possible physiological roles of these biological polycations which have been recognized to be of fundamental importance in a variety of processes linked to cell growth and division.

Registry No. Putrescine, 110-60-1; spermidine, 124-20-9; spermine, 71-44-3; cholesterol, 57-88-5; Mg, 7439-95-4; Ca, 7440-70-2.

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