Spermine as a Modulator of Membrane Fusion: Interactions with Acidic Phospholipids†

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ABSTRACT: The interaction of spermine with acidic phospholipids was investigated for its possible relevance to membrane fusion. Equilibrium dialysis was used to measure the binding of spermine and calcium to large unilamellar vesicles (liposomes) of phosphatidate (PA) or phosphatidylserine (PS). Spermine bound to isolated PA and PS liposomes with intrinsic association constants of approximately 2 and 0.2 M^{-1}, respectively. Above the aggregation threshold of the liposomes, the binding of spermine increased dramatically, especially for PA. The increased binding upon aggregation of PA liposomes was interpreted as evidence for the formation of a new binding complex after aggregation. Spermine enhanced calcium binding to PA, while it inhibited calcium binding to PS, under the same conditions. This difference explained the small effect of spermine on the overall rate of calcium-induced fusion of PS liposomes as opposed to the large effect on PA liposomes. The rate increase could be modeled by a spermine-induced increase in the liposome aggregation rate. The preference for binding of spermine to PA over PS suggested a preference for accessible monoesterified phosphate groups by spermine. This preference was confirmed by the large effects of spermine on aggregation and overall fusion rates of liposomes containing phosphatidylinositol 4,5-diphosphate. The large spermine effects on these liposomes compared with phosphatidate- or phosphatidylinositol-containing liposomes suggested that spermine has a strong specific interaction with phosphatidylinositol 4,5-diphosphate. Clearly, phosphorylation of phosphatidylinositol can lead to a large change in the spermine sensitivity of membrane fusion.

The mammalian polyamines spermine, spermidine, and putrescine are polycations which are found at levels as high as millimolar in many cell types (Bachrach, 1973). They have been implicated as regulators of cell growth, acting at the level of DNA replication (Cohen, 1971; Bachrach, 1973; Tabor & Tabor, 1976; Mamont et al., 1976, 1978; Jänne et al., 1978; Heby & Jänne, 1981). The binding of these polycations to DNA has been demonstrated and studied in detail (Johnson & Bach, 1968; Quigley & Cohn, 1969; Bloomfield & Wilson, 1981; Braunlin et al., 1982). Until recently, little was known about the interaction of polyamines with phospholipids. It is likely that these polycations interact with acidic phospholipids as well as nucleic acids. Indeed, such interactions have been observed.

The membrane-stabilizing effects of polyamines have been known for many years (Tabor, 1962; Harold, 1964; Spisni, 1976; Chun et al., 1976; Sechi et al., 1978; Ballas et al., 1983). It has been suggested that some of the intracellular effects of polyamines may be due to binding to membranes with concomitant displacement of Ca^{2+} (Koenig et al., 1983a,b). Only recently has binding been directly measured by a dye binding technique (Tadolini et al., 1984) and microelectrophoresis of multilamellar vesicles (Chung et al., 1985). In the latter study, relatively strong binding of spermine to phosphatidylserine (PS)[1] and phosphatidylinositol (PI) governed by the Gouy–Chapman–Stern diffuse double layer theory was observed. A strong interaction of spermine with triphosphoinositides such as phosphatidylinositol 4,5-diphosphate (PIP_2) has also been observed by microelectrophoresis (S. McLaughlin, personal communication).

Studies on the effects of polyamines on fusion of liposomes of known phospholipid composition have shown that spermine at physiological levels can be quite active in the promotion of membrane fusion (Hong et al., 1983; Schuber et al., 1983). A particularly interesting finding in these studies was the fact that spermine enhanced the Ca^{2+}-induced fusion of phosphatidate (PA) liposomes but had relatively little effect on the Ca^{2+}-induced fusion of PS liposomes. This suggests that there exists a spermine specificity for interaction with PA, but not with PS, leading to the promotion of Ca^{2+}-induced fusion. Since this effect occurred above the spermine concentrations needed to induce liposome aggregation for both phospholipids, it seems likely that the differences in the spermine effects observed are not solely due to differences in the liposome aggregation rates mediated by spermine binding and surface charge neutralization (Bentz et al., 1983a,b).

In view of the binding of spermine and other polycations leading to effects on fusion of liposomes (Gad et al., 1982, 1986; Hong et al., 1983; Schuber et al., 1983; Nir et al., 1983; Uster & Deamer, 1985), it is conceivable that polyamines could also regulate cellular activities at the level of membrane fusion events involved in membrane trafficking (Morrê, 1977).

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[1] Abbreviations: PS, phosphatidylserine; PI, phosphatidylinositol; PA, phosphatidate; PIP_2, phosphatidylinositol 4,5-diphosphate; PE, phosphatidylethanolamine; DPA, dipicolinic acid; TES, *N*-[tris(hydroxymethyl)methyl]-2-aminoethanesulfonic acid; EDTA, ethylenediaminetetraacetate; NTA, nitrilotriacetic acid; spm, spermine.

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Several observations appear to link the intracellular polyamine level to membrane fusion. First, Harada et al. (1981) have observed that Chinese hamster ovary cells depleted of polyamines by incubation with an ornithine decarboxylase inhibitor accumulate "vacuoles" or intracellular vesicles. Addition of polyamines reverses the effect. Also, inhibition of polyamine synthesis blocks urinary secretion of β-glucuronidase from mouse kidney (Laitinen & Pajunen, 1983). Furthermore, high polyamine levels have been correlated with secretory activity in rat ventral prostate cells (Fuller et al., 1975; Piik et al., 1977; Danzin et al., 1979).

We report here the results of our studies on the relationship of spermine and calcium binding to membrane fusion of liposomes containing acidic phospholipids. Unlike previous studies, our use of equilibrium dialysis has enabled us to measure binding in both aggregated and nonaggregated liposome systems. The results presented suggest explanations for the differential effects of spermine on calcium-induced fusion of liposomes containing different acidic phospholipids. We have also used data from fusion kinetics to determine the step of the fusion process at which spermine acts on Ca^{2+}-induced fusion of PA liposomes. Lastly, we have examined the effect of spermine on the fusion of liposomes containing phosphatidylinositol 4,5-diphosphate.

Materials and Methods

Bovine brain phosphatidylserine (PS), phosphatidate (PA) (derived from egg phosphatidylcholine), phosphatidylinositol (plant), and phosphatidylethanolamine (PE) (transesterified from egg phosphatidylcholine) were purchased from Avanti Polar Lipids (Birmingham, AL). Phosphatidylinositol 4,5-diphosphate (PIP_2), spermine tetrahydrochloride (97%), dipicolinic acid (DPA) (99%), *N*-[tris(hydroxymethyl)-methyl]-2-aminoethanesulfonic acid (TES) (99%), and nitrilotriacetic acid (NTA) (99%) were obtained from Sigma (St. Louis, MO). Ethylenediaminetetracetate (EDTA) and NaCl were obtained from Fisher. $TbCl_3 \cdot 6H_2O$ (99.9%) was obtained from Alfa (Danvers, MA). [^{14}C]Spermine and $^{45}CaCl_2$ were from Amersham (Arlington Heights, IL). Polycarbonate filters were from Nucleopore. Cellulose dialysis tubing was from Spectrapor (Los Angeles, CA).

The purity of phospholipids was assessed by thin-layer chromatography on silica plates in two dimensions using the solvents chloroform/methanol/NH_4OH (65/25/5) and chloroform/acetone/methanol/acetic acid/H_2O (30/40/10/10/5). PIP_2 was tested on oxalate-treated silica plates from Applied Science with the solvent chloroform/methanol/NH_4OH/H_2O (9/7/0.56/1.46). The purity of all phospholipids was estimated as 95% or greater.

Liposome Preparation. Large unilamellar vesicles encapsulating the desired material were prepared by the reverse-phase evaporation method (Szoka & Papahadjopoulos, 1978) as modified by Wilschut et al. (1980). The aqueous solutions for all vesicles contained 100 mM NaCl, 5 mM TES, and 0.1 mM EDTA. EDTA was not used in the buffer for Ca^{2+} binding studies. The pH was 7.4. Liposomes for fusion studies encapsulated either (a) 2.5 mM $TbCl_3$ and 40 mM sodium nitrilotriacetic acid (NTA), (b) 50 mM DPA and 20 mM NaCl, or (c) 1.25 mM $TbCl_3$, 20 mM NTA, 25 mM DPA, and 10 mM NaCl. Each encapsulated solution also contained 5 mM TES, and the pH was adjusted to 7.4 prior to encapsulation. To obtain a relatively uniform size, the vesicles were extruded through polycarbonate filters with a pore size of 0.2 μm followed by 0.1 μm (Szoka et al., 1980). The preparation was centrifuged for 15 min at 10000*g* to remove any remaining very large liposomes. Liposomes were separated from nonencapsulated material by using a 1 × 20 cm column of Sephadex G-75 for each 10 μmol of lipid. Elution was with 100 mM NaCl, 5 mM TES, pH 7.4, and 1 mM EDTA. EDTA was later diluted to a final concentration of 0.1 mM. The phospholipid concentrations were determined by using a phosphate assay (Bartlett, 1959).

Fusion Assay. The fusion assay was that of Wilschut et al. (1980) which is based on the generation of a highly fluorescent chelation complex of Tb^{3+} and DPA upon mixing of the contents of the vesicles when they fuse. The assay mixture contains a 1/1 ratio of Tb^{3+}- and DPA-containing liposomes in a 1-mL final volume of 100 mM NaCl, 5 mM TES, and 0.1 mM EDTA, pH 7.4. A small amount (usually 10–20 μL) of a concentrated solution of calcium or spermine was injected to initiate the fusion. The 100% fusion level was set by lysing the appropriate amount of Tb^{3+}-containing vesicles with 0.5% sodium cholate in medium containing no EDTA and excess DPA. As in Wilschut et al. (1980), the overall fusion rates are expressed as the percentage of the maximum fluorescence (at 100% fusion) attained per minute (% F_{max}/min).

Any mixing of the Tb^{3+} or DPA contents of the vesicles outside of the vesicles due to leakage did not result in the formation of Tb–DPA complexes because of the preferential binding of Tb^{3+} to EDTA and, when Ca^{2+} was present, the preferential binding of Ca^{2+} to DPA (because Ca^{2+} was in vast excess to Tb^{3+}). Leakage was monitored by the dissociation of Tb^{3+} from DPA using liposomes containing both (type c above) under conditions identical with the fusion experiments. Leakage of the preencapsulated Tb^{3+}–DPA complex into the medium or influx of the medium into the fusing liposomes results in dissociation of the complex and a loss of fluorescence (Bentz et al., 1983b, 1985). This assay is quite sensitive to both types of leakage (Bentz & Düzgüneş, 1985). Fusion rates were measured only where corrections for leakage were not necessary.

The fluorescence was measured on an SLM 4000 fluorometer. The Tb–DPA complex was excited at 276 nm and the fluorescence measured at >530 nm through a Corning 3-68 cutoff filter.

Binding Experiments. Equilibrium dialysis was performed in 100 mM NaCl and 5 mM TES, pH 7.4. Spermine binding experiments contained 0.1 mM EDTA. Those with Ca^{2+} did not. Dialysis bags were washed by soaking 2–3 h in 50% ethanol and then washing with ion-exchange cartridge-purified double-distilled water. They were then boiled in 1% sodium bicarbonate, washed again in water, including one wash with 1 mM EDTA, boiled in water, and washed again with water. Dialysis was performed in small vials in which the external volume was 2 mL and the volume in the bag was 1 mL. The bags were placed in the vials in a U shape with both ends at the top and a "flea" magnetic stirrer at the bottom. The appropriate concentration of vesicles was placed inside the bags, and the radioactive cation, along with an appropriate amount of unlabeled cation, was added from a concentrated stock solution to the solution outside the bag just prior to the beginning of the dialysis. The dialysis was performed for 24 h at room temperature in sealed vials with stirring. At the end of the dialysis, samples from inside the bag were withdrawn with a syringe after being mixed as described below for the aggregation experiments. Samples were also taken for phosphate assay to check phospholipid recovery. Samples from outside of the bag were taken to measure the free concentration of the cation of interest. There was significant binding of spermine to the dialysis bags, especially at the lowest con-

centrations. However, since the concentrations inside as well as outside the bag were accurately measured, both free and liposome-bound concentrations were determined directly, eliminating any problems due to nonspecific binding to dialysis tubing.

Equilibrium vesicle aggregation was monitored by subjecting vesicles to the same conditions as for equilibrium dialysis including incubation in the dialysis bag. After 24 h, the solution in the bag was rapidly forced back and forth through the bag to mix any heavy aggregates and withdrawn with a syringe for measurement. Turbidity was measured at 400 nm on a Beckman Model 34 spectrophotometer.

Leakage of contents from liposomes used in equilibrium dialysis was monitored to determine the exposure of the inner leaflet of liposome bilayers to the binding cations. This was accomplished by using vesicles containing both Tb^{3+} and DPA and determining leakage after the dialysis procedure by fluorescence as described above. Only the data on Ca^{2+}–PA binding needed to be corrected for leakage effects. Phosphate assays (Bartlett, 1959) were performed on all samples to check for recovery of lipid.

Evaluation of Binding Constants. Calculations of the amount of Ca^{2+} and spermine bound per PS or PA are based upon 1:1 binding complexes according to the reactions

$$Na^+ + P^- \overset{K_{Na}}{\rightleftharpoons} PNa$$

$$Ca^{2+} + P^- \overset{K_{Ca}}{\rightleftharpoons} PCa^+$$

$$spm^{4+} + P^- \overset{K_{spm}}{\rightleftharpoons} Pspm^{3+}$$

where P^- denotes the negatively charged PS or PA head group and spm^{4+} denotes the tetravalent spermine cation. It should be noted that at pH 7.4 the PA head group could be partially doubly ionized. The details of solving the Poisson–Boltzmann equation to obtain the electrostatic surface potential are given in Bentz (1981, 1982) for up to divalent ions, and the extension to tetravalent ions is straightforward. The same work shows the equations needed to compute the amounts of cations in the double-layer region near the charged membrane. These calculations strictly pertain to isolated surface, i.e., when liposomes remain disperse. However, it is possible to extend these equations to closely apposed surfaces (Bentz, 1982).

We have made two major assumptions in these calculations: that the multivalent cations bind only to one head group and that the cations can be treated as point charges (4+ for spermine, 2+ for Ca^{2+}). McLaughlin et al. (1981) have shown that Ca^{2+} does bind to one head group, although the simultaneous existence of some 2:1 complexes, i.e., one Ca^{2+} complexed to two adjacent PS head groups, is still possible. Other work on Ca–PS binding has been based on 2:1 binding complexes (Nir et al., 1978; Portis et al., 1979; Düzgüneş et al., 1981a; Ohki & Kurland, 1981). In Bentz et al. (1983b), it was shown that the various sets of binding constants gave essentially identical predicted values for bound Ca^{2+}. Here we have reached the same conclusion. The amount of bound Ca^{2+} or spermine is essentially identical, within experimental accuracy, whether one chooses a 1:1 binding with $K_{spm} = 0.2$ M^{-1}, for example, or 4:1 binding with $K_{spm}^{4:1} = 0.7$ M^{-1}, when the spermine concentration is less than 100 μM and the Ca^{2+} concentration is less than 1 mM. Since the binding constant, with its associated binding stoichiometry, is used here primarily as a means of predicting the amounts of bound cations, the particular stoichiometry chosen is not crucial.

The second assumption, that cations can be treated as point charges, is more drastic. While metal ions do behave like point charges, it is known that certain large divalent cations cannot be treated in this fashion (Olivares & MacQuarrie, 1980). Carnie and McLaughlin (1983) have provided a theoretical analysis of large divalent cations treated as two point charges at opposite ends of a rod. Although binding was not explicitly treated, it is clear from their work and our previous work (Bentz & Nir, 1980) that the effect of ignoring finite cation size is to underestimate the binding constant. The more unwieldy the cation, the less its surface concentration and hence, for a given amount of bound cation, the greater the binding constant needed to achieve this level of binding.

The calculations include the amounts of cations concentrated in the double-layer region near the liposome surfaces. However, in all cases treated, the amounts for Ca^{2+} and spermine in the double layer were negligible relative to the amounts bound.

Results

Spermine-Modulated Fusion of PA Liposomes. In the following experiments, the initial rates of fusion of liposomes were measured, and the effects of spermine on these rates were tested. These initial rates are simply the initial slopes of the fusion curves and, as such, represent overall rates which combine both the aggregation rate and the fusion rate, per se (Bentz et al., 1983a,b, 1985). The overall fusion reaction is

$$V_1 + V_1 \underset{D_{11}}{\overset{C_{11}}{\rightleftharpoons}} V_2 \xrightarrow{f_{11}} F_2$$

where V_1 and V_2 denote the liposome and the aggregated dimer, respectively, and F_2 denotes the fused doublet. Note that we refer to the rate of the second step in this process as the fusion rate while the rate of the whole process is called the *overall* fusion rate.

Figure 1 shows the initial overall rates of the Ca^{2+}-induced fusion of PA and PS liposomes as a function of spermine concentration. Equivalent data were obtained by Schuber et al. (1983). In both cases, the Ca^{2+} concentration is subthreshold for fusion (approximately half the concentration needed for observable fusion on the time scale of 5–10 min). It is evident that the spermine effect on the overall rate of PS liposome fusion is negligible compared to the large effect on the overall rate of PA liposome fusion (Figure 1A). The rate is increased from 0 to about 70% F_{max}/min for PA. In Figure 1B, it can be seen that there *is* a very small effect of spermine on the rate of PS liposome fusion, but it is increased from 0 to only 1–1.5% F_{max}/min.

The large effect of spermine on the overall rate of calcium-induced fusion of PA liposomes was investigated under conditions which allowed determination of the contribution of spermine to the rate constant for an individual step of the overall fusion process. Using the above reaction as a model, we applied the previously described methodology for obtaining these primary rate constants (Nir et al., 1980; Bentz et al., 1983a, 1985) to analyze fusion of PA liposomes in the presence of Ca^{2+} and spermine. The results of these analyses are shown in Table I.

For a given spermine concentration, the value of C_{11} increases as the Ca^{2+} concentration increases. Likewise, for a given lipid and Ca^{2+} concentration, the aggregation rate increases as the spermine concentration increases. These results are expected according to the fact that C_{11} is strongly dependent upon the surface charge neutralization by cation binding (Nir & Bentz, 1978; Bentz & Nir, 1981a,b; Bentz et al., 1983b, 1985). However, for a given Ca^{2+} concentration, increasing the spermine and lipid concentrations simultaneously does not necessarily lead to an increase in C_{11}. For

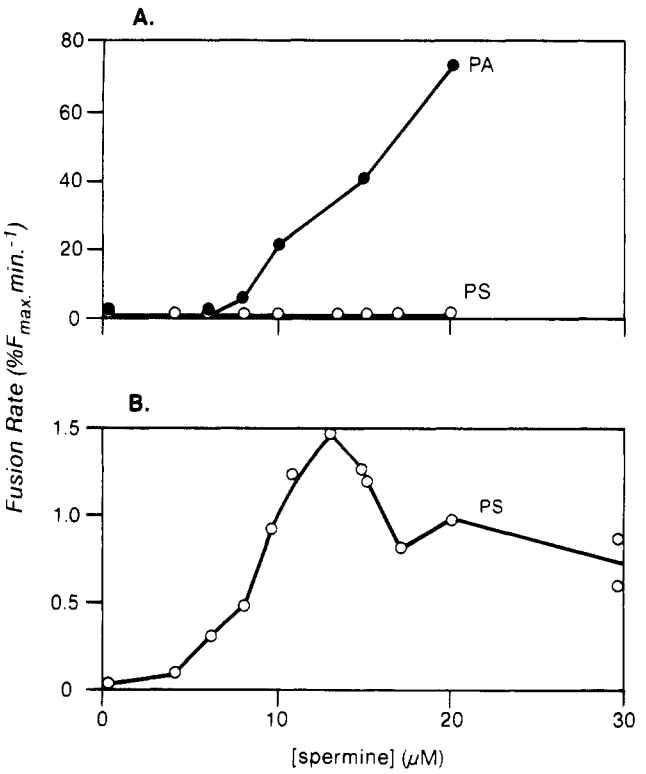

FIGURE 1: (A) Initial overall fusion rates of PA (●) and PS (○) liposomes as a function of spermine concentration. Spermine and Ca^{2+} were added simultaneously at time zero. Total phospholipid concentration was 50 μM. The Ca^{2+} concentration was 0.2 mM for PA and 1 mM for PS. Other conditions are given under Materials and Methods. (B) Initial overall fusion rate of PS (○) liposomes at a constant Ca^{2+} concentration (0.2 mM) as a function of spermine concentration.

Table I: Effect of Spermine and Ca^{2+} on Aggregation Rate Constants for PA Liposome Fusion[a]

[PA] (μM)	[spm] (μM)	[Ca^{2+}] (mM)	C_{11} (M^{-1} s^{-1})
10	4.25	0.2	3×10^6
10	4.25	0.4	1×10^7
20	5.5	0.15	1×10^6
20	5.5	0.2	6×10^6
20	5.5	0.4	1×10^7
20	5.5	0.6	2.5×10^7
50	9.5	0.2	5×10^6
50	9.5	0.6	8×10^6

[a] Aggregation rate constants (C_{11}) for fusion of PA liposomes. Liposome preparation and fusion conditions were described under Materials and Methods. Spermine and Ca^{2+} were added simultaneously at time zero. Rates were measured, and C_{11} was calculated as described under Materials and Methods.

example, at 0.2 mM Ca^{2+} with 5.5 μM spermine and 20 μM PA, the rate constant for aggregation, C_{11}, is larger than with 9.5 μM spermine and 50 μM PA. The simplest explanation for this fact is that there is actually somewhat less spermine bound per liposome in the latter case than in the former. The spermine concentrations are low compared to the phospholipid concentration. Therefore, unlike the situation with Ca^{2+} (Nir et al., 1982; Bentz et al., 1983a, 1985), it is difficult to fix the free concentration of spermine with varying phospholipid concentrations in the absence of knowledge of the exact spermine binding constant. It is clear that experiments which precisely determine the *initial* binding constant of spermine to the liposomes without aggregation will be required to confirm this explanation.

The aggregation step was rate limiting in all these experiments. Under these conditions, the fusion curves are fitted

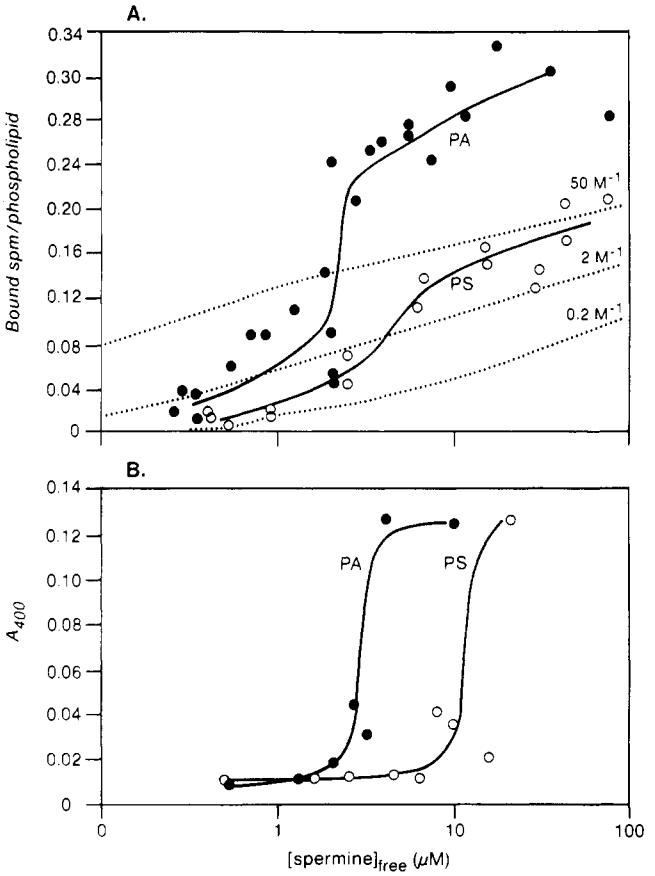

FIGURE 2: (A) Binding of spermine to PA (●) or PS (○) liposomes as determined by equilibrium dialysis. Phospholipid concentration was 50 μM in all cases. Conditions for dialysis measurements were described under Materials and Methods. Spermine bound per phospholipid is given as a function of the measured free spermine concentration. Theoretical binding curves, calculated as described under Materials and Methods, are shown as dotted lines with binding constants in M^{-1} beside them. Bound spermine per phospholipid shown on the *y* axis is in terms of *exposed* phospholipid, i.e., only the outer monolayer of the liposomes in this case. The solid lines are drawn arbitrarily through the data to guide the eye. (B) Aggregation of PA (●) and PS (○) liposomes as a function of free spermine concentration. Conditions were identical with those used in Figure 2A including 24-h incubation in a dialysis bag. Aggregation was measured by the absorbance (turbidity) of the liposome suspension at 400 nm.

by a single parameter, i.e., C_{11}. Hence, the use of only one lipid concentration is sufficient to fix C_{11} (Bentz et al., 1983a). In other words, when aggregation is rate limiting, the fusion rate is too fast to contribute to the overall rate. This is equivalent to saying that the observed effect of spermine in Figure 1 is due solely to the enhancement of the aggregation rate (i.e., C_{11}) presumably because of its binding on the liposome surface. Spermine alone does not induce fusion of PA or PS liposomes.

Spermine Binding to PA and PS Liposomes. The equilibrium binding of spermine alone to PA and PS liposomes under conditions similar to those used for fusion reactions (Schuber et al., 1983; this paper) is shown in Figure 2A. Theoretical curves, modeled on 1:1 binding and a point charge of 4+, are shown for a range of spermine binding constants. We assume that $K_{Na} = 0.6$ M^{-1}, which is its value for PS, although for PA the value may be slightly lower (Eisenberg et al., 1979). These calculations also assume that the liposomes remain monodisperse at all spermine concentrations. This condition is met at low spermine concentrations where the experimental values are approaching the theoretical curves. However, at higher concentrations, aggregation of liposomes occurs. At the low concentrations, the approximate intrinsic binding

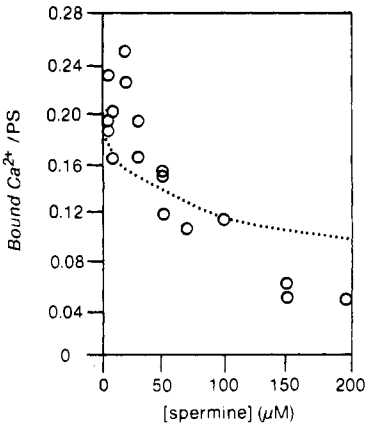

FIGURE 3: Calcium binding to PS (○) as a function of spermine concentration. Phospholipid concentration was 500 μM in all cases. A theoretical curve, calculated as described under Materials and Methods, is shown as a dotted line. The binding constants used for the calculations were 0.2 M^{-1} for spermine, 12 M^{-1} for Ca^{2+}, and 0.6 M^{-1} for Na^+. All Ca^{2+}/phospholipid ratios are expressed in terms of exposed phospholipid.

constants for PA and PS based on this model are 2 and 0.2 M^{-1}, respectively. At higher concentrations, the binding isotherms deviate significantly from the theoretical predictions.

Figure 2B shows the spermine aggregation curves for PA and PS liposomes under the conditions of the equilibrium dialysis as determined by turbidity. Aggregation of liposomes occurs at a free spermine concentration of about 2.5 μM for PA and about 4.2 μM for PS. Comparing these aggregation data to the binding curves, a particularly large shift away from the theoretical binding curves can be seen around the spermine concentration which causes aggregation of PA or PS liposomes. The two most likely explanations for this behavior are an increase in cation concentration at the membrane surface or the generation of a new binding configuration as the bilayers approach apposition.

When the liposomes simply aggregate without the formation of a new binding configuration, it is known that the amount of bound spermine, as well as Na^+, will increase due to the interaction of the charged surfaces (Bentz, 1982). According to theoretical calculations, if we assumed that the PS bilayers were in close apposition, then a binding constant of K_{spm} = 0.2 M^{-1} would yield 0.17 spermine bound per phospholipid in 100 μM spermine and 0.13 spermine bound per phospholipid in 10 μM spermine. That these numbers are similar to the amounts found for PS liposomes when they are aggregated after dialysis suggests that the simple electrostatic model may well account for the observed enhancement of spermine bound per PS upon aggregation. Similar calculations for PA liposomes, assuming a spermine binding constant of 2 M^{-1}, predict 0.19 spermine bound per phospholipid in 100 μM spermine and 0.17 spermine bound per phospholipid in 10 μM spermine, much less than the measured levels of 0.24–0.32 spermine bound per PA. Clearly, the simple electrostatic model cannot explain the enhanced spermine binding to PA under conditions of aggregation. Thus, the emergence of a new strong binding complex between the spermine and and the apposed PA bilayers seems evident.

Calcium Binding. In order to elucidate the nature of the new binding complexes of spermine for PA, and to correlate Ca^{2+} binding with spermine effects on Ca^{2+}-induced fusion rates, the binding of Ca^{2+} to PA and PS liposomes was measured under conditions where the liposomes were aggregated in the presence of spermine. Figures 3 and 4 show the results of these studies for PS and PA liposomes, respectively.

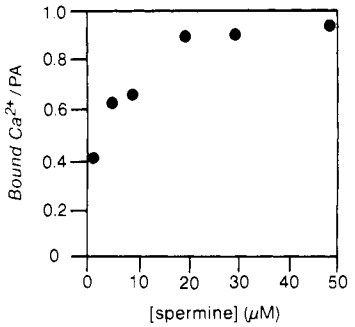

FIGURE 4: Calcium binding to PA (●) as a function of spermine concentration. Phospholipid concentration was 500 μM in all cases. Other conditions are described under Materials and Methods. All Ca^{2+}/phospholipid ratios are expressed in terms of exposed phospholipid.

It is quite evident that Ca^{2+} and spermine show a direct competition for binding to the PS head group. The theoretical curve in Figure 3 shows the expected values for the amount of bound Ca^{2+} assuming that the liposomes remained monodisperse during the binding experiments and assuming a spermine binding constant of 0.2 M^{-1}. The fit to the data is adequate and could be improved if one were to assume close apposition of the bilayers at the higher spermine concentrations.

In contrast to PS, the enhanced binding of Ca^{2+} seen in Figure 4 indicates that the apposition of PA bilayers not only induces the formation of a new binding complex for spermine but also must allow more Ca^{2+} to bind either through a new Ca^{2+} complex or by increasing the number of available binding sites. One possible origin of the increased Ca^{2+} binding in the presence of spermine may be fusion and collapse of the PA liposomes to flat multilamellar structures and appropriate rearrangement of ions and head groups to allow maximum binding between membranes. Leakage of contents observed under these conditions suggests that fusion *is* occurring. Leakage was not observed in any binding experiments involving other conditions. At 0.2 mM Ca^{2+}, where leakage (and probably fusion) was not occurring, binding experiments (data not shown) with spermine in the same concentration range (0–200 μM) revealed almost no effect on the amount of Ca^{2+} bound to PA. Though the increase of Ca^{2+} binding is not observed at this concentration, the result is still clearly different from that with PS where spermine competes off bound Ca^{2+}. It is quite possible that the new Ca^{2+} complex that emerges with PA liposomes at 1 mM Ca^{2+} also occurs at a local level at all Ca^{2+} and spermine concentrations where membranes are apposed in the fusion process. These differences in the spermine effect on Ca^{2+} binding to PA and PS liposomes are therefore undoubtedly related to the differences in the spermine effect on the overall rates of Ca^{2+}-induced fusion of PA and PS liposomes, as discussed below.

Spermine Interaction with Triphosphoinositides. The observation of the strong complex of spermine with the accessible monoesterified phosphate group of PA would predict a strong interaction with other phospholipids containing such groups. To test this prediction, the interaction of spermine with phosphatidylinositol 4,5-diphosphate (PIP_2) was investigated. The content of acidic phospholipid was systematically varied by using liposomes of PIP_2/PS/PE (10/15/75), PI/PS/PE (10/15/75), PA/PS/PE (10/25/75), and PS/PE (25/75). Because the charge of PIP_2 at pH 7.4 is about 3–, the composition PIP_2/PS/PE (3.6/16.1/80.3) was also used to compare PIP_2-containing liposomes to other liposomes at approximately the same surface charge density.

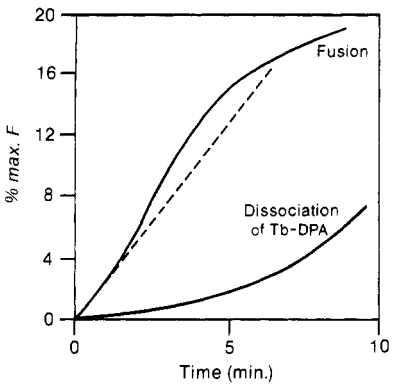

FIGURE 5: Typical fusion and dissociation curves for PE-containing liposomes. 3 mM Ca^{2+} was added at time zero to PIP_2/PS/PE (10/15/75) liposomes. Fusion of Tb^{3+}-loaded liposomes with DPA-loaded liposomes and dissociation of Tb^{3+} from DPA upon leakage from liposomes in which they were coencapsulated are shown as a function of time (see Materials and Methods). The dashed line shows the initial fusion rate measured for this particular case.

The spermine threshold concentration for aggregation of liposomes (as defined by an observable change in the 90° light scattering on the time scale of 1–2 min) decreased from approximately 100 μM for the PI-containing liposomes (10 mol %) to about 15 μM for the PIP_2-containing liposomes (10 mol %). This decrease cannot be explained by increased spermine concentration in the double layer due to increased surface charge. Experiments with only 3.6 mol % PIP_2 bear out this point since the surface charge of these liposomes is about the same as the PA- and PI-containing liposomes, yet the aggregation threshold was still almost as low as for the 10 mol % PIP_2 liposomes. Therefore, the phosphate groups of PIP_2 must provide a site for strong specific binding of spermine to the membrane, thereby mediating aggregation.

Figure 5 shows an example of a typical fusion curve for liposomes containing a small proportion of acidic phospholipid and a large proportion of PE. As for most liposomes with a high PE content (Düzgüneş et al., 1981b), the fusion curves are somewhat biphasic. Note that the rates measured are the *initial* rates, not the maximal rates, and that there is minimal leakage at this point in the fusion curve. Figure 6 shows the effect of varying Ca^{2+} concentration on the initial overall fusion rate for such liposomes. Two classes of liposomes are observed by using these measurements for the Ca^{2+}-induced fusion rates. For one class, PA- and PIP_2-containing liposomes, the overall fusion rates are significantly higher in the range of 0.5–3 mM Ca^{2+} than those for the second class, PI- and PS-containing liposomes.

In Figure 7, the observed overall fusion rates in the presence of both Ca^{2+} and spermine are shown. Since the data were taken under aggregation rate-limiting conditions, the changes in overall fusion rates reflect changes in aggregation rates (C_{11}) directly. To identify the specific spermine effect on the overall fusion rates, the rates of Figure 7 minus the rates due to Ca^{2+} alone and spermine alone were plotted in Figure 8. Clearly, there was a larger effect of 200 μM spermine on the PIP_2-containing liposomes, both at 3.6 and at 10 mol %, than on liposomes of other compositions. These curves show a maximal spermine effect in the range of 1–2 mM Ca^{2+}, where the spermine effect added 10–12% F_{max}/min to the overall rate for PIP_2-containing liposomes as opposed to only 1–2% F_{max}/min for the other compositions. Even the PA-containing liposomes, which like PIP_2-containing liposomes showed a higher Ca^{2+} sensitivity, were not affected by spermine as much as the PIP_2-containing liposomes. The Ca^{2+} threshold for the initial rate of fusion of PIP_2-containing liposomes was lowered

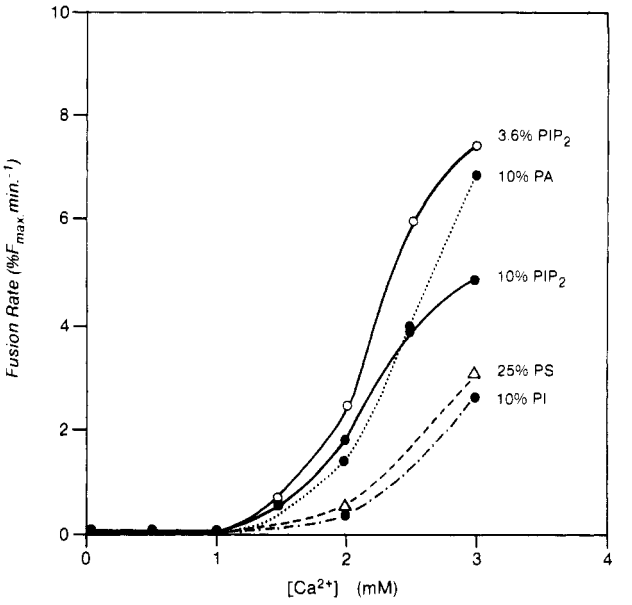

FIGURE 6: Initial overall fusion rate of liposomes composed of PIP_2/PS/PE (10/15/75) (●—●), PA/PS/PE (10/15/75) (●···●), PI/PS/PE (10/15/75) (●-·-●), PS/PE (25/75) (△---△), or PIP_2/PS/PE (3.6/16.1/80.3) (○—○) as a function of Ca^{2+} added at time zero. Total phospholipid concentration in all experiments was 20 μM. Other conditions are described under Materials and Methods. The encapsulated volumes per phospholipid for all liposomes were similar as determined by the fluorescence of coencapsulated Tb^{3+} and DPA, suggesting a similar average size for all liposomes.

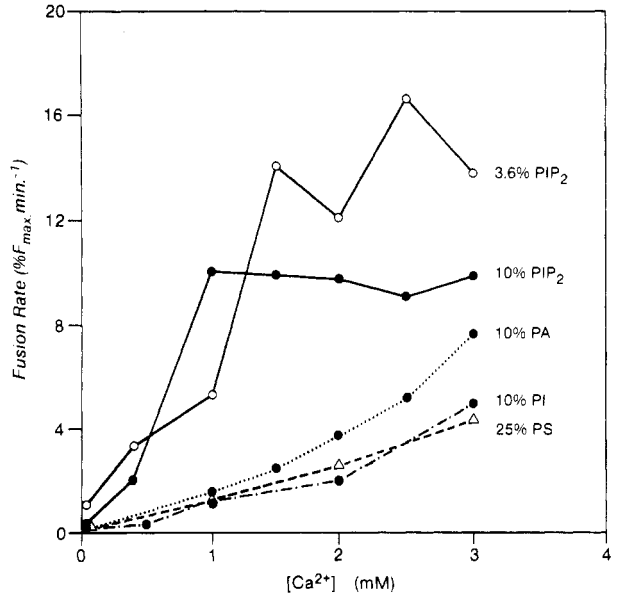

FIGURE 7: Initial overall fusion rate as a function of Ca^{2+} added simultaneously with 200 μM spermine at time zero: PIP_2/PS/PE (10/15/75) (●—●), PA/PS/PE (10/15/75) (●···●), PI/PS/PE (10/15/75) (●-·-●), PS/PE (25/75) (△---△), PIP_2/PS/PE (3.6/16.1/80.3) (○—○). Total phospholipid concentration in all experiments was 20 μM. Other conditions are described under Materials and Methods.

only moderately by spermine, but it is important to note the *maximal* rate of fusion, not shown in these measurements, was still quite high even at only 0.5 mM Ca^{2+} in the presence of 200 μM spermine. The spermine effect for the PIP_2-containing liposomes was even observable at only 20 μM spermine and was just as large as the 200 μM spermine effect on some of the other compositions. Therefore, very low concentrations of spermine have quite significant effects in this system.

Though the average surface charge density is higher in the 10 mol % PIP_2 liposomes than in all the other compositions,

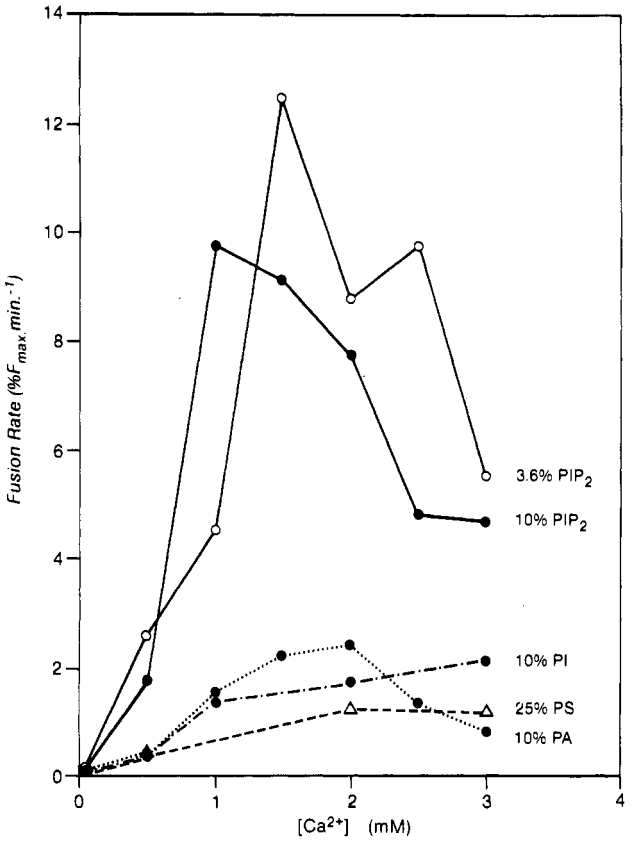

FIGURE 8: Spermine effect on initial Ca^{2+}-induced fusion rates. The spermine effect was defined as the observed overall fusion rate in the presence of spermine and Ca^{2+} minus the rate with Ca^{2+} alone minus the rate with spermine alone (nearly zero in all cases). It is expressed in terms of the change in overall fusion rate (dependent variable). The effect is plotted as a function of the Ca^{2+} concentration. The lipid compositions are the same as those in Figure 7 and are labeled in this figure.

the competing effects of increased local spermine concentration at the membrane and more charge to neutralize would tend to cancel and could not explain the very low spermine concentrations needed for large effects on the rate. The fact that the liposomes containing only 3.6 mol % PIP_2 required no more spermine for rate enhancements than the 10 mol % liposomes suggests that spermine's effect is brought about by a specific interaction with the PIP_2 and is not affected by differences in the surface charge density. Therefore, one must conclude that a specific interaction of spermine with PIP_2 and aggregation mediated mainly through PIP_2 are responsible for the observed rate effects.

These results confirm the suggestion that spermine has a particularly strong interaction with accessible phosphomonoesters on the surface of membranes. It is clear from the experiments with 10 mol % PI vs. those at 10 mol % PIP_2 that phosphorylation of PI can have a very large effect on the spermine interactions of a PI-containing membrane.

Discussion

Spermine and Ca^{2+} Binding. In this study, we have discovered two interesting binding complexes which occur upon the aggregation of PA liposomes. One occurs when spermine alone aggregates PA liposomes, leading to a large increase in the binding constant. The second is a tight Ca^{2+}–PA complex which is generated in the presence of spermine. These complexes do not appear for PS liposomes under the same conditions. In contrast to PA, the magnitude of the increase in spermine binding to PS upon liposome aggregation can be explained solely by the electrostatic interactions of the two double layers when two membranes approach, and the Ca^{2+} binding to PS undergoes simple direct competition with spermine. It is clear from our results that binding of spermine to PA and PS is very different.

There are several possible reasons for the generation of the avid spermine binding site between aggregated PA membranes. One important factor may be the exposure of the phosphate moiety of PA (and PIP_2) as opposed to PS (and PI). The serine moiety on the PS head group (or inositol on the PI head group) could block direct access to the phosphate group. Also, the PS carboxyl group could compete for binding of parts of the spermine molecule. Chung et al. (1985) have suggested that the phosphate moiety is a favored binding site for the amine-containing molecule gentamicin on the basis of similarities they observed in gentamicin binding to PS and PI. This also appears to be true for spermine. One might further speculate that it is possible that spermine, which is 16 Å long in extended configuration, could span the aqueous gap between bilayers in the aggregated state. The exposed phosphate monoester of PA may be favorable for this bridging interaction while the PS head group may not be. It is also likely that the spacing of the phospholipid head groups in the bilayer plays a role in spermine binding because of the relatively fixed spacing of charges in the spermine molecule. Chung et al. (1985) demonstrated such a dependence for gentamicin binding to membranes. The spacing of fixed phosphate groups in PIP_2 could play a role in the avid binding of spermine as well. This spacing could prove important in mixed-composition liposomes if spermine exerts part of its effect by phase separation of acidic phospholipids.

The binding constant determined for the spermine complex with nonaggregated PS liposomes in our study (0.2 M^{-1}) is smaller than that determined by microelectrophoresis (10 M^{-1}) (Chung et al., 1985); however, the values are not radically different. The assumption of a binding constant of 1 M^{-1} for Na^+ as in Chung et al. (1985) would increase our binding constant to some extent. Also, as in the case of gentamicin (Chung et al., 1985), it is likely that the surface area occupied per head group of PS may affect the binding of molecules such as spermine. Since the large unilamellar liposomes used in our studies (approximately 0.1–0.2 μm in diameter) may have a somewhat larger spacing between head groups than multilamellar liposomes used for microelectrophoresis (approximately 10 μM in diameter), the binding constant may be lower due to the increased spacing.

It is important to recognize that since the new binding complexes discussed here emerge only after close apposition of bilayers, it is unlikely that measurements of binding to isolated membrane surfaces by techniques such as microelectrophoresis would show these new binding complexes. The proposal of a tight cation complex betwen apposed acidic bilayers has a precedent in the "trans" complex of Ca^{2+} and PS (Portis et al., 1979; Ekerdt & Papahadjopoulos, 1982).

Spermine also affects the amount of bound Ca^{2+} in preaggregated systems at equilibrium. This is demonstrated by competition in the case of PS or creation of a new tight binding complex in the case of PA. This difference underscores the difference between PS and PA binding of spermine alone. In the following section, the importance of the spermine modulation of Ca^{2+} binding to fusion is discussed.

Spermine Effects on Fusion. The binding of spermine and the binding of Ca^{2+} are intimately related to the effects of spermine on the overall rates of fusion. Two characteristics of spermine are important in the overall fusion process. First, spermine speeds the aggregation of liposomes under the con-

ditions of our experiments. Second, spermine affects the amount of bound Ca^{2+}.

The spermine effect on the rate of aggregation of liposomes was evident in the aggregation rate-limited fusion experiments performed with pure PA liposomes and Ca^{2+}. It is important to distinguish between aggregation promoters (which increase C_{11}) and agents that produce an increase in the fusion rate (f_{11}) in the membrane fusion process. A "fusogen" is an agent that affects the *overall* fusion rate and could affect either aggregation or fusion. Spermine obviously falls into the category of aggregation promoters for the liposomes tested here; i.e., its effects are primarily on C_{11}. Spermine alone aggregates, but does not fuse, PA or PS liposomes. This suggests, but does not prove, that it does not affect the fusion rate. In this respect, it probably interacts with PS liposomes like Mg^{2+} (Wilschut et al., 1981), high concentrations of Na^+ (Nir et al., 1980a; Bentz & Nir, 1981a), or other cations which cause aggregation, but not fusion. Like most cationic species which only aggregate PS and/or PA liposomes, spermine is able to cause fusion of liposomes containing these phospholipids and high proportions of PE (Düzgüneş et al., 1981b; Sundler et al., 1981; Sundler & Papahadjopoulos, 1981; Schuber et al., 1983). This may be due to the fusogenic nature of PE after aggregation (Ellens et al., 1986) more than a direct effect of spermine. It is possible that spermine does not possess the special characteristics of Ca^{2+} such as the ability to dehydrate the surfaces of membranes and the flexibility of bond angles, allowing optimal apposition of bilayers (Portis et al., 1979; Ekerdt & Papahadjopoulos, 1982).

The effect of spermine on Ca^{2+} binding also influences the overall fusion rate in our experiments. The tight binding complex of spermine and/or the enhanced Ca^{2+} binding, observed with aggregated PA liposomes, may not be relevant to fusion rates limited by aggregation. This is because the equilibrium binding of an already aggregated system is measured in the binding experiments. However, the apposed bilayers of the aggregated systems at equilibrium may serve as a model for a prefusion complex (i.e., V_2). In a fusion rate-limited system, the amounts of various cation species bound to such a putative prefusion complex could be crucial. Since the spermine alone can only aggregate PS or PA liposomes, the fusion step of the overall process must be dependent on the amount of Ca^{2+} bound to the membrane prior to fusion (Bentz et al., 1983b). The data in Figures 3 and 4 show that spermine is more likely to compete with Ca^{2+} for binding to aggregated PS liposomes than to aggregated PA liposomes. As spermine concentrations are increased in Figure 1, the observed overall fusion rate is more and more likely to be limited by the fusion rate, assuming that spermine does not itself increase the rate of the fusion step. Therefore, it seems likely that the observed differences in spermine effects on overall Ca^{2+}-induced fusion rates of PA and PS liposomes may be explained by differences in the spermine effect on Ca^{2+} binding to aggregated liposomes (to the extent that the aggregated systems are a model for a prefusion complex).

The striking effect of spermine on the rate of fusion of PIP_2-containing liposomes echoes the result observed with pure PA. By replacement of part of the PS in PS/PE (25/75) liposomes with PIP_2, a large spermine effect on the overall rate of fusion is observed. As with pure PA liposomes, the binding of spermine and Ca^{2+} may be relevant to the observed fusion effects. It is possible that the two exposed monoesterified phosphate groups of PIP_2 also bind spermine quite avidly. As with pure PA, the spacing of the phosphate groups and the possibility of cross-bridging between membranes may also help to make PIP_2 a particularly suitable target for spermine effects.

Despite the different spermine effects observed with 100% PA liposomes vs. 100% PS liposomes, the spermine-modulated rate increases for 10 mol % PA liposomes fell into the same range as those for 10 mol % PI or PS liposomes in which the remainder of the phospholipid was 15 mol % PS and 75 mol % PE. On the other hand, a large spermine effect was observed on the 10 mol % PIP_2 liposomes. Both the head group of PIP_2 and pure PA membranes contain closely spaced phosphate groups. Since we know from equilibrium dialysis that pure PA membranes bind spermine strongly, PIP_2 may provide a similar binding site. However, 10 mol % PA may not generate this avid binding site because the PA head-group phosphates are dispersed in the PE. Furthermore, the projection of the inositol moiety away from the membrane surface may allow aggregation of liposomes containing 10 mol % PIP_2 to occur at a greater separation than for liposomes containing 10 mol % PA. This would allow faster aggregation and lower the spermine threshold for aggregation as we have observed.

Biological Relevance. Though biological membranes are not composed of pure PA or pure PS, these characteristics of spermine illustrate how polyamines may interact with intracellular membranes. Spermine may be involved in bringing intracellular vesicles together or in the binding of exocytic granules to the cytoplasmic side of the plasma membrane. Binding to PA created at the expense of phosphatidylinositol (PI) in the "phospholipid effect" (Hokin & Hokin, 1953; Hokin-Neaverson, 1974; Michell, 1975; Lapetina & Cuatrecasas, 1979) may be important. Binding to PIP_2 may also be important (Griffin & Hawthorne, 1978; Allan & Michell, 1979; Shukla & Hanahan, 1982; Billah & Lapetina, 1982). Though PIP_2 is thought to be catabolized to triphosphoinositol (IP_3) and diacylglycerol during stimulation of cells for secretion, it is possible that some residual PIP_2 remains and could be involved in membrane fusion. It has been shown that polyamino-containing molecules, such as neomycin, inhibit the breakdown of PIP_2 (Orsulakova et al., 1977). Spermine could also control PIP_2 turnover through binding to PIP_2 and consequently may regulate intracellular Ca^{2+} release (Berridge & Levine, 1984).

Polyamines could also exert effects through the attachment or expulsion of certain proteins at the surface of the membrane. The presence of spermine binding sites such as PA or PIP_2 could be important in such situations. Several studies have shown evidence for spermine-modulated protein binding to membranes (Sechi et al., 1978; Jamdar, 1979; Nandi et al., 1981; Meers et al., 1985).

It is clear that the role of polyamines in the interaction of membranes cannot be ignored. We have investigated some of the details of the interactions of spermine with acidic phospholipids. The results suggest that regulation of polyamine levels in cells could play an important role in the control of intracellular fusion and predicts a new aspect of control which should now be investigated at the cellular level.

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Identification of Three Sites of Proteolytic Cleavage in the Hinge Region between the Two Domains of the β_2 Subunit of Tryptophan Synthase of *Escherichia coli* or *Salmonella typhimurium*†

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ABSTRACT: The β_2 subunit of tryptophan synthase is composed of two independently folding domains connected by a hinge segment of the polypeptide that is particularly susceptible to limited proteolysis by trypsin [Högberg-Raibaud, A., & Goldberg, M. (1977) *Biochemistry 16*, 4014-4019]. Since tryptic cleavage in the hinge region inactivates the β_2 subunit, the spatial relationship between the two domains is important for enzyme activity. However, it was not previously known whether inactivation results from cleavage of the chain or from the loss of internal fragment(s) subsequent to cleavage at two or more sites. We now report comparative studies of limited proteolysis by three proteinases: trypsin and endoproteinases Lys-C and Arg-C. Our key finding that endoproteinase Arg-C inactivates the β_2 subunit by cleavage at a single site (Arg-275) demonstrates the important role of the hinge peptide for enzymatic activity. We have also identified the sites of cleavage and the time course of proteolysis by trypsin at Arg-275, Lys-283, and Lys-272 and by endoproteinase Lys-C at Lys-283 and Lys-272. Sodium dodecyl sulfate gel electrophoresis, Edman degradation, and carboxypeptidases B and Y have been used to identify the several fragments and peptides produced. Our finding that the β_2 subunit and F1 fragments have a heterogeneous amino terminus (Met-1 or Thr-2) indicates that the amino-terminal methionine is incompletely removed during posttranslational modification. Our results show that Edman degradation can be effectively used with a protein of known sequence to analyze proteolytic digests that have at least four different amino-terminal sequences. Our analysis of the hinge region may lead to further understanding of the importance of this region of the β_2 subunit for activity, allosteric control, and subunit interaction.

Some proteins consist of two independently folding domains interconnected by a hinge peptide that is particularly susceptible to proteolysis (Neurath, 1980; Wetlauffer, 1981; Richardson, 1981). Two such proteins are the tryptophan synthase β_2 subunit (Högberg-Raibaud & Goldberg, 1977a,b) and the tryptophan synthase α subunit (Miles & Higgins, 1978; Higgins et al., 1979; Miles et al., 1982). Although proteolytic cleavage of the α subunit has little effect on enzyme activity, cleavage of the β_2 subunit results in inactivation, loss of cooperative binding of two pyridoxal phosphate molecules to the apo-β_2 subunit, and loss of interaction with the α subunit (Högberg-Raibaud & Goldberg, 1977a,b; Tschopp & Kirschner, 1980; Chaffotte & Goldberg, 1984). These studies and others have provided evidence that the limited flexibility between domains provided by the hinge peptide is often crucial to substrate binding, allosteric control, and assembly of large structures (Richardson, 1981).

Previous studies of the limited proteolysis of the β_2 subunit of tryptophan synthase of *Escherichia coli* and of *Serratia marcescens* have demonstrated the formation of two proteolytic fragments, F1 and F2, with N-terminal sequences starting at Thr-2 and at Val-276 or Ala-284 (Crawford et al., 1978, 1980; Rocha et al., 1979). The observation that the F1 and F2

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