Free Fatty Acid Enhancement of Cation-Induced Fusion of Liposomes: Synergism with Synexin and Other Promoters of Vesicle Aggregation†

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ABSTRACT: The effect of free fatty acids on the cation-induced fusion of large unilamellar vesicles (liposomes) was investigated by using fluorescent assays which monitor the mixing of aqueous contents of liposomes. Overall fusion was modeled as a two-step process involving aggregation of vesicles followed by actual fusion. Different experimental conditions were used which favored either aggregation or fusion as the rate-limiting step in the overall process. When phosphatidylserine liposomes were induced to fuse by 4 mM Ca^{2+} plus 5 mM Mg^{2+}, preincubation with arachidonic acid showed a dramatically increased overall rate of fusion compared to the same liposomes not treated with fatty acid. When fusion was induced by 3 mM Ca^{2+}, arachidonic acid had little effect. These results were interpreted in terms of the action of arachidonic acid only at the fusion step per se and not the aggregation step. Therefore, the enhancement of the overall fusion rate would be observed solely under conditions where the actual fusion of liposomes was rate limiting (Ca/Mg) rather than the aggregation of liposomes (Ca alone). When other liposome systems were tested, the effect of arachidonic acid was observed only under fusion rate-limiting conditions. Arachidonic acid was found to act synergistically with promoters of liposomal aggregation, such as Mg^{2+}, spermine, and synexin, to enhance the overall rate of liposome fusion, as would be expected from action at separate kinetic steps. The dependence of the fusion rates on arachidonic acid concentration demonstrated an apparently cooperative effect. The structure of the fatty acid is of critical importance in determining its effects, as shown by the fact that 16-doxylstearic acid always increased the rate of fusion while 5-doxylstearic acid always decreased the rate of fusion under all conditions tested. A number of different fatty acids, including oleic acid, elaidic acid, 16-doxylstearic acid, myristic acid, and stearic acid, were effective at increasing the fusion rate to varying extents. In general, unsaturated fatty acids were more effective than saturated ones, either due to partitioning into the membrane or because of structural requirements for promotion of fusion.

Free fatty acids have a wide variety of effects on many types of cells. Upon stimulation of many secretory cell types, a transient rise in fatty acid concentration is observed (Irvine, 1982) as well as a transient rise in intracellular Ca^{2+} concentration. For instance, stimulation of human neutrophils to degranulate is accompanied by release of free arachidonic acid (Stenson & Parker, 1979; Waite et al., 1979; Walsh et al., 1981). Arachidonic acid is also produced during secretion by chromaffin cells (Frye & Holz, 1984). Therefore, fatty acids may play a role in the fusion of membranes occurring during exocytosis or degranulation.

Several studies have demonstrated the potential of free fatty acids to promote the overall rate of membrane fusion. The fusion of hen erythrocytes was shown to be promoted by free fatty acids (Ahkong et al., 1973), as was the slow rate of fusion of small, highly curved unilamellar vesicles of phosphatidylcholine near the phase transition temperature of the phospholipid acyl chains (Kantor & Prestegard, 1975, 1978). Free fatty acids are necessary for the fusion of chromaffin granules preaggregated by Ca^{2+} and synexin (Creutz, 1981) or calpactin (Drust & Creutz, 1988). They are also necessary for Ca^{2+}- and synexin-dependent fusion of specific granules from human neutrophils with liposomes (Meers et al., 1987).

In order to study the effects of free fatty acids on fusion of a system that is simple, yet retains some of the features of cellular exocytosis, we examined the relatively rapid cation-induced fusion of large unilamellar vesicles containing acidic phospholipids. The fusion kinetics of liposomes have been analyzed in the past with a procedure based on a mass action model (Nir et al., 1980; Bentz et al., 1983a) in which two fusion steps are postulated theoretically: liposomal aggregation and actual membrane fusion. We have attempted to determine experimentally the role of free fatty acids in these two steps of membrane fusion. By testing the effect of free fatty acids under conditions where one step or the other was the rate-limiting step, we have been able to determine which step is affected. Furthermore, synergism between free fatty acids and other molecules in promotion of the overall rate of membrane fusion has been explained in these kinetic terms. We also examined some of the structural requirements for potency of the fatty acids and the amount of fatty acid needed in liposomal membranes to exert effects on fusion.

Materials and Methods

Bovine brain phosphatidylserine (PS),[1] phosphatidate (PA) (derived from egg phosphatidylcholine), and phosphatidylethanolamine (PE) (transesterified from egg phosphatidylcholine) were purchased from Avanti Polar Lipids (Birming-

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[1] Abbreviations: AA, arachidonic acid; OA, oleic acid; EA, elaidic acid; MA, myristic acid; SA, stearic acid; 5-DSA, 5-doxylstearic acid; 16-DSA, 16-doxylstearic acid; PS, phosphatidylserine; PA, phosphatidate; PE, phosphatidylethanolamine; PI, phosphatidylinositol; ANTS, 8-aminonaphthalene-1,3,6-trisulfonate; DPX, *p*-xylylbis(pyridinium bromide); DMPC, dimyristoylphosphatidylcholine.

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ham, AL). Spermine tetrahydrochloride, *N*-[tris(hydroxymethyl)methyl]-2-aminoethanesulfonic acid (TES), dipicolinic acid (DPA) (99%), nitrilotriacetic acid (99%), myristic acid (MA, 14:0), and stearic acid (SA, 18:0) were obtained from Sigma (St. Louis, MO). $CaCl_2$ (>99%), $MgCl_2$ (>99%), and NaCl (>99%) were from Fisher (Pittsburgh, PA). Oleic acid (OA, $18:1^{\Delta 9cis}$), arachidonic acid (AA, $20:4^{\Delta 5,8,11,14}$), and elaidic acid (EA, $18:1^{\Delta 9trans}$) were from NuChek Prep and were used without further purification. 8-Aminonaphthalene-1,3,6-trisulfonate (ANTS), *p*-xylylbis(pyridinium bromide) (DPX), 2-(3-carboxypropyl)-4,4-dimethyl-2-tridecyl-3-oxazolidinyloxy free radical (5-doxylstearic acid or 5-DSA), and 2-(14-carboxytetradecyl)-2-ethyl-4,4-dimethyl-3-oxazolidinyloxy free radical (16-doxylstearic acid or 16-DSA) were from Molecular Probes (Eugene, OR). [^{3}H]arachidonic acid (>98.5%) and [^{14}C]sucrose (>98%) were from New England Nuclear (Boston, MA). Polycarbonate filters were from Nuclepore (Pleasanton, CA).

Vesicle Preparation and Fusion Assay. 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). Liposomes were extruded through 0.2-μm and then 0.1-μM polycarbonate filters during this procedure (Szoka et al., 1980). The aqueous solutions for all vesicles contained either (a) 25 mM 8-aminonaphthalene-1,3,6-trisulfonate (ANTS), 40 mM NaCl, and 10 mM TES at pH 7.4, (b) 90 mM *p*-xylylbis-(pyridinium bromide) (DPX) and 10 mM TES, pH 7.4, or (c) 12.5 mM ANTS, 45 mM DPX, 20 mM NaCl, and 10 mM TES at pH 7.4 (Ellens et al., 1985). All liposome preparations were diluted into 100 mM NaCl, 5 mM TES, and 0.1 mM EDTA, pH 7.4 (buffer A). The phospholipid concentrations were determined by using a phosphate assay of Bartlett (1959) as modified by Morrison (1964).

The ANTS/DPX assay for mixing of aqueous contents is based on the collisional quenching of 8-aminonaphthalene-1,3,6-trisulfonate (ANTS) by *p*-xylylbis(pyridinium bromide)(DPX) upon mixing of contents when liposomes fuse (Ellens et al., 1985). A 1:1 ratio of liposomes encapsulating ANTS and liposomes encapsulating DPX was used for fusion assays. In this assay, fluorescence decreases when the fluorophore ANTS mixes with the quencher DPX. Any mixing of the ANTS or DPX contents of the vesicles outside of the vesicles due to leakage during the fusion assays does not result in the quenching of ANTS because the large dilution of the DPX makes quenching negligible. The 100% fluorescence level (0% fusion) was merely the initial fluorescence before fusion. Liposomes with coencapsulated ANTS and DPX and were used to set 0% fluorescence (100%) fusion) as well as to measure leakage. A small amount (usually 10–20 μL) of a concentrated solution of the appropriate cations (containing buffer A adjusted to pH 7.4) was injected to initiate fusion. Initial rates of fusion were measured by determining the time taken for the fluorescence to decrease by 10% of this total fluorescence range. Leakage was measured by the increase in fluorescence due to relief of quenching upon dilution of the coencapsulated material (ANTS and DPX in the same liposomes) into the medium. When necessary, corrections of the initial rates for leakage were made by the method of Bentz et al. (1983a). These corrections were less than 10% of the uncorrected rate in most cases.

In all representations of the time course of this fusion assay in the text, the rate of fusion is shown as the absolute value of the change in fluorescence, so that % F_{max} is actually the *decrease* in fluorescence as a percentage of the maximum possible decrease. F_{max} is the maximal fluorescence change at 100%, but is actually zero fluorescence experimentally. Therefore, time course of fusion are increasing functions as in Figure 1.

For the experiments presented in Figure 3, the Tb/DPA assay was used. The Tb/DPA assay 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 (Wilschut et al., 1980). Vesicle preparation and the assay were carried out as previously described (Meers et al., 1988).

The fluorescence was measured on an SLM 4000 fluorometer. The ANTS/DPX reaction mixtures were excited at 360 nm, and the fluorescence was measured at >530 nm through a Corning 3-68 cutoff filter. All experiments were performed at 25 °C. Fluorescence and 90° light scattering were monitored simultaneously using the "T-format" of the fluorometer.

Analysis of Mass Action Model of the Fusion Reaction. We model the data on overall fusion as a mass action process following the initial reaction (see eq 2 under Results) where two monomer vesicles (V_1) aggregate to a dimer (V_2) and then fuse into a doublet (F_2). Higher order reactions will occur, but we monitor only the early events where the relative amount of higher order aggregation/fusion products is small. It has already been shown that integration of these kinetic yields predicted curves for the Tb/DPA or ANTS/DPX fluorescence which depend upon the primary rate constants (C_{11}, D_{11}, and f_{11}) and the lipid concentration (Nir et al., 1980, 1983; Bentz et al., 1983a,b, 1985; Düzgüneş & Bentz, 1988). Approximate rate constants were fit to the data by employing the approximation to the exact numerically integrated equations from Bentz et al. (1983a). This approximation takes into account aggregates of vesicles and fused multimers of vesicles up to an aggregate size of 4. With only one ratio of ANTS- to DPX-containing vesicles or Tb- to DPA-containing vesicles, it is not possible to rigorously fix all three primary rate constants in the time regime where higher order reactions are negligible (Bentz et al., 1983a; Düzgüneş & Bentz, 1988). However, we can obtain $\hat{C}_{11} = C_{11}/(1 + D_{11}/f_{11})$ and $\hat{f}_{11} = f_{11}(1 + D_{11}/f_{11})$. When aggregation is irreversible, then $\hat{C}_{11} = C_{11}$ and $\hat{f}_{11} = f_{11}$.

Fatty Acid Partitioning. Fatty acid was first dispersed from an ethanolic solution into buffer A. This stock was then added to the liposome solution before fusion experiments. A sufficient incubation period was allowed for the partitioning of the fatty acids into membranes. This was determined by measuring the amount of time necessary for the fatty acids to reach their maximum effects on the overall fusion rate. Less than 0.1% ethanol remained in the final solution. Such concentrations of ethanol do not affect the fusion rate. The final concentrations of fatty acids used in all experiments appeared to be below the critical micelle concentration based on the reported values in the literature and our measurements of turbidity at various fatty acid concentrations.

Partitioning of fatty acids was measured by using centrifugation to separate membrane-bound from aqueous fatty acid. Large unilamellar vesicles were formed as described above for fusion experiments, but without the encapsulated material. Ten microliters of a 1 mM solution of sucrose, containing 1 μCi of ^{14}C-labeled sucrose per millimole of sucrose, was added to 1 mL of the vesicles as an aqueous marker. A stock of 10 μCi of [^{3}H]arachidonic acid per 1 μmol of arachidonic acid was prepared in ethanolic solution to a final concentration of 9.3 mM in arachidonic acid. A 10-μL aliquot of this stock was dispersed by vortexing into 990 μL of buffer A immediately before use. This second stock was added to a final

concentration of 10 μM in 1 mL of a 1 mM liposomal phospholipid dispersion buffer A. It was then incubated for 15 min followed by centrifugation for 3 h at 150000*g*. This method pelleted about 70–80% of the liposomal phospholipid. We made the assumption that the partition coefficient of the fatty acid into the vesicles in the supernatant was the same as into those vesicles remaining in the pellet. The partition coefficient was calculated with a correction for phospholipid in the supernatant as well as for aqueous volume left in the pellet.

The partitioning of 5- and 16-doxylstearic acid was measured by electron spin resonance. The 5 or 16 label was added to a final concentration of 50 μM to 2-mL samples of 1 mM liposomal phospholipid in buffer A. This solution was centrifuged at 150000*g* for 3 h to separate liposomal from aqueous fatty acid. Pellets were resuspended into 2 mL of buffer A. The electron spin concentration of all samples was measured by dispersing 180-μL aliquots in a final volume of 200 μL of 2% sodium cholate. The phospholipid concentration of each aliquot was measured by the phosphate assay. Results were corrected for phospholipid concentration assuming that the partition coefficient for the fatty acids was the same for liposomes in the supernatant as those in the pellet. Electron spin resonance was performed on a Varian E-4 X-band spectrometer with 100-kHz field modulation, equipped with a variable-temperature accessory and interfaced to a DEC PDP-11/23 computer. Experiments were performed at 25 °C with 100-μL samples in flat quartz aqueous cells (Wilmad, Buena, NJ).

Partition coefficients were calculated from the data by using

$$K_p = \frac{[C_p + (C_p[PL]_s/[PL]_p)](V_s/V_m)}{C_s - (C_p[PL]_s/[PL]_p)} \quad (1)$$

where K_p is the partition coefficient defined as the ratio of the amount of fatty acid in the membrane phase to the amount of fatty acid in the aqueous phase at equal volumes of each phase, C_p is the counts or spins in the centrifuged pellet, C_s is the counts or spins in the supernatant, $[PL]_s$ is the amount of phospholipid remaining in the supernatant, $[PL]_p$ is the amount of phospholipid in the pellet, V_s is the volume of the aqueous phase, and V_m is the volume of the membrane phase, which is taken to be 1 mL/g of phospholipid as an approximation. The molecular weight of the phospholipid is taken as 800.

Synexin Preparation. Synexin was purified by a modification of the technique of Creutz et al. (1978) described in Meers et al. (1988). Briefly, bovine liver was homogenized and cleared of debris by centrifugation, and the remaining material was precipitated twice by 22% saturated $(NH_4)_2SO_4$ and resuspended twice. This material was further purified by affinity chromatography on Sepharose 4B covalently linked to small unilamellar vesicles of PS/PC/PE (2:2:1). The purity of this synexin was estimated to be approximately 90% from polyacrylamide gel electrophoresis of the protein.

RESULTS

Kinetic Mechanism of Fatty Acid Effect. In the following experiments, the initial rates of fusion of liposomes were measured, and the effects of fatty acids 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 up to the dimer stage is given by eq 2 where V_1 and V_2 denote

$$V_1 + V_1 \underset{D_{11}}{\overset{C_{11}}{\rightleftharpoons}} V_2 \xrightarrow{f_{11}} F_2 \quad (2)$$

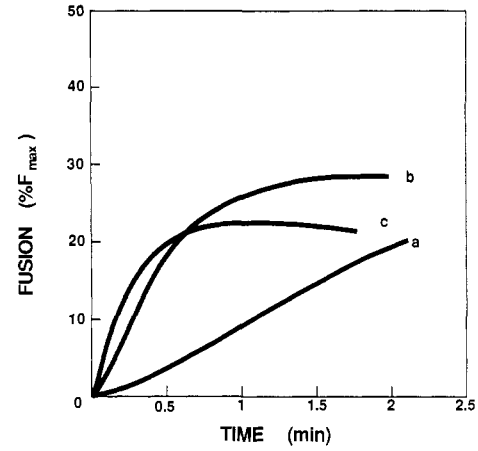

FIGURE 1: Time course of the fusion of phosphatidylserine liposomes. Fusion of 10 μM total phospholipid was induced by addition of 4 mM Ca^{2+} and 5 mM Mg^{2+} at time zero. Experiments were performed in 1 mL of buffer A at 25 °C. Liposomes were untreated (curve a) or incubated with 5 μM (curve b) or 11.5 μM (curve c) arachidonic acid. Fusion was measure by the ANTS/DPX fusion assay as described in the text.

the liposome and the aggregated dimer, respectively, and F_2 denotes the fused doublet. We refer to the rate of the first step in this process as the aggregation rate and the second step as the fusion rate, while the rate of the whole process is called the *overall* fusion rate. The overall rate of fusion is obviously controlled by whichever step is rate limiting.

Figure 1 shows typical time courses for the fusion of PS liposomes in the presence and absence of arachidonic acid. It can be seen that under the conditions of this experiment, arachidonic acid greatly increases the overall rate of fusion. In order to analyze the relative effects of arachidonic acid on both steps of the fusion process, we designed systems in which the change of one parameter could set either the aggregation or the fusion step as rate limiting. One way to achieve this goal was to vary the ionic composition of the fusion-inducing medium (Bentz et al., 1983b; Bentz & Düzgüneş, 1985; Meers et al., 1988). Therefore, the comparative effect of Ca^{2+} or Ca^{2+}/Mg^{2+} on the fusion of liposomes composed of phosphatidylserine (PS) was studied. Fusion induced by 3 mM Ca^{2+} was largely aggregation rate limited, so that the reaction was of the order 1.6 in lipid concentration (data not shown), while fusion induced by 4 mM Ca^{2+} plus 5 mM Mg^{2+} was of the order 1.3 (data not shown) and largely limited by the rate of the fusion per se. It is known that since Mg^{2+} only aggregates but does not fuse large unilamellar vesicles of PS (Wilschut et al., 1981, 1985), it only increases $\hat{C}_{11}$ and diminishes the $\hat{f}_{11}$ slightly due to some competition with Ca^{2+} for binding to PS (Bentz et al., 1983b; Bentz & Düzgüneş, 1985). Therefore, Mg^{2+} converts the mostly aggregation rate-limited system of Ca^{2+} and PS to a mostly fusion rate-limited system.

In Figure 2, a series of experiments are presented by plotting the initial rate of change of ANTS/DPX fluorescence as a function of arachidonic acid concentration. The open squares show the data when fusion of pure PS liposomes was initiated by addition of 3 mM Ca^{2+}, and the closed squares show the data when fusion was initiated by addition of a mixture of 4 mM Ca^{2+} plus 5 mM Mg^{2+}. There is a dramatic difference in the effect of this fatty acid in the two circumstances. Clearly, the arachidonic acid is much more effective in the fusion rate-limited situation than in the largely aggregation rate-limited case. Similar results were also obtained with the Tb/DPA assay (not shown). The comparison in Figure 2 also shows that arachidonic acid and Mg^{2+} appear to act syner-

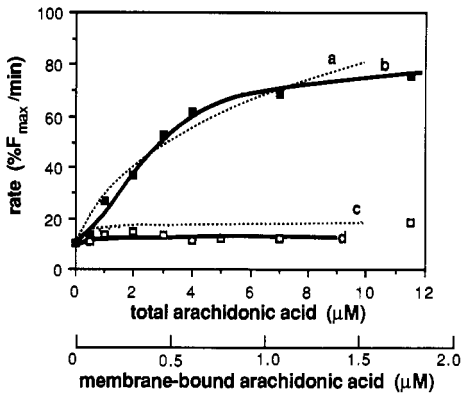

FIGURE 2: Effect of arachidonic acid on the rate of fusion of PS liposomes. PS liposomes (10 μM total phospholipid) were preincubated with varying amounts of arachidonic acid. At time zero, 3 mM Ca^{2+} (□) or 4 mM Ca^{2+} with 5 mM Mg^{2+} (■) was added to initiate fusion. All experiments were performed at 25 °C in 1 mL of buffer A. Solid curves represent theoretical rates calculated by the method of Bentz et al. (1983a). In curve a (dotted line), $\hat{C}_{11} = 2.4 \times 10^8$, $\hat{f}_{11_0} = 5.0 \times 10^{-3}$, and $\hat{f}_{11}$ increased by $6\hat{f}_{11_0}$ per micromolar arachidonic acid added. Constants are defined in the text. In curve c (dotted line), $\hat{C}_{11} = 2.0 \times 10^7$, $\hat{f}_{11_0} = 3.0 \times 10^{-1}$, and $\hat{f}_{11}$ increased by $6\hat{f}_{11}$ per micromolar arachidonic acid added. In curve b, $\hat{C}_{11} = 2.4 \times 10^8$, $\hat{f}_{11_0} = 5.0 \times 10^{-3}$, and the arachidonic acid effect was treated as cooperative as described in eq 3 in the text with $n = 2$, $\hat{f}_{11_{max}} = 0.32$, and $K = 5.0 \times 10^{-2}$. In curve d, $\hat{C}_{11} = 2.0 \times 10^7$, $\hat{f}_{11_0} = 3.0 \times 10^{-1}$, and the arachidonic acid effect was treated as cooperative as described in eq 3 in the text with $n = 2$, $\hat{f}_{11_{max}} = 30$, and $K = 5.0 \times 10^{-2}$. Fusion was measured by the ANTS/DPX fusion assay as described in the text.

gistically to increase the overall rate of fusion. In other words, in the absence of Mg^{2+}, there is little effect of the fatty acid, while in the presence of Mg^{2+}, the fatty acid becomes effective. Fusion of these vesicles induced by Mg^{2+} alone is negligible with or without arachidonic acid (data not shown).

In order to prove that the differences observed between Ca^{2+}- and Ca^{2+}/Mg^{2+}-induced fusion were not due to changes in the membrane-bound concentration of arachidonic acid, the approximate membrane–aqueous partition coefficients for the arachidonic acid were measured by centrifugation of liposomes with [^{3}H]arachidonic acid under various conditions. The partition coefficient was defined in terms similar to Pjura et al. (1984), and the calculations are detailed under Materials and Methods. K_p in buffer in the absence of divalent cations is approximately 21 000:1 (lipid/water). In the presence of 3 mM Ca^{2+} or 4 mM Ca^{2+} plus 5 mM Mg^{2+}, the partition coefficient changes only marginally to $(24 \pm 3) \times 10^3$ and $(23 \pm 6.5) \times 10^3$, respectively. In the presence of Mg^{2+} alone, K_p was higher at $(33 \pm 1.5) \times 10^3$. The difference between the partitioning of arachidonic acid in the presence of Ca^{2+} alone or Ca^{2+} with Mg^{2+} is too small and in the wrong direction to explain the differences observed in overall rates of fusion. Furthermore, the amount of arachidonic acid in the membranes used for fusion experiments was initially the same, since the fatty acid was added before the divalent cations. It can be concluded that the effect of Mg^{2+} in fusion experiments was simply to increase the aggregation rate constant of the liposomes (Wilschut et al., 1981, 1985; Bentz & Düzgüneş, 1985), thereby making the fusion step more rate limiting.

The determination of the partition coefficient also allowed us to scale approximately the effect of arachidonic acid in terms of the amount actually present in the membrane. Therefore, the lower scale on the horizontal axis of the graph in Figure 2 is in terms of the molarity of arachidonic acid in the membrane, assuming a partition coefficient of 21 000. Since the total phospholipid concentration is 10 μM, it is noteworthy that as little as 1–2 mol % of fatty acid in the membrane enhances fusion significantly.

The kinetic behavior of the fusion systems discussed above can be modeled by using the law of mass action to calculate the theoretical values for overall fusion rates under various circumstances (Nir et al., 1980; Bentz et al., 1983a) and by using previously published data on the fusion of PS liposomes (Bentz & Düzgüneş, 1985; Bentz et al., 1983b, 1985). These data were used for an approximation to the exact solution to the integrated kinetic equations (Bentz et al., 1983a). Theoretical rates calculated in this way were used to determine whether the data on the fusion of PS liposomes could be fit by using the hypothesis that arachidonic acid only increases $\hat{f}_{11}$ and not $\hat{C}_{11}$. Starting with the previously determined values for $\hat{C}_{11}$ and $\hat{f}_{11}$ in the absence of free fatty acid, the effects of increasing $\hat{f}_{11}$ or $\hat{C}_{11}$ were tested. As shown in Figure 2 the dotted lines fairly closely model the initial overall rates obtained experimentally if an approximately linear 6-fold increase in $\hat{f}_{11}$ per micromolar arachidonic acid is assumed (curves a and c). This is an increase of approximately 4.2-fold in $\hat{f}_{11}$ per mole percent arachidonic acid in the membrane. An even closer fit is possible by treating the arachidonic acid effect as cooperative (Hill, 1910) and assuming a maximum $\hat{f}_{11}$ (curves b and d). This was done by taking

$$\frac{\hat{f}_{11} - \hat{f}_{11_0}}{\hat{f}_{11_{max}} - \hat{f}_{11_0}} = \frac{K[AA]^n}{1 + K[AA]^n}$$

$$\hat{f}_{11} = \frac{\hat{f}_{11_{max}} K[AA]^n + \hat{f}_{11_0}}{1 + K[AA]^n} \quad (3)$$

where [AA] is the total arachidonic acid concentration, K is an arbitrary constant, $\hat{f}_{11_0}$ is the fusion rate constant in the absence of arachidonic acid, $\hat{f}_{11_{max}}$ is an arbitrary maximum fusion rate constant assuming that the arachidonic acid effect reaches saturation eventually, and n is a cooperativity coefficient. The same $\hat{f}_{11}$ values used for the noncooperative case were then used for generating rates and arachidonic acid concentrations for the cooperative case. The values for $\hat{f}_{11}$ and $\hat{C}_{11}$ in the absence of fatty acids were taken from previously published data (Bentz et al., 1983b; Bentz & Düzgüneş, 1985). Other cooperative models may also describe the data shown. However, it is clear that the general behavior of this system in the presence of arachidonic acid can only be described by a significant effect of fatty acid on $\hat{f}_{11}$. It was impossible to model the difference between Ca^{2+}- and Ca^{2+}/Mg^{2+}-induced fusion by assuming that arachidonic acid increases $\hat{C}_{11}$, as this would tend to increase curves c and d more than curves a and b in Figure 2.

In order to further test the hypothesis that arachidonic acid only increases $\hat{f}_{11}$, the effect of this fatty acid was tested in other liposomes systems. It is known that spermine increases the overall rate of fusion of certain liposomes by increasing the rate of aggregation (Meers et al., 1986). Spermine alone does not fuse PS/PE and PS/PI/PE liposomes. In Figure 3, the effect of arachidonic acid on the spermine-induced fusion of these liposomes is demonstrated. The overall fusion of PS/PE and PS/PI/PE liposomes initiated by 200 μM spermine alone is clearly fusion rate limited, since changes in light scattering indicated spermine-induced aggregation had occurred (not shown), while no fusion was observed on the time scale of the experiments (curves a and c). Addition of arachidonic acid to the liposomes allowed spermine-induced fusion to occur (curves b and d), indicating a direct effect of arachidonic acid on the fusion rate per se.

A similar effect was shown for spermine-induced fusion of PA/PE liposomes, where some fusion is observable in the

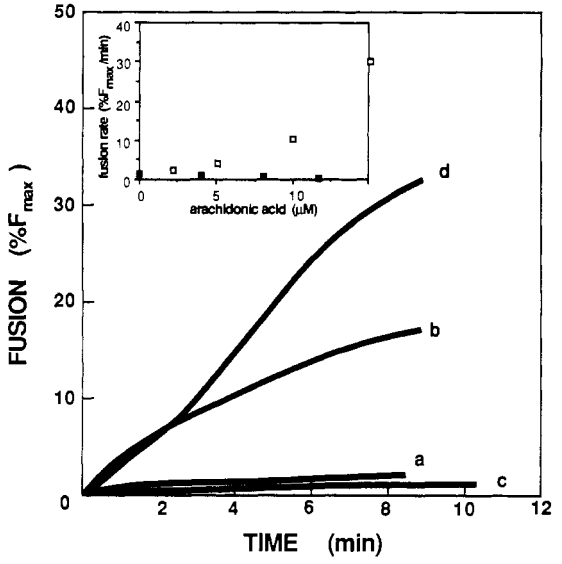

FIGURE 3: Time courses of spermine-induced fusion of liposomes. Fusion of 10 μM total phospholipid was induced by addition of 200 μM spermine at time zero. Experiments were performed in 1 mL of buffer A at 25 °C. Liposomes composed of phosphatidylserine/phosphatidylethanolamine 25:75 were untreated (curve a) or incubated with 11.5 μM arachidonic acid (curve b). Liposomes composed of phosphatidylserine/phosphatidylinositol/phosphatidylethanolamine 15:10:75 were untreated (curve c) or incubated with 11.5 μM arachidonic acid (curve d). Fusion was measured by the Tb/DPA fusion assay as described in the text. Inset: Effect of arachidonic acid on the rate of spermine-induced fusion of phosphatidate/phosphatidylethanolamine (1:3) liposomes. 10 μM total phospholipid was preincubated with varying amounts of arachidonic acid. At time zero, 200 μM spermine (□) or 3 mM Ca^{2+} (■) was added to initiate fusion. All experiments were performed at 25 °C in 1 mL of buffer A. Overall rates of fusion were determined by measuring the amount of time taken to reach 10% maximal fluorescence. Fusion was measured by the ANTS/DPX fusion assay as described in the text.

absence of fatty acids. This is also largely a fusion rate-limited system because increasing concentrations of the aggregation promoter, spermine, do not increase the overall rate of fusion significantly (not shown). In the Figure 3 inset, the dependence of the overall rate of spermine-induced fusion on arachidonic acid concentration is shown. Clearly, the rate increases with arachidonic acid concentration. In contrast, the overall rate of Ca^{2+}-induced fusion of PA/PE liposomes is strictly aggregation rate limited (Meers et al., 1988). In this case, there was almost no effect at all of arachidonic acid. Hence, this is another example of the effect of arachidonic acid on $\hat{f}_{11}$, but not on $\hat{C}_{11}$.

Fatty Acid Effect on Synexin-Mediated Fusion. Free fatty acids have been shown to be necessary for synexin-mediated fusion of chromaffin granules (Creutz, 1981) and synexin-mediated fusion of liposomes with specific granules from human neutrophils (Meers et al., 1987). Therefore, it was of interest to study the effect of arachidonic acid on synexin-mediated fusion of liposomes. Synexin appears to exert its effect on Ca^{2+}-induced liposome fusion by increasing the rate of aggregation of liposomes (Meers et al., 1988). Therefore, arachidonic acid is expected to act synergistically with the aggregation promoter synexin as it did in the case of PS in the presence of the aggregation promoter Mg^{2+}, for example. In Figure 4A, the effect of arachidonic acid on the Ca^{2+}-induced fusion of PA/PE liposomes in the presence and absence of synexin is shown. It is clear that while arachidonic acid had no effect on the overall rate of fusion of these liposomes as induced by Ca^{2+} alone, it significantly enhanced the rate of fusion in the presence of synexin. This apparent synergy

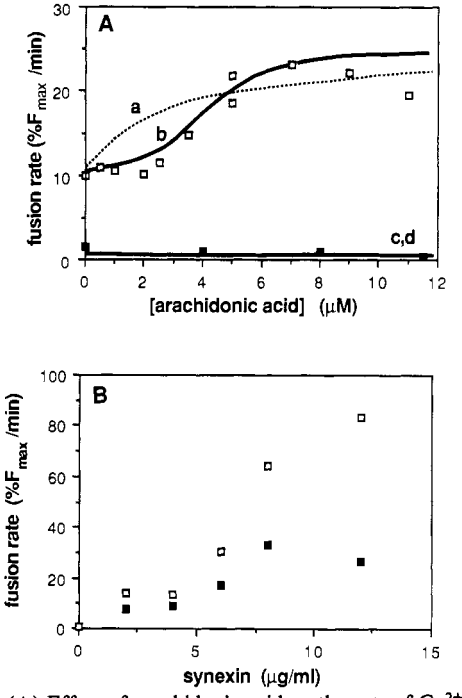

FIGURE 4: (A) Effect of arachidonic acid on the rate of Ca^{2+}-induced fusion of PA/PE liposomes. PA/PE liposomes (10 μM total phospholipid) were preincubated with varying amounts of arachidonic acid and 2 μg/mL synexin (□) or no synexin (■). At time zero, 2 mM Ca^{2+} was added to initiate fusion. All experiments were performed at 25 °C in 1 mL of buffer A. Overall rates of fusion were determined by measuring the amount of time taken to reach 10% maximal fluorescence. Solid curves represent theoretical rates calculated by the method of Bentz et al. (1983a). Rates were determined by calculating the time necessary to reach 10% of maximal fluorescence. In curve a (dotted line), $\hat{C}_{11} = 4.0 \times 10^7$, $\hat{f}_{11_0} = 3.0 \times 10^{-2}$, and $\hat{f}_{11}$ increased by $\hat{f}_{11_0}$ per micromolar arachidonic acid added. Constants are defined in the text. In curve c (dotted line under curve d), $\hat{C}_{11} = 1.0 \times 10^6$, $\hat{f}_{11_0} = 2.0 \times 10^{-1}$, and $\hat{f}_{11}$ increased by $\hat{f}_{11}$ per micromolar arachidonic acid added. In curve b, $\hat{C}_{11} = 4.0 \times 10^7$, $\hat{f}_{11_0} = 3.0 \times 10^{-2}$, and the arachidonic acid effect was treated as cooperative as described in eq 3 in the text with $n = 4$, $\hat{f}_{11_{max}} = 3.2$, and $K = 1.0 \times 10^{-4}$. In curve d, $\hat{C}_{11} = 4.0 \times 10^7$, $\hat{f}_{11_0} = 3.0\ 10^{-2}$, and the arachidonic acid effect was treated as cooperative as described in eq 3 in the text with $n = 4$, $\hat{f}_{11_{max}} = 22$, and $K = 1.0 \times 10^{-4}$. (B) Synexin dependence of overall rate of fusion of PA/PE liposomes in the presence and absence of arachidonic acid. 10 μM total phospholipid was preincubated with varying amounts of synexin and 11.5 μM arachidonic acid (□) or no arachidonic acid (■). At time zero, 2 mM Ca^{2+} was added to initiate fusion. All experiments were performed at 25 °C in 1 mL of buffer A. Overall rates of fusion were determined by measuring the amount of time taken to reach 10% maximal fluorescence. Fusion was measured by the ANTS/DPX fusion assay as described in the text.

between synexin and arachidonic acid can be modeled by effects at separate kinetic steps, just as in the previous case of PS liposomes. In Figure 4A, the effect of synexin is modeled using the previously determined 40-fold increase in $\hat{C}_{11}$ (Meers et al., 1988) for a 1:200 ratio of synexin to phospholipid. The $\hat{C}_{11}$ value used to calculate overall fusion rates in the absence of synexin was 1×10^6 compared with the previously determined value of 0.55×10^6. The $\hat{f}_{11}$ values were the same as those previously determined (Meers et al., 1988). The effect of arachidonic acid in this case was modeled either as a linear increase of $\hat{f}_{11}$ per micromolar arachidonic acid (curve a) or as a cooperative effect (curve b). The linear treatment describes the rate increase; however, a sigmoidal shape of the data curve indicates an apparent cooperativity of the arachidonic acid effect. The data are fit better by eq 3 with a cooperativity coefficient of 4 and a maximum $\hat{f}_{11}$ of 20. For Ca^{2+} alone, either the linear treatment (curve c) or the co-

Table I: Effect of Arachidonic Acid on the Leakage of PS Liposomes during Fusion[a]

	AA concn (μM)	fusion (% F_{max}/min)	leakage (% F_{max}/min)	fusion: leakage ratio
PS, Ca^{2+}/Mg^{2+}[b]	0	8.5	5.46	1.6
	5	61.6	23.2	2.6
	7	68.9	14.3	4.8
	11.5	76	2.26	33.6
PA/PE, synexin[c]	0	0.4	0.63	0.63
	5	21.8	0.64	34.1
	11.5	14	0.47	29.8
PA/PE, spermine[d]	0	1.6	1.88	0.85
	2	2.3	1.8	1.3
	5	4.0	3.0	1.3
	10	10.8	7.0	1.5

[a] Fusion rate was measured as described in the text. Leakage rate was defined by using the amount of time required to reach 10% of maximal fluorescence due to leakage and dilution of the contents of liposomes containing coencapsulated ANTS and DPX. The ratio of leakage to fusion is simply the ratio of the values in column 4 to those in column 3. The appropriate amount of AA was preincubated with the samples several minutes before initiation of fusion and leakage. [b] Assays were performed with 10 μM PS at 25 °C. Fusion or leakage was initiated by addition of 4 mM Ca^{2+} and 5 mM Mg^{2+}. [c] Assays were performed with 10 μM PA/PE at 25 °C. Fusion or leakage was initiated by addition of 2 mM Ca^{2+}. Because leakage was very slow in this case, the rate of leakage was obtained from lower amounts of leakage extrapolated to 10% maximal. [d] Assays were performed with 10 μM PA/PE at 25 °C. Fusion or leakage was initiated by addition of 200 μM spermine. The rate of leakage was obtained from lower amounts of leakage extrapolated to 10% maximal.

operative treatment with a maximal $\hat{f}_{11}$ also about 100-fold greater than the original and a cooperativity coefficient of 4 (curve d) fit adequately. As in Figure 2, other models of cooperativity may also fit the data. We discuss other possible interpretations of the data below.

In Figure 4A, the amount of synexin used probably did not increase $\hat{C}_{11}$ enough to make fusion alone rate limiting. The theoretical reaction order would be approximately 1.6, using the theoretically derived $\hat{C}_{11}$ and $\hat{f}_{11}$, where 1.0 is completely fusion rate limited. Therefore, increasing the amount of synexin so as to increase $\hat{C}_{11}$ would make the reaction more dependent on $\hat{f}_{11}$. Therefore, if synexin and fatty acids exclusively promote aggregation and fusion, respectively, then the effect of a given amount of arachidonic acid on the overall rate of fusion is expected to increase with synexin concentration up to the point where fusion becomes completely rate limiting or there is a saturation of synexin binding. Figure 4B shows the effect of synexin concentration on Ca^{2+}-induced fusion of PA/PE liposomes. At low synexin concentrations, the fatty acid effect is almost nonexistent, while the effect of the fatty acid (comparing open squares with closed squares) increases significantly with synexin concentration, as expected. At 2 μg/mL synexin, arachidonic acid only increases the overall rate of fusion approximately 1.7-fold, while at 12 μg/mL synexin the same amount of arachidonic acid increases the rate over 3-fold.

Leakage of Liposomal Contents. The leakage of liposomes is also affected by arachidonic acid. At the times necessary to reach 10% maximal fluorescence change in our fusion assays (less than 0.2 min), the leakage of coencapsulated ANTS and DPX from fusing liposomes was negligible, except in the case of spermine-mediated fusion. However, at later times, fluorescence due to leakage increased more and more rapidly. As a qualitative index of leakage, we measured the amount of time taken for fluorescence due to leakage of coencapsulated ANTS and DPX to reach 10% of maximal. This time was used to calculate a rate of leakage and compared to the rate

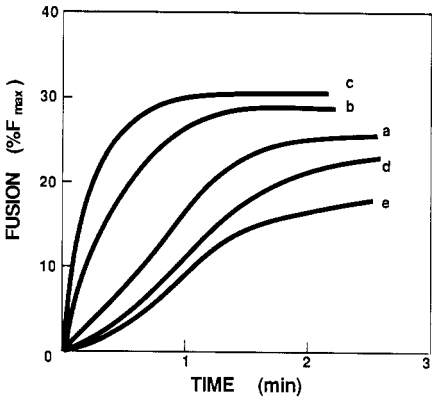

FIGURE 5: Effect of 5- and 16-doxylstearic acid on the time course of Ca^{2+}-induced fusion of PS liposomes. 10 μM total phospholipid was preincubated with buffer only (a), 2 μM 16-doxylstearic acid (b), 5 μM 16-doxylstearic acid (c), 2 μM 5-doxylstearic acid (d), or 5 μM 5-doxylstearic acid (e). At time zero, 4 mM Ca^{2+} with 5 mM Mg^{2+} was added to initiate fusion. All experiments were performed at 25 °C in 1 mL of buffer A. Fusion was measured by the ANTS/DPX fusion assay as described in the text.

of fusion, as defined above, under the same conditions. Data for Ca^{2+}/Mg^{2+}-mediated fusion of PS liposomes, Ca^{2+}/synexin-mediated fusion of PA/PE liposomes, and spermine-mediated fusion of PA/PE liposomes are shown in Table I. Under the conditions shown, increasing fatty acid concentrations increased the rate of fusion. In contrast, the rate of leakage did not increase as rapidly as the rate of fusion, as shown by the ratio of fusion to leakage rates in the last column. Leakage of contents is lower per fusion event at least to some extent under all of the conditions studied when free fatty acids are present. Thus, fusion is promoted by fatty acids while leakage per fusion event is lower in the presence of free fatty acids.

Effects of Other Fatty Acids. Fatty acids other than arachidonic acid were also effective at increasing the rate of fusion and decreasing the rate of leakage of liposomes. The difference between the effects of 5- and 16-doxylstearic acids was particularly noteworthy and quite striking in all cases. Figure 5 shows the effect of these fatty acids on Ca^{2+}/Mg^{2+}-mediated fusion of PS liposomes. 16-DSA had a large positive effect on the rate of fusion while 5-DSA had a negative effect. We estimated partition coefficients of appproximately 1.2×10^4 and 5×10^4 for the 16 and 5 label, respectively, using ESR to assay the amount of spin-label in the supernatant and pellets of centrifuged liposome preparations. Similar membrane-bound concentrations of these two fatty acids are compared in Figure 5 at 2 μM 5-DSA and 5 μM 16-DSA. Even when 5-DSA was present in the membrane at a larger concentration than 16-DSA, it did not promote but actually suppressed fusion. Therefore, the position of the doxyl label on the fatty acid chain is crucial to its effect on membrane fusion. This comparative difference is not a result of differing partition coefficients but differences in the actual structures of the fatty acids.

The effects of 5- and 16-DSA on the synexin-mediated and spermine-mediated fusion of PA/PE liposomes are shown in Figures 6 and 7. A pattern similar to that of PS liposomes is observed. The effect of 16-DSA in each case is a substantial increase in the rate of fusion, while 5-DSA inhibits fusion. The estimated partition coefficients into PA/PE membranes for the 16 and 5 label are 0.5×10^4 and 8×10^4, respectively. Therefore, much more of the 5 label partitions into the membrane under equimolar conditions. So, equal amounts of these fatty acids are in the membrane at 1.2 μM 5-DSA compared to 12 μM 16-DSA (Figure 7). Again, opposite effects of these

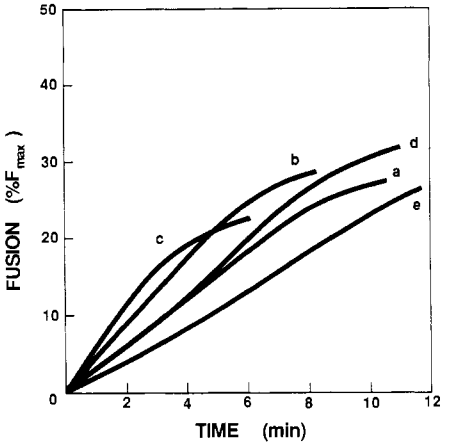

FIGURE 6: Effect of 5- and 16-doxylstearic acid on the time course of Ca^{2+}-induced fusion of PA/PE liposomes as mediated by synexin. 10 μM total phospholipid was preincubated with 2 μg/mL synexin and buffer only (a), 5 μM 16-doxylstearic acid (b), 11.5 μM 16-doxylstearic acid (c), 5 μM 5-doxylstearic acid (d), or 11.5 μM 5-doxylstearic acid (e). At time zero, 2 mM Ca^{2+} was added to initiate fusion. All experiments were performed at 25 °C in 1 mL of buffer A. Fusion was measured by the ANTS/DPX fusion assay as described in the text.

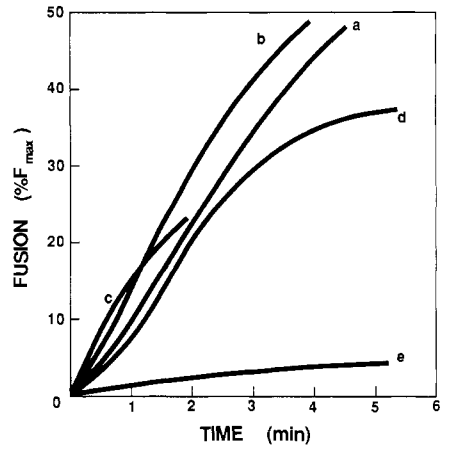

FIGURE 7: Effect of 5- and 16-doxylstearic acid on the time course of spermine-induced fusion of PA/PE liposomes. 10 μM total phospholipid was preincubated with buffer only (a), 5 μM 16-doxylstearic acid (b), 12 μM 16-doxylstearic acid (c), 1.5 μM 5-doxylstearic acid (d), or 5 μM 5-doxylstearic acid (e). At time zero, 200 μM spermine was added to initiate fusion. All experiments were performed at 25 °C in 1 mL of buffer A. Fusion was measured by the ANTS/DPX fusion assay as described in the text.

spin-labels are observed, suggesting an important structure–function relationship.

A number of other fatty acids were tested for activity in promoting fusion. Fusion of 10 μM PS liposomes induced by 4 mM Ca^{2+} and 5 mM Mg^{2+} was promoted by several fatty acids with an order of apparent effectiveness OA $\geq$ 16-DSA $\geq$ EA $\geq$ AA > MA > SA (see abbreviations). In these experiments, 5 μM fatty acid was added to each sample for comparison. A true assessment of the potency of each fatty acid would require precise measurement of the membrane–aqueous partition coefficient for each fatty acid under the experimental conditions used for fusion assays. No matter what the real order of effectiveness in increasing the fusion rate, it is clear that the fatty acid effect seems to be somewhat nonspecific, although unsaturated fatty acids were more effective than saturated fatty acids. Similar results were obtained for fusion of 10 μM PA/PE (1:3) liposomes induced by 2 mM Ca^{2+} in the presence of 2 μg/mL synexin (EA > OA > 16-DSA > MA > AA > SA). For fusion of the same liposomes by 200 μM spermine, the apparent order of effectiveness was OA > AA > EA $\geq$ 16-DSA > MA > SA. In all cases, the order of effectiveness of four of the fatty acids was OA > 16-DSA > MA > SA. EA and AA varied somewhat in relative effectiveness, depending on the system.

The various fatty acids also differed as to the rate of partitioning, as measured by the amount of time required for the fatty acids to reach their maximum effects on the rate of Ca^{2+}/Mg^{2+}-induced fusion of PS liposomes. In general, those fatty acids that increased the fusion rate most at a given concentration also partitioned most rapidly into the liposomal membranes. For instance, it takes only about 1–3 min for arachidonic acid to reach its maximum effect, while it takes over 15 min for stearic acid to reach its maximum effect. In order to eliminate this variable in all experiments, fatty acid was added to vesicles long enough before initiating fusion to allow complete partitioning.

Discussion

Fatty Acid Effects on the Fusion Rate Constant. The results presented here show for the first time that free fatty acids can be powerful promoters of rapid membrane fusion induced by cations in a system consisting of large unilamellar vesicles of acidic phospholipids. The assay for mixing of aqueous contents used in our experiments precludes the possibility of fatty acid induced vesicle size enlargement as a result of release or exchange of phospholipid monomers (Massari et al., 1980a,b). By separating the kinetic steps of membrane fusion, our experiments show that the effect of fatty acids is directly on the rate of the actual fusion of membranes and not the aggregation of vesicles.

The systems tested here are much different from those previously shown to be sensitive to fatty acid mediated fusion; however, there are some interesting similarities in results. Using small, highly curved vesicles composed of dimyristoylphosphatidylcholine (DMPC), Kantor and Prestegard (1975, 1978) showed that free fatty acids potentiate a slow fusion which is fastest at the primary phase transition temperature of the pure DMPC. This fusion of DMPC vesicles was first order in phospholipid concentration, suggesting that the rate of fusion may be limited by the actual fusion process rather than vesicle aggregation. Thus, the fusion-promoting properties of the free fatty acid become manifest under fusion rate-limited conditions, consistent with our observations that fatty acids increase the magnitude of $\hat{f}_{11}$ for rapid cation-induced fusion.

We have further shown that synergistic effects between two agents on the overall rate of liposome fusion may simply be a result of action at separate kinetic steps. Because of the action of fatty acids as promoters of fusion, they can act synergistically with promoters of aggregation. In the case of PS liposomes, Mg^{2+} is a strong promoter of aggregation but does not cause fusion. In combination with Ca^{2+}, it accelerates liposomal aggregation enough such that fusion begins to become rate limiting. Under these conditions, the effects of fatty acids can be observed. Therefore, Mg^{2+} and fatty acids can be considered as acting synergistically. The same is true for spermine, which promotes aggregation of liposomes (Meers et al., 1986), while fatty acids promote fusion. Hence, it is important to consider that action at separate kinetic steps of a process can give synergistic rate effects without direct interaction between the two promoters of the rate. These results demonstrate the importance of designing fusion systems that isolate the effect of modulators at a particular kinetic step.

Synexin-Mediated Fusion. Our earlier kinetic analysis of the effect of synexin on liposome fusion had shown that synexin

is a protein that enhances the overall rate of fusion by promoting liposomal aggregation in the presence of Ca^{2+} (Hong et al., 1983; Meers et al., 1988). The results presented here indicating synergy between synexin and fatty acids can be quantitatively explained, with several assumptions, by separate action of synexin and fatty acids on $\hat{C}_{11}$ and $\hat{f}_{11}$. This explanation is consistent with our other data on fatty acid effects. Another possible interpretation of this synergism is that fatty acids bind directly to synexin and activate it so that it can further accelerate the aggregation rate of liposomes. Such a fatty acid dependent increase in $\hat{C}_{11}$ due to more active synexin could match equally well the data in Figure 4A. Sterner et al. (1985) have shown that certain free fatty acids promote the Ca^{2+}-dependent polymerization of synexin and have suggested that this effect may be responsible for the observed effects of fatty acids on synexin-mediated membrane fusion (Creutz, 1981). While this is possible, it seems more likely that the data presented here are due to the distinct and separate kinetic effects of synexin and free fatty acids, for the following reasons.

The data in Figure 4B showing the progressively greater enhancement of overall fusion rate by the same amount of arachidonic acid at progressively higher synexin concentrations suggest that these factors act at separate kinetic steps. The fatty acid is in great excess to synexin, yet using more synexin increases the rate-enhancing effect of arachidonic acid (compare closed squares to open squares in Figure 4B). Therefore, it is likely that increasing the aggregator (synexin) concentration increases the contribution of fatty acids to $\hat{f}_{11}$ by increasing $\hat{C}_{11}$, just as in the case of Mg^{2+} and PS.

Furthermore, the effects of arachidonic acid occur in the same concentration range (1–20 μM) for synexin-mediated fusion of PA/PE liposomes, Ca^{2+}/Mg^{2+}-mediated fusion of PS liposomes, and spermine-mediated fusion of PA/PE liposomes, suggesting a common mechanism distinct from synexin. The relative effectiveness of various fatty acids is also similar in all three systems (16-DSA, positive effect; 5-DSA, negative; unsaturated > saturated). The specificity for fatty acids is rather low compared to most protein binding sites.

It has also been shown that prepolymerization of synexin decreases its activity (Hong et al., 1983; Meers et al, 1988). Unless the fatty acids exert direct action on synexin after its binding to the membrane, they would be expected to decrease synexin activity by promoting its polymerization.

Therefore, it is reasonable to suggest that synexin and fatty acids act as spatially separate modulators of fusion kinetics of liposomes in the experiments presented here. Measurement of binding of fatty acids to synexin in the presence of liposomal phospholipid may help to settle this question, as suggested by Sterner et al. (1985). 16-Doxylstearic acid may be a useful probe for determining the binding to synexin using spin-label quenching of intrinsic protein fluorescence. However, it would still remain to be shown that any synexin-bound fatty acid, rather than fatty acid bound to the membrane, is actually responsible for increased fusion rates.

Fatty Acid Partitioning. The partition coefficients of various fatty acids are quite relevant in this investigation. Since our experiments were performed at much lower phospholipid concentrations than those of Kantor and Prestegard (1975, 1978), not all of the added fatty acid partitioned into the liposomal membrane. Therefore, some estimate of the partition coefficient of the fatty acid was necessary to ascertain its true potency. For arachidonic acid, we obtained a partition coefficient of approximately 2×10^4. This parameter is defined in a manner similar to Pjura et al. (1984) and falls in the range of partition coefficients (10^3–10^5) they had determined for a number of fatty acids. Therefore, as little as 1–2 mol % of arachidonic acid promotes fusion in the PS system. This relative amount of fatty acid is similar to that found by Kantor and Prestegard (1975, 1978) to potentiate fusion (1–10 mol % of the phospholipid). Our partition coefficients determined by ESR for 5- and 16-doxylstearic acid also fall into the range determined for similar doxyl-fatty acids (Gaffney et al., 1983). On the basis of these partition coefficients, 16-doxylstearic acid may be a very potent fusion promoter. Even at only 2 mol % of the phospholipid in the membrane (Figure 5), it gave a very high overall fusion rate for the Ca^{2+}/Mg^{2+}-induced fusion of PS liposomes. It appears as though very little fatty acid may be necessary for the observed effects on the fusion rate.

It is not clear at present how the partitioning of fatty acids into the bilayer is connected with the fusion-promoting effects. An increase in bilayer area could lead to stress, making liposomes more susceptible to fusion. Indeed, such an increase in area is consistent with preliminary studies where fatty acids were added to pyrene-labeled dimyristoylphosphatidylcholine liposomes (Usher et al., 1978) or fluorescently labeled PS liposomes (our unpublished results). In the experiments presented here, the fatty acid is added to the outside of preformed liposomes. If all the fatty acid remains bound to the outer monolayer of the liposomal bilayer, it could put a particularly large stress on the membrane. However, it is likely that there is very rapid movement of the fatty acid to the inner monolayer of liposomes, because it is likely that much of the fatty acid residing in the membrane is uncharged at the experimental pH of 7.4. Magnetic resonance studies have shown that the effective pK for fatty acids bound to phosphatidylcholine liposomes is in the range of 7–8 (Barratt & Laggner, 1974; Sanson et al., 1976; Kantor & Prestegard, 1978; Ptak et al., 1980). In the negatively charged liposomes used in our studies, the effective pK could be even higher (Ptak et al., 1980), possibly well above the pH at which our experiments were performed.

Leakage. The leakage of liposomes during fusion was dramatically altered by arachidonic acid. It is quite interesting that even though the fusion rate is increased by arachidonic acid, the ratio of fusion to leakage rates also increases. Therefore, the efficiency, in terms of contents mixed, as well as the rate of fusion may actually be increased by arachidonic acid. This result is not consistent with the suggestion of Kantor and Prestegard (1978) that free fatty acids may promote fusion by stabilizing breaks in the liposomal membrane. In contrast, it seems more likely that fatty acids may anneal stressed portions of the membrane that lead to leakage, as well as promote the formation of fusion-producing bilayer defects. However, further studies will be necessary to determine whether the fusion and leakage effects of fatty acids are part of the same mechanism or separate phenomena.

Fatty Acid Structure. Using 5- and 16-doxylstearic acids, we have also found that the structure of the fatty acid is of some importance. The existence of structural specificity is consistent with the observations of Kantor and Prestegard (1975) showing that lauric and myristic acids were more effective than palmitic acid in promoting fusion of small unilamellar vesicles of DMPC. The fact that the position of the doxyl label on the fatty acid makes a very large difference in the effect on fusion suggests that perturbation of the bilayer at specific depths may be important.

A localized kind of expansion effect may be operant, where 16-doxylstearic acid would be expected to increase the area

in the hydrocarbon interior of the membrane, decreasing the headgroup to acyl chain area ratio for the membrane, since the large nitroxide-bearing group is located near the terminal methyl end of this fatty acid analogue. On the other hand, the 5-doxylstearic acid might do the opposite. This putative effect would be analogous to the "molecular geometry" factor (Israelachvili et al., 1977; Cullis & DeKruijff, 1979; Siegel, 1986, 1987) suggested as a possible explanation for the dependence of the formation of the H_{II} phase and other aqueous/lipid phases on lipid structure. Muranushi et al. (1981) suggested this geometrical factor as an explanation for their observations of the effects of free fatty acids on the permeability of liposomes. Marsh and Seddon (1982) found that dipalmitoylphosphatidylcholine at pH 4 goes from the L_β phase directly to the H_{II} phase with increasing temperature in the presence of 66 mol % palmitic acid. There is also evidence that PS (Hauser et al., 1982) and PS-containing bilayers (Tilcock et al., 1984) can form H_{II} phase structures under appropriate conditions, raising the possibility of a tendency toward such phenomena in our PS liposome systems in the presence of fatty acids. Free fatty acids alone in aqueous dispersions also form the H_{II} phase as well as lamellar phases under appropriate conditions (Gebicki & Hicks, 1976; Hargreaves & Deamer, 1978; Cistola et al., 1986). It will be of interest to determine whether free fatty acids can promote H_{II} phase formation in the systems investigated here.

Another possible reason for structural specificity may be that some fatty acids are more fluidizing to the bilayer than others (Klausner et al., 1980). Increased fluidity of the membrane has been correlated with increased fusogenicity (Wilschut et al., 1985). Fluidity is a term which must be defined carefully and differs depending on the technique of measurements. Usher et al. (1978) found no effect of free fatty acids on the apparent microviscosity of dimyristoylphosphatidylcholine bilayers using pyrene excimer to monomer ratios as a probe. On the other hand, there is evidence from ESR and NMR that cis-unsaturated fatty acids fluidize bilayers while saturated fatty acids have the opposite effect (Muranushi et al., 1981; Pauls et al., 1983). It has also been suggested (Seelig & Niederberger, 1974; Stockton et al., 1976) that the doxyl-labeled fatty acids may disorder membranes more than saturated-chain fatty acids substituted with ^2H.

Propensity of fatty acids to form separate phases may also explain differential fusogenicity. Pure fatty acid domains may lead to membrane defects that may be particularly fusogenic. Usher et al. (1978) found evidence for fatty acid induced formation of pyrene-rich domains below the phase transition temperature of dimyristoylphosphatidylcholine. Lateral separations of methyl esters of fatty acids, including doxyl-fatty acids, into fluid phases in the presence of solid Ca-$(PS)_2$ have also been observed in apposed bilayers of PS in the presence of Ca^{2+} (Florine & Feigenson, 1987) even at very low membrane-bound fatty acid concentrations. Other data suggest that free fatty acids and their methyl esters show differing preferences for fluid and solid phases depending on their structure and the composition of the membranes involved (Bashford et al., 1976; Sklar et al., 1979; Klausner et al., 1980). The doxyl-labeled fatty acids used here will be particularly useful for determining the possible existence of fatty acid phase separation under fusion-promoting conditions.

Cooperativity of the Fatty Acid Effect. Another parameter that may relate to the mechanism of the fatty acid effect is its aggregation state in or out of the membrane. In this respect, our observation of an apparent cooperativity of the fatty acid effect is quite interesting. An unambiguous interpretation of the meaning of the cooperativity coefficient, n, is not possible on the basis of our data, but it does provide a means for comparison between experiments. Cooperativity would not be surprising in light of the conclusions of several investigators that dimers (Mukerjee, 1967) and larger aggregates (Smith & Tanford, 1973) of long-chain fatty acids exist in aqueous solution below the critical micelle concentration. This is based on the nonlogarithmic increase in partition coefficient per methylene unit above a certain length (Goodman, 1958). There is also evidence suggesting that some membrane-bound fatty acids may exist as acid–soap dimers or higher aggregates (Cistola et al., 1986), at the physiological pH used in our studies. Therefore, it is possible that arachidonic acid exists in equilibrium as monomers as well as small aggregates with only the aggregates showing activity, consistent with the apparent cooperativity observed in our experiments. Cooperativity would also be consistent with formation of larger separate phases of fatty acid in the membrane. As discussed above, there is abundant evidence for formation of fatty acid rich domains. Broadened phospholipid phase transitions in some systems suggest that fatty acid rich domains probably remain small (Usher et al., 1978; Fodor & Epand, 1981; Schullery et al., 1981).

Biological Significance. Free fatty acids could also play an important role in the fusion of biological membranes. Arachidonic acid is released upon stimulation of degranulation in neutrophils (Stenson & Parker, 1979; Waite et al., 1979; Walsh et al., 1981) and also potentiates the fusion of liposomes with specific granules isolated from neutrophils (Meers et al., 1987). Similarly, arachidonic acid is released during secretion by chromaffin cells (Frye & Holz, 1984) and also potentiates fusion of chromaffin granules (Creutz, 1981; Drust & Creutz, 1988). The synergy between fatty acids and aggregation promoters may be important biologically in that it suggests a mechanism for biological fusion that may involve Ca^{2+}-dependent aggregation-promoting proteins, such as synexin, and fusion promoters that are lipid metabolites, such as free fatty acids. In such a model, we do not require a protein to perform the whole fusion function, as may be the case for viral proteins. The relatively fusogenic lipid composition of the cytoplasmic faces of many biological membranes may allow fusion of membranes to be facilitated following small perturbations of the lipid structure of the membrane by agents such as free fatty acids. Such free fatty acids may be generated by phospholipids in the vicinity of the fusion site. The local concentration of free fatty acid near such enzymes could be transiently quite high depending on the concentration of phospholipases, their turnover rate, and the rate of diffusion of the products. It will be of great interest to determine whether free fatty acids in the presence of phospholipase products, such as equimolar amounts of lysophospholipids or monoacylglycerols, will also be effective at increasing the fusion rate of liposomes.

Finally, the importance of using a fusion rate-limited system for studying the effects of various agents must be emphasized. Because many of the model systems used thus far to study membrane fusion have been aggregation rate limited, contributions to the fusion rate have not been observed. Therefore, fusogens may have been overlooked. It is not currently known whether most intracellular membrane fusion events are limited by aggregation or fusion. Therefore, it is of utmost importance to study lipids or proteins under conditions where it becomes evident whether they affect the fusion rate or the aggregation rate. Determining the kinetic step of importance may also help to better elucidate the physical principles underlying the mechanism of membrane fusion. The fact that the free fatty

acids are such potent promoters of membrane fusion makes further study of their action quite important.

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Relationship between the Transverse Distribution of Phospholipids in Plasma Membrane and Shape Change of Human Platelets†

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ABSTRACT: ESR spectroscopy was used to investigate the distribution of spin-labeled analogues of sphingomyelin, phosphatidylcholine, phosphatidylethanolamine, and phosphatidylserine in the presence of human platelets. Three rates were determined: hydrolysis of the ester bond at position 2, reduction of labels by cytoplasm, and internalization of labels situated in the outer leaflet of the plasma membrane. We found that the half-time for transverse diffusion of added phospholipids was shorter for aminophospholipids (40 min and less than 10 min for PE and PS, respectively) than for the choline derivatives (>120 min for PC, not measurable for SM). Addition of any of the phospholipids led to a considerable change in the initial platelet shape (assessed by electron microscopy) from a discoid form to a smaller body with very long pseudopods. When aminophospholipids were used, the platelets quickly returned to the initial shape [half-time of 20 min and less than 5 min for (0,2)PE and (0,2)PS, respectively]. Conversely, there was no relaxation after (0,2)PC or (0,2)SM was added. We conclude that there is a relationship between the excess of phospholipids in the outer leaflet of the plasma membrane and cytoskeletal organization presumably via actin polymerization, which is responsible for platelet shape.

It has been accepted for some time that the transverse distribution of the phospholipids in plasma membranes is very dissymmetric (Bretscher, 1972). The clearest example is the erythrocyte. The determination of the percentages of different polar heads in its bilayer is unambiguous because of the presence of a single membrane. The choline head group derivatives are mostly situated outside, whereas the aminophospholipids are preferentially situated inside (Verkleij et al., 1973). Although it has been claimed that dissymmetry applies to other cell types, it has only actually been demonstrated in blood cells, i.e., erythrocytes typical of falciform anemia (Zachowski et al., 1985a), malaria-infected erythrocytes (Van Der Schaft et al., 1987), platelets (Chap et al., 1977), and erythroleukemic Friend cells (Rawyler, 1984). This dissymmetry can only be explained if there is a difference in speed between the outside–inside ("flip") and the inside–outside diffusion ("flop"). Phospholipids move very slowly between the two halves of the bilayer in pure phospholipid systems (Kornberg & McConnell, 1971). In biological membranes, however, it appears that the transfer rate can be high, depending strongly on polar heads of the phospholipids involved (Seigneuret & Devaux, 1984; Seigneuret et al., 1984; Sune et al., 1987) and the fatty acid composition (Middelkoop et al., 1986). By use of phospholipids labeled with fluorescent probes or nitroxide groups, it has been demonstrated that aminophospholipids are quicker than choline derivatives in entering different cell types such as normal or pathological erythrocytes (Seigneuret & Devaux, 1984; Seigneuret et al., 1984; Zachowski et al., 1985a), human platelets (Sune et al., 1987), and fibroblasts (Martin & Pagano, 1987). The transverse diffusion of these phospholipids may be easier in the presence of ATP-dependent carrier having a higher specificity for PS than for PE (Seigneuret and Devaux, 1984; Seigneuret et al., 1984, Zachowski et al., 1986).

In the case of erythrocytes, for example, the addition of exogenous phospholipids results in a very large modification of cell form (Ferrel et al., 1985), which first becomes echynocytic and then relaxes with kinetics comparable to that of marker internalization. After addition of (0,2)PC[1] and (0,2)SM, the cells stay crenated for several hours. In contrast, erythrocytes return to the discocyte form in less than 1 h at 37 °C after addition of (0,2)PE or (0,2)PS (Seigneuret & Devaux, 1984). The addition of exogenous phospholipids to platelets results in more drastic shape changes, with the appearance of micrometer long pseudopods. These pseudopods may become vesicularized by specifically losing membrane material (Kobayashi, 1984). Daleke and Huestis (1985) have attributed this erythrocyte shape change following alteration of the membrane composition to a physical cause. According

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[1] Abbreviations: (0,2)PC, 1-palmitoyl-2-(4-doxylpentanoyl)phosphatidylcholine; (0,2)PE, 1-palmitoyl-2-(4-doxylpentanoyl)phosphatidylethanolamine; (0,2)PS, 1-palmitoyl-2-(4-doxylpentanoyl)phosphatidylserine; (0,2)SM, [*N*-(4-doxylpentanoyl)-*trans*-sphingen-1-yl]phosphocholine; (0,2)FA, 4-doxylpentanoic acid; (*m*,*n*), general nomenclature of spin-labeled chains, *m* and *n* being respectively the number of methylene groups after and before the labeled position on the acyl chain; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; BSA, bovine serum albumin.

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