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Articles

Ca^{2+}-Induced Fusion of Phospholipid Vesicles Containing Free Fatty Acids: Modulation by Transmembrane pH Gradients

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ABSTRACT: The influence of a transmembrane pH gradient on the Ca^{2+}-induced fusion of phospholipid vesicles, containing free fatty acids, has been investigated. Large unilamellar vesicles composed of an equimolar mixture of cardiolipin, dioleoylphosphatidylcholine, and cholesterol, containing 20 mol % oleic acid, were employed. Fusion was measured using a kinetic assay for lipid mixing, based on fluorescence resonance energy transfer. At pH 7.5, but not at pH 6.0, in the absence of a pH gradient, oleic acid stimulates the fusion of the vesicles by shifting the Ca^{2+} threshold concentration required for aggregation and fusion of the vesicles from about 13 mM to 10 mM. In the presence of a pH gradient (at an external pH of 7.5 and a vesicle interior pH of 10.5), the vesicles exhibit fusion characteristics similar to vesicles that do not contain oleic acid at all, consistent with an effective sequestration of the fatty acid to the inner monolayer of the vesicle bilayer induced by the imposed pH gradient. The kinetics of the fusion process upon simultaneous generation of the pH gradient across the vesicle bilayer and initiation of the fusion reaction show that the inward movement of oleic acid in response to the pH gradient is extremely fast, occurring well within 1 s. Conversely, dissipation of an imposed pH gradient, by addition of a proton ionophore during the course of the fusion process, results in a rapid enhancement of the rate of fusion due to reequilibration of the oleic acid between the two bilayers leaflets.

Membrane fusion is a fundamental process in cell biology. It plays a key role in cell–cell fusion phenomena such as fertilization and myogenesis. It is also the basis of intracellular trafficking and sorting processes, involving fusion of shuttle vesicles derived from one cellular compartment with the limiting membrane of another compartment or, as in the process of exocytosis, with the plasma membrane of the cell. Obviously, these membrane fusion processes must be highly specific and strictly controlled, at the level of the initial recognition and attachment of the interacting membranes as well as that of the actual fusion reaction. However, very little is known about the molecular mechanisms involved.

Much of our current knowledge of the molecular mechanisms of membrane fusion has been derived from investigation of fusion in lipid vesicle (liposome) systems [for reviews, see Bentz and Ellens (1988), Düzgünes (1985), Nir et al. (1983), Wilschut and Hoekstra (1986), Wilschut (1988, 1990), and Wilschut et al. (1988)]. From these studies, it has become apparent that the lipid composition of interacting membranes is a crucial determinant for the propensity of membranes to fuse. Accordingly, transverse asymmetry in the distribution of lipid components between the inner and outer bilayer leaflets may be expected to profoundly influence the fusion properties of mixed lipid membranes. However, in model systems, this is not readily demonstrated, as it is usually difficult to achieve transbilayer phospholipid asymmetry in large unilamellar vesicles (Nordlund et al., 1981; Lentz et al., 1982; Hope & Cullis, 1987; Hope et al., 1989). Nevertheless, it is generally accepted that, in biological membranes, not only the protein components but also the phospholipids are asymmetrically distributed between the two bilayer leaflets [for a review, see Op den Kamp (1979)]. Therefore, transverse lipid asymmetry and transient changes in the lipid distribution between the two bilayer halves may well be important factors contributing to

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the specificity and control of membrane fusion in cell biology.

It has been demonstrated recently that, in lipid vesicle systems, the transbilayer distribution of amphiphiles exhibiting weak acid or base characteristics, such as fatty acids or alkylamines, is highly sensitive to a transmembrane pH gradient (Hope & Cullis, 1987; Eastman et al., 1989). For example, in phosphatidylcholine (PC)[1] large unilamellar vesicles, containing 10 mol % oleic acid, in the presence of a pH gradient of 3 units across the bilayer (pH 10 in the vesicle interior, pH 7 externally), the fatty acid is no longer available for removal by external bovine serum albumin (Hope & Cullis, 1987). This indicates an effective sequestration of the fatty acid to the inner monolayer of the vesicles, in a manner dictated by a Henderson–Hasselbalch equilibrium between protonated and charged fatty acid species in each of the bilayer halves, on the one hand, and rapid transbilayer movement of the protonated vs slow movement of the charged species, on the other (Gutknecht, 1988; Deamer & Nichols, 1989). On the basis of a similar mechanism, transbilayer asymmetry of a phospholipid, phosphatidylglycerol, in response to a transbilayer pH gradient has also been achieved (Hope et al., 1989), although in this case the asymmetry was not complete and the establishment of the asymmetric distribution required incubation at elevated temperature.

In the present paper, we demonstrate that a transmembrane pH gradient provides a way to modulate the fusion characteristics of lipid vesicles through rapid redistribution of free fatty acids between the two bilayer leaflets. It is shown that the Ca^{2+}-induced fusion of cardiolipin-containing vesicles (Wilschut et al., 1982, 1985) is stimulated by free fatty acids, in agreement with earlier reports on the enhancement of lipid vesicle fusion (Kantor & Prestegard, 1975, 1978; Meers et al., 1987, 1988) or fusion of biological membranes (Ahkong et al., 1973; Cullis & Hope, 1978; Hope & Cullis, 1981; Creutz, 1981; Drust & Creutz, 1988; Meers et al., 1987) by free fatty acids. The fatty acid induced stimulation of the fusion of the vesicles is shown to be strongly affected by a pH gradient across the vesicle bilayer, consistent with a transbilayer movement of the fatty acids in response to the imposed pH gradient. This movement of fatty acids across the bilayer occurs extremely fast and, thus, provides for an effective and rapid modulation of the fusion characteristics of the vesicles.

Experimental Procedures

Materials. Bovine heart cardiolipin (CL),[1] dioleoylphosphatidylcholine (DOPC), *N*-(7-nitro-2,1,3-benzoxadiazol-4-yl)phosphatidylethanolamine (*N*-NBD-PE), and *N*-(lissamine Rhodamine B sulfonyl)phosphatidylethanolamine (*N*-Rh-PE) were obtained from Avanti Polar Lipids (Alabaster, AL). Cholesterol (Chol), oleic acid (OA), nigericin, and valinomycin were from Sigma Chemical Co. (St. Louis, MO).

Phospholipid Vesicles. Large unilamellar vesicles were prepared from a mixture of CL, DOPC, and Chol in a molar ratio of 1:1:1, with or without oleic acid (20 mol %). Lipid mixtures were dried down from a chloroform solution under a stream of nitrogen, and residual solvent was removed under vacuum for at least 1 h. The lipids were dispersed in the appropriate buffer by vortex mixing, and the dispersions of multilamellar vesicles were passed through five cycles of freezing and thawing (Mayer et al., 1985) using liquid nitrogen and warm water, alternately. Subsequently, the preparations were extruded (Hope et al., 1985) 10 times through two stacked polycarbonate membrane filters with a pore diameter of 100 nm (Nuclepore, Pleasanton, CA), using the Extruder from Lipex Biomembranes, Inc. (Vancouver, British Columbia). In control experiments (results not shown), the fusion characteristics of vesicles composed of an equimolar mixture of CL and DOPC and prepared by the above freeze–thaw/extrusion procedure were found to be identical to those of vesicles of the same composition, but prepared by reverse-phase evaporation and subsequent extrusion (Wilschut et al., 1982, 1985).

Vesicles were prepared in either one of the following two buffers: (i) 150 mM NaCl, 5 mM KCl, and 10 mM HEPES (pH 7.5); (ii) 150 mM boric acid (final borate plus boric acid concentration) containing 5 mM KOH (final K^+ concentration, 5 mM) and further adjusted to pH 10.5 with NaOH (final Na^+ concentration, approximately 130 mM; note that the boric acid is converted partially to $B_4O_7^{2-}$). Phospholipid concentrations in the vesicle preparations were determined by phosphate analysis (Bartlett, 1959). Vesicle preparations were stored on ice until use. Under these conditions, phospholipid hydrolysis in vesicles made and kept at pH 10.5 was found to be approximately 1% in 24 h by HPTLC analysis and phosphate determination of the lipids extracted from the vesicles using standard methodologies. The vesicles were used within 48 h after preparation, during which period no change in their fusion properties was detected.

Fusion Measurements. Fusion of the vesicles was monitored using the fluorescence resonance energy transfer (RET) assay, described by Struck et al. (1981). *N*-NBD-PE and *N*-Rh-PE were incorporated in a population of vesicles at a concentration of 0.7 mol % each. A small volume (50 μL) of a concentrated mixture (4.0 mM total lipid), containing fluorescently labeled vesicles and unlabeled vesicles (of otherwise the same composition and prepared in the same way as the unlabeled vesicles) in a ratio of 1:3, was injected into a cuvette containing 1.95 mL of 150 mM NaCl, 5 mM KCl, and 10 mM HEPES (pH 7.5), at the desired final concentration of $CaCl_2$. The final lipid concentration in the cuvette was 0.1 mM. The medium in the cuvette was stirred continuously and maintained at 25 °C. Complete mixing of the vesicle aliquot with the medium in the cuvette was achieved within 1 s. The increase of *N*-NBD-PE fluorescence, due to dilution of the fluorophores into the unlabeled vesicle bilayers during fusion, was monitored continuously in an SLM/Aminco SPF 500C or an SLM 8000 fluorometer. Excitation and emission wavelengths were 465 and 530 nm, respectively, and a cutoff filter (<520 nm) was placed between the sample and the emission monochromator. The fluorescence scale was set such that the zero level corresponded to the residual fluorescence of the labeled vesicles and the 100% value to complete mixing of all the lipids in the system. The latter value corresponds, at a 1:3 ratio of labeled to unlabeled vesicles, to 75% of the fluorescence intensity at infinite probe dilution, which was determined by addition of 0.1% (v/v) Triton X-100 to the system and appropriate correction for the effect of Triton X-100 on the quantum yield of *N*-NBD-PE (Struck et al., 1981). In the concentration range of the fluorophores used, the *N*-NBD-PE fluorescence intensity increases linearly with the dilution of the probes (Struck et al., 1981; Driessen et al., 1985). On the other hand,

[1] Abbreviations: Chol, cholesterol; CL, cardiolipin (bovine heart); DOPC, dioleoylphosphatidylcholine; DPA, dipicolinic acid; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; HPTLC, high-performance thin-layer chromatography; MES, 2-(*N*-morpholino)ethanesulfonic acid; *N*-NBD-PE, *N*-(7-nitro-2,1,3-benzoxadiazol-4-yl)phosphatidylethanolamine; *N*-Rh-PE, *N*-(lissamine Rhodamine B sulfonyl)-phosphatidylethanolamine; OA, oleic acid; PC, phosphatidylcholine; PE, phosphatidylethanolamine; RET, resonance energy transfer.

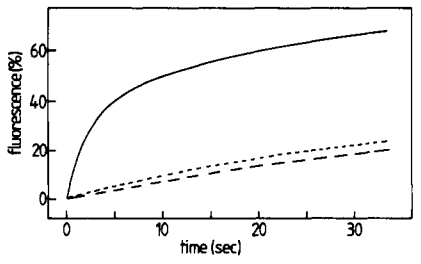

FIGURE 1: Effect of oleic acid on the Ca^{2+}-induced fusion of CL/DOPC/Chol (1:1:1) vesicles. $CaCl_2$ concentration was 12 mM. Drawn line, vesicles containing 20 mol % OA at pH 7.5 in the absence of a ΔpH; dotted line, vesicles containing 20 mol % OA at pH 6.0 in the absence of a ΔpH (in this case, the standard 10 mM HEPES inside the vesicles and in the fusion medium was replaced by 10 mM MES, adjusted to pH 6.0); dashed line, vesicles without OA at pH 7.5 in the absence of a ΔpH.

it should be noted that, at a ratio of labeled to unlabeled vesicles of 1:3, the fluorescence intensity is increasing non-linearly with the extent of vesicle fusion.

Generation of a pH Gradient. A pH gradient across the vesicle bilayer was generated, in the cuvette of the fluorometer simultaneous with the induction of vesicle fusion, by injection of a small volume of a concentrated vesicle suspension prepared and kept in the borate buffer at pH 10.5, into the NaCl/KCl/HEPES/$CaCl_2$ medium at pH 7.5. This simple (40-fold) dilution of the vesicles into the neutral-pH medium creates a pH gradient of approximately 3 units across the vesicle bilayer (basic inside). It has been demonstrated before (Hope & Cullis, 1987; Eastman et al., 1989) that borate does not readily permeate vesicle bilayers and that, thus, in the presence of borate inside the vesicles a pH gradient across the vesicle bilayer is maintained for a relatively long period of time (at least up to 30 min). Dissipation of the gradient was achieved by injection of 10 μL of a solution of nigericin and valinomycin in ethanol (1.0 and 10 μM, respectively) into the cuvette, resulting in a molar ratio of ionophores to total lipid of 1:20000 and 1:2000, respectively. The combination of nigericin (an H^+/K^+-exchanger) and valinomycin (a K^+-ionophore; added to avoid K^+ depletion of the vesicle interior) has been used before (Hope & Cullis, 1987; Hope et al., 1989; Eastman et al., 1989) to induce a rapid and effective dissipation of transbilayer pH gradients in similar systems. Preincubation of concentrated vesicle suspensions with the ionophores was done at the same molar ratio of ionophores to lipid.

Aggregation Measurements. For the determination of vesicle aggregation, liposomes were prepared in 150 mM NaCl, 5 mM KCl, and 10 mM HEPES (pH 7.5). Measurements were made in the same buffer, containing the desired concentration of $CaCl_2$, at a lipid concentration of 0.1 mM and a temperature of 25 °C. Aggregation was followed continuously as an increase of the turbidity at 450 nm in a Shimadzu UV-160 spectrophotometer.

Results

Effect of Oleic Acid on the Fusion of CL/DOPC/Chol Vesicles. Figure 1 shows that the Ca^{2+}-induced fusion of CL/DOPC/Chol is markedly enhanced by the presence of OA in the vesicle bilayer. Fusion of the vesicles was measured with the resonance energy transfer (RET) assay, described by Struck et al. (1981). This assay involves labeling of one population of vesicles with the fluorophores *N*-NBD-PE and *N*-Rh-PE, and measurement of their dilution into an unlabeled vesicle population during fusion of the vesicles. Dilution of the fluorophores results in an increase of the *N*-NBD-PE fluorescence due to a decrease of the energy transfer efficiency between the two probes. It has been demonstrated repeatedly that *N*-NBD-PE and *N*-Rh-PE do not exchange between lipid vesicles, even when the vesicles are aggregated (Struck et al., 1981; Nichols & Pagano, 1983).

The stimulation of the Ca^{2+}-induced fusion of CL/DOPC/Chol vesicles by OA was confirmed using the Tb/DPA assay (Wilschut et al., 1980, 1982, 1985), which registers the mixing of internal aqueous contents during lipid vesicle fusion (results not shown).

It is only the ionized form of OA that exerts a stimulatory effect on the Ca^{2+}-induced fusion of CL/DOPC/Chol vesicles. This is demonstrated by the dotted line in Figure 1, representing the fusion of CL/DOPC/Chol/OA vesicles at pH 6.0 in the absence of a pH gradient across the vesicle bilayer. Clearly, the stimulatory effect of OA on the fusion process was minimal at this pH value, while the fusion of CL/DOPC/Chol vesicles without OA was not affected by the pH being either 7.5 or 6.0 (results not shown). The apparent pK_a of free fatty acids incorporated in a PC bilayer has been demonstrated to be approximately 7–8 (Kantor & Prestegard, 1978; Ptak et al., 1980; Von Tscharner & Radda, 1981; Rooney et al., 1983; Hamilton & Cistola, 1986; Ortiz & Gómez-Fernández, 1988; Cevc et al., 1988; Sankaram et al., 1990), while in the negatively charged vesicles used here this value may well be even higher (Ptak et al., 1980; Samkaram et al., 1990). Accordingly, the lack of stimulation of vesicle fusion by OA at pH 6.0, well below the effective pK_a of the fatty acid, indicates that it is only the charged fatty acid species that enhances the rate of the fusion process, at least in this particular vesicle system. In addition, since even pH 7.5, at which we have carried out our experiments, is likely below the effective pK_a of the OA, the concentration of the charged fatty acid involved in the stimulation of vesicle fusion is considerably lower than 20 mol %.

It is to be noted that in the fusion measurements, upon dilution of the concentrated vesicle suspension into the cuvette, the partitioning of the OA between the aqueous and the membrane phase in principle shifts into the direction of the aqueous phase. Yet, we think that extensive dissociation of the fatty acid from the vesicles under these conditions is not likely to occur. The partition coefficient, K_p, of OA between PC bilayers and an aqueous medium at pH 7.4 (defined as the molar concentration of the fatty acid in the membrane divided by the molar concentration in water phase) has been reported to be 10^4–10^5 (Rooney et al., 1983; Pjura et al., 1984; Gutknecht, 1988). On this basis we estimate that, prior to dilution of the vesicles (at a lipid concentration of 4.0 mM), essentially all of the fatty acid will be in the membrane phase while after dilution (to a lipid concentration of 0.1 mM), at equilibrium, 50–90% of the fatty acid will remain in the membrane phase. Presumably, due to the likely upward shift of the apparent pK_a of OA in the negatively charged vesicle system used here (Ptak et al., 1980), an even larger proportion of the OA will remain vesicle-associated (Brecher et al., 1984; Hamilton & Cistola, 1986). In addition, the desorption of fatty acids from lipid bilayers has been reported to be a relatively slow process (Doody et al., 1980; Hamilton & Cistola, 1986), that is not likely to play a significant role at the time scale of the fusion experiments reported here. We do not believe, therefore, that fatty acid partitioning into the aqueous phase occurs to a significant extent under the conditions of our experiments. Moreover, if it did, we would only underestimate the actual potency of the fatty acid to stimulate vesicle fusion.

Oleic Acid Induces a Downward Shift in the Threshold Ca^{2+} Concentration Required for Fusion. The stimulation of

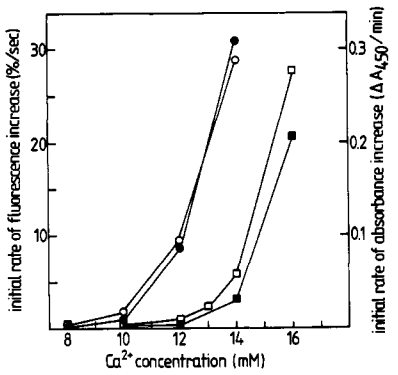

FIGURE 2: Effect of oleic acid on the initial rates of fusion and aggregation of CL/DOPC/Chol vesicles at different Ca^{2+} concentrations. Fusion was measured with the RET assay (open symbols). Aggregation was measured, under the same conditions with unlabeled vesicles, as the initial rate of absorbance increase at 450 nm (closed symbols); the absorbance of the vesicles in the absence of Ca^{2+} was approximately 0.015. The measurements were carried out at pH 7.5 in the absence of a ΔpH. Circles, vesicles containing 20 mol % OA; squares, vesicles without OA.

the fusion of CL/DOPC/Chol vesicles by OA is accompanied by a downward shift of the threshold Ca^{2+} concentration required to initiate the process. It is well established that, in a 100 mM NaCl medium, fusion of CL/DOPC (1:1) vesicles in the presence of Ca^{2+} requires a threshold concentration of the divalent cation of 9–10 mM (Wilschut et al., 1982, 1985). Above that value, the initial rate of fusion increases steeply with increasing Ca^{2+} concentrations. Figure 2 shows that, for CL/DOPC/Chol (1:1:1) vesicles in the 150 mM NaCl medium used, the Ca^{2+} threshold is about 13 mM. In the presence of 20 mol % OA, at pH 7.5, this Ca^{2+} threshold was found to be shifted downward to approximately 10 mM.

The shift of the Ca^{2+} threshold concentration required for fusion of the OA-containing CL/DOPC/Chol vesicles is also reflected in a shift of the Ca^{2+} threshold required for the aggregation of the vesicles. Aggregation was monitored as an increase of the apparent absorbance at 450 nm (turbidity) of the vesicle suspension. Figure 2 shows that the initial rate of aggregation (closed symbols) parallels that of the fusion of the vesicles determined with the RET assay (open symbols).

Effect of a Transbilayer pH Gradient on Fusion of OA-Containing Vesicles. Figure 3 shows that a pH gradient across the vesicle bilayer of OA-containing CL/DOPC/Chol vesicles (basic inside) results in fusion characteristics of the vesicles very similar to those of corresponding vesicles without OA. This is entirely consistent with an efficient sequestration of the fatty acid to the inner monolayer of the vesicles in response to the imposed ΔpH (Hope & Cullis, 1987; Eastman et al., 1989). Figure 3A shows fusion curves obtained when small volumes of a concentrated suspension of OA-containing vesicles, made and kept in a buffer of pH 10.5, were injected into a medium of pH 7.5 containing various Ca^{2+} concentrations. By injection of the vesicles into the neutral pH buffer, a transbilayer pH gradient of approximately 3 units is generated. Obviously, just before injection of the vesicles, the internal and external pH values are equal (10.5) and the concentrations of OA in the inner and outer vesicle monolayers the same. The fatty acid transbilayer asymmetry is induced only upon establishment of the pH gradient when the vesicles are injected into the neutral pH medium in the cuvette, i.e., at the time the vesicles are exposed to Ca^{2+} as well. Accordingly, the fusion curves are biphasic, consisting of an initial steeply increasing part followed by a secondary much slower phase. The initial part likely reflects the fusion rate of the vesicles prior

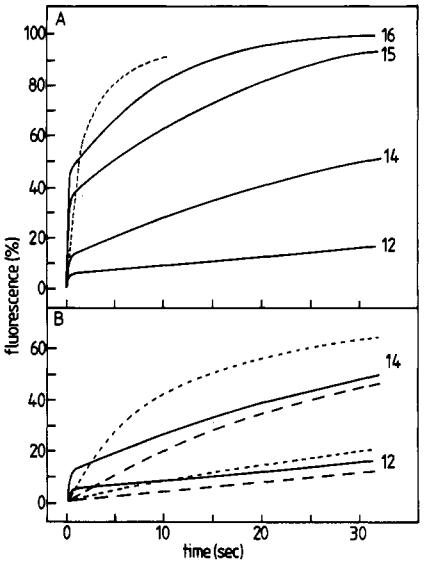

FIGURE 3: Effect of a transbilayer pH gradient on the Ca^{2+}-induced fusion of CL/DOPC/Chol vesicles containing 20 mol % oleic acid. (Panel A) Vesicles, prepared in pH 10.5 buffer, were injected into a medium of pH 7.5, containing the millimolar $CaCl_2$ concentrations indicated; the dotted line represents fusion at 14 mM Ca^{2+} and pH 7.5 in the *absence* of a ΔpH. (Panel B) Conditions as in panel A: drawn lines, OA-containing vesicles in the presence of a ΔpH; dashed lines, vesicles without OA in the presence of a ΔpH; dotted lines, OA-containing vesicles at pH 7.5 in the *absence* of a ΔpH.

to completion of the sequestration of OA to the inner vesicle monolayer in response to the ΔpH. This is indicated by the dotted line in Figure 3A, representing, for comparison, an example of fusion of the OA-containing vesicles in the absence of a ΔpH at one of the Ca^{2+} concentrations used (14 mM). In the secondary much slower phase of the fusion process, after the point of inflection (drawn lines, Figure 3A), the vesicles fused at a rate very similar to that observed for vesicles that did not contain free fatty acid at all. To illustrate this latter point, Figure 3B presents a direct comparison between fusion of the OA-containing vesicles (solid lines) with fusion of vesicles without OA (dashed lines) at 12 and 14 mM Ca^{2+} in the presence of a ΔpH.

Importantly, at all Ca^{2+} concentrations, the point of inflection in the fusion curves (drawn lines in Figure 3A) was reached well within 1 s. In fact, it was reached in the time required for complete mixing of the injected vesicle suspension with the medium in the cuvette. This indicates that the sequestration of the OA to the inner vesicle monolayer is completed within 0.5–1 s after the pH gradient is generated by the injection of the vesicles into the neutral pH medium. Therefore, the transbilayer movement of the fatty acid in response to a pH gradient appears to be an extremely fast process.

A transbilayer pH gradient appeared to also have an effect, albeit of limited magnitude, on the fusion of CL/DOPC/Chol vesicles without OA. Comparison of the dotted (no ΔpH) and dashed lines (+ΔpH) in Figure 3B shows that, in the presence of a ΔpH, the fusion of these vesicles was inhibited, which was particularly evident at 14 mM Ca^{2+}. At present, we have no explanation for this effect, which does not seem to be mediated by transbilayer movement of any of the membrane lipids.

Figure 4 presents the initial rates of fusion of vesicles with and without OA at different Ca^{2+} concentrations in the absence or presence of a transbilayer pH gradient. In the case of OA-containing vesicles in the presence of a ΔpH, the rate after the inflection point was taken as the "initial" rate; for Ca^{2+} concentrations above 14 mM, this fusion rate is not

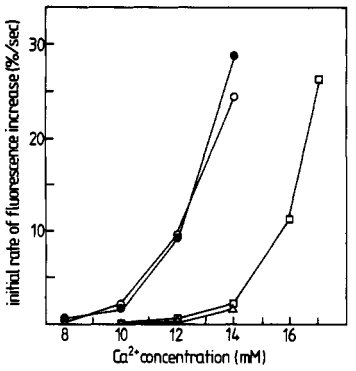

FIGURE 4: Effect of a transbilayer pH gradient on the initial rate of the Ca^{2+}-induced fusion of CL/DOPC/Chol vesicles with or without oleic acid. Triangles, vesicles with 20 mol % OA in the presence of a ΔpH (rates after the points of inflection, see Figure 3A); squares, vesicles without OA in the presence of a ΔpH; open circles, OA-containing vesicles, pretreated with nigericin/valinomycin, in the presence of an imposed ΔpH (the rate of fusion was determined from the steepest part of the curve, see Figure 5); closed circles, OA-containing vesicles at pH 7.5 in the *absence* of a ΔpH.

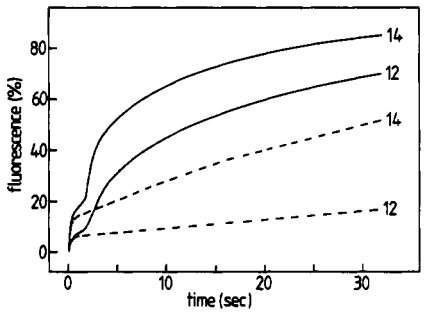

FIGURE 5: Effect of pretreatment of CL/DOPC/Chol vesicles, containing oleic acid, with nigericin/valinomycin on their fusion in the presence of Ca^{2+} and an imposed transbilayer ΔpH. Final millimolar Ca^{2+} concentrations are indicated. Drawn lines, ionophore-pretreated vesicles; dashed lines, untreated vesicles.

indicated in Figure 4, as its value represents a significant underestimate due to the advanced stage of fusion that is already reached in the very first fast phase of the process (see Figure 3A). In the presence of a ΔpH, the Ca^{2+} threshold concentration was the same (approximately 14 mM), irrespective of whether the vesicles contained fatty acid or not (Figure 4). Due to the effect of a ΔpH per se on the fusion of the vesicles, apart from its effect on fatty acid movement (Figure 3B), the threshold concentration of 14 mM represents a slight upward shift relative to the Ca^{2+} threshold of about 13 mM for vesicles without OA in the absence of a ΔpH (Figure 2).

Ionophore Pretreatment of the Vesicles. Figure 5 (drawn lines) presents fusion curves at 12 and 14 mM Ca^{2+} for vesicles pretreated with nigericin and valinomycin. Also in this case, by injection of the vesicles, containing pH 10.5 buffer, into a neutral pH medium, a pH gradient of approximately 3 units was imposed on the vesicles. However, the ionophore-pretreated vesicles are unable to maintain the gradient; they fuse, after a short delay, with rates equal to the rates seen with OA-containing vesicles in the absence of a ΔpH (Figure 4, closed and open circles, respectively). For comparison, in Figure 5, we also show fusion curves for OA-containing vesicles, not pretreated with nigericin and valinomycin, in the presence of a ΔpH (dashed lines, see also Figure 3).

In the curves in Figure 5 for the ionophore-containing vesicles (drawn lines), the short delay, after the initial rapid fluorescence increase, is readily explained by the time required for dissipation of the pH gradient generated by the injection

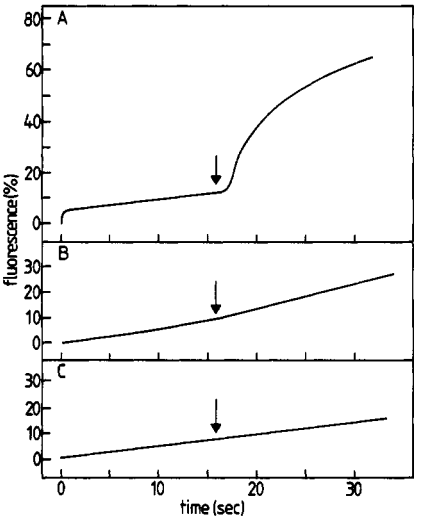

FIGURE 6: Effect of ionophore-induced dissipation of an initially imposed transbilayer ΔpH on the fusion of CL/DOPC/Chol vesicles with or without oleic acid. During the course of the fusion process, 10 μL of an ethanolic solution of nigericin and valinomycin was injected into the cuvette (arrows). (Panel A) OA-containing vesicles in the presence of 12 mM Ca^{2+} and a ΔpH. (Panel B) Vesicles without OA in the presence of 12 mM Ca^{2+} and a ΔpH. (Panel B) Vesicles without OA in the presence of 12 mM Ca^{2+} and a ΔpH. (Panel C) OA-containing vesicles, prepared in a pH 7.5 buffer and, therefore, in the absence of a ΔpH, at a Ca^{2+} concentration of 8 mM.

of the vesicles into the medium at pH 7.5. It appears that complete dissipation of the gradient takes approximately 2 s under the conditions employed (it should be noted that the buffering capacity of the borate medium inside the vesicles is quite significant, which implies that a considerable flux of protons is required for complete dissipation of the ΔpH). The fact that the fusion of the vesicles slowed down temporarily after 0.5–1 s indicates that the transbilayer movement of OA occurred faster than the dissipation of the pH gradient. Thus, within 0.5–1 s after the injection of the vesicles, the fatty acid in all likelihood moved to the inner half of the vesicle membrane in response to the ΔpH, established initially, while after the subsequent dissipation of the ΔpH it rapidly reequilibrated between the two bilayer halves, resulting in the secondary increase of the rate of fusion.

Dissipation of the pH Gradient during the Fusion Process. The above results, demonstrating that transbilayer movement of fatty acids in response to a ΔpH is a very fast process, suggest the possibility of modulating the rate of vesicle fusion by dissipation of an imposed pH gradient during the course of the process. Indeed, addition of nigericin and valinomycin to OA-containing vesicles, with an imposed ΔpH in the presence of 12 mM Ca^{2+}, resulted in an enhancement of the rate of fusion due to a rapid redistribution of the fatty acid (Figure 6A). In the control experiment, shown in Figure 6B, vesicles without OA also exhibited a minor increase of the rate of fusion upon addition of the ionophores, consistent with the results in Figure 3B regarding the effect of a transbilayer ΔpH on the fusion of these vesicles. Figure 6C demonstrates that the ionophores did not affect the fusion process in the absence of an imposed pH gradient.

Spontaneous Dissipation of the pH Gradient as a Result of the Interaction of the Vesicles with Ca^{2+}. It is well established that Ca^{2+} induces relative permeability increases in CL-containing vesicles (Mandersloot et al., 1981; Smaal et al., 1987) and the formation of inverted micellar or hexagonal (H_{II}) structures (Cullis et al., 1978; Cullis & De Kruijff, 1979; De Kruijff et al., 1982). It is important to note that our present results, indicating that a transbilayer ΔpH can be maintained

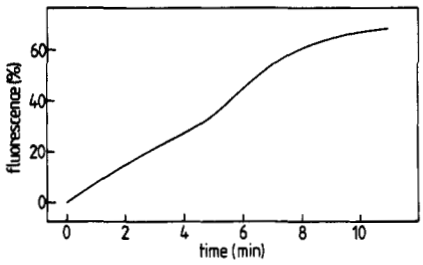

FIGURE 7: Spontaneous collapse of an imposed transbilayer ΔpH during continued slow fusion of CL/DOPC/Chol vesicles containing oleic acid. Ca^{2+} concentration was 10 mM. Note the difference in time scale relative to that in Figures 1, 3, 5, and 6.

for a significant period of time during fusion of the vesicles (see, e.g., the curve at 14 mM Ca^{2+} in Figure 3A), underline previous (Wilschut et al., 1982, 1985) and more recent (A. Ortiz, J. L. Nieva, J. A. Killian, A. J. Verkleij, and J. Wilschut, unpublished results) conclusions that the initial Ca^{2+}-induced fusion events in CL vesicle systems are relatively nonleaky. In this respect, the inclusion of cholesterol in the vesicle bilayer did not significantly influence the fusion behavior of the vesicles in the presence or absence of a ΔpH; vesicles composed of CL, DOPC, and OA exhibited essentially the same fusion characteristics (results not shown).

Figure 7 shows a curve for OA-containing CL/DOPC/Chol vesicles, fusing slowly in the presence of a transbilayer ΔpH at a relatively low Ca^{2+} concentration (10 mM). Clearly, only after 5–6 min was there an indication of a spontaneous collapse of the pH gradient, resulting in an enhancement of the rate of the process. Apparently, the ΔpH is maintained for a considerable period of time up to a stage in the fusion process corresponding to about 35–40% lipid mixing, under which condition the majority of the vesicles have fused at least once.

Discussion

Transbilayer Movement of Fatty Acids Induced by a pH Gradient. This study demonstrates that the transverse movement of free fatty acids across lipid bilayer membranes in response to the establishment of a transmembrane pH gradient (Hope & Cullis, 1987; Eastman et al., 1989) is a very fast process, occurring on the time scale of seconds to milliseconds. Capitalizing on the stimulation of phospholipid vesicle fusion by free fatty acids, we show that the kinetics of such vesicle fusion can be rapidly modulated by the establishment, and also by the subsequent dissipation, of a transbilayer pH gradient. In the presence of a ΔpH (basic inside), the vesicles fuse as though they did not contain fatty acid at all, consistent with sequestration of the fatty acids to the inner half of the vesicle bilayer. This sequestration appears complete within 1 s after establishment of the pH gradient (Figure 3). Conversely, after dissipation of the pH gradient in such a system, the fatty acids rapidly reequilibrate between the two bilayer halves, resulting in an enhancement of the rate of fusion (Figure 5).

Transmembrane pH gradients exist intracellularly across the limiting membranes of several cell compartments, such as the endosomal/lysosomal compartment and secretory vesicles. The results of the present and previous (Hope & Cullis, 1987; Eastman et al., 1989) studies suggest that any free fatty acid in the membranes of these organelles will be rapidly and effectively transferred to the cytoplasmic membrane leaflet. In lysosomes, this may facilitate the intracellular transport of fatty acids generated by lipid hydrolysis. In the case of secretory vesicles, translocation of fatty acids to the cytoplasmic membrane leaflet may augment their possible function in the fusion of the secretory vesicles with the cellular plasma membrane during secretion, as discussed below.

A remarkable implication of the pH-induced transbilayer movement of the fatty acid in our present vesicle system relates to the apparent stability of the vesicles under conditions where essentially all of the fatty acid is sequestered to the inner leaflet of the bilayer. In the case of vesicles containing 20 mol % OA, complete sequestration of the fatty acid to the vesicle interior implies that the fatty acid content of the inner monolayer is about 40 mol %. It is surprising that such vesicles maintain their stability, as one would expect considerable packing constraints under these conditions. It is possible that the vesicles change shape to compensate for the induced packing imbalance. Alternatively, the movement of fatty acid to the inner monolayer may be accompanied by a reverse movement of other membrane components, such as, e.g., cholesterol.

Stimulation of Phospholipid Vesicle Fusion by Free Fatty Acids. This study underlines earlier observations (Kantor & Prestegard, 1975, 1978; Meers et al., 1987, 1988; Cevc et al., 1988) regarding the stimulatory effect of free fatty acids on the fusion of phospholipid vesicles. Although the precise nature of this stimulatory effect remains to be established, and may in fact depend on the particular system involved, it would appear that it is primarily the uncharged fatty acid species that can exert a destabilizing action on lipid bilayer membranes. Specifically, there is evidence, elaborated below, suggesting that fatty acids below their apparent pK_a value, or fatty acid anions complexed to divalent cations, have the capacity to induce the formation of inverted hexagonal (H_{II}) or inverted cubic structures in phospholipid systems. Such inverted structures may well favor fusion (Ellens et al., 1989).

Vesicles consisting of unsaturated phosphatidylethanolamine (PE) and free fatty acids or other acidic amphiphiles have been observed to become destabilized at a mildly acidic pH and/or in the presence of divalent cations (Connor et al., 1984; Düzgünes et al., 1985; Ellens et al., 1985). In these systems, the charged amphiphile anion stabilizes the lamellar configuration of the lipids and prevents vesicle aggregation by electrostatic repulsion. At pH values below the pK_a of the amphiphile, the vesicles aggregate and are destabilized in contact-dependent fashion (Ellens et al., 1985), primarily due to the tendency of the PE to adopt an H_{II} structure. Like protons, divalent cations also bind to and thus neutralize the charge of the fatty acid anions. However, divalent cations probably have an effect beyond mere charge neutralization, Ca^{2+} or Mg^{2+} at neutral pH being considerably more potent than low pH in inducing fusion in PE/OA vesicle systems (Düzgünes et al., 1985). In this respect, the 1:2 complexes formed between divalent cations and fatty acid anions, by virtue of their structural properties, may well act synergistically with PE in the formation of H_{II}-related inverted structures.

Even in systems consisting of the notorious bilayer-forming PC as the phospholipid component, free fatty acids have been demonstrated to promote the formation of H_{II} or inverted cubic structures (Marsh & Seddon, 1982; Cevc et al., 1988; Heimburg et al., 1990; Rama Krishna & Marsh, 1990; Seddon, 1990). Again, this bilayer-destabilizing activity of free fatty acids is evident primarily at pH values below their pK_a (Cevc et al., 1988). In view of the possible formation of a 1:2 complex between divalent cations and fatty acid anions, favoring the formation of inverted structures and fusion, it would be of interest to examine the effect of Ca^{2+} on PC vesicles containing free fatty acids at pH values above their pK_a.

Within the context of the present study, the notion that fatty acids may promote vesicle fusion through the induction of

inverted structures is of particular interest, since such structures have been suggested to play a crucial role in the Ca^{2+}-induced fusion of CL-containing vesicles (Verkleij et al., 1979; Verkleij, 1984; De Kruijff et al., 1982; Wilschut et al., 1982, 1985; Frederik et al., 1989). Clearly, the bilayer-destabilizing effect of the OA anion in the present system requires Ca^{2+}, the OA anion as such being not destabilizing to the vesicles at all, as discussed above. Thus, our results are consistent with the formation of a complex between Ca^{2+} and OA, promoting fusion on the basis of its structural characteristics. It is interesting that in other lipid vesicle systems the facilitation of Ca^{2+}-induced fusion by free fatty acids has been shown to be dependent on the structure of the fatty acid as well; specifically, unsaturated fatty acids were more effective than saturated ones (Meers et al., 1988). A further evaluation of the effects of fatty acids in the present vesicle system will require a detailed characterization of the physical structure of the lipid mixture in the presence of Ca^{2+}.

Possible Role of Fatty Acids and Transmembrane pH Gradients in Biological Membrane Fusion. Since only relatively high levels of OA had significant effects on the particular fusion process studied in the present system, the physiological relevance of the data may not be directly apparent. However, it should be noted that free fatty acids have been observed to promote fusion of biological membranes in several systems (Ahkong et al., 1973; Cullis & Hope, 1978; Hope & Cullis, 1981; Creutz, 1981; Drust & Creutz, 1988; Herbette et al., 1984). In these studies, unsaturated fatty acids were more potent than saturated ones, while also a mildly acidic pH and/or the presence of Ca^{2+} was needed. These conditions are consistent with the requirements for fusion in vesicle systems and underline the above notion that fatty acids may promote fusion through the formation of inverted structures (Cullis & Hope, 1978).

It is well established that arachidonic acid is released during degranulation of neutrophils (Walsh et al., 1981), and during secretion by adrenal medullary chromaffin cells (Frye & Holz, 1984), while in in vitro systems this fatty acid also promotes fusion of neutrophils with lipid vesicles (Meers et al., 1987) and fusion of chromaffin granules (Creutz, 1981; Creutz & Pollard, 1982; Drust & Creutz, 1988). If arachidonic acid plays a role in the exocytic fusion of secretory vesicles, one may speculate on the involvement of the pH gradient across the vesicle membrane (Pollard et al., 1979), likely inducing translocation of the fatty acid to the outer membrane leaflet, which interacts with the plasma membrane.

Registry No. Chol, 57-88-5; DOPC, 4235-95-4; OA, 112-80-1; Ca^{2+}, 7440-70-2.

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Concerted Modulation by Myelin Basic Protein and Sulfatide of the Activity of Phospholipase A_2 against Phospholipid Monolayers†

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ABSTRACT: The effect of myelin basic protein (MBP) on the activity of phospholipase A_2 (PLA_2, EC 3.1.1.4) against monolayers of dilauroylphosphatidylcholine (dlPC) or dilauroylphosphatidic acid (dlPA) containing different proportions of sulfatide (Sulf) and galactocerebroside (GalCer) was investigated. MBP was introduced into the interface by direct spreading as an initial constitutive component of the lipid–protein film or by adsorption and penetration from the subphase into the preformed lipid monolayers. The effect of MBP on PLA_2 activity depends on the type of phospholipid and on the proportion of MBP at the interface. At a low mole fraction of MBP, homogeneously mixed lipid–protein monolayers are formed, and the PLA_2 activity against dlPC is only slightly modified while the degradation of dlPA is markedly inhibited. This is probably due to favorable charge–charge interactions between dlPA and MBP that interfere with the enzyme action. The PLA_2 activity against either phospholipid is increased when the mole fraction of MBP exceeds the proportion at which immiscible surface domains are formed. GalCer has little effect on the modulation by MBP of the phospholipase activity. The effect of Sulf depends on its proportions in relation to MBP. The individual effects of both components balance each other, and a finely tuned modulation is regulated by the interactions of MBP with Sulf or with the phospholipid.

In experimental models of demyelination and remyelination and in the pathological lesions of multiple sclerosis and related diseases, there are changes of the content of sulfatide (Sulf),[1] cerebrosides (GalCer), or gangliosides and losses of myelin-specific proteins, particularly myelin basic protein (MBP)

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[1] Abbreviations: PLs, phospholipids; GSLs, glycosphingolipids; MBP, myelin basic protein; dlPC (dilauroylphosphatidylcholine), didodecanoyl-*sn*-glycero-3-phosphocholine; dlPA (dilauroylphosphatidic acid), didodecanoyl-*sn*-glycero-3-phosphate; dpPC (dipalmitoylphosphatidyl choline), dihexadecanoyl-*sn*-glycero-3-phosphocholine; PLA_2, porcine pancreas phospholipase A_2 (phosphatidylcholine 2-acylhydrolase, EC 3.1.1.4); PLC, *Clostridium perfringens* phospholipase C (phosphatidylcholine cholinephosphohydrolase, EC 3.1.4.3); Cer (ceramide), *N*-acylsphingosine; GalCer (galactocerebroside), Gal(β1-1)Cer; Sulf (sulfatide), Gal(3-sulfate)(β1-1)Cer.

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