Siegel, R. C., Schumaker, V. N., & Poon, P. H. (1981) *J. Immunol. 127*, 2447-2452.
Sim R. B., Porter, R. R., Reid, K. B. M., & Gigli, I. (1977) *Biochem. J. 163*, 219-227.
Spycher, S. E., Nick, H., & Rickli, E. E. (1986) *Eur. J. Biochem. 156*, 49-57.
Summers, M. D., & Smith, G. S. (1987) A manual of methods for baculovirus vectors and insect cell culture procedures, *Tex. Agric. Exp. Stn.*, [*Bull.*], 1555.
Tarentino, A. L., Gomez, C. M., & Plummer, T. H., Jr. (1985) *Biochemistry 24*, 4665-4671.
Thielens, N. M. (1990) *J. Biol. Chem. 265*, 14469-14475.
Thielens, N. M., Van Dorsselaer, A., Gagnon, J., & Arlaud, G. J. (1990) *Biochemistry 29*, 3570-3578.
Tosi, M., Duponchel, C., Meo, T., & Julier, C. (1987) *Biochemistry 26*, 8516-8524.
Towbin, H., Staehelin, R., & Gordon, J. (1979) *Proc. Natl. Acad. Sci. U.S.A. 76*, 4350-4354.
Tschopp, J., Villiger, W., Fuchs, H., Kilchherr, E., & Engel, J. (1980a) *Proc. Natl. Acad. Sci. U.S.A. 77*, 7014-7018.
Tseng, Y., Poon, P. H., Zavodszky, P., & Schumaker, V. N. (1991) *J. Immunol. 147*, 1884-1890.
Villiers, C. L., Arlaud, G. J., & Colomb, M. G. (1985) *Proc. Natl. Acad. Sci. U.S.A. 82*, 4477-4481.
Weiss, V., Fauser, C., & Engel, J. (1986) *J. Mol. Biol. 189*, 573-581.
Ziccardi, R. J. (1982a) *J. Immunol. 128*, 2500-2504.
Ziccardi, R. J. (1982b) *J. Immunol. 128*, 2505-2508.

Influence of Phospholipid Asymmetry on Fusion between Large Unilamellar Vesicles†

Simon J. Eastman,* Michael J. Hope, Kim F. Wong, and Pieter R. Cullis
Department of Biochemistry, University of British Columbia, 2146 Health Sciences Mall, Vancouver, British Columbia, Canada V6T 1W5
Received September 6, 1991; Revised Manuscript Received December 3, 1991

ABSTRACT: The ability of lipid asymmetry to regulate Ca^{2+}-stimulated fusion between large unilamellar vesicles has been investigated. It is shown that for 100-nm-diameter LUVs composed of dioleoylphosphatidylcholine, dioleoylphosphatidylethanolamine, phosphatidylinositol, and dioleoylphosphatidic acid (DOPC/DOPE/PI/DOPA; 25:60:5:10) rapid and essentially complete fusion is observed by fluorescent resonance energy transfer techniques when Ca^{2+} (8 mM) is added. Alternatively, for LUVs with the same lipid composition but when DOPA was sequestered to the inner monolayer by incubation in the presence of a pH gradient (interior basic), little or no fusion is observed on addition of Ca^{2+}. It is shown that the extent of Ca^{2+}-induced fusion correlates with the amount of exterior DOPA. Further, it is shown that LUVs containing only 2.5 mol % DOPA, but where all the DOPA is in the outer monolayer, can be induced to fuse to the same extent and with the same rate as LUVs containing 5 mol % DOPA. These results strongly support a regulatory role for lipid asymmetry in membrane fusion and indicate that the fusogenic tendencies of lipid bilayers are largely determined by the properties of the monolayers proximate to the fusion interface.

The asymmetric transbilayer distributions of lipids commonly observed in biological membranes may be expected to play a role in regulating membrane fusion in vivo. Model membrane systems composed of unsaturated phosphatidylethanolamine (PE) and phosphatidylserine (PS), approximating the inner monolayer composition of the erythrocyte membrane, for example, fuse readily in the presence of physiological stimuli such as Ca^{2+} (Hope et al., 1983). Alternatively, vesicles composed of phosphatidylcholine (PC) and sphingomyelin, the outer monolayer composition, are resistant to fusion. It may therefore be expected that membranes whose external monolayers contain fusogenic lipids such as PE and PS will fuse more readily than membranes with identical lipid compositions but where the fusogenic lipids are localized to the inner monolayer. These speculations are supported by several observations. For example, Sessions and Horwitz (1981, 1983) have shown that the external leaflet of the plasma membrane of myoblasts, which undergo fusion to form myotubes, contains more PE and PS than the outer monolayer of the erythrocyte. Further, the concentrations of these lipids in the outer monolayer increase prior to fusion (Santini et al., 1990). It may also be noted that erythrocytes which have lost lipid asymmetry fuse more readily than erythrocytes exhibiting asymmetric lipid distributions with PE and PS in the inner monolayer (Tullius et al., 1989).

The regulatory role of lipid asymmetry in fusion has proven difficult to investigate, due in part to the lack of an appropriate model system. However, recent work from this laboratory has shown that lipid asymmetry can be generated in large unilamellar vesicles (LUVs) by imposing transmembrane pH gradients (Hope et al., 1989; Redelmeier et al., 1990; Eastman et al., 1991). Here we utilize this phenomenon to investigate the role of lipid asymmetry in the regulation of Ca^{2+}-induced membrane fusion. It is shown that lipid asymmetry can profoundly regulate fusion phenomena between LUV systems and that the composition of the outer monolayer plays a dominant role in determining the rate and extent of fusion.

Materials and Methods

Lipids and Chemicals. Dioleoylphosphatidylcholine (DOPC), dioleoylphosphatidylethanolamine (DOPE), dioleoylphosphatidic acid (DOPA), bovine liver phosphatidylinositol (PI), *N*-(7-nitro-2,1,3-benzoxadiazol-4-yl)phosphatidyl-

† This work was supported by the Medical Research Council of Canada.

0006-2960/92/0431-4262$03.00/0

ethanolamine (NBD-PE), and *N*-(lissamine rhodamine B sulfonyl)phosphatidylethanolamine (Rh-PE) were obtained from Avanti Polar Lipids (Pelham, AL). ANTS (1-aminonaphthalene-3,6,8-trisulfonic acid) and DPX [*N*,*N*'-*p*-xylenebis(pyridinium bromide)] were from Molecular Probes (Junction City, OR). TNS [2-(*p*-toluidinyl)naphthalene-6-sulfonic acid] and all buffers were obtained from Sigma Chemical Co. (St. Louis, MO).

Preparation of Large Unilamellar Vesicles. Lipids were mixed in chloroform and dried down under a stream of nitrogen, and residual chloroform was removed under high vacuum for 1 h. The resulting lipid film was then hydrated by the addition of the appropriate buffer followed by vortexing to form multilamellar vesicles (MLVs). These MLVs were freeze–thawed 5 times, employing alternating liquid nitrogen/cold water cycles (<20 °C), in order to obtain equilibrium transmembrane solute distributions (Mayer et al., 1985). The freeze–thawed MLVs were then extruded 10 times through two stacked polycarbonate filters with 100-nm pore size, employing an extrusion device (Lipex Biomembranes, Vancouver, British Columbia, Canada) to produce a homogeneous population of large unilamellar vesicles (LUVs) with an average diameter of 90 nm as determined by quasi-elastic light scattering and freeze–fracture techniques (Hope et al., 1985).

Detection of Fusion by Lipid Mixing. Vesicle fusion, as assayed by lipid mixing, was monitored using resonance energy transfer (RET) as described by Struck et al. (1981). Briefly, unlabeled vesicles of the appropriate lipid composition were mixed with similar vesicles containing 0.7 mol % each of NBD-PE and Rh-PE, in a 3:1 ratio. The vesicles were prepared in 300 mM HEPES, pH 7.5, and were subsequently run down a Sephadex G-25 column preequilibrated with 300 mM sucrose/1 mM HEPES, pH 7.5. All buffers were adjusted to the appropriate pH with arginine-free base. The vesicles were then diluted to a concentration of 10 mM total phospholipid. A small aliquot of the vesicles (25 μL) was added to a cuvette containing 1.9 mL of 100 mM sucrose/50 mM MES, pH 5.5. Fusion was initiated by the injection of 80 μL of a 200 mM $CaCl_2$ solution to obtain a final Ca^{2+} concentration of 8 mM. The increase in NBD-PE fluorescence due to fluorescence dequenching as the fluorophores exchanged with lipids on the unlabeled vesicles was monitored using a Perkin-Elmer LS-50 spectrofluorometer equipped with a thermostated, stirred cuvette holder. An excitation wavelength of 465 nm and an emission wavelength of 535 nm were employed, and a cutoff filter for light less than 530 nm was used to reduce the effects of light scattering. Zero fluorescence was set at the residual fluorescence of the NBD-PE in the labeled vesicles while 100% fluorescence corresponded to complete mixing of the lipids. Using a 3:1 ratio of unlabeled to labeled vesicles, complete mixing yields a fluorescence intensity of 75% of the infinitely diluted probe. The fluorescence intensity for the infinitely diluted probe was obtained by the addition of Triton X-100 to a final concentration of 0.1 mM and correcting for its effects on the quantum yield of NBD fluorescence by measuring the decrease in fluorescence of vesicles containing 0.7 mol % NBD-PE after the addition of TX-100 to a 0.1 mM concentration.

In order to determine the effect of Ca^{2+} concentration on the fusion of the LUVs, a sufficient amount of a stock solution of 200 mM $CaCl_2$ was added to a cuvette containing 100 mM sucrose/50 mM MES, pH 5.5, to give the desired Ca^{2+} concentration in a final volume of 2 mL. A 50-μL aliquot of the LUVs (5 mM total phospholipid) was injected into the cuvette, and the increase in NBD fluorescence due to fusion was monitored as described above.

Detection of Fusion by Contents Mixing. Vesicle fusion, as assayed by mixing of aqueous contents, was monitored employing the ANTS/DPX assay (Ellens et al., 1985). Fusion was detected as a decrease in ANTS fluorescence due to quenching by DPX upon mixing of internal contents. Vesicles, DOPC/DOPE/PI/DOPA (25:60:5:10), were prepared in either 25 mM ANTS/40 mM NaCl/10 mM HEPES, pH 7.5, or 90 mM DPX/10 mM HEPES, pH 7.5. Untrapped solute was removed by passing the vesicles over Sephadex G-25 columns equilibrated in 300 mM sucrose/1 mM HEPES, pH 7.5. The fluorescence scale was set such that vesicles containing only ANTS exhibited 100% fluorescence while vesicles containing both ANTS and DPX represented 0% fluorescence.

To monitor fusion, equal concentrations of vesicles containing either ANTS or DPX (125 μM final phospholipid concentration) were added to a cuvette containing 100 mM sucrose/50 mM MES, pH 5.5. At the appropriate time, an aliquot of 200 mM CaCl was added to the cuvette to bring the Ca^{2+} concentration to 8 mM. The ANTS fluorescence was followed continuously on a Perkin-Elmer LS-50 fluorometer employing an excitation wavelength of 272 nm and an emission wavelength of 545 nm, and using an emission cutoff filter for wavelengths <530 nm.

To monitor leakage of vesicle contents, vesicles containing ANTS and DPX were added to a cuvette containing 100 mM sucrose/50 mM MES, pH 5.5, to a final lipid concentration of 125 μM. ANTS fluorescence was monitored, and Ca^{2+}-induced leakage of contents was detected as an increase in fluorescence since the quenching of ANTS fluorescence by DPX is strongly dependent on the concentration of DPX. The fluorescence scale was set such that vesicles containing ANTS and DPX represented 0% fluorescence and 100% fluorescence was obtained by the addition of cholate to these vesicles to achieve a final concentration of 0.5% (w/v).

Induction of DOPA Asymmetry. The transport of phosphatidic acid to the inner monolayer was monitored using TNS as described by Eastman et al. (1991). Briefly, unlabeled vesicles containing 10 mol % DOPA were prepared in 300 mM HEPES, pH 7.5, and passed down a Sephadex G-25 column equilibrated in 300 mM sucrose/1 mM HEPES, pH 7.5. In order to induce DOPA transport, the external pH of the vesicles was reduced by diluting an equal volume of vesicles with a buffer containing 100 mM sucrose/20 mM citrate, pH 4.0. To stop transport, 100 μL of the vesicles was added to 500 μL of ice-cold 100 mM citrate/100 mM ammonium acetate, pH 6.0, and the vesicles were stored on ice. Asymmetry was assessed by adding 2 mL of 3 μM TNS in 5 mM HEPES/5 mM acetate, pH 7.0, and reading the fluorescence using excitation and emission wavelengths of 321 and 445 nm, respectively. This value was normalized with respect to vesicles containing no DOPA (100% fluorescence). The external concentration of DOPA over time was calculated by comparing the fluorescence of the samples to a standard curve prepared using vesicles containing 0–10 mol% DOPA.

For the transport of DOPA to the outer monolayer, LUVs were prepared in 300 mM citrate, pH 4.0 (with arginine), and passed down Sephadex G-25 columns equlibrated in 300 mM sucrose/1 mM citrate, pH 4.0. Transport of DOPA to the outer monolayer was initiated by the addition of an equal volume of 100 mM sucrose/20 mM HEPES, pH 7.5. The ability of Ca^{2+} to induce vesicle fusion was monitored using the RET assay.

^{31}P NMR Studies. Frozen and thawed multilamellar vesicles were prepared by hydration in a 100 mM surcrose/50

mM MES, pH 5.5 buffer and then were freeze–thawed 5 times. The proton-decoupled 81.0-MHz ^{31}P NMR spectra were then recorded employing a Bruker MSL 200 spectrometer under the following conditions. The free induction decay (FID) was collected using a pulse width of 3.7 μs (90°) with an interpulse delay of 1.0 s. After 1000 scans, the FID was Fourier-transformed employing a line-broadening function of 25 Hz. Sufficient 1 M $CaCl_2$ was added to the liposomes to bring the Ca^{2+} concentration to 100 mM, a value in large excess of the DOPA concentration (100 mM total lipid, 10 mM DOPA). The MLVs were freeze–thawed 2 additional times to achieve transbilayer equilibration of the Ca^{2+}, and spectra were then re-recorded.

Freeze–Fracture Electron Microscopy. Vesicles used for freeze–fracture studies were prepared as for fusion assays. Vesicles (100 mM total lipid) containing either no DOPA or 10 mol % DOPA were prepared in 300 mM HEPES and passed down Sephadex G-25 columns equilibrated in 300 mM sucrose/1 mM HEPES, pH 7.5. The vesicles were then mixed with an equal volume of 100 mM sucrose/20 mM citrate, pH 4.0, and incubated for times up to 4 h at pH 4.0 and then passed down Sephadex G-50 columns equilibrated in 100 mM sucrose/50 mM MES, pH 5.5.

To induce fusion, a small aliquot of 200 mM $CaCl_2$ was added to the vesicles to bring the Ca^{2+} concentration to 8 mM. Vesicles were incubated with Ca^{2+} for times t = 0, 5, 10, 15, and 30 s before fusion was stopped. Fusion was stopped by adding 100 μL of the vesicle solution to a tube containing 25 μL of 200 mM EGTA. Glycerol was then added to the samples (10 mM phospholipid) to a final concentration of 25%. Samples were then frozen in Freon cooled by liquid nitrogen and fractured. Platinum/carbon replicas of the samples were prepared as previously described (Fisher & Branton, 1974).

Results

Vesicle Composition. Previous studies have shown that vesicles composed of phosphatidic acid (PA) or mixtures of PE and PA fuse readily in the presence of Ca^{2+} (Hope et al., 1983). The incorporation of PC into the membrane increases their stability and prevents Ca^{2+}-induced fusion at high PC contents (Düzgunes, 1985). In the studies performed here, LUVs composed of various mixtures of DOPC, DOPE, PI, and DOPA were first tested for their ability to fuse in the presence of Ca^{2+}. PI was incorporated to prevent aggregation of vesicles containing little or no DOPA. It was also chosen because earlier studies have shown that it is not transported to the inner monolayer of vesicles under conditions where PA is transported (Eastman et al., 1991) and that it is a nonfusogenic lipid (Sundler & Papahadjopoulos, 1981). PC and PE are zwitterionic lipids that are also not transported in response to transmembrane pH gradients. It was found that LUVs formed from mixtures of these lipids in a DOPC:DOPE:PI ratio of 25:60:5 did not fuse appreciably in the presence of Ca^{2+} but did when 10 mol % DOPA was present. Further, this system did not fuse in acidic environments which was important in order to allow the generation of lipid asymmetry by the transmembrane pH gradient.

Effect of Ca^{2+} on LUV Fusion. The effect of Ca^{2+} concentration on the fusion of LUVs containing 10 mol % PA (DOPC:DOPE:PI:DOPA ratio of 25:60:5:10) is shown in Figure 1A. The extent and initial rate of fusion are influenced greatly by the concentration of Ca^{2+} up to about 8 mM Ca^{2+}, where nearly 100% fusion occurs within 30 s. Higher concentrations of Ca^{2+} had little further effect on the extent of fusion and led to very rapid fusion rates which were inconvenient to measure. The initial rate of vesicle fusion for vesicles

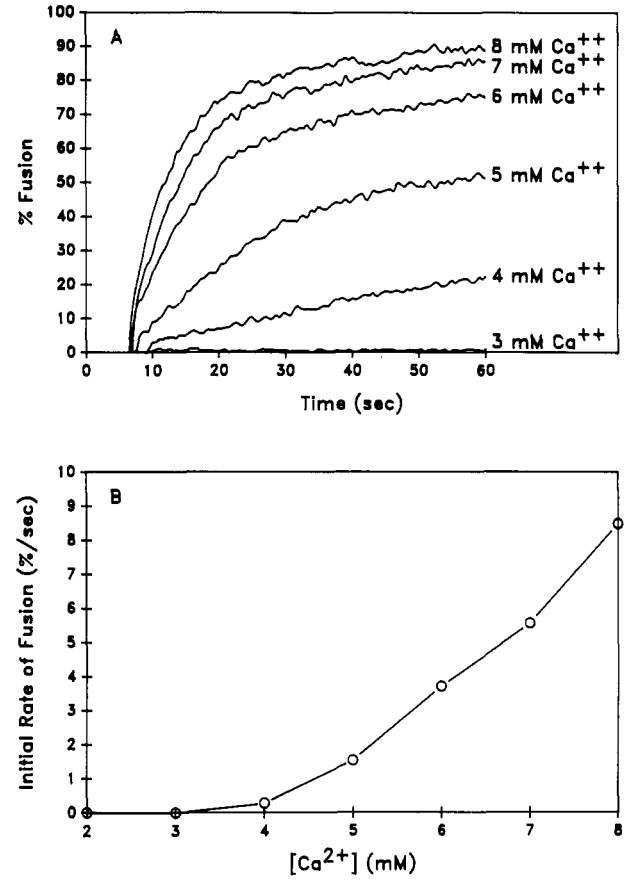

FIGURE 1: (A) Fluorescence traces of the effect of Ca^{2+} concentration on the fusion of 100-nm LUVs composed of DOPC/DOPE/PI/DOPA (25:60:5:10). Labeled and unlabeled vesicles were prepared in 300 mM HEPES, pH 7.5, and mixed in a 1:3 ratio. The vesicles were passed down a Sephadex G-25 column equilibrated in 300 mM sucrose/1 mM HEPES, pH 7.5. The vesicles were diluted to a concentration of 5 mM total phospholipid. Fifty microliters of the vesicles was injected into a cuvette containing the desired Ca^{2+} concentration, and fusion was monitored by the resonance energy transfer (RET) assay (seem Materials and Methods). (B) Initial rate of vesicle fusion.

containing 10 mol % DOPA as a function of Ca^{2+} concentration is shown in Figure 1B.

LUV Fusion Assayed by Mixing of Internal Contents. In order to establish that the lipid mixing assay reflects vesicle fusion, the mixing of internal aqueous contents was monitored employing the ANTS/DPX assay (see Materials and Methods) developed by Ellens et al. (1985). When the LUV system containing 10 mol % DOPA was employed, the addition of 8 mM Ca^{2+} resulted in rapid mixing of internal contents as reflected by a decrease in ANTS fluorescence of approximately 30%. Subsequently, the ANTS fluorescence increased. This was attributed to leakage of contents as demonstrated in LUVs containing both ANTS and DPX. It was noted that the onset of leakage was delayed with respect to the mixing of aqueous contents and that the kinetics of fusion were similar to those observed by the lipid mixing assay.

Effect of DOPA Content on LUV Fusion. Vesicles were prepared with DOPA concentrations ranging from 0 to 10 mol % (in 1% increments). These vesicles were tested for fusion in the presence of 8 mM Ca^{2+} using the resonance energy transfer (RET) assay. Fluorescence traces of the LUVs containing various concentrations of DOPA are shown in Figure 2. The vesicles containing no DOPA fuse to only a small extent, whereas the addition of as little as 1% DOPA greatly enhances fusion. The extent of fusion continues to increase with increasing contents of DOPA and approaches

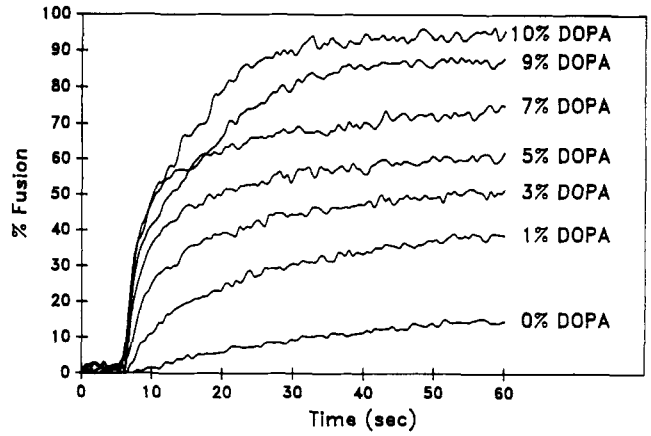

FIGURE 2: Fluorescence traces indicating the effect of DOPA concentration on fusion of 100-nm-diameter LUVs composed of DOPC, DOPE, PI, and varying amounts of DOPA. Vesicles, both labeled and unlabeled, were prepared containing various amounts of DOPA (0–10 mol %). The vesicles were hydrated in 300 mM HEPES, pH 7.5, mixed in a 1:3 ratio, and passed down a Sephadex G-25 column equilibrated in 300 mM sucrose/1 mM HEPES, pH 7.5. The vesicles were diluted to 10 mM total phospholipid, and 25 μL was added to a cuvette containing 1.9 mL of 100 mM sucrose/50 mM MES, pH 5.5. To initiate fusion, 80 μL of 200 mM $CaCl_2$ was added to bring the final Ca^{2+} concentration to 8 mM. Fusion was monitored by the resonance energy transfer assay.

100% at concentrations of DOPA greater than 7 mol % of the total phospholipid.

Effect of DOPA Asymmetry on LUV Fusion. Previous work has shown that transmembrane pH gradients can induce asymmetric transbilayer distributions of DOPA in LUVs (Hope et al., 1989; Eastman et al., 1991). In particular, in LUVs with a basic interior, DOPA will move to the inner monolayer to the extent that more than 95% of the total DOPA is present in this monolayer. This asymmetry can be readily assayed employing TNS (see Materials and Methods), and the ability of a transmembrane pH gradient (pH_i = 7.5; pH_o = 4.0) to induce the inward movement of DOPA is shown in Figure 3A. It is important to note that the other lipids in this sytem (DOPE, DOPC, and PI) are not transported to the inner monolayer of LUVs under these conditions (Eastman et al., 1991). The transport of DOPA to the inner monolayer is rapid with essentially all the external DOPA being transported to the inner monolayer after a 4-h incubation at room temperature (21 °C).

The ability of Ca^{2+} to induce fusion of LUVs containing 10 mol % DOPA was monitored at various times during the induction of DOPA asymmetry (Figure 3B). Immediately after the transmembrane ΔpH was established, the vesicles fused at the same rate and to the same extent as vesicles containing 10 mol % DOPA in the absence of a pH gradient. However, the rate and extent of fusion decreased markedly as the external DOPA concentration decreased, indicating that the fusion of the LUVs was regulated by the amount of DOPA in the outer monlayer. These results are consistent with the time course of DOPA transport (Figure 3A) and the dependence of fusion on the DOPA content of these LUVs (Figure 2).

The ability of DOPA transbilayer asymmetry, where DOPA is preferentially localized to the inner monolayer, to inhibit fusion of DOPA-containing LUVs is clearly demonstrated in Figure 3. These results show that the characteristics of the outer monolayer can regulate the fusion characteristics of the bilayer as a whole. In order to study this ability further, LUVs were prepared such that all of the DOPA from the inner monolayer was transported to the external monolayer. The

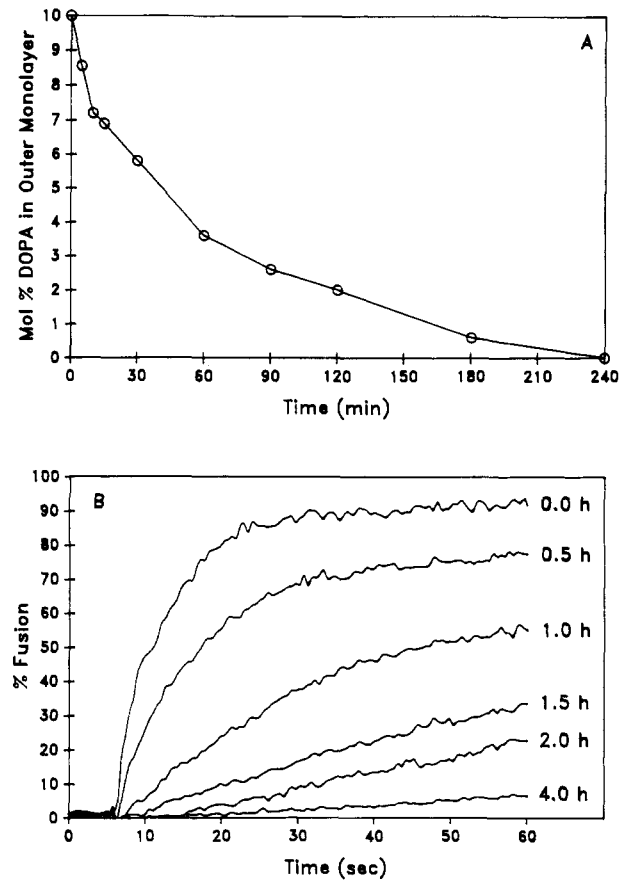

FIGURE 3: (A) Detection of the transbilayer transport of DOPA in response to a transmembrane ΔpH employing TNS (see Materials and Methods). (B) Effect of this transbilayer asymmetry on the fusion of DOPC/DOPE/PI/DOPA (25:60:5:10) LUVs. Vesicles were prepared with an internal pH of 7.5 as described under Materials and Methods. A transmembrane pH gradient was established across the LUVs, and the ability of the LUVs to fuse was monitored at various times after the induction of the ΔpH, ranging from t = 0 to t = 4 h.

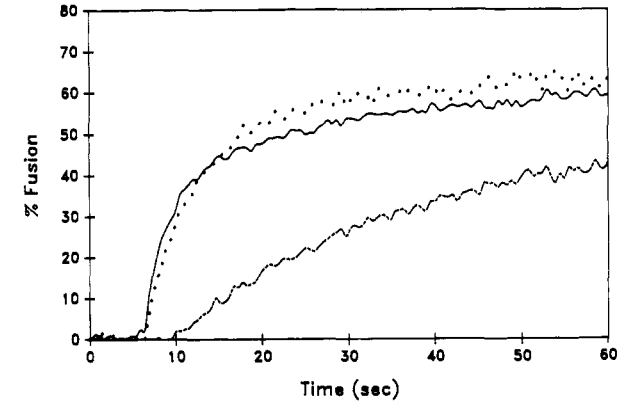

FIGURE 4: Fluorescence traces showing the extent of vesicle fusion for vesicles containing 2.5 mol % DOPA at times t = 0 (---) and t = 4 h (···) after inducing transport of the DOPA to the outer monolayer (see Materials and Methods) and of vesicles containing 5 mol % DOPA (—).

fusion characteristics of these vesicles were then compared to vesicles containing symmetrically distributed DOPA at the same external DOPA concentration. This was performed by preparing LUVs containing 2.5 mol % DOPA, inducing the asymmetric distribution of the DOPA in response to a transmembrane pH gradient (inside acidic) to achieve 5 mol % DOPA in the outer monolayer and comparing the rate and extent of fusion of these LUVs to LUVs containing 5 mol %

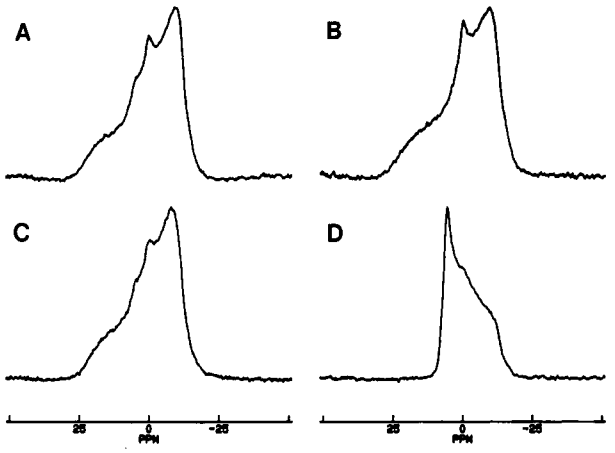

FIGURE 5: ^{31}P NMR spectra of MLVs composed of DOPC/DOPE/PI (25:60:5; mol/mol/mol) in the absence (A) and presence (B) of excess Ca^{2+} or of DOPC/DOPE/PI/DOPA (25:60:5:10) in the absence (C) and presence (D) of excess Ca^{2+}. Spectra were first recorded in the absence of Ca^{2+}. Following the addition of excess Ca^{2+}, the MLVs were subjected to two freeze–thaw cycles, and the spectra were re-recorded.

DOPA symmetrically distributed between the two monolayers. The results of this experiment are shown in Figure 4. After all the DOPA was transported from the inner monolayer to the outer monolayer (final DOPA concentration in the outer monolayer 4.9 mol %), the vesicles containing only 2.5 mol % DOPA are shown to fuse at the same rate and to the same extent as vesicles containing 5 mol % DOPA.

Polymorphic Phase Preferences. Intermediates in bilayer to hexagonal (H_{II}) phase transitions, such as inverted vesicles and interlamellar attachment sites, are likely intermediates in membrane fusion processes (Cullis & Hope, 1978; Verkleij, 1984; Siegel et al., 1989; Ellens et al., 1989). ^{31}P NMR experiments were performed to determine the structural preferences of lipids comprising the outer monolayer of fusing and nonfusing LUVs, in the absence and presence of excess calcium. As shown in Figure 5, MLVs composed of DOPC/DOPE/PI (25:60:5) exhibit bilayer ^{31}P NMR spectra both in the presence and in the absence of Ca^{2+}. However, the addition of 10 mol % DOPA resulted in MLVs which exhibit bilayer structure in the absence of Ca^{2+} but undergo a transition to the H_{II} phase in the presence of excess Ca^{2+}. This is indicated by the transition from a ^{31}P NMR line shape with a low-field shoulder and high-field peak to a spectrum which exhibits reversed asymmetry and is a factor of 2 narrower (Cullis & de Kruijff, 1979).

Freeze–Fracture Studies of Vesicle Fusion. Freeze–fracture studies were performed to visualize the fusogenic behavior of vesicle systems exhibiting symmetric and asymmetric transbilayer lipid distributions. As shown in Figure 6, LUVs which do not contain DOPA do not fuse appreciably either in the presence or in the absence of Ca^{2+} (8 mM). This is in agreement with the RET assay results (see Figure 2). On the other hand, LUVs containing 10 mol % DOPA (in the absence of a pH gradient) fuse very quickly in the presence of 8 mM Ca^{2+}. After 5 s, many large structures are visible, and almost all the LUVs appear to have undergone at lease one round of fusion. After 30 s, all the vesicles have fused into huge complexes. These results correspond well with the fluorescence data which show a very fast initial rate of fusion for the 10% DOPA vesicles and nearly 100% lipid mixing. In contrast, vesicles containing 10 mol % DOPA but where the DOPA has been sequestered to the inner monolayer due to a pH gradient (interior basic) show very little fusion in the presence of 8 mM calcium even after 30 s.

Discussion

The results of this investigation clearly demonstrate the potential regulatory role of lipid asymmetry in membrane fusion processes. This is shown by the strong correlation between the amount of DOPA in the outer monolayer of the LUVs employed here and fusion detected by the resonance energy transfer technique and freeze–fracture procedures. Three aspects of this work which are of interest concern the influence of the inner monolayer on the fusion process, the mechanism of fusion, and the relation to regulation of fusion processes in vivo. We discuss each of these points in turn.

The results presented here show that as that amount of DOPA in the outer monolayer of DOPC/DOPE/PI/DOPA (25:60:5:10) LUVs decreases due to net transport of DOPA to the inner monolayer, the fusion rate decreases correspondingly (Figure 3). This is perhaps not surprising given the strong influence of DOPA content on fusion; however, it clearly establishes the importance of the outer monolayer lipid composition of the external monolayers proximate to the fusion interface, rather than the total lipid composition, as a determinant of fusion. A related question concerns the role of the inner monolayer, and whether the composition of the inner monolayer exerts any influence on the propensity of the LUV as a whole to fuse. The results of Figure 4 where LUVs containing only 2.5% DOPA, but where all the DOPA is located in the outer monolayer, fuse at effectively the same rate as LUVs containing 5% DOPA symmetrically distributed across the bilayer suggest that it does not. The lack of influence of the composition of the inner monolayer on the rate and extent of fusion argues for the validity of lipid asymmetry as a regulatory agent in fusion in vivo and has interesting implications for generating more controlled fusion processes. In particular, fusion between model membrane LUV systems in vitro is, in contrast to membrane fusion in vivo, generally a leaky process. This is at least in part because the stimuli employed to initiate fusion in vitro commonly promote the hexagonal (H_{II}) phase in the lipid mixture (Hope & Cullis, 1981; Burger & Verkleij, 1990). End-stage formation of hexagonal structure is not compatible with maintenance of a permeability barrier. However, systems exhibiting asymmetric transbilayer distributions of lipid clearly have the potential to be self-regulating and possibly to exhibit leak-tight fusion. For example, in asymmetric LUV systems exhibiting a fusogenic outer monolayer but where the overall lipid mixture will not support fusion, fusion will presumably proceed until the fusogenic potential of the outer monolayer is dissipated by mixing with inner monolayer lipids. It will be of particular interest to determine the leakiness of fusion events in such systems.

Regarding the mechanism of fusion, the results presented here are consistent with a fusion process with relies on the ability of component lipids to adopt transitory nonbilayer structures. This is indicated by the fact that MLVs composed of DOPC/DOPE/PI (25:60:5) did not adopt the H_{II} phase in the presence of excess Ca^{2+} and LUVs with the same lipid composition did not fuse appreciably in the presence of Ca^{2+}, whereas the addition of 10% DOPA resulted in the ability of excess Ca^{2+} to induce the H_{II} phase and also stimulated fusion between corresponding LUVs. As has been well discussed elsewhere (Cullis & Hope, 1978; Siegel et al., 1989; Ellens et al., 1989), the actual intermediary in fusion is unlikely to be the hexagonal phase per se. More logical structures include the inverted micelle and interlamellar attachment sites, which are likely intermediates in the bilayer-to-H_{II} and bilayer-to-inverted cubic phase transition processes, respectively (Cullis

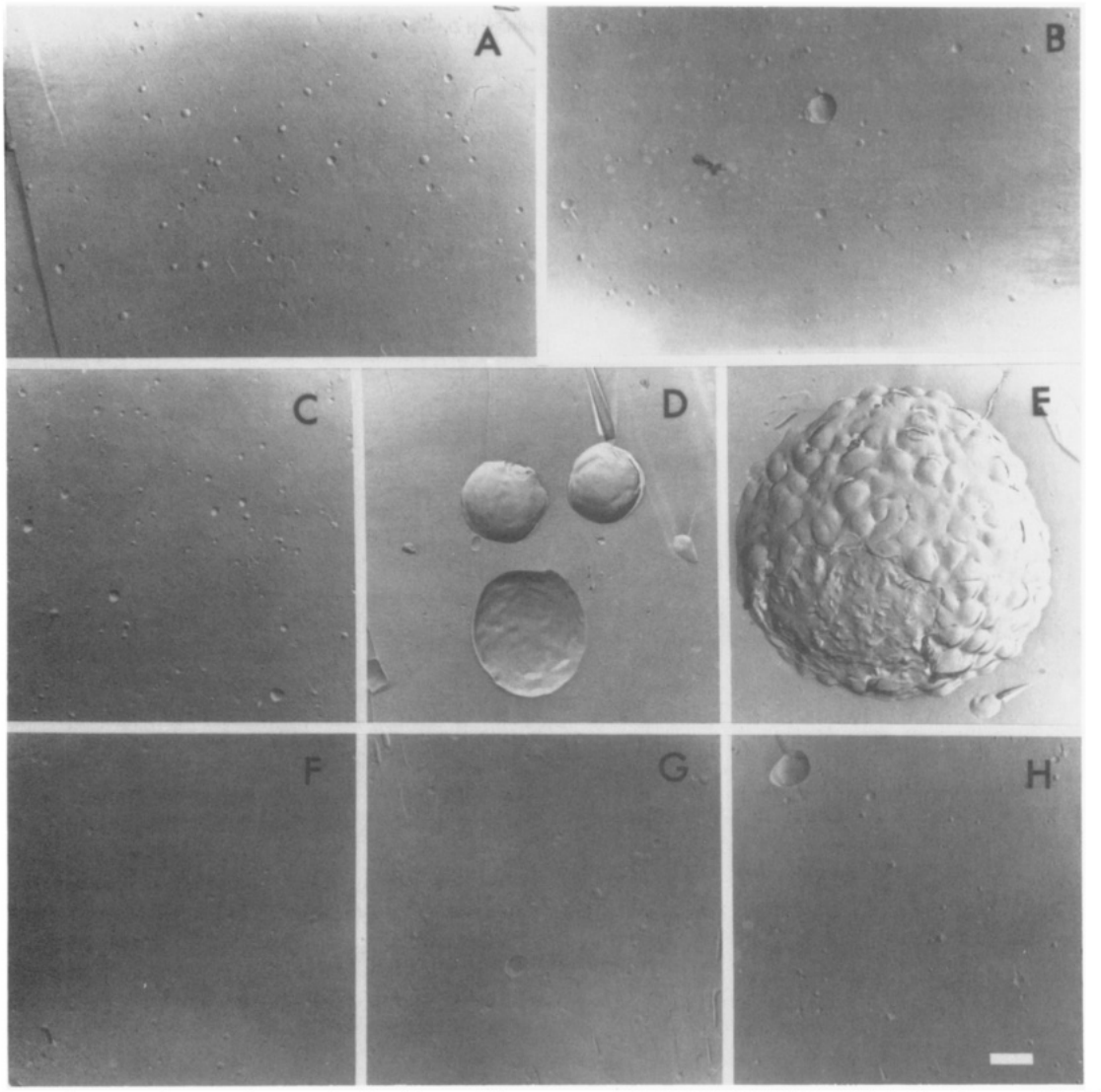

FIGURE 6: Freeze–fracture electron micrographs of various vesicle systems. DOPC/DOPE/PI (25:60:5) in the absence of Ca^{2+} (A) and 30 s after the addition of 8 mM Ca^{2+} (B). DOPC/DOPE/DOPA (25:60:5:10) in the absence of Ca^{2+} (C) and at various times after the addition of 8 mM Ca^{2+} [5 s (D) and 30 s (E)]. DOPC/DOPE/PI/DOPA (25:60:5:10) vesicles which have been exposed to a transmembrane pH gradient for 4 h in order to induce PA asymmetry. In the absence of Ca^{2+} (F) and at various times after the addition of Ca^{2+} [5 s (G) and 30 s (H)]. Fusion reactions were terminated during the time course by the addition of excess EGTA (see Materials and Methods).

& Hope, 1978; Siegel et al., 1989).

The regulatory role that lipid asymmetry could play in vivo is of major interest. Two types of regulation are clearly possible—a passive form where the previously generated static asymmetry determines whether fusion can proceed and a more active regulation where the local generation of asymmetry promotes or inhibits fusion. An example of passive regulation could be the stable transbilayer asymmetry observed for plasma membranes of eukaryotes. The presence of phosphatidylcholine and/or sphingomyelin in the outer monolayer inhibits fusion with extracellular entities except under exceptional conditions. Alternatively, the inner monolayer composed predominantly of phosphatidylethanolamine and phosphatidylserine would be expected to fuse more readily with internal organelles or secretory vesicles in response to local stimuli such as increased Ca^{2+} concentrations.

The possibility that lipid asymmetry may actively, locally regulate fusion is more speculative and requires that lipids can be quickly mobilized from one side of the bilayer to the other as appropriate, or can be rapidly generated on demand. The relatively slow rates of transbilayer lipid movement in plasma membranes suggest that transbilayer mobilization would not be a feasible regulatory process in vivo. However, in membranes such as the endoplasmic reticulum membrane, where transbilayer flip-flop rates are rapid (Zilversmit & Hughes, 1977; Bishop & Bell, 1985), it is possible that fusion could be regulated by such a mechanism. Alternatively, of course, the local enzymatic generation of fusogenic lipids such as diglycerides or phosphatidic acid could also result in a similar end.

In summary, the results of this investigation clearly demonstrate the potential regulatory role of lipid asymmetry in membrane fusion phenomena. Further, the properties of the outer monolayers nearest the fusion interface appear to determine the fusogenic tendencies of the bilayer as a whole.

Registry No. Ca^{2+}, 7440-70-2; DOPC, 4235-95-4; DOPE, 4004-05-1; DOPA, 61617-08-1.

REFERENCES

Bishop, W. R., & Bell, R. M. (1985) *Cell 42*, 51–60.
Burger, K. N. J., & Verkleij, A. J. (1990) *Experientia 46*, 631–644.
Cullis, P. R., & Hope, M. J. (1978) *Nature 271*, 672–674.
Cullis, P. R., & deKruijff, B. (1979) *Biochim. Biophys. Acta 559*, 399–420.
Düzgunes, N. (1985) *Subcell. Biochem. 11*, 195–286.

Eastman, S. J., Hope, M. J., & Cullis, P. R. (1991) *Biochemistry 30*, 1740-1745.
Ellens, H., Bentz, J., & Szoka, F. C. (1985) *Biochemistry 24*, 3099-3106.
Ellens, H., Siegel, D. P., Alford, D., Yeagle, P. L., Boni, L., Lis, L. J., Quinn, P. Q., & Bentz, J. (1989) *Biochemistry 28*, 3692-3703.
Fisher, K., & Branton, D. (1974) *Methods Enzymol. 32*, 35.
Hope, M. J., & Cullis, P. R. (1981) *Biochim. Biophys. Acta 640*, 82-90.
Hope, M. J., Bally, M. B., Webb, G., & Cullis, P. R. (1985) *Biochim. Biophys. Acta 812*, 55-65.
Hope, M. J., Walker, D. C., & Cullis, P. R. (1983) *Biochem. Biophys. Res. Commun. 110*, 15-22.
Hope, M. J., Redelmeier, T., Wong, K. F., Rodrigueza, W., & Cullis, P. R. (1989) *Biochemistry 28*, 4181-4187.
Mayer, L. D., Hope, M. J., & Cullis, P. R. (1985) *Biochim. Biophys. Acta 817*, 193-196.
Redelmeier, T. E., Hope, M. J., & Cullis, P. R. (1990) *Biochemistry 29*, 3046-3053.
Santini, M. T., Indovina, P. L., Cantafora, A., & Blotta, I. (1990) *Biochim. Biophys. Acta 1023*, 298-304.
Sessions, A., & Horwitz, A. F. (1981) *FEBS Lett. 134*, 75-78.
Sessions, A., & Horwitz, A. F. (1983) *Biochim. Biophys. Acta 728*, 103-111.
Siegel, D. P., Burns, J. L., Chestnut, M. H., & Talmon, Y. (1989) *Biophys. J. 56*, 161-169.
Struck, D. K., Hoekstra, D., & Pagano, R. E. (1981) *Biochemistry 20*, 4093-4099.
Sundler, R., & Papahadjopoulos, D. (1981) *Biochim. Biophys. Acta 649*, 743-750.
Tullius, E. K., Williamson, P., & Schlegel, R. A. (1989) *Biosci. Rep. 9(5)*, 623-633.
Verkleij, A. J. (1984) *Biochim. Biophys. Acta 779*, 43-63.
Zilversmit, D. B., & Hughes, M. E. (1977) *Biochim. Biophys. Acta 469*, 99-110.

Synapsin IIa: Expression in Insect Cells, Purification, and Characterization†

Yaw L. Siow,*,‡ Tamie J. Chilcote,‡ Fabio Benfenati,‡,‖ Paul Greengard,‡ and Gerald Thiel‡,§
Laboratory of Molecular and Cellular Neuroscience, The Rockefeller University, New York, New York 10021, and Institute of Human Physiology, University of Modena, 41110 Modena, Italy
Received November 12, 1991; Revised Manuscript Received February 14, 1992

ABSTRACT: Synapsin IIa belongs to a family of neuron-specific phosphoproteins called synapsins, which are associated with synaptic vesicles in presynaptic nerve terminals. In order to examine the biochemical properties of synapsin IIa, and ultimately its physiological function, purified protein is required. Since attempts to purify significant quantities of synapsin IIa, an isoform of the synapsins, from mammalian brain have proven difficult, we undertook the production of recombinant synapsin IIa by utilizing the baculovirus expression system. Rat synapsin IIa cDNA was introduced into the baculovirus genome via homologous recombination, and the recombinant baculovirus was purified. *Spodoptera frugiperda* (Sf9) cells infected with this virus expressed synapsin IIa as 5% of the total cellular protein. The recombinant protein was extracted from the particulate fraction of the infected Sf9 cells with salt and a nonionic detergent and purified by immunoaffinity chromatography. The purified synapsin IIa was phosphorylated by the catalytic subunit of cAMP-dependent protein kinase to a stoichiometry of 0.8 mol of phosphate/mol of protein. Metabolic labeling with [^{32}P]P_i demonstrated synapsin IIa phosphorylation in infected Sf9 cells. Using a homogenate of uninfected Sf9 cells, a cAMP-dependent protein kinase activity which can phosphorylate synapsin IIa was detected. Limited proteolysis of recombinant synapsin IIa phosphorylated in vitro and in vivo resulted in identical phosphopeptide maps. Further, synapsin IIa, like synapsin I, binds with high affinity in a saturable manner to synaptic vesicles purified from rat cortex.

The synapsins are a family of neuronal phosphoproteins that are localized on the cytoplasmic surface of small synaptic vesicles [for a review, see De Camilli et al. (1990)]. This family is comprised of four proteins, synapsins Ia and Ib (collectively termed synapsin I) and synapsins IIa and IIb (collectively termed synapsin II). The mRNAs of synapsins Ia and Ib and of synapsins IIa and IIb are generated by alternative splicing of transcripts from two different genes (Südhof et al., 1989). A comparison of the nucleotide and deduced amino acid sequences of the synapsins reveals a high degree of identity in more than half of each protein. The NH_2-terminal domains, A, B, and C, of synapsin I and synapsin II show an overall sequence identity of 70% covering 420 residues (Südhof et al., 1989). The synapsins differ extensively, however, in their COOH-termini, except for synapsin Ia and synapsin IIa which contain 76% identical amino acids in the final 50 residues.

The functional behavior of the synapsins is affected by their phosphorylation states, which are regulated by a variety of physiological and pharmacological stimuli that alter synaptic efficacy (Nestler & Greengard, 1983). All four synapsins share an NH_2-terminal phosphorylation site (site 1) for cAMP-dependent protein kinase and Ca^{2+}/calmodulin-dependent protein kinase I; synapsins Ia and Ib have two ad-

† This work was supported by U.S. Public Health Service Grant MH-39327, by Cooperative Agreement CR-813286 from the Environmental Protection Agency, and by the Rose M. Badgeley Charitable Trust.
* Address correspondence to this author at the Laboratory of Molecular and Cellular Neuroscience, The Rockefeller University, 1230 York Ave., Box 296, New York, NY 10021-6399.
‡ The Rockefeller University.
‖ University of Modena.
§ Present address: Institute for Genetics, University of Cologne, D-5000 Cologne, Germany.

0006-2960/92/0431-4268$03.00/0