Intermembrane Molecular Contacts by Polymyxin B Mediate Exchange of Phospholipids†

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ABSTRACT: Direct intermembrane exchange of dimyristoylphosphatidylmethanol is mediated by polymyxin B (PxB), a cationic amphipathic cyclic decapeptide. The possibility that the phospholipid exchange is mediated by solubilization of phospholipids or by fusion of vesicles is ruled out. By kinetic and spectroscopic methods it is shown that the exchange occurs directly through vesicle−vesicle contacts formed by a few PxB molecules. The contact is stable on the time scale of several minutes such that neither PxB nor the vesicles in the pair forming a contact exchange with excess vesicles. Several contacts may be formed on a vesicle, which leads to the formation of a cluster of vesicles, and the lipid molecules on the outer monolayers of vesicles exchange throughout the cluster. Kinetics of substrate replenishment during processive interfacial catalysis suggests that the exchange of anionic lipids over the contact occurs at a rate considerably faster than 300 s^{-1}. The exchange through the contact is specific for certain lipids, and phospholipids with a modified head group or phospholipase A_2 bound to a vesicle are not transferred to the other vesicle in contact. Since this phenomenon has not been described before, possible implications of direct vesicle−vesicle exchange of phospholipids through peptide-mediated molecular contacts are discussed. Such a mechanism for intermembrane transfer of phospholipids could be responsible for intracellular trafficking and sorting of phospholipids; it could be a necessary first step for the sequence of events leading to budding, vesiculation, and secretion; and PxB-mediated transfer between the inner and outer membranes of Gram-negative bacteria could also account for its antibiotic action.

Formation of intermembrane contacts is believed to be a necessary first step for several cellular processes. Some of the well-studied cases [reviewed in Sowers (1987), Hoekstra (1994), and Moreau and Cassagne (1994)] include the following. Intracellular transport and sorting of phospholipids requires mechanisms for a selective and rapid transfer from the site of synthesis to the designated targets. Similarly, endocytosis, fertilization, and entry of enveloped viruses requires formation of contacts leading to vesiculation of plasma membrane and uptake. Secretion by regulated exocytosis must also depend on contacts between intracellular vesicles and plasma membrane leading to the formation of fusion pores. In the light of the fact that membranes of a cell have a remarkably constant complement of lipids and proteins, constraints of changes following the intermembrane interaction are critical for cellular function.

Intermembrane contacts could play a role in directed transfer of phospholipids between cellular compartments. Spontaneous transfer of phospholipids between membranes is slow (Brown, 1992) because the rate is limited by the low concentration of solitary monomers in the aqueous phase. Collision-dependent direct vesicle−vesicle transfer of phospholipids is not favored, because such a process involves exposure of phospholipid chains to the aqueous phase. Other mechanisms for rapid transfer of phospholipids include lipid mixing by fission of micelles (Fullington et al., 1990) or fusion of vesicles (Struck et al., 1981). Transfer of phospholipids is also mediated by exchange proteins which form water-soluble complexes with specific phospholipids (Wirtz, 1991). Such mechanisms randomly mix the contents of the donor and acceptor membranes, and their role for transfer of phospholipids to specific targets in trafficking, targeting, and sorting remains doubtful. A novel conceptual and experimental model for a direct and rapid intermembrane exchange of specific phospholipids follows from the results described in this paper: It is shown that direct and rapid exchange of phospholipids is possible through stable vesicle−vesicle contacts formed by a peptide. Preliminary results have been reported elsewhere (Cajal et al., 1995).

A dramatic increase in the apparent initial rate of hydrolysis of DMPM vesicles by phospholipase A_2 (PLA2)[1] or a bacterial lipase is observed in the presence of PxB (Jain et al., 1991b, 1994). The basis for the apparent activation of the observed rate of hydrolysis by PxB is due to a rapid PxB-mediated replenishment of the substrate from excess vesicles to the enzyme-containing vesicles through stable

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[1] Abbreviations: DTPE-DNS, *N*-dansylated 1,2-ditetradecylglycero-*sn*-3-phosphoethanolamine; DMPM, 1,2-dimyristoylglycero-*sn*-3-phosphomethanol; DTPM, 1,2-ditetradecylglycero-*sn*-3-phosphomethanol; PLA2, phospholipase A2 from pig pancreas; NBD-PE, *N*-(7-nitro-2,1,3-benzoxadiazol-4-yl)-1,2-dioleoylphosphatidylethanolamine; PxB, polymyxin B; PyPM, 1-hexadecanoyl-2-(1-pyrenedecanoyl)glycero-*sn*-3-phosphomethanol; Rh-PE, *N*-(lissaminerhodamine-B-sulfonyl)-1,2-dioleoylphosphatidylethanolamine.

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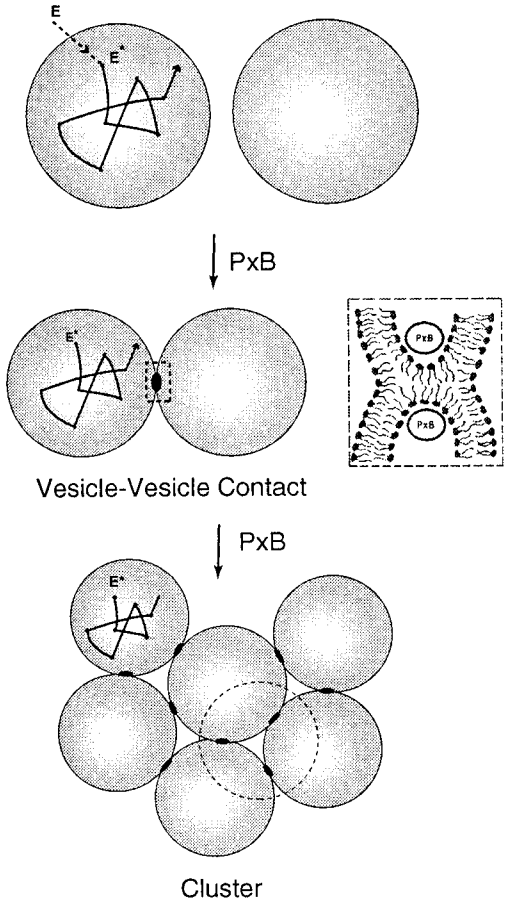

FIGURE 1: Postulated sequence of events to account for the effect of PxB on the interfacial catalysis on DMPM vesicles by PLA2. PLA2 added to an excess of vesicles distributes according to Poisson statistics, such that there is at most 1 enzyme/vesicle. Addition of PxB to vesicles makes vesicle−vesicle contacts which serve as bridges for direct intervesicle exchange of phospholipids. With increasing amounts of PxB, multiple contacts are formed, resulting in larger clusters of vesicles where exchange of outer membrane phospholipids occurs at a fast rate (>300 s^{-1}). Details of the molecular organization of the contact are shown in the inset to the right. Fusion of vesicles occurs only at >0.05 mole fraction of PxB (>250 PxB/vesicle).

vesicle−vesicle contacts formed by PxB. For example, with about 35 PxB molecules/vesicle and at 6 vesicles/enzyme, virtually all the excess substrate becomes available for the hydrolysis by the enzyme added initially. Under these conditions the reaction progresses at a rate that is limited by the chemical step of the interfacial catalytic turnover cycle (Jain et al., 1992). These kinetic features and the effect of PxB on the reaction progress are observed with all secreted PLA2 (Jain et al., 1991c) as well as for the phospholipase activity of a bacterial lipase (Jain et al., 1994). This suggests that the effect of PxB is primarily on vesicles and that the rate of PxB-mediated transfer of phospholipids is >300 s^{-1}.

In this paper we show that PxB mediates direct vesicle−vesicle exchange of DMPM by establishing stable contacts between vesicles. The results are consistent with the sequence of events illustrated in Figure 1. Since this phenomenon has not been described before, this model should be useful for the design of experiments, interpretation of results, and discussion of possible alternatives. An analytical model for the formation of cluster of vesicles is developed in the Appendix.

MATERIALS AND METHODS

PyPM (Molecular Probes), octadecylrhodamine (Molecular Probes), NBD-PE (Avanti), Rh-PE (Avanti), and PxB (Sigma) as pentasodium hydrogen sulfate salt (MW 1815) were used as obtained from the supplier. DTPE-DNS was synthesized as described (Jain & Vaz, 1987). Preparation of PLA2 from pig pancreas, DMPM, DTPM, and protocols for monitoring the time course of hydrolysis of DMPM vesicles in the highly processive scooting mode by PLA2 have been described in detail (Jain et al., 1986a−c, 1991a,b). All spectroscopic and kinetic studies reported in this paper were carried out on small unilamellar vesicles obtained by sonication in a bath-type sonicator (Lab Supplies, Hickesville, NY; Model G112SPIT), of DMPM in water above 35 °C. Covesicles of DTPM containing 2 mol % DTPE-DNS were prepared by evaporating a mixture of the lipid solutions in $CHCl_3$/MeOH (1:1); the dried film was hydrated and then sonicated until clear (typically 2−4 min).

Purity of PxB was checked by HPLC and mass spectrometry. Concentration of the stock solution of PxB in water was determined by amino acid analysis on a Beckman (System 6300) autoanalyzer after 24 h of hydrolysis with 6 M HCl in vacuum at 110 °C. Diluted solutions were obtained from the stock solution in 1 M NaCl so as to avoid adsorption of PxB on the glass walls. The tendency of PxB to adsorb on the glass surfaces also requires care in washing cuvettes and the reaction vessel for pH-stat titration. For example, to prevent carryover of PxB activity, the quartz cuvettes used for spectroscopic measurements were soaked overnight in 60% perchloric acid and then washed thoroughly with distilled water and ethanol.

Kinetic Measurements in the Scooting Mode. Reaction progress curves for the hydrolysis of DMPM were obtained on a Radiometer pH-stat titration assembly with a stirrer speed of about 2000 rpm. The sequence of addition of reactants was critical due to the tight and essentially irreversible binding of PxB to the vesicles. Rapid mixing rates are required to ensure a uniform distribution of the components. Additional precautions are necessary because the binding of PLA2 and PxB to anionic vesicles is rapid and essentially irreversible; therefore, it is not possible to randomly mix the components in the reaction mixture. In fact, the strict sequence of addition of the components to vigorously stirred reaction mixtures forms a critical basis for several protocols designed to obtain specific information about the direct vesicle−vesicle exchange of phospholipids as mediated by PxB. Unless stated otherwise, PxB was added first to the reaction mixture containing 4 mL of 0.5 mM $CaCl_2$ and 1 mM NaCl solution equilibrated at pH 8.0 in a stream of nitrogen. Stock solution of vesicles containing 0.6−1 mg of DMPM was added and the reaction mixture was allowed to equilibrate. The reaction was initiated by adding the enzyme solution, typically 2−30 pmol of PLA2 in 1−10 μL of water. The number of substrate molecules present in the outer monolayer of the vesicles (N_s) was obtained from the extent of hydrolysis under the conditions where there was at most 1 enzyme/enzyme-containing vesicle. The total amount of substrate available in the reaction mixture for the hydrolysis by PLA2 was obtained by adding excess enzyme, so that there was at least one enzyme on every vesicle. On the basis of the total substrate present in the reaction mixture, it was found that typically

about 65% of the total substrate was accessible to the enzyme added to the reaction mixture. As shown elsewhere (Berg et al., 1991), this is expected only if the lipid in the outer monolayer of vesicles is hydrolyzed, the lipid present in the inner monolayer of vesicles does not flip to the outer layer, and the vesicle remains intact even after all the substrate in its outer monolayer is hydrolyzed by PLA2. Results reported in this paper show that these conditions are also satisfied in the presence of PxB.

Exchange of Labeled Phospholipids. Fluorescence spectroscopic measurements were carried out at 25 °C in 10 mM Tris at pH 8.0 on an AB-2 spectrophotometer (SLM-Aminco). When necessary, wavelength scans were corrected for instrumental anomalies by a program provided by the manufacturer. Typically the slit widths were kept at 4 nm each and the sensitivity was adjusted to 1% for the Raman peak from the buffer blank. The relative change in the fluorescence, δF, is defined as $(F - F_0)/F_0$, where F_0 is the intensity without PxB and F is the intensity in the presence of PxB. In some cases only net changes in the fluorescence intensity (on an arbitrary scale) are reported.

Protocols for resonance energy transfer from Trp-3 of PLA2 to DTPE-DNS are described elsewhere (Jain & Vaz, 1987). In these experiments we used nonhydrolyzable phospholipid ether analogue (DTPM) so as to avoid complications due to a somewhat rapid rate of exchange of products of hydrolysis. The assay for lipid mixing and for the formation of vesicle−vesicle contact (or aggregation) was based on the resonance energy transfer between NBD-PE and Rh-PE, with an energy transfer distance of about 50 Å (Struck et al., 1981). Typically, DMPM vesicles containing 0.6 mol % probe were prepared from the lipid film obtained from evaporation of the mixture of lipids in chloroform/methanol (3:2). The probe concentration was 0.3 mol % each if both probes were present in the same vesicle.

The exchange or transfer of lipid between vesicles upon the addition of PxB was also assessed by using PyPM as donor vesicles and 100-fold excess DMPM vesicles as acceptors. The assay is based on the increase of the pyrene monomer fluorescence intensity at 395 nm (excitation 346 nm) resulting from the transfer of PyPM to DMPM vesicles. After vesicles of PyPM are mixed with vesicles of DMPM, it is often necessary to allow time for equilibration, possibly of free pyrenedecanoic acid present as a trace impurity, before the addition of PxB.

Light Scattering. Changes in the 90° scattering were measured at 360 nm (slit widths 1 nm each) on the same spectrophotometer, and the relative change in the intensity of the scattered light, δI, is also defined as for the fluorescence intensity.

RESULTS

Unique features of the highly processive reaction progress for interfacial catalysis by PLA2 on vesicles of DMPM provide a basis to monitor vesicle−vesicle exchange of phospholipids. During the hydrolysis of DMPM vesicles, the enzyme (as well as the substrate and products of hydrolysis) does not exchange with excess vesicles (Figure 1, top). Reaction progress for the hydrolysis at a vesicle-to-enzyme ratio of >5 exhibits several characteristic features (Jain et al., 1986a, 1995; Jain & Berg, 1989; Berg et al., 1991) that are not observed for the hydrolysis of soluble

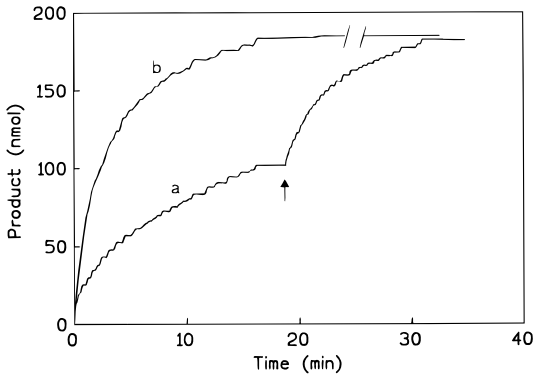

FIGURE 2: Reaction progress curves for the hydrolysis of small sonicated vesicles of DMPM (951 nmol or 176 pmol of vesicles) by PLA2 (21.4 pmol) in 4 mL of 0.5 mM $CaCl_2$ and 1 mM NaCl, pH 8.0, at 23 °C. In all cases, the reaction was initiated by the addition of PLA2. (a) After cessation of hydrolysis, 0.55 nmol of PxB was added to the reaction mixture (at arrow). (b) 0.55 nmol of PxB was added to the reaction mixture 60 s before the reaction was initiated by PLA2. Under the conditions used in this study, each vesicle contains on the average 3 PxB and at most 1 molecule of PLA2.

substrate by soluble enzyme: only a small fraction of the total available substrate is hydrolyzed; in the presence of excess vesicles the extent of hydrolysis is proportional to the amount of enzyme; and the number of substrate molecules hydrolyzed per enzyme is the same as the number of substrate molecules present in the outer monolayer of the enzyme-containing vesicles. Within these constraints the decrease in the rate and cessation of hydrolysis occurs due to local depletion of the substrate on the enzyme-containing vesicles even though excess vesicles are not hydrolyzed.

Reaction Progress Curves in the Presence of PxB. At about 7 vesicles/PLA2 molecule in the reaction mixture, the effect of PxB on the hydrolysis of sonicated DMPM vesicles is shown in Figure 2. In the absence of PxB (first part of curve a) the reaction progress is observed till all the available substrate, about 3500 molecules/enzyme ($= N_S$), is hydrolyzed and the excess substrate is not accessible to the bound enzyme (cf. top panel in Figure 1). Upon the addition of 3 PxB molecules/vesicle at the end of the reaction progress, the reaction is reinitiated immediately and the hydrolysis progresses until another 3200 molecules of the substrate are hydrolyzed. As also shown in this figure (curve b), if the same amount of PxB is added before the addition of the enzyme, about 6700 substrate molecules are hydrolyzed by each enzyme molecule before the reaction stops. These results show that the total number of DMPM molecules hydrolyzed is the same whether PxB is added before or after the initiation of the reaction progress. This interpretation of the reaction progress corresponds to the events of the top and middle panels, or the first step, in Figure 1.

The extent of hydrolysis observed under the conditions of Figure 2 with increasing amounts of PxB is shown in Figure 3. The extent of hydrolysis increases until about 65% of the total substrate present in the reaction mixture is hydrolyzed. About 67% of the total substrate is hydrolyzed if excess enzyme is added in the absence of PxB. These results indicate that, at about 35 PxB molecules/vesicle, virtually all the substrate molecules present in the outer monolayer of the vesicles are hydrolyzed by the same amount of enzyme which hydrolyzed less than 15% of the substrate in the absence of PxB. Therefore, results in Figure 2 and 3

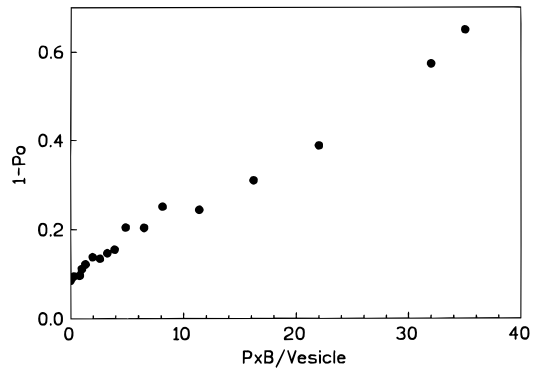

FIGURE 3: Dependence of the extent of hydrolysis of DMPM (951 nmol) vesicles as a function of the number of PxB per vesicle. Vesicles were added to the reaction mixture containing PxB, and PLA2 was added after 60 s. Po represents the amount of substrate that is not hydrolyzed. Maximum product formation corresponds to the hydrolysis of all the available substrate (628 nmol), as also obtained experimentally by adding excess enzyme to the vesicles. Other conditions were as given in the legend of Figure 2. It may be noted here that in the presence of excess PxB the last part of the reaction progress exhibited a slow steady state (~5% of the initial rate) which is probably due to an enhanced transbilayer movement of phospholipids from the inner monolayer of vesicles in a cluster. In such cases, the extent of hydrolysis was obtained by extrapolation of the steady-state phase of the reaction progress to the start of the reaction progress.

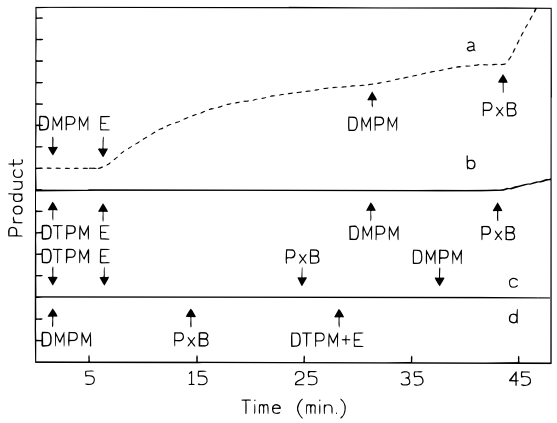

FIGURE 4: Reaction progress for the hydrolysis of DMPM under different sequences of addition of the components that influence the reaction progress. (a, dashed curve) DMPM (2.3 μmol) + E (5.3 pmol) + DMPM (1.1 μmol), and then PxB (12 nmol or 31 PxB/vesicle) after about 20 min. (b) Same as for (a) except that the reaction sequence was initiated with DTPM (300 nmol) followed by E (36 pmol) + DMPM (1.3 μmol) and PxB (3 nmol or 11 PxB/total vesicle in the mixture). (c) Same as for (b) except that the order of addition of PxB and DMPM was reversed. (d) PxB (3 nmol or 16 PxB/vesicle) was added to DMPM (1.1 μmol) vesicles, and a mixture of DTPM (0.2 μmol) and E (5.3 pmol) was added last. It may be noted that hydrolysis was observed only in experiments a and b. Each division on the ordinate corresponds to 45 nmol of products of hydrolysis.

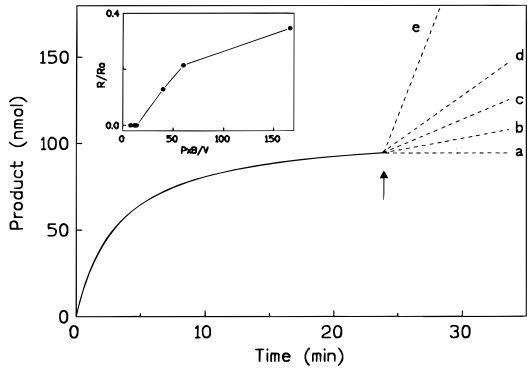

FIGURE 5: Irreversibility of the binding of PxB to anionic vesicles by monitoring intervesicle exchange of PxB. At the end of the reaction progress for the hydrolysis of DMPM vesicles (951 nmol) by PLA2 (20 nmol), the reaction was reinitiated by adding 2.23 nmol of PxB premixed with varying amounts of DTPM vesicles (incubation time 4 min) at (a) 14, (b) 40, (c) 60, or (d) 166 PxB/vesicle. Control: (e) DTPM was added to the reaction mixture followed by 2.23 nmol of PxB. Inset: Plot of the ratio between the rate of hydrolysis (R) corresponding to the reaction curves a−d and the rate when the same amount of free PxB is added to the reaction at different numbers of PxB molecules/vesicle of DTPM.

show that in the presence of PxB more substrate becomes accessible for hydrolysis. This interpretation is consistent with the second step in Figure 1, where all the substrate present in the outer monolayers of the vesicles forming a cluster is now accessible to the enzyme initially present on one of the vesicles in the cluster. This interpretation of reaction progress for the hydrolysis of DMPM vesicles is based on the constraints of catalysis by PLA2 in the highly processive scooting mode (Berg et al., 1991; Jain et al., 1995): the enzyme bound to DMPM vesicles does not exchange with excess vesicles; even under the conditions where the reaction progress has stopped, the enzyme is catalytically functional; and the vesicles which have been hydrolyzed as well as the excess substrate vesicles remain intact. As shown by direct experiments described below, these results are also inconsistent with other interpretations based on the possibility of PxB-mediated intervesicle transfer or exchange of the bound enzyme, or fusion of vesicles, or solubilization of DMPM vesicles.

PxB Does Not Exchange between Vesicles. Kinetic results in Figure 4 show that neither PxB nor PLA2 exchanges between vesicles. As shown in sequence *b*, PLA2 on DTPM vesicles is not available for the hydrolysis of DMPM vesicles; however, hydrolysis is seen on the addition of PxB. The rate of hydrolysis observed under these conditions is slower than that observed if only DMPM vesicles were present in the reaction mixture (sequence *a*). This is an expected result because DTPM is a competitive inhibitor of PLA2 (Jain et al., 1986d). As shown in sequence c, no hydrolysis is seen if PxB is added before the addition of DMPM. Similarly, as shown in sequence d, addition of a mixture of DTPM and PLA2 to DMPM vesicles containing PxB did not initiate the hydrolysis. These results show that PxB added last to a mixture of vesicles immediately establishes a contact between vesicles that permits vesicle−vesicle exchange of phospholipid. Sequences c and d also show that PxB bound to vesicles does not hop from vesicle to vesicle, nor it is available to make contact with vesicles added last. All other permutations of this protocol yielded results (not shown) expected on the basis of the conclusion that PLA2 and PxB bound to anionic vesicles are not transferred to vesicles added afterward.

It may be remarked here that at most only 40 PxB/vesicle can be utilized to form a cluster from which vesicles, enzyme, or PxB are not able to exchange. As shown in Figure 5, with a larger excess of PxB subsequent addition of vesicles can make new contacts, as if excess PxB (beyond 40 PxB/vesicle) were available, on the surface of the cluster or by exchange, to make contact with vesicles added afterward. The relative rate of hydrolysis of DMPM (R/R_0) at different PxB per DTPM vesicle ratios is shown as an inset in Figure

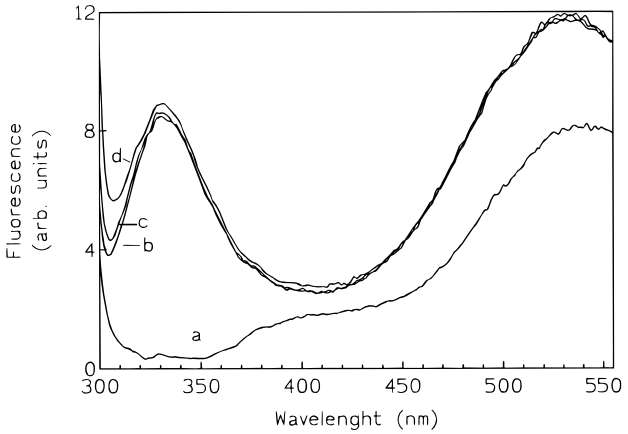

FIGURE 6: Corrected fluorescence emission spectra of (a) 2% DTPE-DNS/DTPM covesicles (0.425 mM); (b) 2% DTPE-DNS/DTPM covesicles (0.425 mM) + PLA2 (0.95 μM); (c) addition of DMPM (0.211 mM) vesicles to (b); and (d) same as (c) after the addition of PxB (2.33 μM). These experiments were carried out in 10 mM Tris/0.5 mM $CaCl_2$, pH 8.0, at 25 °C. Excitation was set at 292 nm. Excitation and emission band passes were 4 nm.

5. *R* is the rate observed when PxB is added premixed with DTPM, and R_0 is the rate when the same amount of PxB is added alone. Although we have not investigated such effects of PxB in detail, these and other results (see below) are consistent with the general conclusion that at low mole fractions, PxB forms contacts which lead to clustering of vesicles. Only excess PxB that is not present in vesicle−vesicle contacts is probably exchangeable. This is because the maximum number of vesicle−vesicle contacts that can be formed will depend on the constraints of stacking of vesicles in a cluster.

PxB Does Not Promote Hopping of PLA2. If the rapid onset of hydrolysis after the addition of PxB (Figure 2) was due to PxB-mediated hopping of the bound PLA2, then all excess vesicles would have been hydrolyzed at the end of the reaction progress. That the intervesicle exchange of PLA2 is not promoted by PxB was also shown by two independent methods.

That PxB does not cause desorption of PLA2 from the interface to the aqueous phase is shown by the fact that the increase in the tryptophan fluorescence intensity observed on the binding of PLA2 to vesicles is not altered in the presence of PxB [data not shown here; see, however, Jain et al. (1991b)]. The possibility of direct PxB-mediated intervesicle transfer of bound PLA2 was ruled out on the basis of resonance energy transfer measurements carried out with nonhydrolyzable DTPM. As shown in Figure 6, PLA2 bound to DTPM vesicles containing 2% DTPE-DNS showed substantial energy transfer from the emission of Trp-3 (Jain & Vaz, 1987). On the addition of DMPM or DTPM vesicles to the probe-containing vesicles the emission intensity at 340 nm (excitation at 292 nm) from Trp-3 of PLA2, or at 495 nm from the dansyl fluorophore acceptor on the bilayer surface, did not change. This is an expected result if the probe or the bound enzyme on the probe-containing vesicle does not exchange with excess vesicles. A change in the fluorescence emission was not detected at low mole fractions of PxB, which indicated that under these conditions there is no net transfer of probe or the bound enzyme. Net transfer of DTPM or DMPM to or away from the probe-containing vesicles is also ruled out by these results because a change in the average separation between Trp and dansyl fluorophore would have resulted in a change in the emission due to energy transfer. On the other hand, fusion induced by calcium resulted in the dilution of the probe or the enzyme, and a similar change was also observed with PxB at mole fractions >0.06; in both cases a substantial decrease in the emission intensity at 495 nm was observed (results not shown).

PxB Does Not Induce Exchange of Head-Group-Labeled Phospholipids. Additional control experiments were carried out with 6% DTPE-DNS in DTPM, where the dansyl fluorescence is self-quenched. No change in the fluorescence emission of these vesicles was observed upon mixing with DMPM vesicles, or after the addition of PxB to this mixture of vesicles. These results imply that DNS-phospholipids are not transferred through vesicle−vesicle contacts induced by PxB. Since both populations of vesicles contain excess DTPM or DMPM, their exchange between the vesicles will not be detected by this protocol. This is an expected result in terms of the sequence of events shown in Figure 1; it is also consistent with results shown in Figures 2−4, and it is further substantiated by results described later in this paper.

The fact that PxB does not mediate a transfer of PLA2 or DTPE-DNS also rules out nonspecific lipid mixing due to fusion of vesicles by PxB. Not only are the results shown in Figures 2−4 inconsistent with the fusion mechanism, but direct measurements (see below) also show that PxB promotes fusion of anionic vesicles only at >0.05 mole fraction, which is at least 100-fold above the concentrations of PxB used for the substrate replenishment.

Mixing of probes accompanied by fusion of vesicles can be measured by release of self-quenching or a change in the emission due to resonance energy transfer (RET). For example, mixing of DMPM vesicles with covesicles containing 4% octadecylrhodamine (self-quenched) in DMPM did not cause an increase in the rhodamine emission on the addition of PxB below 0.02 mole fraction (results not shown). An increase is expected if the probe was surface-diluted due to the transfer of the probe (Stegmann et al., 1993; Jain et al., 1986a). This conclusion is reinforced by results shown in Figure 7. Resonance energy transfer from NBD-PE to Rh-PE is significant when these probes are present in the same vesicle. As shown in Figure 7A, the RET spectrum of covesiculated probes mixed with DMPM vesicles (1:50) does not change on the addition of <0.01 mole fraction of PxB. These results show that average separation between NBD-PE and Rh-PE is not altered under these conditions, as would be the case in the event of fusion (spectrum c, at 0.06 mole fraction PxB).

The RET from NBD-PE vesicles to Rh-PE vesicles is seen in the presence of PxB. As shown in Figure 7B, PxB (<0.01 mole fraction) added to a mixture of vesicles containing NBD-PE and vesicles containing Rh-PE induces a change in the spectrum that results from enhanced resonance energy transfer, i.e., an increase in the rhodamine acceptor emission (at 590 nm) and a decrease in the NBD donor emission (at 535 nm). These results clearly show that PxB induces formation of a contact between the vesicles. Not only is this consistent with steps 1 and 2 (Figure 1), but the fact that energy transfer is seen at all with a pair with energy transfer distance of about 50 Å suggests that some of the donor−acceptor probes present in separate vesicles are within the transfer distance. Of course, as also shown in Figure

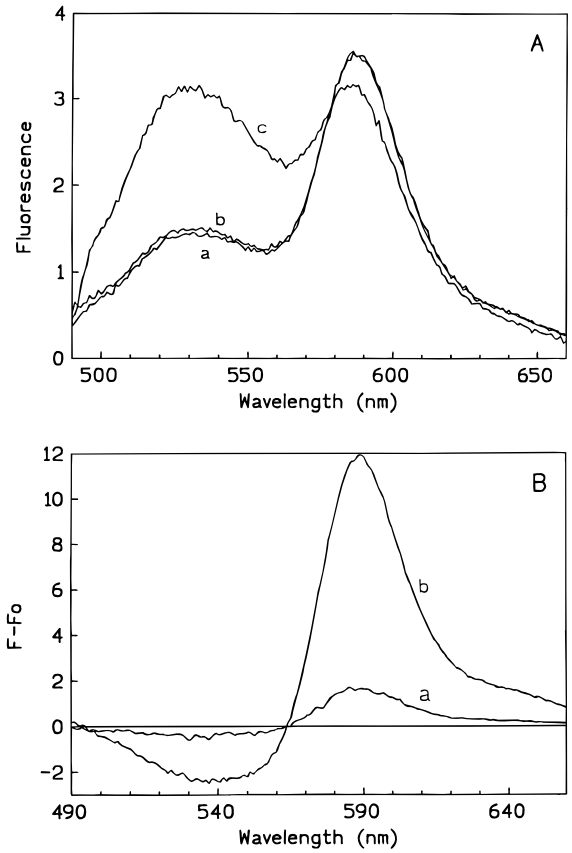

FIGURE 7: PxB-induced vesicle−vesicle contacts detected by RET. (Panel A) (a) Emission spectra of a 1:50 mixture of covesicles of 0.3%NBD-PE/0.3% Rh-PE/DMPM with DMPM (832 μM). (b) No change is detected after addition of PxB (2.75 μM). (c) A decrease in RET is seen at higher amounts of PxB (51 μM), which induces fusion of vesicles. (Panel B) Change in the spectrum due to RET between (a) an equimolar mixture of covesicles of 0.6%NBD-PE/DMPM and 0.6%Rh-PE/DMPM (81.3 μM total lipid), mixed with PxB (0.275 μM) and (b) the same lipid mixture with 4.4 μM PxB, which causes fusion of vesicles. Excitation 460 nm, excitation and emission band passes 4 nm.

7B, the magnitude of the energy transfer is considerably higher as fusion and nonspecific lipid mixing is induced at >0.04 mole fraction PxB.

Taken together, results described thus far not only rule out PxB-mediated hopping of PLA2 or a net transfer of DMPM or DTPM but also imply that once PxB binds to anionic vesicles and forms vesicle−vesicle contacts, it is not available to form new contacts. PLA2 and phospholipids labeled in the head group (amino group of PE) are not transferred across the vesicle−vesicle contact; however, kinetic experiments show that the contact does mediate the rapid exchange of DTPM, DMPM, and the products of hydrolysis of DMPM by PLA2.

PxB Does Not Promote Intervesicle Exchange of DMPM by Solubilization. It is possible that PxB promotes solubilization of phospholipids, and a water-soluble species thus formed could act as a carrier to promote exchange of phospholipids between the enzyme-containing and excess vesicles. If so, in the presence of PxB ultimately all the available substrate would be hydrolyzed, i.e., during the reaction progress the main effect of PxB would be on the rate at which maximum hydrolysis is achieved, not in the extent of hydrolysis. This expectation is based on the detergent-like effect of PxB; however, it is inconsistent with virtually all the results shown in Figures 2−7. Also, PxB

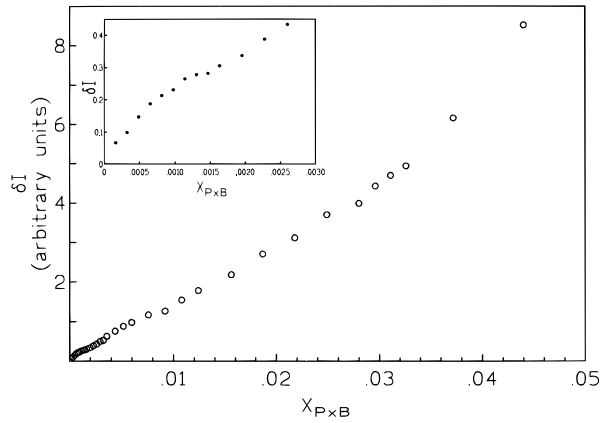

FIGURE 8: Changes in the intensity of 90° scattered light at 360 nm of DMPM vesicles (0.238 mM in 10 mM Tris buffer, pH 8.0) caused by the addition of PxB (mole fraction). Temperature 25 °C. The initial part of the titration curve is shown in the inset.

does not show any tendency to form micellar aggregates at least up to 2 mM, which provides a lower limit for its critical micelle concentration.

A direct effect of PxB on the size of DMPM vesicles was also monitored. As shown in Figure 8, the rapid increase in the 90° scattered intensity of DMPM vesicles exhibits a linear increase at low mole fractions of PxB (below 0.04 mole fraction), and an additional slow time-dependent increase in the scattering is seen only above 0.04 mole fraction of PxB. On the basis of experiments shown in Figures 6 and 7, the onset of the slow change in the scattering at >0.04 mole fraction PxB is most probably due to the fusion of vesicles. Thus, results at low mole fractions of PxB rule out the possibility of solubilization of DMPM vesicles by PxB to form particles smaller than the DMPM vesicles. In addition, even at very low mole fractions of PxB (inset in Figure 8) the 90° scattering increases, which is consistent with the formation of small clusters of vesicles in which the decrease in the number of vesicles is more than compensated by an increase in the size of the cluster.

Lipid Transfer Assay. PxB-mediated exchange of anionic phospholipids was determined by monitoring the change in the "monomer" fluorescence emission of a mixture of PyPM vesicles and a 100-fold excess of DMPM vesicles. As shown in Figure 9A, with less than 0.02 mole fraction PxB there is a rapid increase in the fluorescence, indicating that PyPM molecules are transferred into DMPM vesicles. At >0.04 mole fraction PxB the increase in the intensity also has a slower time-dependent component which is characteristic of fusion-related processes. The increase in the monomer fluorescence emission intensity δF versus mole fraction of PxB (Figure 9B) shows biphasic behavior, and the intercept of the two slopes corresponds to the onset of vesicle fusion. This interpretation is consistent with the fact that with the onset of fusion there is a significant decrease in the excimer fluorescence. Furthermore, no transfer of PyPM was observed when PyPM vesicles were added to a mixture of DMPM and PxB (PxB/vesicle <40). These results indicate that PxB does not exchange freely after it is bound to DMPM (results not shown); however, additional PxB makes new contacts between DMPM and PyPM in the mixture.

It may also be noted that at about 0.05 mole fraction PxB the fluorescence intensity at 395 nm is about 65% of the maximum monomer emission observed at about 0.15 mole

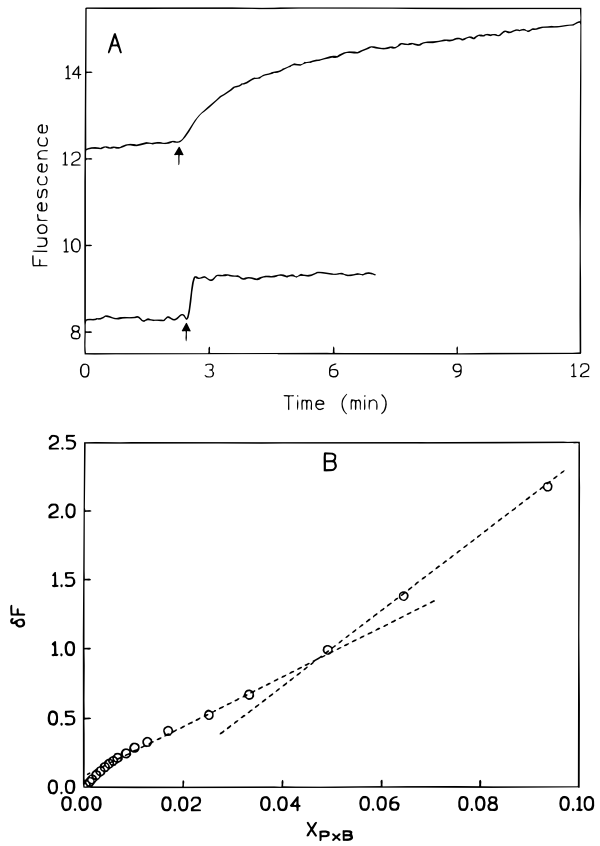

FIGURE 9: Changes in the pyrene monomer fluorescence intensity as a result of the PxB-mediated transfer of PyPM from donor vesicles (PyPM 1.25 μM) to acceptor vesicles (DMPM 159 μM). PxB was successively added from a stock solution in water (2.75 mM) to a preequilibrated mixture of PyPM and DMPM vesicles in 10 mM Tris at 25 °C. (A) Time course of the fluorescence change at 0.018 (lower curve) and 0.084 (upper curve) mole fractions of PxB (marked with arrows). (B) Increase in fluorescence as a function of the mole fraction of PxB. Excitation wavelength 346 nm, monitor wavelength 395 nm.

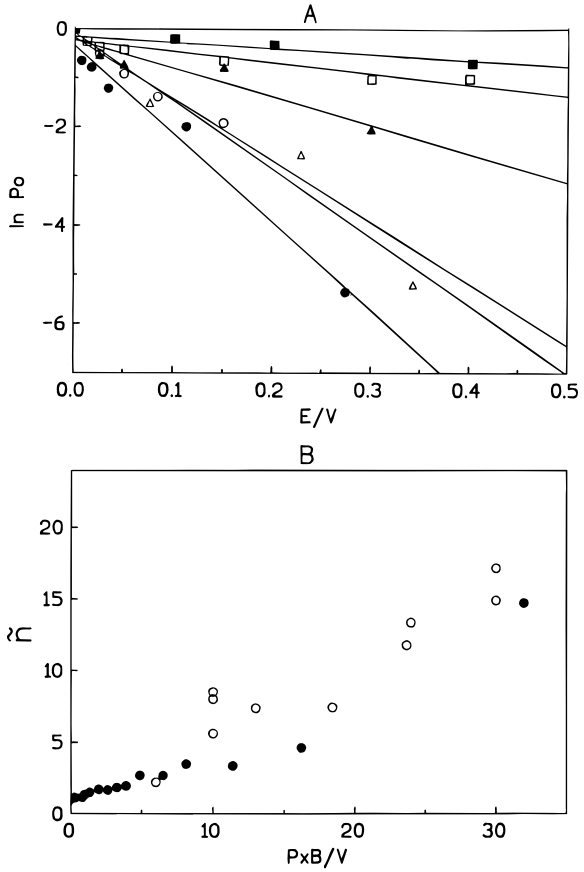

FIGURE 10: Formation of clusters of DMPM vesicles is induced by PxB. (A) Poisson plot of the extent of hydrolysis, ln P_0 (expressed in accordance with eq 5 in Appendix) as a function of enzyme to vesicle ratio at different PxB per vesicle (PxB/V) ratios: (●) PxB/V = 30, slope 17.8; (△) PxB/V = 24, slope 13.9; (○) PxB/V = 15, slope 12.6; (▲) PxB/V = 10, slope 5.8; (□) PxB/V = 6, slope 2.4; (■) no PxB (slope = 1.1). The order of addition for the determination of the extent of hydrolysis was PxB followed by DMPM (475 nmol) and PLA2 (in 0.2−10 μL from 4.96 μM solution) to initiate the reaction. Data was fitted by linear regression. (B) Average cluster size ($\bar{n}$) at different PxB/V. Here we combine the calculated slopes in panel A (○) plotted versus the amount of PxB per vesicle, with the average cluster size from the extent of hydrolysis in Figure 3 using eq 10 (●). In this experiment the sequence of addition of the components is critically important; so is the volume of the reagent added.

fraction PxB (36.6 versus 59 arbitrary units of emission, respectively) where the excimer peak at 480 nm also disappears completely. These results are consistent with the fact that under fusion conditions both the inner and outer monolayer phospholipids of the vesicle are able to mix. These results are also consistent with the kinetic results, where only 65% of the total lipid is hydrolyzed at <0.03 mole fraction of PxB (Figure 3); however, virtually all (100%) the substrate is hydrolyzed if the mole fraction of PxB exceeds 0.08 (data not shown), as transbilayer movement of phospholipids is also promoted.

From these experiments alone, it can be concluded that the mechanism by which PxB mediates intermembrane lipid transfer is not the formation of a soluble molecular complex that allows the transfer of one molecule of lipid at a time, because this would lead to a time-dependent increase in monomer fluorescence until all the lipid in the outer monolayer is transferred [e.g., see van Paridon (1987)].

Modeling the Vesicle−Vesicle Contacts and Clusters. The sequence of PxB-induced events illustrated in Figure 1 provides a unique basis for a rapid substrate replenishment during interfacial catalysis by PLA2 in the highly processive scooting mode. Results described so far show that PxB mediates a direct vesicle−vesicle exchange of DMPM (with itself or with its products of hydrolysis) without any complications by other possible competing mechanisms. Such an exchange would necessarily require the formation of an irreversible contact by PxB between two vesicles, where PxB or the companion vesicles do not exchange with excess vesicles. Although the bound enzyme is not transferred across a PxB contact, anionic lipids like DMPM or PyPM present in the outer monolayer are transferred. A cluster of vesicles will result if more than one PxB contact is formed per vesicle. Thus intervesicle exchange of DMPM can occur among a large number of vesicles forming a cluster, although only one of these vesicles may contain an enzyme molecule. Within these constraints it is possible to model the kinetic features of catalysis in the scooting mode to obtain information about the distribution of cluster sizes. This has been carried out in the Appendix where all the equations referred to below are given. The main result is an expression for the fraction, P_0, of vesicles (or accessible substrate) that cannot be hydrolyzed determined as a function of the enzyme-to-vesicle ratio *E/V*.

Figure 10A shows that the plot of ln P_0 versus *E/V* is roughly linear over a wide range of *E/V*. With no PxB, the

upper line (slope = 1.1), the cluster size is $\bar{n} = 1$ and $P_0 = p_0$ from eq 5. This is consistent with independent evidence that a single PLA2 monomer is fully catalytically active on a vesicle (Jain et al., 1991c). With PxB the slope increases as larger clusters form and the extent of hydrolysis is best described by eq 7 or 9. This indicates that either the cluster size distribution is fairly narrow, satisfying the relations given in eq 8, or that the enzymes have a Poissonian distribution over the clusters rather than over the vesicles.

The average slopes in Figure 10A give rough estimates of the average cluster sizes ($\bar{n}$) at various concentrations of PxB. These have been plotted in Figure 10B. In this figure are also plotted average cluster sizes estimated from the extent of hydrolysis data in Figure 3 using eq 10. The scatter in this plot, apart from the experimental uncertainty, is due to differences in analysis where the data points based on Figure 10A are averages over several values of *E/V*, while those from Figure 3 are calculated at a single value of *E/V*. In some cases there is also a difference in the order of addition of the components which could influence both the cluster distribution and the distribution of enzymes over clusters. If it is assumed that at the lowest PxB concentrations at most dimers form and that all PxB molecules participate in the vesicle−vesicle contacts of the dimers, it can be estimated that 5−6 PxB molecules are required for each contact.

The same stoichiometry for PxB contact is predicted at excess PxB. From the results of the type shown in Figure 3, it can be shown that with about 35 PxB/vesicle, all vesicles are in large clusters where all the available substrate in the outer monolayer of 180 pmol of vesicles can be hydrolyzed by about 20 pmol of enzyme. This shows that the integrity of the vesicles is maintained although the outer layer lipid can be transferred from vesicle to vesicle. If we assume that the stoichiometry of the contact is 6 PxB/contact (or 3 PxB/vesicle making the contact), and that the cluster is close packed (like a stack of marbles) so that every vesicle is in nearest-neighbor contact with at most 12 others, therefore 6 × 6 PxB molecules/vesicle would lead to a maximum cluster formation.

DISCUSSION

On the basis of the results described in this paper, it is clear that stable contacts between apposed membranes can indeed be established by specific peptides or proteins. PxB-induced vesicle−vesicle contacts described in this paper are stable species where two vesicles are bought in close apposition and the outer monolayer phospholipids exchange, while the continuity of the inner monolayers is preserved. Selective, rapid, and direct vesicle−vesicle exchange of phospholipids mediated by a peptide has not been described before. Formation of clusters is interpreted in terms of the binding of PxB to vesicles, PxB-mediated formation of vesicle−vesicle contact, and clustering of vesicles with multiple contacts. There are indications that ultimately, at high mole fraction of PxB, vesicles in contact fuse.

The main thrust of this paper is on the surprising observation that even a few molecules of PxB per vesicle (mole fraction 0.0002) promote a rapid intervesicle exchange of DMPM. Results show that stable vesicle−vesicle contacts are formed through which DMPM exchanges rapidly with DMPM, or with DTPM, or with the products of hydrolysis of DMPM present in the companion vesicle in contact. Diacylphosphatidylethanolamines labeled in the head group or PLA2 are not transferred through such contacts, although PyPM (with the label in the *sn*-2 position of the phospholipid) is readily transferred. The possibility that the exchange is due to some nonspecific process (like hemifusion) is also ruled out by the fact that phospholipids with probes in the head group do not exchange with DMPM.

Virtually all earlier studies on the effect of PxB on the properties of bilayer have focused on its effects at >0.02 mole fraction [for example, in Kubesh et al. (1987)]. Under these conditions, our results are in complete accord: PxB at >0.04 mole fraction in anionic vesicles promotes fusion accompanied by nonspecific lipid mixing. PxB mole fraction dependence for fusion and nonspecific lipid mixing is nonlinear as these processes are not seen at <0.02 mole fraction PxB. In this sense, the effect of higher mole fraction PxB is similar to the fusogenic effect of several other peptides and ions (Walter & Siegel, 1993; Hoekstra, 1990; Nieva et al., 1994; Suenaga et al., 1989).

Our success in identifying the selective transfer of phospholipids through vesicle−vesicle contacts formed by a few molecules of PxB lies in a combination of protocols that exploit some of the well-established kinetic and spectroscopic methods in conjunction with certain unique characteristics of tight binding of PLA2 to the interface. Specific lipid transfer without fusion has not been described before; however, nonspecific lipid mixing is implicated as a possibility in the hemifusion intermediate that precedes formation of the fusion pore (Bonderson & Sundler, 1990; Török et al., 1994; Moreau & Cassagne, 1994).

We are still investigating the structural and molecular aspects of PxB-mediated exchange of DMPM. Since this novel phenomenon could serve as a useful model to understand intermembrane transfer of phospholipids between cellular compartments, it is probably relevant to consider some of the basic properties of such intermembrane contacts:

(a) Vesicle−vesicle contacts formed by PxB are stable and essentially irreversible on the time scale of several minutes. These could serve as models for unfused vesicles as docked or localized on membranes (Schweizer et al., 1995; Valtorta & Befenati, 1995).

(b) The rate of transfer of DMPM through PxB-mediated contacts is rapid, and the transfer is complete within the mixing time (<10 s). In contrast, apparent lipid mixing time under the fusion conditions is slower, as if it is limited by the kinetics of the fusion step which requires mixing of the inner lamellae of the two apposed bilayers.

(c) Only certain lipid molecules (but not others) in the outer monolayer exchange through the contacts.

(d) It appears that the transfer occurs only under exchange conditions because in symmetrical contacts there is no net driving force. If, however, small sonicated vesicles were metastable, it is conceivable that the propensity to form larger vesicles would drive lipid mixing. Since only the outer layer lipids exchange through the contact, the propensity to form larger vesicles may not be able to overcome the barrier that exists for the mixing of the inner layer. However, it is conceivable that net transfer across a contact may occur if a suitable driving force can be established or if the process is coupled to an energy source.

We are still investigating the molecular, structural, and organizational aspects of vesicle−vesicle contacts which

provide a mechanism to overcome the barrier for intermembrane transfer of phospholipids. A significance of the observations described in this paper relates to the fact that PxB is bactericidal against Gram-negative bacteria and its mechanism of action is not well-established (Storm et al., 1977). If PxB could establish contacts between the inner and outer membranes, exchange of anionic lipids would lead to a loss of the compositional specificity and therefore the viability of the organism. This assertion is consistent with a range of observations which suggest that at antibacterial concentrations PxB could indeed change the properties of the bacterial membrane [to be published; however, see Cajal et al. (1995)].

Broader significance of the protein-mediated vesicle−vesicle transfer of phospholipids lies in the fact that intermembrane contacts are the necessary first step for apparently diverse cellular processes including membrane flow (Moreau & Cassagne, 1994), sorting, secretion, budding, and vesiculation (Palade, 1975, Brown & Greene, 1991). Vesicular membrane traffic and fusion between membranes is also implicated in many other physiological functions, i.e., intracellular membrane fusion events occur during movement of secretory material between the stacks of the Golgi apparatus, exocytosis of neurotransmitters and hormones, endocytosis, viral infection, cell division, fertilization, etc. If specificity of transfer or exchange through such contacts is considered, it is possible to begin to account for some unusual features of membrane biogenesis. For example, synaptic vesicles are retrieved following exocytosis, without intermixing between vesicles and plasma membrane components; this is possibly due to the rapidity of the retrieval or to the absence of complete lipid fusion (Valtorta & Befenati, 1995; Bergeron, 1973). The mechanisms conferring regulation and specificity have not been articulated, yet it is widely accepted that proteins play a fundamental role in membrane fusion by fulfilling two basic requirements, cross-linking and destabilization of bilayers (White, 1992; Zimmerberg, 1993; Hoekstra, 1990); many of these proteins are currently being characterized (Schweizer et al., 1995).

A major difficulty in modeling such multistep processes is that the intermediate structures involved are putatively transient and localized (Blumenthal, 1987; Schweizer et al., 1995). Two different approaches have been taken to explain membrane fusion through different intermediates. One group (Verkleij, 1979; Siegel, 1984) advocates that fusion proceeds by way of inverted micelles, yet lately the formation of an initial local point contact that expands, forming a stalk connecting the two membranes, is favored (Markin & Hudspeth, 1993; Kozlov et al., 1989; Siegel, 1993; White, 1992; Zimmerberg, 1993), where in the hemifused state only the outer lamellae are in contact to mix their contents. Although it is tempting to consider a potential relationship between the hypothetical stalk and the PxB-mediated contact experimentally characterized here, the differences between their predicted and observed characteristics certainly preclude the possibility that they are one and the same.

APPENDIX

Cluster Distributions. Under the conditions of the experiments, clusters of vesicles form irreversibly in the presence of PxB. Assume that the fraction f_n of the vesicles is present in clusters containing n vesicles ($n = 1, 2, 3, ...$). If the total concentration of vesicles is V, then the concentration of clusters of size n will be

$$C_n = \frac{f_n}{n} V \tag{1}$$

and the total concentration of clusters is

$$C = \sum_{n=1}^{\infty} C_n = V \sum_{n=1}^{\infty} \frac{f_n}{n} \tag{2}$$

The mean cluster size can be calculated as the average over the probabilities, C_n/C, that clusters are of size n:

$$\bar{n} = \sum_{n=1}^{\infty} n \frac{C_n}{C} = \frac{1}{\sum_{n=1}^{\infty} \frac{f_n}{n}} \tag{3}$$

The mean square cluster size is

$$\overline{n^2} = \sum_{n=1}^{\infty} n^2 \frac{C_n}{C} = \bar{n} \sum_{n=1}^{\infty} n f_n \tag{4}$$

The enzymes are irreversibly bound to the vesicles according to some distribution. For simplicity, we will consider only two limiting cases: either the enzymes have a Poissonian distribution over the vesicles or they have a Poissonian distribution over clusters.

(i) Poissonian over Vesicles. This will be the case if an enzyme has equal probability to bind to any of the vesicles and it will be the distribution expected if the enzymes bind to the vesicles before clusters are formed. If clusters are formed first, it is the expected distribution if an enzyme binds to a cluster with a rate that is proportional to the cluster size n. Then the probability that a vesicle does not have an enzyme bound is (Berg et al., 1991)

$$p_0 = \exp(-E/V) \tag{5}$$

where E is the concentration of enzymes. The probability that a cluster of size n does not have any enzyme bound is $(p_0)^n = \exp(-nE/V)$. Thus the fraction of vesicles that cannot be hydrolyzed is

$$P_0 = \sum_{n=1}^{\infty} f_n \exp(-nE/V) \tag{6}$$

By studying P_0 as a function of the enzyme-to-vesicle ratio E/V, one can gain information about the cluster distribution as given by f_n.

If cluster sizes are not too disperse, eq 6 can be approximated as

$$P_0 \approx \exp(-\bar{n}E/V)\left[1 - \frac{E}{V}\frac{\sigma_n^2}{\bar{n}} + \frac{1}{2}\left(\frac{E}{V}\right)^2 {\sigma_n}^2\right] \tag{7}$$

This holds as long as the variance, $\sigma_n^2 = \overline{n^2} - \bar{n}^2$, satisfies

$${\sigma_n}^2 < \bar{n}\frac{V}{E} \quad \text{and} \quad {\sigma_n}^2 < \left(\frac{V}{E}\right)^2 \tag{8}$$

(ii) Poissonian over Clusters. This is the expected distribution if enzymes bind to a cluster with a rate that is independent of the cluster size *n*. In this case, the probability that a cluster does not have any enzyme bound is determined by the ratio *E*/*C* of enzyme-to-cluster concentrations in analogy with eq 5. The total cluster concentration *C* is given by eq 2 and one finds

$$P_0 = \exp(-E/C) = \exp(-\bar{n}E/V) \qquad (9)$$

where $\bar{n}$ is the average cluster size from eq 3. In this case, a plot of ln (P_0) versus *E*/*V* would be a straight line with slope $-\bar{n}$; this is approximately the case also from eq 7 at least for small *E*/*V*, except that the slope would be $-(\bar{n} + \sigma_n^2/\bar{n})$. Alternatively, the average cluster size could also be estimated from eq 9 as

$$\bar{n} = -\frac{V}{E}\ln(P_0) \qquad (10)$$

This result would hold only if enzymes have a Poissonian distribution over clusters, or from eq 7 if cluster sizes are not too disperse.

The association rate of enzymes to clusters is expected to increase with cluster size but, at least for large clusters, more slowly than linearly with *n*. Thus, when clusters are formed first, the distribution of enzymes is expected to be intermediate between the two extremes discussed above.

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