

Visualization of Domain Formation in the Inner and Outer Leaflets of a Phospholipid Bilayer

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Abstract. Large vesicles (5–10- μm in diameter) were formed in the presence of phospholipids fluorescently labeled on the acyl chain and visualized using a fluorescence microscope, charge-coupled-device camera and digital image processor. When such vesicles contained a fluorescent phosphatidic acid (PA) and were exposed to 2 mM CaCl_2 or 0.5 mM PrCl_3 , it was possible to visualize PA-enriched domains within the vesicles. Calcium-induced domain formation was reversible in the presence of 4 mM EGTA. Vesicles were formed containing fluorescent PA on either the inner or outer leaflet of the bilayer and the patching and dissolution of patching were studied under conditions where calcium was present on the outside of the vesicle and where calcium was distributed across the

bilayer. In addition, vesicles were formed with two different fluorescent PA's, one on the inner leaflet and a different one on the outer leaflet of the bilayer. The results of the experiments show that in vesicles formed primarily with naturally occurring phospholipids such as egg phosphatidylcholine or brain phosphatidylethanolamine, there was no coordinate action of the two leaflets of the bilayer. An exception to this was found, however, if the vesicles were formed in the presence of primarily dioleoyl phospholipids (>95 mol %). In these vesicles there was a coordinate or coupled response to calcium by the two leaflets of the bilayer. In most cases, however, the two leaflets of the bilayer showed independent or uncoupled domain formation.

THE Fluid Mosaic Model of membrane structure proposes that the components of cell membranes are capable of lateral movement within the bilayer (26). One question not directly addressed in this model is whether or not the two leaflets of the bilayer communicate with one another or are coupled in some fashion such that motion or structure of the components of one leaflet is reflected by motion or structure of the components of the other. Such information would be quite valuable in understanding a number of phenomena such as transmembrane signaling when occupancy of a receptor results in a change in the intracellular milieu. Sheetz and Singer (22, 23) formulated the bilayer couple hypothesis from a series of elegant experiments on erythrocyte membrane-drug interactions. Briefly stated, their hypothesis proposed that the two leaflets of the bilayer could respond independently to various drugs causing either crenation (interaction of drugs with the exterior leaflet) or cup formation (interaction of drugs with the cytoplasmic leaflet).

Previous work to study the direct interaction of the two leaflets of the bilayer has provided mixed results. Nuclear Magnetic Resonance has been a good technique to study the

phase transition temperatures of the inner and outer leaflets of the bilayer in phospholipid vesicles. By using trivalent lanthanide ions both as shift reagents and to alter the transition temperatures, different transitions could be observed for the two leaflets of N-stearoylsphingomyelin vesicles indicating an uncoupled response (19). However, the same study showed a coupled response for N-lignocerylsphingomyelin. In similar studies using saturated phosphatidylcholine's (PCs)¹, no coupling was observed across the bilayer (25), although some interactions of metal ions with the head groups of one monolayer can be transmitted to the other monolayer (8). In cell membranes a number of studies have concluded that the physical properties of the two leaflets are different which may reflect an asymmetric distribution of lipids across the membrane (18, 20, 27, 28, 30, 31).

This laboratory has previously described a system for visualization of phospholipid phase separation or domain formation using fluorescence microscopy and digital image analysis (7). Large vesicles composed of phospholipids with a fluorophore on one of the acyl chains were used to show

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1. *Abbreviations used in this paper:* C_r -dansyl, N-(5-dimethylaminonaphthalene-1-sulfonyl)amino-butyric acid; DOPA, dioleoylphosphatidic acid; DOPC, dioleoylphosphatidylcholine; DOPE, dioleoylphosphatidylethanolamine; NBD, 1-acyl-2-[N-4(-nitrobenzo-2-oxa-1,3-diazole)amino-caproyl]; PA, phosphatidic acid; PC, phosphatidylcholine; PE, phosphatidylethanolamine.

the formation of domains when acidic phospholipids such as phosphatidic acid (PA) interacted with divalent cations such as Ca^{2+} . Such domains are areas of the vesicle that were greatly enriched in the acidic phospholipid, although other phospholipids are still present. Ca^{2+} -induced domain formation was demonstrated also in a simple cell system, rabbit erythrocytes (7). There is evidence that discrete lipid regions or domains exist in biological membranes (1, 9, 10, 13, 17, 21, 24, 29, 32).

The study presented here extends the earlier study and addresses the question of whether or not the two leaflets of the vesicle bilayer are acting in a coupled or uncoupled manner. The results suggest that both situations are possible, depending upon the fatty acid composition of the phospholipids.

Materials and Methods

Phospholipids

Dioleoylphosphatidylcholine (DOPC), dioleoylphosphatidylethanolamine (DOPE), and 1-acyl-2-[N-4(nitro-benzo-2-oxa-1,3-diazole)amino caproyl] phosphatidylcholine (NBD-PC) were purchased from Avanti Polar Lipids (Birmingham, AL). C_4 -dansyl ([N-(5-dimethylaminonaphthalene-1-sulfonyl)amino] butyric acid) was purchased from Chemical Dynamics Corp., South Plainfield, NJ. All other phospholipids were purchased from Sigma Chemical Company (St. Louis, MO). C_4 -dansyl-PC was synthesized as previously described (7) by first forming a fatty acid anhydride with the C_4 -dansyl and then attaching the fatty acid to L- α -lyso-PC (Sigma Chemical Co.). Phosphatidic acid compounds (C_4 -dansyl-PA, NBD-PA and DOPA) were all synthesized by digestion of the parent PC with the phospholipase D according to Comfurius and Zwaal (3). Concentrations were based on phosphate content after perchloric acid digestion (11). All compounds were routinely checked by thin layer chromatography for purity and repurified as necessary.

Instrumentation

The instrumentation has been previously described (7). All figures are photographs taken from the computer monitor and represent 150 video frames averaged (30 frames/s) to decrease background and random electrical noise. All viewing images have had a pseudo color program applied to them. In essence the 256 gray tones viewed by the charge-coupled-device have been assigned colors by the computer ranging from blue (0) to green to yellow to red (256).

Slide Preparation

Slides for viewing were prepared by mixing the vesicle preparations with agarose as previously described (7).

Area Measurements

All area measurements are given as a percentage of the total area. Area measurements were calculated by the arbitrary area program contained within the Quantex Imaging System. That area of the vesicle that was visibly patched was encircled using an overlay menu and the area within the circle was calculated in pixels. The entire vesicle was then encircled and taken as 100% of the vesicle.

Vesicle Preparations

Large vesicles for viewing were prepared by hydrating a dried lipid film in deoxygenated water according to the method of Darzon et al. (4). Vesicles so prepared have been shown to be unilamellar (4). Such viewing vesicles, unless further treated, were assumed to be uniformly labeled in terms of equal distribution of fluorescent label and all phospholipids across the two monolayers. No further additions were made to the vesicle preparations except such compounds as indicated for the specific experiments.

Smaller (sub-visible) vesicles for use in labeling or removing label from the viewing vesicles were made by the ethanol injection methods of Kreamer et al. (12). Specific composition of all vesicles is given below.

To address the question of whether or not there is coupled movement of the two phospholipid leaflets in this system, protocols were designed to make vesicles with label specifically on either the inner or outer leaflet of the bilayers.

Uniformly Labeled Vesicles. Uniformly labeled vesicles were made by hydrating a phospholipid film containing 4 mol % DOPA, 1 mol % NBD-PA and 95 mol % PC to a final concentration of 0.5 mM phospholipid. For the remaining vesicles with specific phospholipid labels on either the inner or outer leaflets, use was made of the methods of Denkins and Schroit (5) for synthesis of asymmetrically labeled vesicles. Short chain phospholipids or more polar phospholipids, in this case the fluorescent PA's, exchange at significant rates between populations of vesicles containing long acyl chain phospholipids (16). During the time of the experiments there is no exchange of the long acyl chain phospholipids, allowing for the specific removal of a fluorescent phospholipid from the external leaflet of the bilayer and the subsequent insertion of a different fluorescent phospholipid.

NBD-PA Only on the Inner Leaflet. Vesicles with NBD-PA only on the inner leaflet were made by hydrating a phospholipid film containing 1 mol % NBD-PA and 99 mol % PC to a final concentration of 0.5 mM, and incubating the resulting vesicles with an equal volume of 100 mol % DOPC ethanol-injected vesicles (1.0 mM final phospholipid concentration) for 30 min. The resulting viewing vesicles, while still fluorescent, had an ~50% reduction in fluorescence intensity as would be expected for unilamellar vesicles after the back-exchange of the NBD-PA from the outer leaflet onto the smaller, unlabeled ethanol-injected vesicles (14). This procedure resulted in a 5% increase in the background intensity of the images although the ethanol-injected vesicles were too small to be seen as individual vesicles.

DOPA in Both the Inner and Outer Leaflets, but NBD-PA Only in the Inner Leaflet. Vesicles containing DOPA in both the inner and outer leaflets, but NBD-PA only in the inner leaflet were prepared as above for the vesicles containing NBD-PA only in the inner leaflet, except the viewing vesicles were hydrated from a phospholipid film containing 1 mol % NBD-PA, 4 mol % DOPA and 95 mol % PC (final concentration 0.5 mM). Back exchange of the NBD-PA from the outer leaflet was again carried out in the presence of 100 mol % DOPC ethanol-injected vesicles. It was assumed that only the NBD-PA exchanged out of the outer leaflet and not the DOPA (15). It was determined that in these vesicles the presence of DOPA in the bilayer did not affect the exchange of the NBD-PA from the outer leaflet. This determination was based on exchange rates and amounts (data not shown) as measured using the methods of Pagano (14).

NBD-PA Only in the Outer Leaflet. Vesicles containing NBD-PA only in the outer leaflet were made by hydrating a phospholipid film containing 100 mol % PC (0.5 mM phospholipid) and incubating 100 μ l of the viewing vesicles with 100 μ l of ethanol-injected vesicles containing 25 mol % NPD-PA and 75 mol % DOPC (1.0 mM phospholipid, final concentration) for 30 min. Although initially the viewing vesicles were nonfluorescent, after incubation with the NBD-PA labeled ethanol-injected vesicles they were visible, as might be expected from the exchange of NBD-PA into the 100 mol % PC viewing vesicles (14).

DOPA on the Inner and Outer Leaflet, but with NBD-PA Only on the Outer Leaflet. Vesicles with DOPA on the inner and outer leaflet, but with NBD-PA only on the outer leaflet were made in the same manner but the initial viewing vesicles were hydrated from a phospholipid film containing 5 mol % DOPA and 95 mol % PC (0.5 mM phospholipid). Again, these vesicles were not visible until after incubation with the NBD-PA donor ethanol-injected vesicles.

Double-labeled Vesicles. The protocol for forming double labeled vesicles took advantage of the vesicles formed with NBD-PA just on the inner leaflet of the bilayer. Vesicles so formed were then further incubated with an equal volume of ethanol-injected vesicles containing 35 mol % C_4 -dansyl-PA and 65 mol % DOPC (1.0 mM final phospholipid concentration) for 30 min. Based on fluorescence intensity, it was estimated that the viewing vesicles contained 4-6 mol % C_4 -dansyl-PA after this procedure. In this manner vesicles were formed with NBD-PA on just the inner leaflet and C_4 -dansyl on the outer leaflet. By starting with vesicles which had NBD-PA on just the inner leaflet and DOPA on both the inner and outer leaflet, similar double-labeled vesicles but with DOPA on both leaflets were also formed.

Results

Methods have been developed previously to visualize the distribution of phospholipids in large vesicles or cells and to demonstrate that calcium will induce the formation of rela-

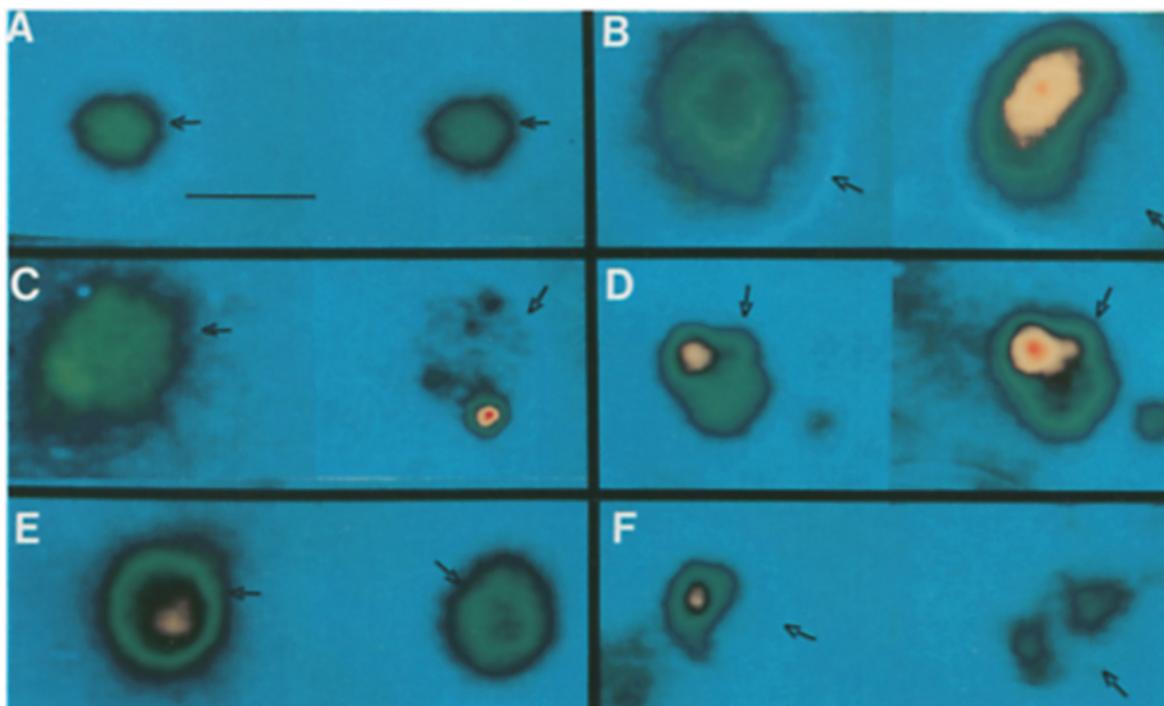


Figure 1. Domain formation in vesicles formed with egg-PC. Vesicles were formed containing DOPA, egg-PC, and DOPC evenly distributed across the two leaflets. The vesicles were fluorescently labeled with NBD-PA (inner leaflet) and C_4 -dansyl-PA (outer leaflet). For each panel, the left image is the vesicle viewed for NBD fluorescence and the right image is the same vesicle viewed for dansyl fluorescence. Arrows indicate the edges of vesicles. (A) A representative vesicle at zero time. (B) The same vesicle as in A after an incubation with 2.0 mM $CaCl_2$ for 30 min. (C) A similar vesicle as in A after incubation with 0.5 mM $PrCl_3$ for 30 min. (D) A similar vesicle as in A after incubation with 0.5 mM A23187 and 2.0 mM $CaCl_2$. (E) A similar vesicle as in D followed by a 30-min incubation with 4.0 mM EGTA. (F) A vesicle as in E subsequently incubated with 0.5 mM $PrCl_3$ for 30 min (total incubation time 90 min). Bar, 10 μm .

tively large domains enriched in specific phospholipids when the bilayers contain acidic phospholipids (7). The most direct manner in which to determine whether or not the two leaflets of the vesicle bilayer were acting independently of one another or in a coupled manner was the construction of vesicles with two different fluorescent PAs, one on the inner leaflet and a different one on the outer leaflet of the bilayer. Such vesicles were synthesized as outlined in detail in the Methods section by first making uniformly labeled vesicles containing 2 mol % NBD-PA as the fluorescent phospholipid, 4 mol % DOPA, 25 mol % egg-PC, and the balance as DOPC. The NBD-PA was removed from the outer leaflet and replaced with C_4 -dansyl-PA. When such a vesicle was viewed at zero time using either excitation and emission filters for NBD or C_4 -dansyl (Fig. 1 A), the vesicles were visible and both fluorophores appeared to be uniformly distributed (see also Table I). However, after 30 min in the presence of 2 mM $CaCl_2$, it could be seen that while the NBD fluorescence was still uniformly distributed, the C_4 -dansyl fluorescence was now greatly increased in one area of the vesicle (Fig. 1 B). This result indicated that the C_4 -dansyl-PA on the outer leaflet of the vesicle bilayer had patched in response to calcium, while the NBD-PA on the inner leaflet was still randomly distributed. Thus, the two leaflets of the bilayer were acting in an uncoupled manner.

To ensure that these results were not due to differences in the partitioning of PA due to the acyl-linked fluorophores, the same experiment was repeated but in vesicles with C_4 -

dansyl-PA on the inner leaflet and NBD-PA on the outer leaflet of the bilayer. In such vesicles, again, only the PA on the outer leaflet of the vesicles patched in response to calcium addition (not shown).

In an effort to determine how widespread this uncoupled response might be, a similarly double-labeled vesicle was treated with 0.5 mM $PrCl_3$, another cation that induces phospholipid phase separations. Pr^{3+} also was advantageous

Table I. Domain Formation in Double-labeled Vesicles*

Incubation conditions (time)	NBD-inner leaflet	Dansyl-outer leaflet
2.0 mM $CaCl_2$ (30 min)	—	+
0.5 mM $PrCl_3$ (30 min)	—	++
2.0 mM $CaCl_2$ (30 min) then 4.0 mM EGTA (30 min)	—	—
0.5 mM A23187 and 2.0 mM $CaCl_2$ (30 min)	+	+
0.5 mM A23187 and 2.0 mM $CaCl_2$ (30 min) then 4.0 mM EGTA (30 min)	+	—
2.0 mM $CaCl_2$ (30 min) then 4.0 mM EGTA (30 min) then 0.5 mM $PrCl_3$ (30 min)	+	++

* Vesicles were composed of NBD-PA, DOPA, egg-PC and DOPC on the inner leaflet and C_4 -dansyl-PA, DOPA, egg-PC and DOPC on the outer leaflet. Patch formation on the inner leaflet was monitored by NBD fluorescence. Patch formation on the outer leaflet was monitored by C_4 -dansyl fluorescence. (—) Indicates no patch, (+) a single patch, and (++) multiple patches.

to use since it has been reported that PA can act as a Ca^{2+} ionophore (2). After 30 min in the presence of Pr^{3+} , the C_4 -dansyl-PA was again unevenly distributed indicating domain formation of the external leaflet PA while the inner leaflet PA, as monitored by NBD fluorescence was still randomly and uniformly distributed (Fig. 1 C). These results gave further evidence that the two leaflets of the bilayer were uncoupled. Interestingly, the type of patching seen in the presence of Pr^{3+} was very different from that seen with calcium. Pr^{3+} induced multiple domains that were smaller than the calcium-induced domains. However, when area measurements were taken, the percent of the total area of the vesicle that was patched was similar under the two conditions ($\text{Ca}^{2+} = 11\%$, $\text{Pr}^{3+} = 11\%$). The domains were enriched two- to threefold in the concentration of the acidic phospholipid. Precise quantitation of the phospholipid concentration is difficult, however, due to self-quenching of the fluorophore and other technical problems.

The results of the previous experiments suggested that the reason the NBD-PA did not patch was that neither the Ca^{2+} nor the Pr^{3+} reached the inside of the vesicle as it had previously been shown that NBD-PA will patch in response to calcium (7). To rule out the possibility that for some reason the NBD-PA was simply not reacting to the presence of Ca^{2+} or Pr^{3+} in this instance, calcium was introduced into the inside of the vesicle using the calcium ionophore A23187. When a double-labeled vesicle was treated with both calcium and A23187 (2.0 mM and 0.5 mM, respectively), both the NBD-PA and the C_4 -dansyl-PA now formed patches (Fig. 1 D, Table I), indicating that the earlier results of apparent uncoupling of the bilayer were not due to lack of ability of the inner leaflet PA to form a patch, but rather that calcium was not coming into contact with the inner leaflet PA. The addition of the A23187 alone to the vesicle preparations caused no reorganization of the PA.

As a further demonstration of the uncoupling of the two leaflets of the bilayer in these vesicles, the separate patching of the two leaflets of the bilayer in response to Ca^{2+} and Pr^{3+} was attempted. To carry out this experiment, it was first necessary to find conditions where the patching of the PA on the inner leaflet could be observed without the patching of the PA on the outer leaflet. To this end, double-labeled vesicles were formed with the two different fluorophores as above and treated with 0.5 mM A23187 and 2.0 mM CaCl_2 to induce patching of the PA on both leaflets as in Fig. 1 D. When such vesicles were subsequently treated with 4.0 mM EGTA, the chelation of the calcium on the outside of the vesicle caused the dissolution of the PA-enriched domain on the external leaflet of the vesicle only. Under the conditions of the experiment enough calcium apparently remained on the interior of the vesicles to maintain the patch on the inner leaflet. This was evident by the fact that when such a vesicle was viewed with filters for NBD fluorescence, an NBD-PA-enriched domain was still visible while with filters for C_4 -dansyl fluorescence, there was now uniform distribution of the C_4 -dansyl-PA (Fig. 1 E). When vesicles that were thus patched (only on the inner leaflet) were then treated with 0.5 mM PrCl_3 , the type of patching on the inner leaflet was not affected (still a single large patch) while the C_4 -dansyl-PA on the outer leaflet was now reorganized into multiple patches by the Pr^{3+} (Fig. 1 F, cf. Fig. 1 C).

The experiments described above demonstrated that the

Table II. Domain Formation in Vesicles Made with egg-PC*

Inner‡	Outer§	CaCl_2	then EGTA¶	A23187 and CaCl_2 **	then EGTA‡‡	PrCl_3 §§
No PA egg-PC DOPC	NBD-PA egg-PC DOPC	+	-	+	-	++
NBD-PA egg-PC DOPC	No PA egg-PC DOPC	-	-	+	+	-
NBD-PA egg-PC DOPC	NBD-PA egg-PC DOPC	+	-	+	+	++
DOPA egg-PC DOPC	NBD-PA DOPA egg-PC DOPC	+	-	+	-	++
NBD-PA DOPA egg-PC DOPC	DOPA DOPA egg-PC DOPC	-	-	+	+	-
NBD-PA DOPA egg-PC DOPC	NBD-PA DOPA egg-PC DOPC	+	-	+	+	++

* Vesicles were examined for the presence (+) or absence (-) of patching. (++) indicates multiple patches.

‡ Composition of the inner leaflet of the vesicle.

§ Composition of the outer leaflet of the vesicle.

|| Vesicles at 30 min after treatment with 2.0 mM CaCl_2 .

¶ Vesicles as in ||, then treated with 4 mM EGTA for 30 min.

** Vesicles at 30 min after treatment with 0.5 mM A23187 and 2.0 mM CaCl_2 .

‡‡ Vesicles as in **, then treated with 4 mM EGTA for 30 min.

§§ Vesicles treated with 0.5 mM PrCl_3 for 30 min.

two leaflets of the vesicles were acting independently of one another. In support of these data, additional experiments were carried out but with vesicles that contained fluorescent PA either on just the inner or just the outer leaflet of the bilayer. The results of these experiments are outlined in Table II.

Vesicles with NBD-PA only on the outer leaflet of the bilayer were formed by hydrating 100 mol % PC vesicles (75 mol % DOPC and 25 mol % egg-PC) and then incubating them with ethanol-injected NBD-PA containing vesicles. When such vesicles were treated with 2.0 mM CaCl_2 , there was complete reorganization of the fluorescent PA into a single large patch. The size and type of patch was not affected by the presence or absence of the calcium ionophore A23187. However, the patch was fully sensitive to the addition of 4.0 mM EGTA to the system, in that it was completely dissolved after 20 min in the presence of the calcium chelator regardless of whether or not the vesicle had been treated with A23187. These results and the results with the double-labeled vesicles showed that there had been no flip-flop of the NBD-PA to the inner leaflet of the vesicle. A small amount of flip-flop would have been detected as a smaller patch (in the absence of ionophore) since the size of the patch depends on the PA concentration (data not shown). After such calcium/EGTA treatment, the NBD-PA could be "repatched" by the addition of 0.5 mM PrCl_3 . The NBD-PA could also be

patched by the addition of PrCl_3 without any CaCl_2 treatment (Table II).

Vesicles with NBD-PA only on the inner leaflet of the vesicles were formed by back exchanging the NBD-PA from the outer leaflet. When such vesicles were viewed at zero time, there was uniform distribution of the fluorescent PA and this uniform distribution was not affected by the addition of 2 mM CaCl_2 to the vesicle preparation (Table II). Thus, all the NBD-PA on the outer leaflet had been removed and there was no appreciable flip-flop during the time of the experiment. If the calcium ionophore A23187 was added (0.5 mM) at the same time as the calcium, the NBD-PA did reorganize into a single large patch per vesicle. In addition, these patches were stable after the addition of 4.0 mM EGTA, suggesting that the NBD-PA was, in fact, solely on the inner leaflet of the vesicle and not accessible to the EGTA effects. There was no reorganization of the PA in the presence of Pr^{3+} either after this treatment or when Pr^{3+} was added alone, further suggesting that all of the NBD-PA was localized on the inner leaflet of the bilayer.

Similar experiments were carried out in the presence of DOPA on both leaflets of the vesicle (Table II) and with C_4 -dansyl-PA as the fluorescent PA (not shown) with no difference in the results.

The interpretation of the results depends on the fact that calcium was excluded from the vesicles unless A23187 was present and that EGTA fully chelates the calcium on the outside of the vesicles. These points were further examined by using the calcium indicator dye, fura-2. Vesicles were formed from a dried lipid film containing 5 mol % DOPA and 95 mol % PC in deoxygenated water containing 0.5 μM

fura-2. Under conditions of excitation and emission for viewing fura only in the calcium bound state, there was no visible fluorescence present when a slide of these vesicles was prepared with no calcium (Fig. 2 A). When such vesicles were incubated with 2.0 mM CaCl_2 for 60 min, however, well beyond the time course of other experiments outlined above, it was possible to see the fluorescent calcium-bound fura only on the outside of the vesicles (Fig. 2 B). When such vesicles were then exposed to 4.0 mM EGTA, the slide again presented a totally dark field (Fig. 2 C), indicating that all of the calcium had been chelated by the EGTA.

When vesicles prepared in the presence of fura were incubated with both calcium and A23187, the vesicles themselves were fluorescent as well as the external solution, leading to a totally fluorescent image (Fig. 2 D). When such vesicles were then treated with EGTA, only the vesicles themselves remained visible under the conditions for viewing fura in the calcium-bound state (Fig. 2 E), again indicating that all of the calcium on the outside of the vesicles had been chelated. Similar results were seen with vesicles containing 100 mol % DOPC and in vesicles containing 25 mol % egg-PC (not shown).

As indicated above, different systems have been used to address the question of coupling of the leaflets of phospholipid bilayers. One of the variations in these systems has been that phospholipids with different polar head groups have been used. Therefore, the experiments using calcium as the patching agent were repeated using vesicles composed of the various PAs as above, but with sheep brain-PE in place of the egg-PC. The balance of the vesicle was again DOPC. The results of these experiments are outlined in Table III and indi-

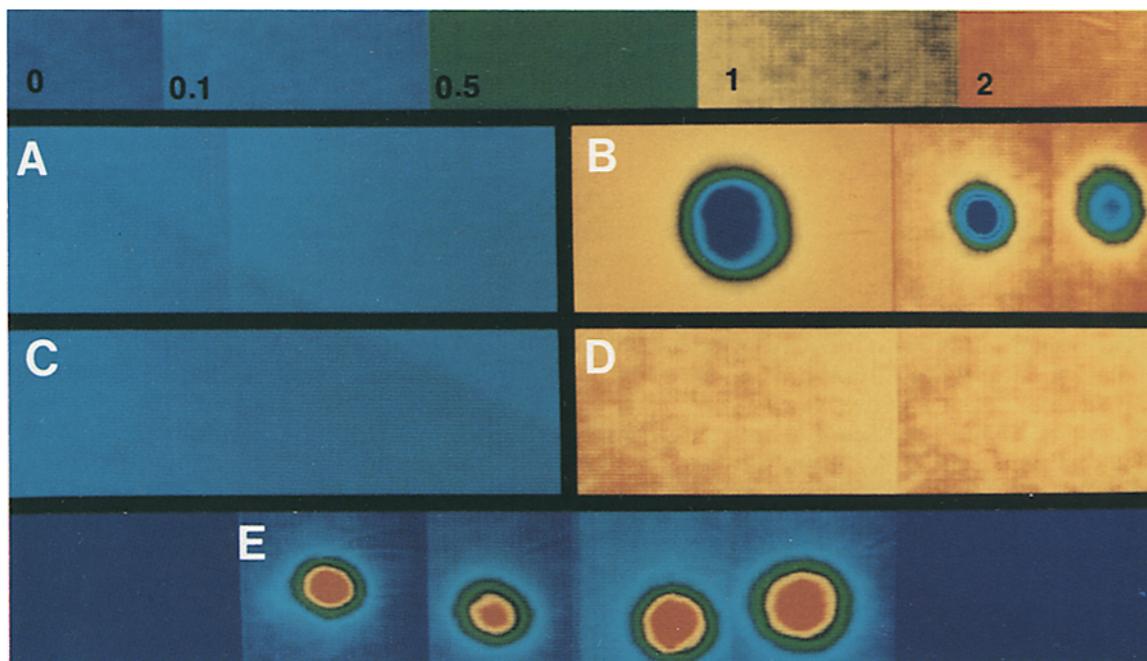


Figure 2. Presence of calcium inside or outside vesicles. Vesicles were formed containing 5 mol % DOPA and 95 mol % DOPC in the presence of 0.5 μM fura-2. Vesicles were viewed using epi-fluorescence at 355 ± 5 nm excitation and 505 ± 5 nm emission, wavelengths for viewing fura in the calcium-bound state. The band across the top of the figure indicates the colors assigned to the fluorescence generated by fura in the presence of the indicated concentrations of calcium (mM). (A) Vesicles at zero time. (B) Vesicles after a 60-min incubation with 2.0 mM CaCl_2 . (C) Vesicles as in B with a subsequent incubation with 4.0 mM EGTA for 30 min. (D) Vesicles incubated for 60 min with 0.5 mM A23187 and 2.0 mM CaCl_2 . (E) Vesicles as in D with a subsequent incubation with 4.0 mM EGTA for 30 min.

Table III. Domain Formation in Vesicles Made with Phosphatidylethanolamine*

Composition		CaCl ₂ ‡		A23187 and CaCl ₂ §		Comments
Inner	Outer	NBD	Dansyl	NBD	Dansyl	
NBD-PA brain-PE DOPC	no PA brain-PE DOPC	-	0	+	0	Control
NBD-PA DOPA brain-PE DOPC	DOPA brain-PE DOPC	-	0	+	0	Uncoupled
NBD-PA brain-PE DOPC	C ₄ -dansyl-PA brain-PE DOPC	-	+	+	+	Uncoupled
NBD-PA DOPA brain-PE DOPC	C ₄ -dansyl-PA DOPA brain-PE DOPC	-	+	+	+	Uncoupled
NBD-PA DOPE DOPC	no PA DOPE DOPC	-	0	+	0	Control
NBD-PA DOPA DOPE DOPC	DOPA DOPE DOPC	+	0	+	0	Coupled
NBD-PA DOPE DOPC	C ₄ -dansyl-PA DOPE DOPC	+	+	+	+	Coupled
NBD-PA DOPA DOPE DOPC	C ₄ -dansyl-PA DOPA DOPE DOPC	+	+	+	+	Coupled

* Vesicles were formed in either the presence of 20 mol % of DOPE or brain-PE and examined for the presence (+) or absence (-) of domain formation. (0) indicates no fluorophore present.

‡ Vesicles viewed after 30 min in the presence of 2.0 mM CaCl₂.

§ Vesicles viewed after 30 min in the presence of 0.5 mM A23187 and 2.0 mM CaCl₂.

cate that the substitution of a portion of the PC by PE did not cause any differences in the lack of coupling between the two monolayers. For example, as in Fig. 1, vesicles labeled with NBD-PA on the inner leaflet and C₄-dansyl-PA on the outer leaflet only showed patching of the C₄-dansyl-PA when treated with calcium. Similarly, vesicles that had been treated with A23187, calcium and EGTA showed patching only of the internal NBD-PA.

The other major variable in previous studies has been the acyl chain composition of the phospholipids. The experiments described above have used vesicles with heterogeneous acyl chains. Therefore, the effect of a more homogeneous system on the uncoupling of the leaflets was investigated. For these experiments, only dioleoyl phospholipids such as DOPA, DOPC and DOPE, (>95 mol %) were used to form the vesicles with the remainder being the fluorescent PA.

The results of the experiments with DOPC are outlined in Table IV and are strikingly different from the previous results. Initial DOPC control vesicles containing NBD-PA on both leaflets of the bilayer showed patching in the presence

Table IV. Domain Formation in Vesicles Made with DOPC*

Inner	Outer	CaCl ₂	A23187		PrCl ₃		
			then EGTA	then EGTA			
NBD-PA DOPC	NBD-PA DOPC	+	-	+	+	++	
No PA DOPC	NBD-PA DOPC	+	-	+	-	++	
NBD-PA DOPC	No PA DOPC	-	-	+	+	-	
NBD-PA DOPA DOPC	NBD-PA DOPA DOPC	+	-	+	+	++	
DOPA DOPC	NBD-PA DOPA DOPC	+	-	+	+	++	
NBD-PA DOPA DOPC	DOPA DOPA DOPC	+	-	+	+	++	
NBD-PA DOPC	C ₄ -dansyl-PA DOPC	NBD‡ Dansyl§	+	-	+	+	++ ++
NBD-PA DOPA DOPC	C ₄ -dansyl-PA DOPA DOPC	NBD‡ Dansyl§	+	-	+	+	++ ++

* All headings are as in Table II.

‡ NBD fluorescence.

§ Dansyl fluorescence.

of calcium as had been demonstrated earlier (7) and such patches dissolved with the subsequent addition of EGTA. However, when A23187 was added as well as calcium, the patches that were formed did not dissolve with EGTA and showed no change in size indicating that the PA on the outer leaflet was still patched, despite the apparent absence of free calcium (as indicated by the fura experiments discussed above). When DOPC vesicles were formed that contained no DOPA and only NBD-PA on the outer leaflet, the calcium-induced patches were reversible by EGTA, regardless of the presence or absence of A23187. However, when vesicles were formed that contained DOPA on both leaflets of the bilayer and NBD-PA only on the other leaflet, patches formed in the presence of calcium were reversible with EGTA only when A23187 had not been added to the preparation. One interpretation of these results was that the DOPA on the inner leaflet of the vesicle, which was presumably patched in response to the internal calcium, was holding the PA on the outer leaflet within a domain, regardless of the absence of calcium. To assess this possibility, vesicles were formed containing DOPA on both leaflets and NBD-PA only on the inner leaflet. Such vesicles, when treated with calcium, showed patching, despite the apparent lack of calcium in contact with the NBD-PA. Again, these results suggested that the patching of the DOPA on the external leaflet of the vesicle induced the patching of the DOPA on the internal leaflet.

To more fully examine this possibility, vesicles were formed containing NBD-PA on the inner leaflet, C₄-dansyl-PA on the outer leaflet, 4 mol % DOPA on both leaflets and the balance of the vesicle as DOPC. With these vesicles, it was possible to see that both the NBD-PA and the C₄-

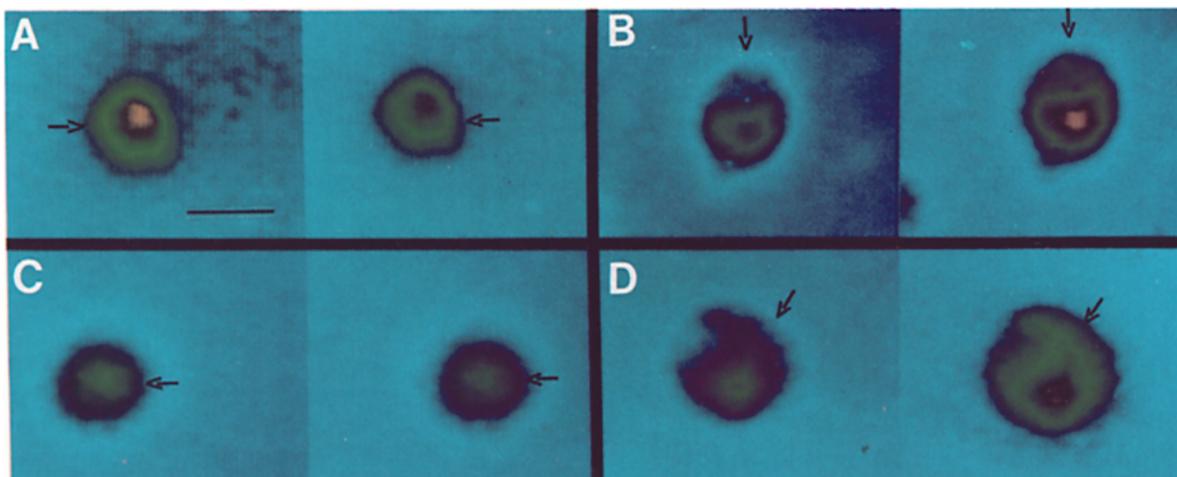


Figure 3. Domain formation in vesicles with DOPC. Vesicles were formed containing DOPA and DOPC and labeled with NBD-PA on the inner leaflet and C_4 -dansyl-PA on the outer leaflet. For each panel, the left image is the vesicle viewed for NBD fluorescence and the right image is the same vesicle viewed for dansyl fluorescence. Arrows indicate the edges of vesicles. (A) A representative vesicle after incubation with 2.0 mM $CaCl_2$ for 30 min. (B) A representative vesicle after incubation with 0.5 mM A23187 and 2.0 mM $CaCl_2$ for 30 min. (C) A vesicle formed as in A with further incubation in the presence of 4.0 mM EGTA. (D) A vesicle as in B with further incubation in the presence of 4.0 mM EGTA. Bar, 10 μ m.

dansyl-PA had patched in response to external calcium (Fig. 3 A). These results were not affected by the presence of A23187 (Fig. 3 B). In vesicles treated only with calcium, the patches dissolved in the presence of EGTA (Fig. 3 C). However, in vesicles treated with both A23187 and calcium, both the NBD-PA and C_4 -dansyl-PA remained patched after EGTA treatment (Fig. 3 D). This result showed that the NBD-PA was holding the C_4 -dansyl-PA in a patch, despite the absence of external calcium (see Fig. 2). Similar results were seen with vesicles that did not contain DOPA (Table IV), indicating that the additional PA present was not responsible for the results. The data presented in Fig. 3 and Table IV suggested that in this particular system with dioleoyl phospholipids, the two leaflets of the bilayer were, in fact, coupled. That is, domain formation in one leaflet induced domain formation in the other.

In order to again address the question of the possible role of head group composition on the coupling or lack thereof between the leaflets of the bilayer, vesicles were formed containing DOPE in place of a portion of the DOPC. The results of these experiments are outlined in Table III and indicate that, as above, in vesicles where the acyl chains are more homogeneous, or at least in the case where the phospholipids are primarily dioleoyl phospholipids, the two leaflets of the bilayer are coupled.

Discussion

There has been little investigation into the forces that might be active between the two leaflets of phospholipids that make up a bilayer. It has been generally assumed that the inter-leaflet forces are not of a sufficient magnitude to affect such processes as transmembrane signaling or lateral phospholipid mobility, although only recently has there been a theoretical study to address how large these inter-leaflet forces and pressures might be (6). One reason for the lack of work in this area has been the difficulty in developing a system

whereby the two leaflets of a bilayer could be studied as separate entities. In this report a direct method of studying the two leaflets of the bilayer has been used.

Through the construction of large (5–10 μ m) vesicles with two different fluorescent labels specifically placed on the inner and outer leaflets of the bilayer, it was possible to demonstrate that the two leaflets of the bilayer responded to agents that caused a lateral phase separation in an independent or uncoupled manner (Fig. 1, Tables I and II). This was true, however, only in vesicles that contained a heterogeneous acyl chain composition. In such vesicles, it was possible to see that the reorganization of the phospholipids on the outer leaflet induced by calcium did not effect the organization of the phospholipids on the inner leaflet. Conversely, when the phospholipids on the inner leaflet were induced to form PA-enriched domains by the presence of calcium, the phospholipids in the outer leaflet were free to reorganize. In the particular vesicles studied here, this was seen by first the dissolution of a PA-enriched domain (in the presence of EGTA) and the subsequent reformation of a slightly different type of PA-enriched domain (in the presence of $PrCl_3$), all within vesicles that showed a continuous PA-enriched domain on the inner leaflet.

In initial experiments studying the effects of a more homogeneous acyl chain composition, dioleoyl phospholipids, the results that were seen with vesicles after EGTA treatment, in the presence of A23187, showed that coupling between the two bilayers occurred (Table IV). The data indicating a lack of change in the size of the domain formed in the presence of A23187 and calcium followed by EGTA treatment showed that the PA on the outer leaflet of the vesicle remained patched, despite the absence of a patching agent in direct contact with the PA. Further evidence for this interpretation came from results with vesicles that contained unlabeled PA (DOPA) on both leaflets of the vesicle but with NBD-PA on only the inner leaflet. In such vesicles the interaction of calcium and DOPA on the outer leaflet caused the subsequent

patching of the DOPA and the NBD-PA on the inner leaflet. In vesicles prepared with two different fluorophores on the two leaflets, it was possible to demonstrate that under conditions where calcium was excluded from the vesicles (see Fig. 2), the patching of the PA on the outer leaflet induced patch formation of the PA on the inner leaflet (Fig. 3).

These results using DOPC vesicles showing the apparent coupling of the two leaflets suggested that the homogeneous acyl chain composition was responsible for this unusual case. In addition to acyl chain composition, the effects of a different polar head group on leaflet interactions were also examined briefly. The results of these experiments (Table III) showed that the substitution of a portion of the DOPC with DOPE did not cause the uncoupling of the bilayers. However, again when the acyl chain composition of the vesicle was altered by the inclusion of brain-PE, no bilayer coupling was seen. Double-labeled vesicles containing DOPC, DOPE, and egg-PC also showed uncoupled behavior (data not shown). In the work of Schmidt et al. (19) the length of the acyl chain in sphingomyelin played a major role in the coupling or lack thereof between the two leaflets of the bilayer. N-lignoceryl-sphingomyelin showed coupling but N-stearoylsphingomyelin showed no coupling. It was suggested that the longer acyl chain of lignoceric acid could extend beyond the midpoint of the bilayer, interdigitating into the other monolayer, to provide a mechanism for coupling of the leaflets. In the studies presented here, homogeneity vs. heterogeneity of the acyl chains appeared to be the critical factor in the coupling or uncoupling of the leaflets of the bilayer. Heterogeneity of the fatty acids might overcome the weak interactions between the two leaflets and lead to an uncoupled response. Also, when Ca^{2+} was present on both sides of vesicles containing egg-PC, which show uncoupled behavior, the PA domains on the two leaflets appeared coincident (Fig. 1 D). In this case a distortion on one leaflet, that is a domain, due to the phase separation may have been compensated by an equal distortion on the other leaflet.

In conclusion, a unique system for examining lateral phase separations in the two leaflets of a phospholipid bilayer vesicle is described in this report. Using large asymmetrically labeled vesicles, it has been possible to visualize specifically either the inner or outer leaflet of the bilayer and examine domain formation within them. Independent phase separation in the two leaflets has been shown with naturally occurring phospholipids as well as an unusual case of coupled interactions with dioleoyl phospholipids.

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