Interaction of Annexin VI with Membranes: Highly Restricted Dissipation of Clustered Phospholipids in Membranes Containing Phosphatidylethanolamine†

Mohammad D. Bazzi and Gary L. Nelsestuen*

Department of Biochemistry, University of Minnesota, St. Paul, Minnesota 55108

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ABSTRACT: Association of annexin VI with membranes induced extensive clustering of acidic phospholipids as detected by self-quenching of fluorescent-labeled acidic phospholipids [Bazzi, M. D., & Nelsestuen, G. L. (1991) *Biochemistry 30*, 7961]. The present study examined the rates of protein-induced clustering of acidic phospholipids in membranes containing 10–15% fluorescent-labeled phosphatidic acid dispersed in phosphatidylcholine (PC) or phosphatidylethanolamine (PE). Both membranes supported similar levels of protein-induced fluorescence quenching. With membranes containing PC, protein–membrane association and fluorescence quenching were rapid, and were virtually complete within seconds after the reagents were mixed. Membranes containing PE exhibited rapid protein–membrane association, but showed a fluorescence quenching that was several orders of magnitude slower than membranes containing PC. Calcium chelation resulted in rapid dissociation of protein–membrane complexes. Subsequent recovery of the fluorescence signal of both membranes was virtually complete, but the rate of fluorescence recovery was very different. The recovery was rapid in membranes containing PC, while PE-containing membranes showed slow recovery that approached the rate at which the fluorescent-labeled phosphatidic acid exchanged between vesicles. Thus, the presence of PE appeared to severely restrict dissipation of clustered phospholipids in membranes. Membranes containing PE, *N*-methyl-PE, *N*,*N*-dimethyl-PE, and PC showed successive increases in the rates of fluorescence quenching and recovery, suggesting that hydrogen bonding between head groups was the basis for this property. If the restricted dissipation of phosphatidic acid in PE membranes is a general property, the relative mobility of membrane components and even diffusion on interior cell membranes may be greatly influenced by this phenomenon.

Annexins are a large family of proteins that share the common property of binding to membranes in a calcium-dependent manner (Burgoyne & Geisow, 1989; Klee, 1988). While the biological functions of these proteins are not known, a variety of possible functions have been proposed, based on protein properties (Pollard et al., 1990; Smith et al., 1990; Zaks & Creutz, 1990). Biochemically, annexin V, annexin VI, and protein kinase C exhibit a unique cooperative membrane binding that produces a highly sequential interaction of proteins on the same membrane particle (Bazzi & Nelsestuen, 1991b). The association of annexins with membranes is very responsive to the nature of the membrane so that annexins constitute very sensitive biological probes of membrane structure. They appear to detect biochemical properties that are largely unanticipated by biophysical probes (Bazzi et al., 1992).

Biological membranes display asymmetrical organization of phospholipids (Devaux, 1991). Most acidic phospholipids are found in intracellular membranes where they are embedded in a matrix composed largely of phosphatidylethanolamine (PE).[1] On the exterior leaflet of the plasma membrane, phosphatidylcholine (PC) is the major phospholipid. For many purposes, PE and PC may appear similar since they are both neutral, zwitterionic phospholipids that may function as a dispersion medium for more bioactive molecules. The association of several cytoplasmic proteins with membranes, however, exhibited a preference for acidic phospholipids dispersed in PE rather than PC (Bazzi et al., 1992; Hoffman & Marjerus, 1982; Kaibuchi et al., 1981). In certain cases, preferences for PE were dramatic and resulted in nearly a 10-fold reduction in the calcium requirement for protein–membrane association or in the ability of proteins to associate with membranes containing physiological levels of acidic phospholipids (Bazzi et al., 1992).

The phospholipid matrix may influence other membrane properties that can be detected by the annexins. For example, the association of annexin VI with membranes containing PC induced extensive clustering of acidic phospholipid components (Bazzi & Nelsestuen, 1991a). Consequently, protein binding appears to involve lateral motion of phospholipids in the membrane. This property may provide a tool for examining the microdiffusibility of phospholipids in membranes and for assessment of the importance of neutral phospholipids on this process.

In this study, we examined protein-induced clustering and declustering of acidic phospholipids in membranes of various compositions using fluorescence quenching or dequenching of fluorescent-labeled acidic phospholipids. While both PE- and PC-containing membranes bound protein rapidly, large differences were observed in the kinetics of clustering with PE producing much slower rates than PC. Recovery of fluorescence upon protein dissociation showed a similar pattern. Slow diffusion in PE-containing membranes may be due to hydrogen

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[1] Abbreviations: EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PMME, *N*-monomethylphosphatidylethanolamine; PDME, *N*,*N*-dimethylphosphatidylethanolamine; PS, phosphatidylserine; NBD-PA, NBD-PE, NBD-PG, and NBD-PS, 1-acyl-2-[12-[7-nitro-2,1,3-benzoxadiazol-4-yl)amino]dodecanoyl]phosphatidic acid, -phosphatidylethanolamine, -phosphatidylglycerol, and -phosphatidylserine, respectively; DMPC and DMPE, dimyristoyl-L-α-phosphatidylcholine and -phosphatidylethanolamine, respectively; DPPC and DPPE, dipalmitoyl-L-α-phosphatidylcholine and -phosphatidylethanolamine, respectively; SUV, small unilamellar vesicle(s) of about 30-nm diameter; LUV, large unilamellar vesicle(s) of 100-nm diameter.

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bonding between head groups. Similar properties may have important implications for biological membranes.

EXPERIMENTAL PROCEDURES

Materials. Egg phosphatidylcholine (PC) and phosphatidylethanolamine (PE) were purchased from the Sigma Chemical Co. or from Avanti Polar Lipids, Inc. Phosphatidyl-*N*-monomethylethanolamine (PMME), phosphatidyl-*N*,*N*-dimethylethanolamine (PDME), and 1-acyl-2-[12-[7-nitro-2,1,3-benzoxadiazol-4-yl)amino]dodecanoyl]phosphatidic acid (NBD-PA) as well as all other fluorescently labeled phospholipids were purchased from Avanti Polar Lipids, Inc. All phospholipids were greater than 98% homogeneous (supplier estimates). Other chemicals were from the Sigma Chemical Co. and were of the highest grade available. Annexin VI, a 64-kDa protein, was purified from bovine brain by a method described previously (Bazzi & Nelsestuen, 1991b) and followed by additional ion-exchange chromatography on a MonoQ column (Pharmacia). The protein preparations were highly homogeneous (>99%) by the criterion of staining patterns of SDS gel electrophoretograms.

Vesicle Preparation. Phospholipid vesicles were prepared by mixing the various phospholipid components in organic solvent. The solutions were dried under a stream of nitrogen and mixed with aqueous buffer (20 mM Tris, pH 7.9, containing 100 mM NaCl) to a final concentration of about 0.5 mg/mL. Single-bilayer vesicles were prepared by two different methods. Sonication involved placing the suspension in an ice–water bath and sonicating at maximum output with a direct probe microtip (Heat Systems Ultrasonic, Inc., Model W385). Cycles of 2 s with 3-s intervals were repeated to give a total sonication time of 2 min. Large unilamellar vesicles were prepared by the extrusion method (Hope et al., 1985) using 0.1-μm polycarbonate filters in the final step. Extrusion is a chemically mild method that produces homogeneous vesicles with a diameter of about 100 nm. Extensive experiments were conducted with vesicles prepared by each method, and the results showed no detected difference. The experiments selected for presentation below were derived primarily from vesicles prepared by sonication.

Fluorescence Measurements. Segregation of acidic phospholipids in membranes was monitored by self-quenching of NBD-PA fluorescence. Measurements were made in a Hitachi-Perkin-Elmer Model MPF44A fluorescence spectrophotometer with excitation at 460 nm and emission at 530 nm. A 500-nm emission cutoff filter was also used. Phospholipid vesicles (12.5–20 μg) containing 12–15% (w/w) fluorescent phospholipid were added to 1.6 mL of buffer (20 mM Tris, pH 7.5, and 100 mM NaCl) followed by calcium. For most of the experiments reported below, the change in the fluorescence signal was examined with time after a single addition of protein (10–30 μg). Other experiments examined the change in the fluorescence signal as a function of protein concentration. In the latter case, protein concentration was increased by several successive additions of protein at time intervals of about 5 min. In all cases, the reversibility of quenching was assessed by the addition of excess EGTA at the end of titrations. Unless indicated, reversibility was always complete within experimental error.

The magnitude of fluorescence changes was reported as a percentage change, $[(I - I_o)/I_o]100$, or as a logarithmic function, $\ln (I/I_o)$, where I represents the fluorescence signal of the sample and I_o represents the original fluorescence of the sample before beginning the experiment described. With some vesicle preparations, addition of protein resulted in an immediate but small (usually less than 4%) fluorescence quenching that did not appear to be related to protein–membrane interaction. This small change of fluorescence was not reversed by EGTA addition and was subtracted from the total signal as a background. Most vesicle preparations did not display this change.

Protein–Phospholipid Binding. Binding of annexin VI to phospholipid vesicles was measured by the light-scattering intensity as described in detail previously (Bazzi & Nelsestuen, 1987). Light-scattering intensity measurements allowed quantitative estimation of the amount of protein bound to small unilamellar vesicles (Nelsestuen & Lim, 1977). The results are reported as a molecular weight ratio, M_2/M_1, where M_2 is the molecular weight of the protein–lipid complex and M_1 is the molecular weight of the lipid only. Light scattering was determined at 90° in the Hitachi-Perkin-Elmer MPF44A fluorescence spectrophotometer with both the excitation and emission wavelengths set at 320 nm.

Other Methods. Phospholipid concentrations were determined from organic phosphate (Chen et al., 1956) using a phosphorus to phospholipid weight ratio of 1:25. Protein concentration was determined according to Bradford (1976) using BSA as a standard.

RESULTS

Binding of protein kinase C, annexin V, and annexin VI to membranes resulted in extensive clustering of acidic phospholipids as detected by self-quenching of NBD-PA dispersed in PC (Bazzi & Nelsestuen, 1991a). Recent results showed that the matrix of the membrane significantly influenced the membrane binding properties of these proteins (Bazzi et al., 1992). Protein-induced clustering of acidic phospholipids in membranes was examined using annexin VI and membranes containing either PE or PC as the matrix. The initial suggestion of a difference between PE and PC was obtained from the experiments shown in Figure 1.

Figure 1A shows titrations of fluorescence quenching as a function of annexin VI conducted at 25 °C. Binding of annexin VI to vesicles containing 12.5% NBD-PA dispersed in either PC or PE/PC (50:37.5) resulted in substantial quenching of the fluorescence signal (Figure 1A). In both cases, protein-induced fluorescence quenching appeared saturable and was reversed by the addition of EGTA. However, the two membranes exhibited different quenching curves, especially at lower concentrations of protein (Figure 1A) where membranes containing 50% PE seemed to display less fluorescence quenching. The difference was similar when both titrations were carried out at 2 mM calcium (data not shown). Figure 1B shows experiments conducted at 37 °C. In this case, protein-induced fluorescence quenching showed relatively similar titration curves with PC- or PE-containing membranes. Thus, an apparent difference between PE and PC could be minimized by conducting the experiments at higher temperatures. This suggested a possible rate-determined difference.

Rates of protein–membrane binding and fluorescence quenching were examined (Figure 2). With membranes containing 15% NBD-PA in PC, both protein–membrane binding and fluorescence quenching were very rapid, and were virtually complete within the time required to mix the reagents (Figure 2A). Protein–membrane binding was also rapid with membranes containing 15% NBD-PA and 50% PE. However, protein-induced fluorescence quenching was a much slower process (Figure 2B). At 25 °C, fluorescence quenching had not reached a maximum after 30 min. Addition of EGTA resulted in rapid dissociation of protein from both membranes

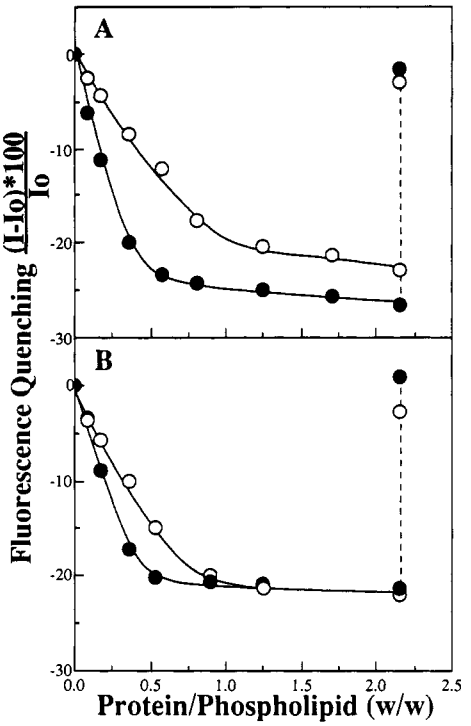

FIGURE 1: Protein-induced fluorescence quenching. The fluorescence intensity of vesicles was measured about 5 min after each addition of annexin VI. The titrations were performed at 25 °C (panel A) or 37 °C (panel B). Both panels showed results obtained with vesicles containing 15% NBD-PA/85% PC in the presence of 2.0 mM calcium (●) or with vesicles containing 15% NBD-PA/50% PE/35% PC in the presence of 0.25 mM calcium (○). All measurements were conducted with 25 μg of vesicles suspended in 1.6 mL of buffer containing 50 mM Tris (pH 7.5), 100 mM NaCl, and calcium as indicated. The dashed lines show the changes observed upon the addition of 5 mM EGTA.

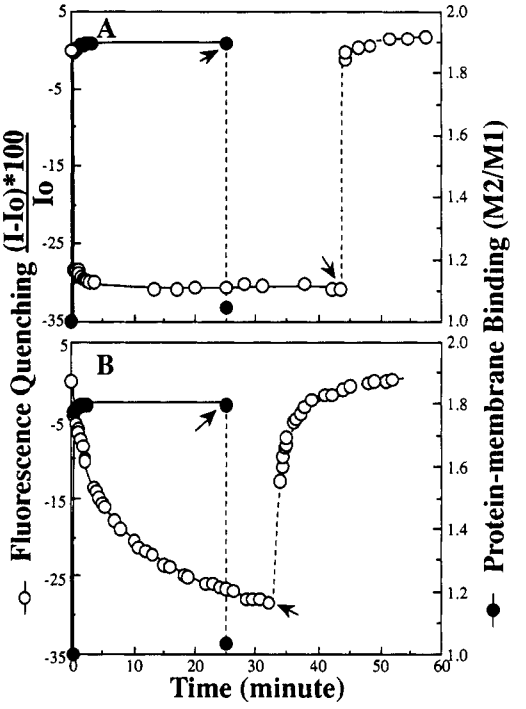

FIGURE 2: Time dependence of protein–phospholipid binding and fluorescence quenching. Protein–membrane binding (●) and protein-induced fluorescence quenching (○) were examined as a function of time using phospholipid vesicles containing NBD-PA/PC/PE at either 15:85:0 (panel A) or 15:35:50 (panel B). Protein–membrane binding was measured by light-scattering intensity using samples of the same phospholipid preparations that were used to measure protein-induced quenching of fluorescence. In both panels, measurements were initiated by the addition of 25 μg of annexin VI to the vesicles (12.5 μg in 1.5 mL of buffer) in the presence of saturating calcium, 5.0 (panel A) or 2.0 mM (panel B). At the times indicated by arrows, EGTA (2 mM in excess over calcium) was added. Dissociation of protein (●, dashed lines) and recovery of fluorescence (○, dashed lines) were monitored with time. All measurements were conducted at 25 °C.

(Figure 2, dashed lines). However, the rate of fluorescence recovery was different. Membranes containing PE exhibited a measurably slower rate of fluorescence recovery than membranes containing PC (Figure 2B).

Comparison of Figure 2A with Figure 2B suggested that the membrane matrix (PE vs PC) did not alter the quantitative aspects of either protein–membrane binding or fluorescence quenching; both membranes bound similar amounts of protein as indicated by the similar M_2/M_1 ratios, and both membranes exhibited similar magnitudes of fluorescence quenching (approximately 30% for both membranes). Also, binding of annexin VI to either membrane did not involve secondary events (Figure 2); light-scattering experiments did not exhibit time-dependent changes that are indicative of vesicle aggregation or fusion. Membranes containing PE simply showed a slower response of fluorescence quenching and dequenching.

The rate of protein-induced fluorescence quenching was examined as a function of the PE content of the membranes (Figure 3). In order to assure bilayer structure and minimum perturbation by divalent ions, the PE content of the vesicles did not exceed 80%. The phosphospholipid matrix influenced the calcium requirement for protein binding (Bazzi et al., 1992), and the experiments shown (Figure 3) utilized similar calcium saturation levels for the different membranes. Phospholipid clustering in membranes containing PC was very rapid (Figure 3A), and accurate determination of this rate was not attempted. Inclusion of as little as 20% PE in the membranes hindered the clustering process so that the rate of protein-induced fluorescence quenching became measurable (Figure 3A). Higher amounts of PE in the membranes had more dramatic effects. For example, membranes containing 80% PE showed fluorescence quenching that was still in its initial stages 1 h after protein–membrane binding (Figure 3A). Conducting these experiments at constant calcium concentration (2.0 mM) did not alter the qualitative differences between PE and PC membranes.

Although the results in Figure 3A suggested that there might be reduction in the magnitude of fluorescence quenching with increasing amounts of PE in membranes, the differences were due to the rates of phospholipid clustering. More complete fluorescence quenching experiments are shown in Figure 3B,C, where time is plotted on a logarithmic scale. At long time periods, similar levels of quenching were obtained in all membranes. Two formats of data presentation were adopted: a simple percentage change in fluorescence (Figure 3B) was useful for some comparisons. However, since the fluorescence of NBD-PA decreased exponentially with its concentration in the membranes (Bazzi & Nelsestuen, 1991a), a logarithmic scale for fluorescence intensity (Figure 3C) may give a closer approximation of the actual rate of clustering.

Regardless of the format, two important observations are evident in Figure 3. First, the magnitudes of protein-induced quenching were similar in all membranes examined. This suggested that annexin VI clustered a similar number of acidic phospholipids per protein, regardless of membrane composition. Second, membranes containing higher amounts of PE exhibited slower rates of fluorescence quenching. While the clustering rates in membranes containing PC could not be determined accurately, the results in Figure 3C suggested

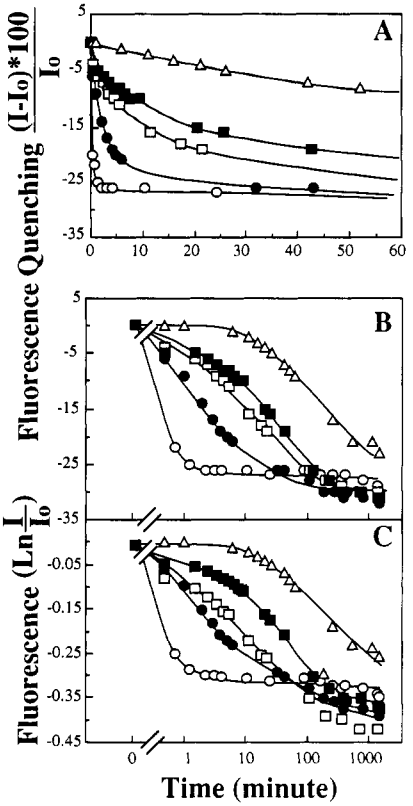

FIGURE 3: Effect of PE on the rate of protein-induced fluorescence quenching. Protein-induced fluorescence quenching was monitored with time after the addition of 22 μg of annexin VI to phospholipid vesicles (25 μg in 1.6 mL of buffer). In all panels, vesicles contained NBD-PA (12.5%) and PE at either 0% (○), 20% (●), 40% (□), 60% (■), or 80% (△). The remaining phospholipid was PC. Panel A shows the initial rates of fluorescence quenching. Fluorescence was monitored for more than 24 h, and more complete data are presented on logarithmic scale in panels B and C. Fluorescence quenching was expressed as a percentage change, $[(I - I_0)/I_0]100$, in panels A and B or as ln (I/I_0) in panel C. Experiments shown were conducted at 25 °C. See Experimental Procedures for other details.

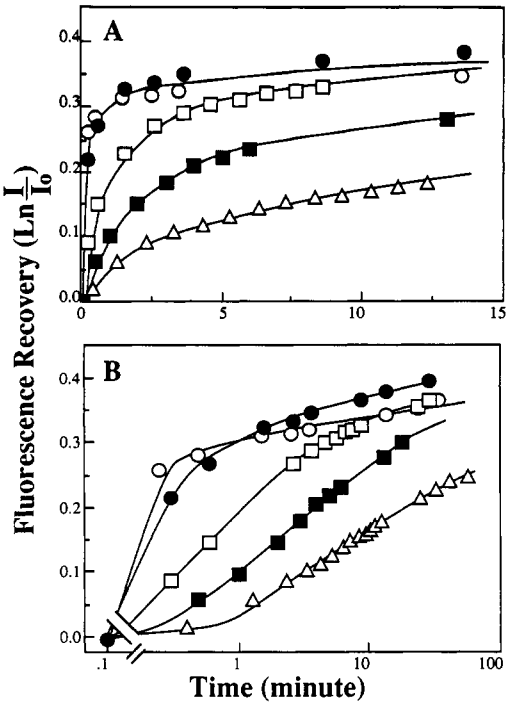

FIGURE 4: Fluorescence recovery as a function of PE content. At the end of the titrations described in Figure 3, excess EGTA was added to dissociate annexin VI. The fluorescence recovery of vesicles containing 12.5% NBD-PA and PE at 0% (○), 20% (●), 40% (□), 60% (■), or 80% (△) was monitored with time. Panel A shows the initial rates of fluorescence recovery, and panel B shows more extended data presented on a logarithmic scale. In both panels, the changes in fluorescence were expressed as ln (I/I_0), where I represents the fluorescence of the sample and I_0 represents the fluorescence of the same sample before addition of EGTA. The temperature was 25 °C.

that the phospholipid clustering rate in membranes containing PE was at least 3 orders of magnitude slower than that observed with membranes containing PC.

Annexins are a group of proteins that bind a variety of acidic or neutral membranes at the appropriate calcium concentrations. Interestingly, annexin V bound to membranes containing PA or PE at similar calcium concentrations (Andree et al., 1990). Consequently, it was possible that the slow clustering rates observed with membranes containing PE were the result of a slow approach to thermodynamic equilibrium. Initially, annexin VI may bind to the membrane, utilizing both PA and PE. Several steps of association and dissociation with individual phospholipid molecules would then be needed to reach thermodynamic equilibrium where PA molecules are preferred over PE. This could produce the slow fluorescence quenching of NBD-PA. Rates of quenching may therefore underestimate the real rate of diffusion of phospholipids in the PE-containing bilayers.

Recovery of the fluorescence signal, upon protein dissociation from membranes with clustered NBD-PA, should represent the rate of dissipation of the clusters without influence by external factors. Addition of EGTA to protein–membrane complexes caused rapid dissociation of protein from all membranes (see Figure 2, for example). Subsequent fluorescence recovery was examined (Figure 4). With membranes containing PC, the fluorescence signal rapidly returned to its original intensity (Figure 4A). This suggested that the rate of cluster dissipation in PC membranes was rapid and could not be determined accurately in these experiments. Membranes containing PE showed slower rates of fluorescence recovery. Half-recovery of the fluorescence signal required 3, 6, or 10 min for membranes containing 40%, 60%, or 80% PE, respectively (Figure 4B). Thus, it appeared that by substitution of PE for PC, the dissipation of NBD-PA clusters decreased by several orders of magnitude. Further studies suggested that the rates of dissipation in PE membranes, as suggested by the results in Figure 4, were upper estimates. The actual rates may be even slower (see below).

Comparison of Figure 3C with Figure 4B suggested that fluorescence quenching occured at slower rates than fluorescence recovery for all membranes containing PE. It was not possible to make such a comparison with membranes containing PC because of the promptness of both processes. However, neither the quenching nor the dequenching of the fluorescence may represent unrestricted motion in the membrane. For example, charge repulsion among PA molecules would hinder the clustering/quenching processes, but would facilitate the declustering/dequenching events. Fluorescence recovery might, therefore, overestimate the rates of cluster dissipation in the membrane.

An additional factor was the possible influence of phospholipid exchange on the quenching and dequenching processes. NBD-labeled phospholipids have a relatively high critical bilayer concentration so that an equilibrium exists between NBD-PA molecules in vesicles and those free in solution (Nichols & Pagano, 1981). This raises the possibility that the slow rates observed with membranes containing PE might arise from some exchange process. For example, quenching might arise from annexin-induced clustering of

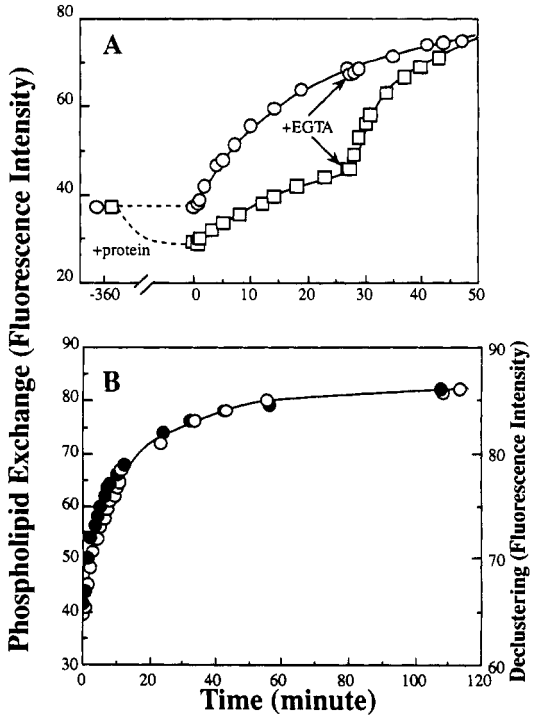

FIGURE 5: Comparison of phospholipid transfer rates and declustering rates. Panel A compares the rates of phospholipid transfer between labeled and unlabeled vesicles in the presence and the absence of protein. Two samples of phospholipid vesicles (25 μg of NBD-labeled PA/PE/PC, 12.5:80:7.5) in 1.6 mL of buffer containing 0.5 mM Ca^{2+} were used. Annexin VI was added to one sample (□), but not to the other (○), and both samples were incubated for 6 h to generate protein-induced clustering of NBD-PA and consequent fluorescence quenching (dashed lines). The fluorescence was monitored with time after the addition of a 5-fold excess (125 μg) of phospholipid vesicles composed of PC. After approximately 30 min (indicated by the arrows), excess EGTA was added to both samples. Panel B shows the rates of the fluorescence intensity change due to phospholipid transfer and declustering of NBD-PA in membranes containing PE. Phospholipid transfer (○, left scale) was measured as described in panel A: 25 μg of the donating vesicles contained NBD-labeled PA/PE/PC (12.5:80:7.5), and the acceptor vesicles contained PC (125 μg). Fluorescence recovery after adding EGTA to vesicles with protein-induced clustered phospholipids (●, right scale) is from the experiment presented in Figure 4. All experiments were conducted at 25 °C.

NBD-PA monomers in solution. The rate of fluorescence quenching might then be dependent upon dissociation of NBD-PA from membranes, a relatively slow process (Shin et al., 1991). This possibility was examined by measuring the spontaneous transfer of fluorescent phospholipid between labeled and unlabeled vesicles (Figure 5A).

Spontaneous transfer of NBD-PA was examined using two samples of phospholipid vesicles. One contained bound protein with clustered NBD-PA, and the other served as a control. Exchange from these vesicles was monitored by the increase in the fluorescence signal after addition of an excess of PC vesicles. In the absence of protein, there was a normal hyperbolic increase in the fluorescence signal which was indicative of NBD-PA transfer from labeled to the unlabeled vesicles (Nichols & Pagano, 1981). Chelation of calcium did not alter the rate of phospholipid transfer (Figure 5A). Only slightly faster exchange (about 25%) was observed when the donating vesicles were composed of PC rather than PE (data not shown). However, in the presence of protein, the rate of the fluorescence increase was significantly lower. In fact, the protein-induced fluorescence quenching appeared larger as the NBD-PA exchange progressed further (Figure 5A). This suggested that annexin VI clustered NBD-PA in the membrane, thereby preventing or slowing the transfer of these molecules to the unlabeled vesicles. Addition of EGTA dissociated the protein and produced an increase in the fluorescence signal (Figure 5A). Transfer of NBD-PA upon protein dissociation appeared relatively more rapid than the control, presumably because of the high concentrations of NBD-PA of the clustered, donating regions.

Thus, binding of annexin VI to membranes would create regions in the membranes with two different distributions of phospholipids: NBD-PA-rich regions (clustered phospholipids) and NBD-PA-depleted regions. Upon release of the protein, fluorescence recovery could be achieved by two mechanisms: lateral mobility (dispersion of clustered acidic phospholipids within the plane of membrane) or phospholipid transfer (spontaneous association/dissociation of NBD-PA monomers from various regions of the membranes). Because of the slow rate of exchange and rapid rate of fluorescence recovery (compare Figure 4 with Figure 5), lateral mobility was probably the dominant mechanism for fluorescence recovery with membranes containing PC or 20% PE. However, membranes containing 60% or 80% PE showed fluorescence recovery rates that were comparable to that of phospholipid transfer. Figure 5B shows the relative increase in fluorescence upon phospholipid transfer (open circles) and the recovery of fluorescence upon releasing annexing VI from membranes containing 80% PE (closed circles). Rates of fluorescence change were nearly indistinguishable. This suggested that the lateral mobility of phospholipids in PE-containing membranes could only be equal to or slower than that of phospholipid transfer. Thus, the recovery rates shown in Figure 4 represented an upper limit for the NBD-PA diffusion rates in PE membranes.

Hydrogen Bonding and Lateral Mobility of Phospholipids. PE could form an extensive hydrogen-bonding network (Boggs, 1987; Hauser et al., 1981), and this could be the underlying mechanism for the large difference in lateral mobilities of phospholipids in PE vs PC membranes. Successive methylation of PE produces structures that are intermediate between PE and PC, particularly in hydrogen-bonding ability (Gagné et al., 1985). Clustering of phospholipids was examined using membranes containing NBD-PA in a phospholipid matrix that consisted primarily of PE, *N*-monomethylphosphatidylethanolamine (PMME), *N,N*-dimethylphosphatidylethanolamine (PDME), or PC (Figure 6). All these phospholipids were prepared from egg PC by phospholipase D action and should contain similar acyl chain compositions.

Phospholipid matrices with different head groups exhibited different rates of protein-induced fluorescence quenching (Figure 6). These experiments were conducted at 37 °C, a temperature that gave convenient rates of fluorescence change. Initial rates of fluorescence quenching are shown in Figure 6A. Membranes containing PE showed a very slow quenching response to protein binding, but single methylation of the head group resulted in nearly a 10-fold enhancement of this rate (compare PE with PMME). Further methylation of the head group resulted in further enhancement of the clustering rate (compare PMME with PDME, Figure 6). Consistent with the data in the previous figures, membranes containing PC showed very rapid fluorescence quenching at this temperature.

Recovery of fluorescence upon protein dissociation was also examined at 37 °C (Figure 7). Membranes containing PC or PDME showed a very rapid response so that fluorescence recovery was virtually complete within the time required for mixing EGTA with the samples. Even though occurring at

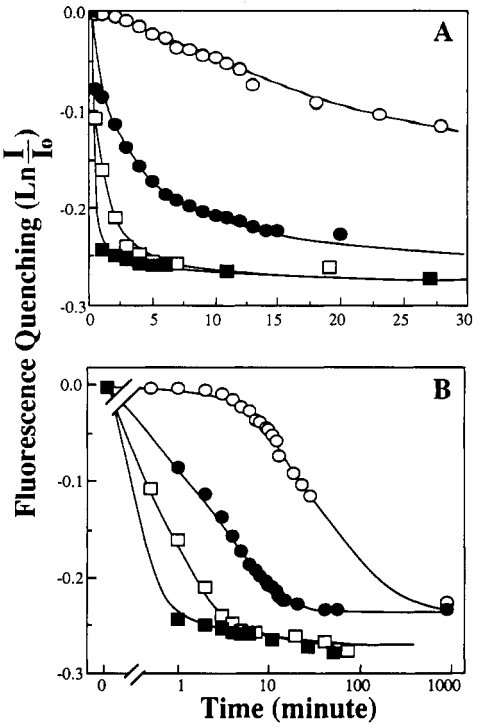

FIGURE 6: Effect of N-methylation on the rate of protein-induced fluorescence quenching. Protein-induced fluorescence quenching was examined using vesicles (20 μg) containing 12.5% NBD-PA, 7.5% PC, and 80% of either PE (○), PMME (●), PDME (□), or PC (■). In each case, fluorescence was monitored with time after the addition of annexin VI (25 μg). Panel A shows the initial rates of fluorescence quenching, and panel B shows the same data presented on a logarithmic scale and monitored for 20 h. These experiments were conducted using 1.6 mL of buffer containing 50 mM Tris, pH 7.5, 100 mM NaCl, and saturating calcium, 2.0 (□, ■) or 0.2 mM (○, ●). The temperature was 37 °C.

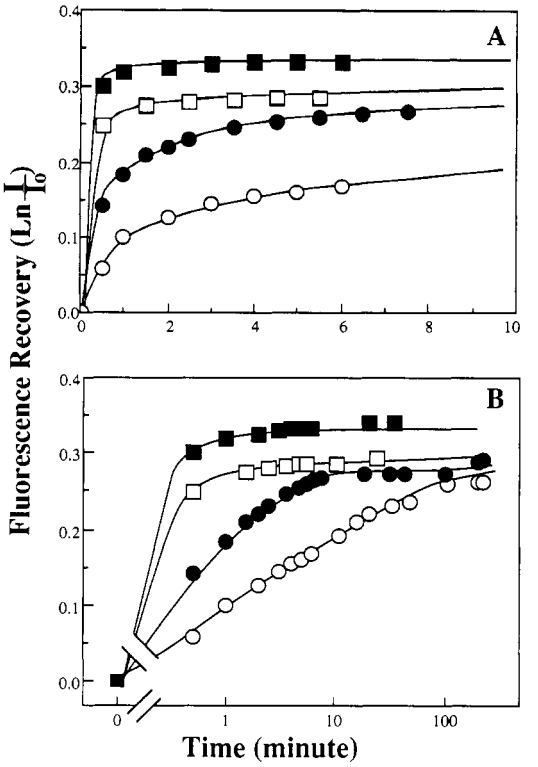

FIGURE 7: Effect of PE methylation on the rates of fluorescence recovery. At the end of the clustering experiments shown in Figure 6, excess EGTA (8 mM) was added to dissociate the protein, and the increase in fluorescence was monitored. Panel A shows the initial rates of fluorescence dequenching. Display of complete fluorescence recovery required use of a logarithmic scale presented in panel B. In both panels, fluorescence recovery is shown for vesicles containing 12.5% NBD-PA, 7.5% PC, and 80% of either PE (○), PMME (●), PDME (□), or PC (■). Data are presented as described in Figure 3.

measurable rates, fluorescence recovery of membranes containing PMME was also relatively fast, and the recovery process was nearly complete in 5 min. Membranes containing PE, however, showed the slowest response, and complete fluorescence recovery required about 2 h.

These data suggested that successive methylation of the head group, which altered the ability to participate in hydrogen bonding, altered the rates of fluorescence quenching. On the time scale of these experiments, the transformation of PE to monomethyl-PE had the most prominent effect on the clustering (Figure 6) or declustering rates (Figure 7); the differences between monomethyl-PE and dimethyl-PE, while significant, were not as dramatic. Comparison between dimethyl-PE and PC was not possible because of the rapid rate of clustering and declustering of both membranes.

DISCUSSION

The fluid mosaic model (Singer, 1974; Singer & Nicolson, 1972) is a cornerstone for description of biological membrane structure. Lateral and/or rotational motion of proteins and other components are regulated by membrane viscosity, protein concentration, protein aggregation number, and/or other specific interactions (Cherry, 1979; Jacobson et al., 1987). The phospholipids are often portrayed as passive components that provide a two-dimensional continuum with random distribution of phospholipids. A common corollary suggests impedance of mobility by virtue of acyl chain viscosity. While many properties are consistent with this detailed description of membranes, some observations need additional explanation. The present study suggested that phospholipid head-group interactions may also play an important role in determining distribution and/or mobility rates of membrane components.

Association of annexin VI with membranes probably involves multiple "calcium bridges" between protein and phospholipids (Bazzi & Nelsestuen, 1991b). An important feature of this interaction was the clustering of acidic phospholipids as detected by quenching of fluorescent phospholipids (Bazzi & Nelsestuen, 1991a). The rate of phospholipid clustering should reflect the lateral mobility of phospholipids in the membrane. The present study suggested that the rates of protein-induced fluorescence quenching of NBD-PA varied tremendously depending on whether the membranes contained PE or PC. However, protein–membrane binding may involve the active participation of PE until the thermodynamically most stable protein–membrane contact, involving all acidic phospholipids, is attained. Thus, a more important measure of phospholipid mobility was the rate of fluorescence recovery after addition of EGTA (Figures 4 and 7).

Upon protein dissociation, the clusters of NBD-PA produced by protein binding should be able to disperse without the influence of external factors. Protein–membrane dissociation was rapid in all cases examined, and, as expected, fluorescence recovery in PC-containing membranes was also rapid on the time scale of the measurement (<10 s). However, dissipation of NBD-PA clusters in PE was exceedingly slow and approached the rate of NBD-PA transfer between different vesicles. The latter process may actually become the method of fluorescence recovery in membranes of high PE content, and the dissipation rate may be so restricted as to render the

NBD-PA clusters virtually permanent membranes features. This property would have many implications for membrane mobility and/or organization, especially pertaining to interior cell membranes that contain high levels of PE (Devaux, 1991).

The dissipation rate of clustered NBD-PA would have to arise partially or wholly from properties of the bulk phospholipid, not the NBD moiety, since slow dissipation was unique to membranes containing PE. Two mechanisms might account for this behavior. First, PE may simply restrict lateral diffusion of phospholipids in membranes. This mechanism should be easily detected by other techniques that measure lateral diffusion. While such a property has not been reported, only a few studies have examined PE (see below), and further work may be needed to fully test this possibility. A second mechanism for restricted dissipation of NBD-PA clusters might consist of a highly restricted phospholipid miscibility; PE may restrict the dispersion of clustered NBD-PA without impeding other types of phospholipid diffusion. Such a situation could arise if there were a thermodynamic barrier for entrance of another phospholipid into a lattice of PE or for penetrating the interface between two regions of pure phospholipids. This second mechanism may not be easily detected by other common methods for measuring lateral diffusion in membranes. Either of these mechanisms would have significant impact on our understanding of mobility in biological membranes, and it is possible that both mechanisms have contributed to the observed results, since they are not mutually exclusive.

Clustering and declustering experiments were performed at temperatures significantly above the melting points of the acyl chains (37 and 25 °C) so that it is unlikely that membrane fluidity was the basis for restricted dissipation of NBD-PA clusters in membranes containing PE. Furthermore, changes in fluidity of the acyl chain region of the membrane do not always correlate with changes in lateral mobility (Kleinfeld et al., 1981). PE is known to have a smaller hydration shell (Rand & Parsegian, 1989) and to exhibit higher thermal transitions than PC despite identical acyl chains. Both of these properties may arise from hydrogen bonding between PE head groups (Boggs, 1987). It is very likely that the restricted NBD-PA dispersion was related to hydrogen-bonding interactions. Extensive arguments support the proposal that PE participates in hydrogen bonding both above and below its gel- to liquid-crystal-phase transition (Boggs, 1987; Hauser et al., 1981).

Other observations support the conclusion that hydrogen bonding by PE was the major impediment to dissipation of clustered NBD-PA. For example, systematic variation of the head groups by successive methylation from PE to PC (all phospholipids possessed similar acyl chain compositions) resulted in faster rates of fluorescence quenching and recovery (Figures 6 and 7). While PMME is still capable of forming a hydrogen-bonding lattice (Boggs, 1987), the methyl group may restrict the range or the strength of the lattice. Quenching and dequenching processes were much faster in membranes containing PDME or PC, neither of which should form multiple simultaneous hydrogen bonds.

Relationship to Other Studies. Lateral mobility in membranes has been the subject of many investigations. Basically, phospholipid bilayers and natural biomembranes exhibit large differences in lateral mobilities and in the extent of an "immobile" fraction. Bilayers of pure phospholipids, sometimes referred to as "artificial" membranes, usually display rapid diffusion coefficients, typically 10^{-7}–10^{-8} cm^2/s, that are regulated by the fluidity of the acyl chain (Fahey & Webb, 1978; Wu et al., 1977). In contrast, phospholipids in biomembranes often display much lower diffusion coefficients (typically $D = 10^{-9}$–10^{-11} cm^2/s). In a similar contrast, proteins reconstituted in artificial membranes often exhibit faster diffusion rates than they have in natural membranes. When examined by fluorescence recovery after photobleaching (FRAP), there is usually a significant immobile fraction in natural, but not in artificial, membranes (Jacobson et al., 1987). Relative to artificial bilayers, lateral mobility in natural membranes is probably influenced by protein concentration and/or specific interactions among membrane components.

PC is the usual choice for studies with artificial membranes, with an extensive literature dedicated to this particular phospholipid. In contrast, there have been very few studies which examine PE-containing membranes. Michaelson et al. (1974) reported that the head-group mobility of PE was slower than that of PC. Galla et al. (1979) reported that the rate of pyrenyl-PC diffusion in PE membranes was slightly slower than that observed with PC membranes. Interestingly, lipids extracted from natural membranes often provide lower diffusion coefficients than PC membranes. For example, it has been reported that the diffusion coefficient of NBD-PE was about 4 times faster in egg PC than in lipids extracted from erythrocytes (Golan et al., 1984). Also, lipids derived from spermatozoa displayed low diffusion coefficients and a significant immobile fraction [see Wolf et al. (1988) and references cited therein]. The latter study concluded that the immobile fraction must arise from segregation of phospholipid components. It is possible that lipids extracted from natural membranes contained some phospholipids that participate in hydrogen bonding and, thus, contribute to various types of hindered lateral motion. Other phospholipids and membrane components may interrupt hydrogen bonding and enhance rates of lateral motion. In this way, cells may be able to create large or small regions of discontinuous or immobile membrane structure.

Diffusibility of phospholipids in the inner and outer leaflets of erythrocytes has been examined (Henis et al., 1982; Morrot et al., 1986). At high temperatures (≥6 °C), the mobile fraction of the inner leaflet exhibited lower viscosity and a higher diffusion rate than the external leaflet. Diffusion of fluorescent phospholipids in erythrocytes apparently is a multicomponent process, and the estimated diffusion rates of both leaflets do not differ greatly. Furthermore, the temperature dependence of diffusion in the inner leaflet of erythrocytes suggested discontinuities that were not due to the gel–liquid-crystal-phase transition of the lipids (Henis et al., 1982). It is possible that discontinuities and other unexplained properties of membrane diffusion in these systems arise from factors related to hydrogen bonding by PE in the membranes.

Evaluation of Protein-Induced Self-Quenching of Fluorescent Phospholipids as a Probe of a Specific Type of Lateral Mobility. Lateral mobility of proteins and/or phospholipids has been examined by a variety of biophysical methods such as NMR, EPR, fluorescence polarization, and FRAP [for a review, see Cherry (1979)]. These techniques measure the average motion of molecules in membranes. They sample the entire range of possible motions, and are usually insensitive to minor heterogeneities that may exist in membranes. In contrast, protein-induced self-quenching of fluorescent phospholipids, as a probe of lateral mobility, samples relative motion of phospholipid and is very sensitive to minor membrane heterogeneities.

The system used in this study appeared unique and capable of measuring the rate at which individual members of a cluster of phospholipids disperse by entering a bulk phase composed of a different phospholipid. Association of annexin VI with a membrane involved clustering of 10–12 acidic phospholipid molecules (Bazzi & Nelsestuen, 1991a) in a small area of the membrane [estimated at 60 phospholipids from light-scattering experiments; also see Meers et al. (1991)]. Given random distribution of phospholipid clusters on the membrane surface, the distance from the center of the clustered phospholipids to the outer edge of the membrane area from which they are derived is about 4 nm. Thus, dissipation of such a cluster of acidic phospholipids can be achieved by diffusion over extremely small distances. In contrast, most previous methods for measuring motion in membranes, such as FRAP, examine movements over long distances (typically micrometers) which can include motion of individual phospholipids as well as larger aggregates. Thus, extremely slow dispersion of phospholipid clusters, observed in this study, will not necessarily translate into virtual membrane immobility on the much larger diffusional scale.

Phospholipids may have a high propensity to exist as small clusters when present in a membrane containing PE. Small clusters may not be detected as separate phases by common physical techniques, but proteins could be valuable tools in detecting or producing such types of microheterogeneities in membranes. For example, preformed clusters of PS in PE might explain the observation that 20% PS in PE was as effective in binding the annexins as 100% PS (Bazzi et al., 1992). Other preliminary studies of protein-induced fluorescence quenching also supported this concept.[2] With membranes containing 15% fluorescent phospholipid dispersed in egg PC, annexin VI quenched the fluorescence of NBD-PA, NBD-PG (Bazzi & Nelsestuen, 1991a), NBD-PS, and N-labeled NBD-PE. However, association of annexin VI with membranes containing 15% NBD-PA dispersed in DMPC, DMPE, DPPE, or DPPC produced little quenching of fluorescence.[2] Since the fluorescence of NBD-PA in these matrices was already highly quenched, it is possible that the phospholipids did not form a homogeneous mixture but already contained clusters of acidic phospholipids. Recktenwald and McConnell (1981) reported the possible coexistent of immiscible fluid–fluid domains in DMPC membranes.

Overall, the approach used appears to examine a unique type of mobility. The results suggested that membranes may have the capability of generating highly restricted diffusion barriers. It seems probable that, under some circumstances, this property will apply to biological membranes as well. The observed properties are consistent with the reported hydrogen bonding of PE and have implications for the functions and properties of biological membranes.

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