Taki, T., Kimura, H., Takatsuka, C., & Matsumoto, M. (1983) *J. Biochem. (Tokyo) 94*, 925-930.
Taki, T., Maeda, H., Arai, K., Matsumoto, M., Kon, K., & Ando, S. (1988) *Cell Struct. Funct. 13*, 61-72.
Tatsumura, T., Ariga, T., Yu, R. K., & Satorelli, A. C. (1988) *Cancer Res. 48*, 2121-2124.
Tsuboyama, A., Takaku, F., Sakamoto, S., Kano, Y, Ariga, T., & Miyatake, T. (1980) *Br. J. Cancer 42*, 908-914.
Vance, D. E., & Sweeley, C. C. (1967) *J. Lipid Res. 8*, 391-395.
Wiels, J., Holm, E. H., Cochran, N., Tursz, T., & Hakomori, S.-I. (1984) *J. Biol. Chem. 259*, 14783-14787.
Xia, X.-J., Gu, X.-B., Satorelli, A. C., & Yu, R. K. (1989) *Lipid Res. 30*, 181-188.
Yamakawa, T., & Nagai, Y. (1978) *Trends Biochem. Sci. 3*, 128-131.
Yohe, H. C., Cuny, C. L., Berenson, C., & Ryan, J. L. (1988) *J. Leukocyte Biol. 44*, 521-528.
Yoshida, K., Seki, M., Maeda, M., Fujita, J., & Tadokoro, K. (1988) *Leuk. Res. 12*, 731-736.
Young, W. W., Hakomori, S.-I., Durdik, J. M., & Henney, C. S. (1980) *J. Immunol. 124*, 199-201.
Yu, R. K., & Ledeen, R. W. (1970) *J. Lipid Res. 11*, 506-516.

Extensive Segregation of Acidic Phospholipids in Membranes Induced by Protein Kinase C and Related Proteins†

Mohammad D. Bazzi and Gary L. Nelsestuen*
Department of Biochemistry, University of Minnesota, St. Paul, Minnesota 55108
Received February 20, 1991; Revised Manuscript Received May 15, 1991

ABSTRACT: Protein kinase C and two other proteins with molecular masses of 64 and 32 kDa, purified from bovine brain, constitute a type of protein that binds a large number of calcium ions in a phospholipid-dependent manner. This study suggested that these proteins also induced extensive clustering of acidic phospholipids in the membranes. Clustering of acidic phospholipids was detected by the self-quenching of a fluorescence probe that was attached to acidic phospholipids (phosphatidic acid or phosphatidylglycerol). Addition of these proteins to phospholipid vesicles containing 15% fluorescently labeled phosphatidic acid dispersed in neutral phosphatidylcholine resulted in extensive, rapid, and calcium-dependent quenching of the fluorescence signal. Fluorescence-quenching requirements coincided with protein–membrane binding characteristics. As expected, the addition of these proteins to phospholipid vesicles containing fluorescent phospholipids dispersed with large excess of acidic phospholipids produced only small fluorescence changes. In addition, association of these proteins with vesicles composed of 100% fluorescent phospholipids resulted in no fluorescence quenching. Protein binding to vesicles containing 5–50% fluorescent phospholipid showed different levels of fluorescence quenching that closely resemble the behavior expected for extensive segregation of the acidic phospholipids in the outer layer of the vesicles. Thus, the fluorescence quenching appeared to result from self-quenching of the fluorophores that become clustered upon protein–membrane binding. These results were consistent with protein–membrane binding that was maintained by calcium bridges between the proteins and acidic phospholipids in the membrane. Since each protein bound eight or more calcium ions in the presence of phospholipid, they may each induce clustering of a related number of acidic phospholipids. This property, which was very striking for this class of proteins, was barely detectable for another class of proteins that display calcium-dependent binding to membranes containing acidic phospholipids. The membrane-altering behavior of protein kinase C and other related proteins may contribute unique features to the total calcium response of the cell.

Protein kinase C (PKC)[1] is a regulatory enzyme that is believed to be involved in many cell functions (Nishizuka, 1986). With appropriate substrates, the activation of this enzyme is dependent on calcium and phospholipids (Bazzi & Nelsestuen, 1987a). Understanding the interaction of PKC with these two cofactors is essential for understanding the process of its activation and its role in the calcium response [reviewed in Nelsestuen and Bazzi (1991)]. PKC binds to membranes containing acidic phospholipids in a calcium-dependent manner. This property is shared with other diverse groups of proteins including various intracellular proteins (Klee, 1988), vitamin K dependent proteins of the plasma (Schwalbe et al., 1989), and others (Creutz et al, 1987; Khanna et al., 1990; Schwalbe, 1990). However, PKC and other recently described proteins (Bazzi & Nelsestuen, 1991a) constitute a category that appears distinguishable from the others by several unusual properties.

The interaction of PKC with calcium was strongly dependent on the presence of phospholipids (Bazzi & Nelsestuen,

†Supported in part by Grant GM 38819 from the National Institutes of Health.

[1] Abbreviations: EGTA, ethylene glycol-bis(β-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid; PC, phosphatidylcholine; PS, phosphatidylserine; PG, phosphatidylglycerol; NBD-PA, 1-acyl-2-[12-[(7-nitro-2,1,3-benzoxadiazol-4-yl)amino]dodecanoyl]phosphatidic acid; NBD-PC, 1-acyl-2-[12-[(7-nitro-2,1,3-benzoxadiazol-4-yl)amino]dodecanoyl]-phosphatidylcholine; NBD-PG, 1-acyl-2-[12-[(7-nitro-2,1,3-benzoxadiazol-4-yl)amino]dodecanoyl]phosphatidylglycerol; PKC, phospholipid- and calcium-dependent protein kinase C; SUV, small unilamellar vesicles of about 30-nm diameter; LUV, large unilamellar vesicles of 100-nm diameter.

0006-2960/91/0430-7961$02.50/0

1990). In fact, little calcium binding to free PKC was detected. However, in the presence of membranes, PKC bound eight to nine calcium ions. Two additional proteins of M_r 64K and 32K, purified from bovine brain, had similar calcium- and phospholipid-binding properties (Bazzi & Nelsestuen, 1991a). Other proteins such as lipocortin I and II also show phospholipid-enhanced calcium binding but bound fewer calcium ions per protein (Glenney, 1986; Glenney et al., 1987; Schlaepfer & Haigler, 1987). Since the free form of these proteins bound little or no calcium, they could not accurately be described as calcium-binding proteins. Instead, PKC and those related proteins would best be described as calcium-interacting systems that are comprised of a protein plus phospholipid.

The interaction between membranes and members of this class of proteins may be supported by multiple calcium "bridges". In this model, the proteins would provide only partial coordination of each calcium while the phospholipid may provide the remainder to form a "complete calcium-binding site". The crystal structue of annexin V, a member of the lipocortin family, has recently provided support for this general model (Huber et al., 1990). The coordination of calcium at different "binding sites" on annexin V was variable, but none was geometrically complete. It is possible that phospholipids may complete the coordination sphere of calcium, thereby supporting protein–membrane association.

The effects that these proteins have on the structure of the membrane is not known. For example, if binding of PKC to membranes involves eight to nine calcium "bridges", a related number of acidic phospholipids may be involved. In membranes with compositions comparable to intracellular membranes (about 20% acidic phospholipids), this large number of contacts may require extensive clustering of acidic phospholipids. This study was initiated to investigate this possibility. The results showed that PKC, the 64-kDa, and the 32-kDa proteins caused changes in the fluorescence yield of a membrane reporter group that were consistent with extensive segregation of acidic phospholipids. Clustering of acidic phospholipids by these proteins was particularly striking due to its virtually quantitative extent. This extreme behavior correlated with another highly unusual behavior of these proteins that is illustrated in an accompanying report (Bazzi & Nelsestuen, 1991b).

Experimental Procedures

Materials. Bovine brain phosphatidylserine (PS) and egg yolk phosphatidylcholine (PC) were purchased from Sigma Chemical Co. or from Avanti Polar Lipids, Inc. 1-Acyl-2-[12-[(7-nitro-2,1,3-benzoxadiazol-4-yl)amino]dodecanoyl]-phosphatidic acid (NBD-PA), phosphatidylglycerol (NBD-PG), and phosphatidylcholine (NBD-PC) were purchased from Avanti Polar Lipids, Inc. Polycarbonate filters (0.1-μm diameter) were purchased from Nucleopore Corp. Other chemicals and reagents were from Sigma Chemical Co. and were of the highest grade available.

Protein Purification. Protein kinase C, the 64-kDa protein, and the 32-kDa protein were purified to apparent homogeneity from bovine brain by methods described previously (Bazzi & Nelsestuen, 1991a). Bovine protein Z and bovine blood-coagulation factor X were purified according to published procedures (Nelsestuen, 1984).

Vesicle Preparation. Phospholipid vesicles were prepared by mixing the various phospholipid components in organic solvent. The solutions were dried under a stream of nitrogen, mixed with aqueous buffer (20 mM Tris, pH 7.9, containing 100 mM NaCl), and sonicated at maximum output with a direct probe microtip (Heat Systems Ultrasonic, Inc., model W385). Cycles of 2 s followed by a 3-s wait were repeated to give a total sonication time of 5 min. The samples were kept in an ice–water bath during the entire procedure. The vesicle preparation was centrifuged at 48000g for 1 h. However, for determination of protein–membrane binding, small unilamellar vesicles that are suitable for light-scattering intensity measurements were obtained by gel filtration of the sonicated vesicles (Bazzi & Nelsestuen, 1987b; Huang, 1969). All vesicle preparations showed properties corresponding to small unilamellar vesicles as determined by light-scattering and gel filtration measurements.

Large unilamellar vesicles were prepared by the extrusion method (Hope et al., 1985) using 0.1-μm polycarbonate filters. This method produces homogeneous vesicles (diameter of about 100 mm) with little or no chemical degradation of the phospholipids.

Fluorescence Measurements. Segregation of acidic phospholipids in membranes was monitored by the quenching of NBD-PA or NBD-PG fluorescence. Measurements were made in a Hitachi–Perkin-Elmer model MPF44A fluorescence spectrophotometer with excitation at 475 nm and emission at 530 nm. A 500-nm cutoff filter was also used. Phospholipid vesicles (12.5–50 μg) containing various percentages of fluorescent phospholipid were added to 1.6 mL of buffer (20 mM Tris, pH 7.5, containing 100 mM NaCl) followed by calcium. The change in the fluorescence signal was then examined as a function of protein addition to the solution. Fluorescence quenching was reported as a percentage change, $(I - I_o) \times 100/I_o$, where I_o represents the fluorescence signal of the phospholipid after adding calcium, and I is the fluorescence signal in the presence of protein and calcium. The reversibility of quenching was assessed by the addition of excess EGTA at the end of titrations. All values were corrected for dilution.

Fluorescence quenching was also examined as a function of calcium concentration at constant protein. In these experiments, phospholipid vesicles and protein (12.5–25 μg of each component) were mixed in 1.6 mL of buffer, and the fluorescence signal was monitored as a function of added calcium. In all cases, a simultaneous experiment was conducted with a phospholipid solution that contained no protein. Fluorescence quenching due to protein (ΔI) was obtained by simple subtraction of the fluorescence intensity of the sample containing protein plus phospholipid from that of the sample containing phospholipid only. The values are reported as percentage changes, $\Delta I \times 100/I_o$, where I_o is the initial fluorescence of the phospholipids in the absence of protein or calcium.

Protein–Phospholipid Binding. Binding of the various proteins to phospholipid vesicles was measured by light-scattering intensity as described in detail previously (Bazzi & Nelsestuen, 1987b). Phospholipid vesicles were added to 1.6 mL of buffer (20 mM Tris, pH 7.5, containing 100 mM NaCl), and the binding was monitored as a function of calcium concentration at a constant protein to phospholipid ratio or as a function of protein concentration at a constant calcium concentration. 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. M_2/M_1 is related to I_2/I_1 by the general relationship

$$I_2/I_1 = (M_2/M_1)^2[(\partial n/\partial c_2)/(\partial n/\partial c_1)]^2$$

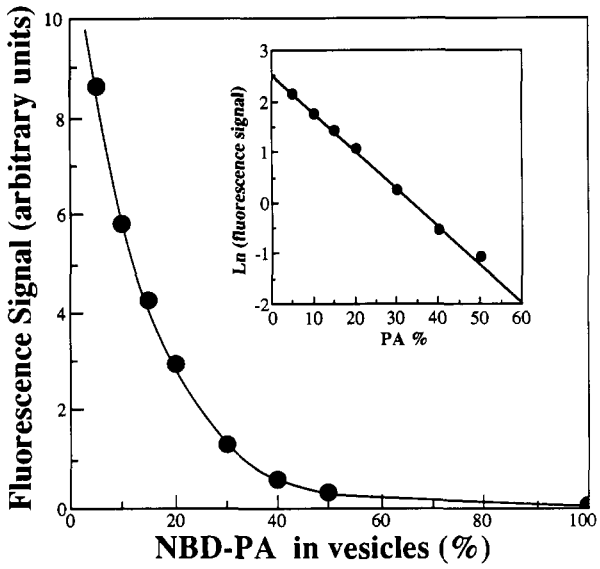

FIGURE 1: Fluorescence of NBD-PA in vesicles. The fluorescence of phospholipid vesicles containing NBD-PA at the indicated level (the remaining phospholipid was PC) was determined for small unilamellar vesicles prepared by sonication and centrifugation. The fluorescence intensity of these vesicles (18 μg) was measured and normalized to equal concentrations of NBD-PA. The inset shows the data plotted on semilogarithmic scale.

where I_2 is the light-scattering intensity of the protein–lipid complex, I_1 is light-scattering intensity of the phospholipid, and $\partial n/\partial c$ is the refractive index increment of each species (Nelsestuen & Lim, 1977). Light scattering was detected at 90° with light of 320 nm in the Hitachi–Perkin-Elmer spectrofluorometer. All experiments were conducted at 25 °C.

Calculation of Fluorescence of Clustered Phospholipids. Although major conclusions could be supported by qualitative analysis of the data, a mathematical model was developed as well. The mathematical model was based on several assumed features. Small unilamellar vesicles (M_r 4 × 10^6) were assumed to contain 5000 phospholipid molecules distributed uniformly into an outer layer (60% of the total phospholipids) and an inner layer (40% of the total phospholipids). This is similar to the distribution proposed previously (Huang & Mason, 1978). The phospholipid vesicles were composed of PC and NBD-PA at various ratios, and it was assumed that both phospholipids occupied similar areas in the membrane. Each phospholipid vesicle was capable of binding a number of protein molecules (Y) that was related to the vesicle composition (see below). At any given composition, the initial fluorescence of the vesicles was proportional to the number and the concentration of NBD-PA molecules. For example, the fluorescence of vesicles containing X% NBD-PA was calculated as 5000 × (X/100) × F(X%), where 5000 × (X/100) is the number of NBD-PA molecules and $F(X\%)$ is the fluorescence yield of NBD-PA at X% composition. The term $F(X\%)$ was obtained from the data in Figure 1 (see below).

Fluorescence quenching was assumed to arise equally from inter- and intralayer quenching events.[2] Binding of each protein molecule was assumed to cluster N acidic phospholipids. The result of binding Y proteins was the clustering of $N \times Y$ NBD-PA molecules in the outer layer of the vesicles. The remaining NBD-PA molecules in the outer layer [3000 × (X/100) − ($N \times Y$)] were assumed to be distributed uniformly [3000 − ($N \times Y$)]. This generated two regions of membrane with different NBD-PA compositions. The average composition of the region containing the clustered NBD-PA would be increased to (100 + X)/2, the average of the outer layer (100% NBD-PA) and the inner layer (X%, the original composition). The average composition of the NBD-PA-depleted region would be decreased to the average of the inner layer composition (X%) and the composition of the depleted region of the outer layer.

The fluorescence signal of vesicles was then calculated as the sum of the fluorescence of the two regions. Changes in the fluorescence upon clustering was reported as a percentage difference: $\Delta F(\%) = (F_c - F_i) \times 100/F_i$, where F_c is the fluorescence of the vesicles after clustering and F_i is the initial fluorescence of the vesicles.

Vesicles with different acidic phospholipid compositions appeared to bind different amounts of protein. Vesicles containing 25% NBD-PA or higher could bind the same amount of protein regardless of their composition. NBD-PA in excess of 25% was therefore assumed to distribute randomly without influence by the proteins. This upper limit closely correlated with the composition where vesicles became saturated with PKC (Bazzi & Nelsestuen, 1987b). Under these conditions, light-scattering measurements indicated that 0.85 g of protein could bind to 1 g of phospholipids. A lower limit of composition was also imposed. At 1.0 mM calcium, protein–phospholipid binding was considered negligible with vesicles containing 2.5% NBD-PA or less. The concept of a minimum composition as well as the actual value used (2.5%) correlated with a report for PKC activation (Newton & Koshland, 1990). The model then assumed that membranes containing 2.5–25% NBD-PA could accommodate protein molecules in direct proportion to their composition.

Other Methods. Phospholipid concentrations were determined from organic phosphate (Chen et al., 1956) by using a phosphorus to phospholipid weight ratio of 1:25. Protein concentration was determined according to Bradford (1976) with BSA as a standard. The buffers used were 20 mM Tris, pH 7.5, containing 0.1 mM NaCl.

RESULTS

The fluorescence of NBD-labeled phospholipids is subject to the self-quenching phenomenon (Graham et al., 1985; Hoekstra, 1982; Nichols & Pagano, 1981). Although the basis for self quenching of NBD is not known and may include several mechanisms (MacDonald, 1990, and references cited therein), this phenomenon provides a basis for detecting phospholipid clustering in bilayer membranes. When dispersed in membranes that contained PC as the inert phospholipid, the fluorescence of NBD-PA appeared to decrease exponentially with its concentration (Figure 1). An apparent exponential increase of self-quenching has been observed with several other fluorescence probes (Sklar et al., 1980; MacDonald, 1990). The linear relationship of the plot shown in the inset of Figure 1 was therefore empirically used to predict the fluorescence intensity of membranes of various compositions (see below).

Effect of the 64-kDa Protein on NBD-PA. Addition of the 64-kDa protein to small unilamellar vesicles containing 10% NBD-PA resulted in substantial quenching of the fluorescence signal in a calcium-dependent process (Figure 2A). Calcium

[2] Assuming an average area of 79 Å²/phospholipid molecule, the diameter of a circle containing five phospholipid molecules is 22.4 Å. Since phospholipid vesicles containing 20% NBD-PA (one probe per five phospholipids) exhibited more than 50% quenching, the critical distance (R_0) for 50% quenching was probably less than the distance between fluorophores in the inner and outer layers of the vesicles. Interlayer quenching was probably important, especially for membranes with low densities of probe.

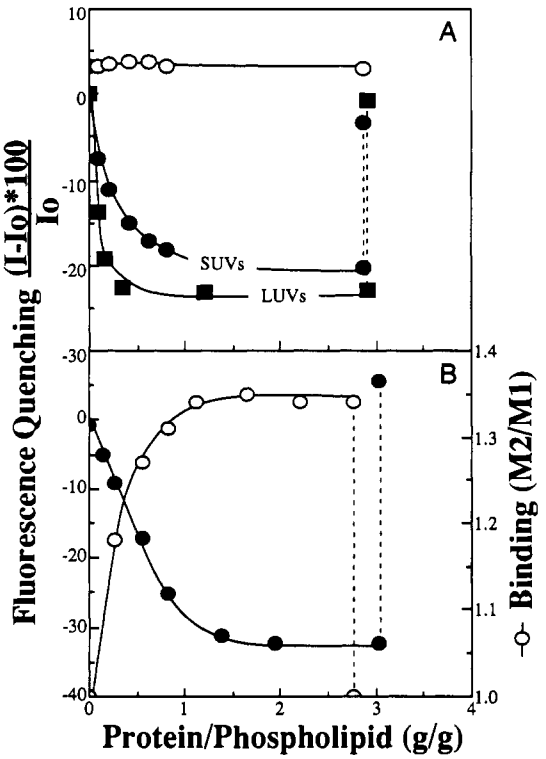

FIGURE 2: Fluorescence quenching induced by the 64-kDa protein. Panel A shows the fluorescence of large (■) or small (●) unilamellar vesicles as a function of added 64-kDa protein in the presence of 1 mM calcium. In both cases, 12.5 μg of NBD-PA/PC (10:90) was used. The panel also shows a titration that was performed with small unilamellar vesicles in the presence of 1 mM EGTA (○). Panel B shows the titration of protein–phospholipid binding (M_2/M_1, ○) and fluorescence quenching (●) using small unilamellar vesicles (25 μg of NBD-PA/PC, 15:85) in the presence of 1 mM calcium. In both panels, the dashed lines signify the changes observed upon the addition of 3 mM EGTA. The sample volume was 1.6 mL.

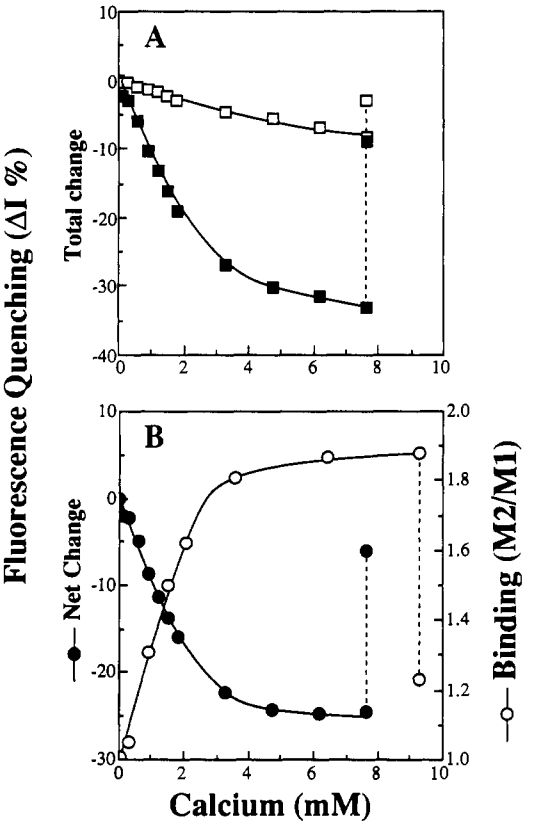

FIGURE 3: Calcium requirements of protein–phospholipid binding and fluorescence quenching. Independent samples of the same phospholipid preparations were used to measure protein–phospholipid binding by light-scattering intensity (M_2/M_1) and protein-induced quenching of fluorescence. Panel A shows the total effect of calcium on the fluorescence signal of phospholipid vesicles (40 μg of NBD-PA/PC, 10:90 in 1.6 mL of buffer) in the presence (■) or the absence (□) of the 64-kDa protein (40 μg). Panel B shows the protein–membrane binding (M_2/M_1, ○) and the protein-induced quenching of fluorescence (●). In this case, the fluorescence signal represents the net protein-induced changes where the contributions of calcium alone (panel A) were subtracted. In both panels, the dashed lines represent the addition of 3 mM EGTA.

alone had very little effect on the signal. Also, protein alone or protein in the presence of EGTA did not alter the fluorescence signal to a detectable extent. Protein-induced fluorescence quenching was largely reversible by subsequent chelation of calcium. There were minor variations in the extent of signal reversibility upon the addition of EGTA; some samples did not fully return to the original level (Figure 2A), while others returned to intensities somewhat greater than the original sample (Figure 2B). These slight deviations showed no consistent pattern in the many experiments conducted (see below) and were viewed as background changes that arose from the multiple manipulations of the sample.

Membrane surface curvature was not a critical factor in the fluorescence quenching. The binding of the 64-kDa protein to large unilamellar vesicles (LUVs) also induced large fluorescence quenching that was reversed by the addition of EGTA (Figure 2A). LUVs were prepared by extrusion and offered substantial contrast to sonicated vesicles in curvature and surface properties. A number of other preparations of LUVs of different composition showed similar quenching to that obtained with SUVs (data not shown). Thus, a principal observation was that protein–membrane binding caused a large fluorescence quenching event that was almost fully reversed by EGTA.

Binding of protein to the membrane and quenching of fluorescence were closely correlated (Figure 2B). Both events were saturable at similar protein to phospholipid ratios and were reversed by the addition of EGTA. In addition, both protein–membrane binding and fluorescence quenching occurred rapidly and were complete within the time required for mixing the reagents (about 15 s). This suggested that quenching of NBD-PA fluorescence was a direct consequence of protein–membrane binding. An important observation was that protein–phospholipid binding, as determined by light-scattering intensity, was of very high affinity and occurred without aggregation or fusion of vesicles. These results are similar to those obtained with vesicles containing phosphatidylserine (Bazzi & Nelsestuen, 1991a). Thus, the fluorescent group attached to the acidic phospholipid did not greatly alter the interaction of these proteins with phospholipids.

Possible Mechanism of Fluorescence Quenching. An attractive explanation for the fluorescence quenching observed in Figure 2 was that the binding of the 64-kDa protein to the membrane induced clustering of the acidic (fluorescent) phospholipids. A number of further experiments and observations supported this explanation.

Calcium can induce changes in the structure of vesicles (Papahadjoulos et al., 1979) including lateral phase separation (Hoekstra, 1982; Nichols & Pagano, 1981; Tilcock et al., 1988). However, calcium alone induced small or negligible quenching of the fluorescence signal at the concentrations and membrane compositions used in this study (Figure 3A). In contrast, calcium-dependent quenching of the fluorescence in the presence of the 64-kDa protein was large, saturable, and correlated closely with the calcium dependence of protein–membrane binding (Figure 3B).

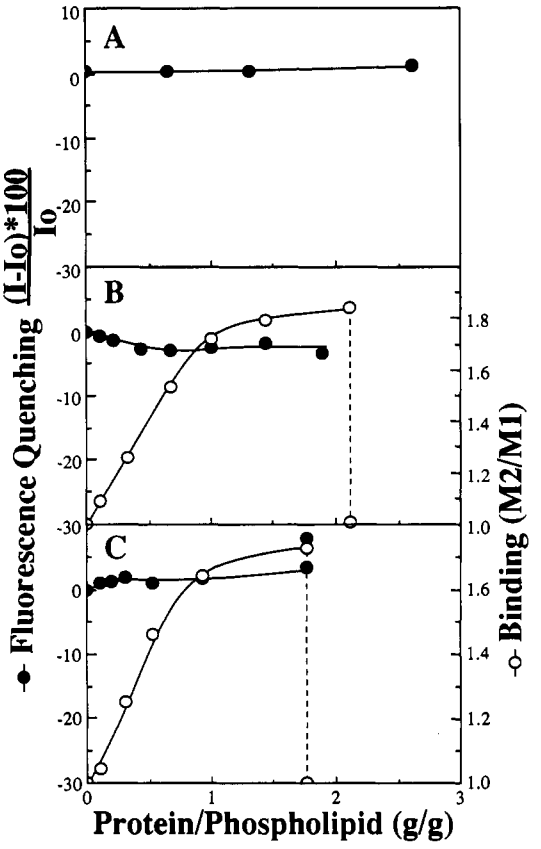

FIGURE 4: Fluorescence changes as a function of phospholipid composition. Protein–vesicle binding (M_2/M_1, ○) and fluorescence quenching (●) of small unilamellar vesicles of different compositions were measured as a function of added protein. Panel A shows the changes in the fluorescence of vesicles (25 μg) composed of NBD-PC/PC (15:85) in the presence of 1 mM calcium. Panel B shows protein–vesicle binding (○) and fluorescence quenching (●) of vesicles (12.5 μg) composed of NBD-PC/PS/PC (15:50:35) in the presence of 0.1 mM calcium. Panel C shows protein–vesicle binding (○) and fluorescence quenching (●) of phospholipid vesicles (50 μg) composed entirely of NBD-PA in the presence of 25 μM calcium. In all panels, the dashed line shows the effect of adding 2 mM EGTA.

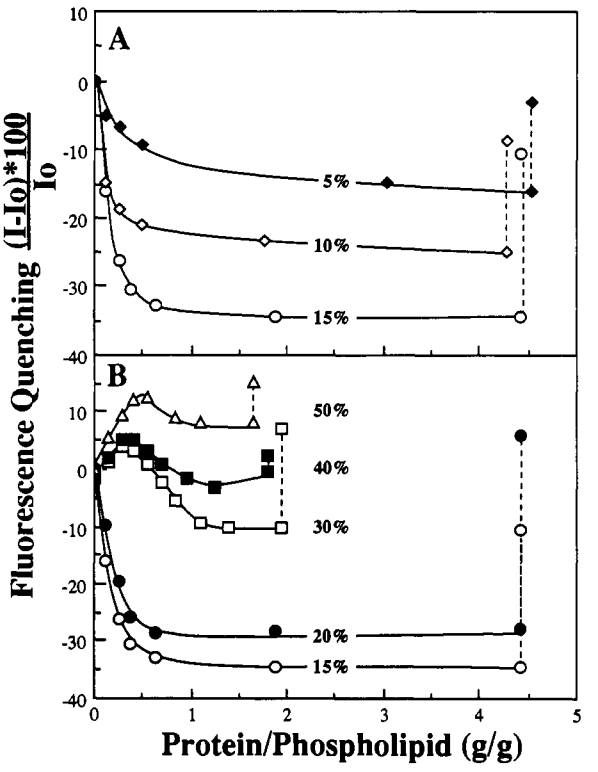

FIGURE 5: Fluorescence quenching as a function of NBD-PA composition. Small unilamellar phospholipid vesicles containing 5–50% NBD-PA (the remaining phospholipid was PC) were prepared by sonication and centrifugation. In each case, vesicles (25 μg) were mixed in 1.6 mL of buffered calcium, and the fluorescence changes were monitored as a function of protein addition. Panel A shows fluorescence changes observed with vesicles containing 5% (◆), 10% (◇), or 15% NBD-PA (○). Panel B shows the fluorescence changes with vesicles containing 15% (○), 20% (●), 30% (□), 40% (■), or 50% (△) NBD-PA. The calcium concentrations used were 1.0 mM with vesicles containing 5–20% NBD-PA, 0.5 mM with vesicles containing 30% NBD-PA, or 0.2 mM with vesicles containing 40 or 50% NBD-PA. In both panels, the dashed lines show the effect of adding 2.0 mM EGTA.

Although the fluorescent group was attached to C12 of the acyl chain of the phospholipid, its average location is probably at the interface of the membrane (Chattopadhyay & London, 1987). While this location might allow direct access of the fluorophore to the protein, several results suggested that direct protein contact was not responsible for the observed quenching. For example, neither protein–phospholipid binding nor fluorescence quenching were observed upon the addition of the 64-kDa protein to phospholipid vesicles containing 15% NBD-PC dispersed in PC (Figure 4A). This suggested that the fluorescent group itself (NBD) was not capable of supporting protein–phospholipid binding. Phospholipid vesicles containing 15% NBD-PC and 50% PS bound the 64-kDa protein with high affinity, but there was little change in the fluorescence of the vesicles (Figure 4B). Similar results were obtained with vesicles containing 15% NBD-PA with 50% of either PS or PG (data not shown). Little quenching in these cases was consistent with preferential clustering of PS or a mixture of PS and NBD-PA. Neither of these conditions would substantially increase the density of NBD-PA to produce large quenching. These results also suggested that protein binding did not significantly alter the general environment in the membrane as to cause extensive quenching of the probe.

Other mechanisms for fluorescence quenching such as changes in the conformation, lateral mobility, or environment of the probe would probably make lesser contributions. Consistent with this was the observation that vesicles composed entirely of NBD-PA supported protein–phospholipid binding with no significant changes in the fluorescence signal (Figure 4C). Vesicles of pure NBD-PA cannot undergo selective clustering but should be sensitive to protein-induced changes in the environment of the highly quenched fluorophore.

Another feature that supported quenching by clustering of the probe was the observation that the magnitude of protein-induced quenching of NBD-PA was dependent on the composition of the phospholipids (Figure 5). The results showed an increase in maximum quenching as the proportion of NBD-PA was increased from 5 to 15% NBD-PA (Figure 5A). This was followed by a reduction in quenching as the vesicle composition was increased from 15 to 50% NBD-PA (Figure 5B). A surprising property was that vesicles containing 30%, 40%, and 50% NBD-PA showed biphasic changes in the fluorescence signal with an initial enhancement of the signal followed by quenching as the protein density on the membrane reached its maximum (Figure 5B). At this maximum density, protein did not significantly alter the fluorescence of vesicles containing 40% NBD-PA, it dequenched the fluorescence of vesicles containing 50% NBD-PA, and it slightly quenched the fluorescence of vesicles containing 30% NBD-PA.

Addition of EGTA at the end of the titrations showed reversal of signal for vesicles containing 5–40% NBD-PA (Figure 5). While the actual change was always small, the direction of change caused by EGTA addition to titrations of 50%

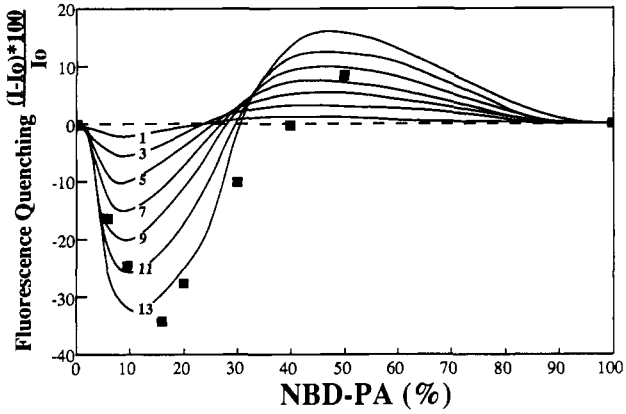

FIGURE 6: Theoretical levels of fluorescence quenching. Theoretical changes in fluorescence intensity due to clustering of NBD-PA were calculated as described in the text. The lines drawn represent fluorescence changes expected as a function of excess phospholipids in a cluster. A value of 1 indicates a cluster of 2 acidic phospholipids per protein and a value of 11 is for a cluster size of 12 per protein. The data points are the maximum changes observed with the 64-kDa protein (■) (data from Figure 5). This model assumed that membranes with a minimum of 2.5% NBD-PA were required for binding and that NBD-PA in excess of 25% did not participate in clustering.

NBD-PA was not consistent; some experiments gave increased while other experiments gave slightly decreased signals. This minor inconsistency was not pursued further. Small variation might arise from multiple physical manipulations of these phospholipids.

The total pattern of behavior observed in Figure 5 was consistant with specific clustering of the fluorophore. Protein added to the outside of vesicles should cluster only those phospholipids in the outer layer to give a maximum quenching of about 50–60%. However, the total signal will include probes that are on the outer layer but are not clustered. These will be in an environment with reduced probe density. Also, probes on the inner layer of the membrane will experience reduced quenching from probes in the outer layer. Consideration of all these factors suggests that actual quenching of 35% (15% NBD-PA, Figure 5) may correspond to a very high degree of clustering of the exposed lipids. That quenching decreased for membranes of high charge density is consistent with clustering of a specific number of phospholipids per protein. As the number of acidic phospholipids increased beyond those needed for binding, the excess acidic phospholipids would not be clustered by the protein and the overall fluorescence quenching would decrease.

Quantitative Model of Fluorescence Quenching. The general behavior shown in Figure 5 was qualitatively consistent with quenching by the clustering of fluorophore groups. A more quantitative model for this behavior was developed based on the number of acidic phospholipid clustered per protein and several simplifying assumptions (see Experimental Procedures).

The model assumed that each protein clustered a constant number of NBD-PA molecules (1–13, Figure 6). It further assumed that some minimum number of NBD-PA molecules were necessary in order to initiate clustering. The theoretical results (Figure 6) showed that maximum fluorescence quenching increased as the membrane composition was increased from 0 to 15% NBD-PA. Smaller magnitudes of fluorescence quenching are expected with membranes containing 15 to about 30% NBD-PA (Figure 6). This model even predicted a net dequenching of the signal for vesicles containing more than 30% NBD-PA. This general pattern, including some dequenching of the signal for membranes of 30–50% NBD-PA, was observed experimentally (Figure 5).

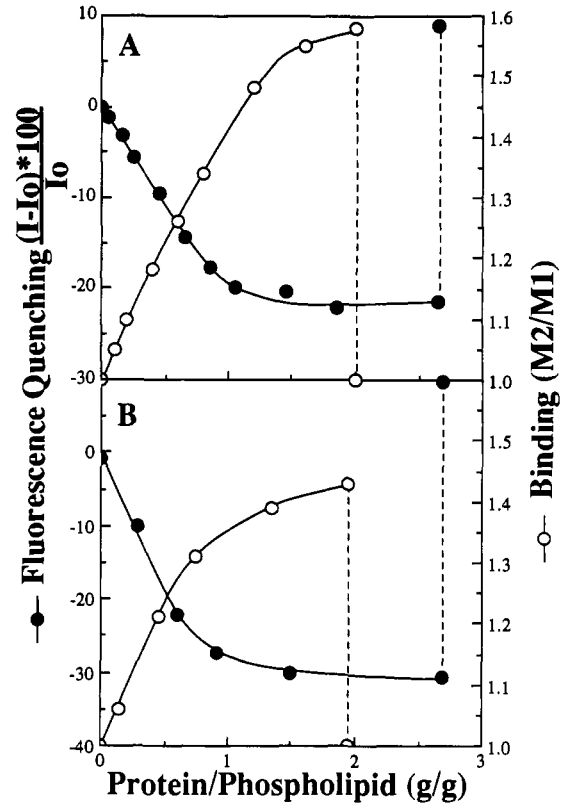

FIGURE 7: Fluorescence quenching induced by PKC and the 32-kDa protein. Panel A shows results obtained with PKC. Panel B shows results obtained with the 32-kDa protein. In both panels, 25 μg of phospholipid vesicles containins (NBD-PA/PC, 15:85) were mixed in 1.6 mL of buffer in the presence of 1.0 mM calcium. Protein–membrane binding (M_2/M_1, ○) and fluorescence changes (●) were measured as described in the legend to Figure 2. The dashed lines show the effect of adding excess EGTA.

The theoretical calculations presented in Figure 6 were viewed as an oversimplification of a complex process. For example, the model was based on vesicles with rigid geometry and clearly separated clusters of phospholipids. The effects of restricted lateral mobility of the phospholipids were not considered. In addition, the model ignored possible small contributions from other quenching mechanisms. Nevertheless, an important aspect of this model was that the general pattern of behavior could readily fit the observed data. Precise agreement was not expected. In keeping with the limitations of the modeling process, clustering of 13 NBD-PA per protein molecule showed the best fit (Figure 6), but the more appropriate conclusion was that the number of phospholipids clustered per protein molecule was large and probably ≥10.

Fluorescence Quenching by Other Proteins. Protein kinase C (Bazzi & Nelsestuen, 1990) displayed calcium- and phospholipid-binding properties that were similar to those of the 64-kDa protein. Like the 64-kDa protein, PKC bound to phospholipid vesicles containing 15% NBD-PA (Figure 7A) and caused large light-scattering intensity changes that reached saturation at M_2/M_1 ratios of approximately 1.6. PKC–membrane binding coincided with extensive quenching of the fluorescence signal of NBD-PA (Figure 7A). The magnitude of quenching was similar to that of the 64-kDa protein, suggesting a similar magnitude of phospholipid clustering.

The 32-kDa protein (Bazzi & Nelsestuen, 1991a) bound to phospholipid vesicles composed of 15% NBD-PA and caused extensive quenching of the fluorescence signal (Figure 7B). Again, fluorescence quenching as well as protein–membrane binding were reversed by addition of EGTA. The fluorescence

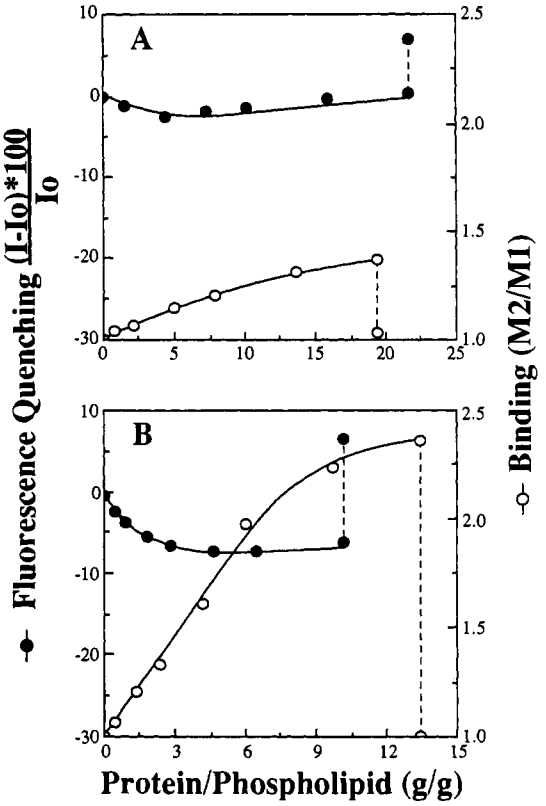

FIGURE 8: Fluorescence changes induced by blood coagulation factor X and protein Z. Panel A shows results obtained with bovine blood coagulation factor X. Panel B shows results obtained with bovine protein Z. In each panel, protein–membrane binding (M_2/M_1, O) and fluorescence changes (●) are shown. The measurements were performed as described in Figure 7 except that the calcium concentration was 5 mM. The dashed lines show the effect of adding excess EGTA.

quenching by the 32-kDa protein was very similar to that induced by the 64-kDa protein.

Vitamin K dependent proteins bind to membranes in a calcium-dependent manner (Schwalbe et al., 1989, and references cited therein). Prothrombin has been reported to cluster a small number of acidic phospholipids (Mayer & Nelsestuen, 1981, 1983). It appears that this does not involve extensive regions of phase-separated phospholipids (Jones & Lentz, 1986). Under the experimental conditions used in this study, neither prothrombin nor its fragment 1 region (156 residues, N-terminal) bound to vesicles containing 15% NBD-PA (data not shown). It appeared that the NBD moiety on the phospholipid side chain had dramatic effects on the interaction of these proteins with phospholipids.

Other vitamin K dependent proteins, factor X and protein Z, did associate with vesicles containing 15% NBD-PA but displayed low affinity. Low affinity was suggested by the amount of protein (protein/phospholipid ratio) needed to support protein–membrane binding (Figure 8). In comparison, a protein to phospholipid ratio of 1.0 was adequate for a high level of factor X association with membranes containing 20% PS (Nelsestuen & Lim, 1977). Binding was reversed by the addition of EGTA (Figure 8). Although protein Z bound to the membrane to a high extent (M_2/M_1, Figure 8), it caused only small changes in the fluorescence intensity of the NBD-PA. This small degree of quenching may correlate with clustering of a smaller number of NBD-PA molecules per protein Z molecule (about four molecules as estimated from the theoretical curves in Figure 6). Alternatively, it is possible that the small quenching observed for protein Z arose from

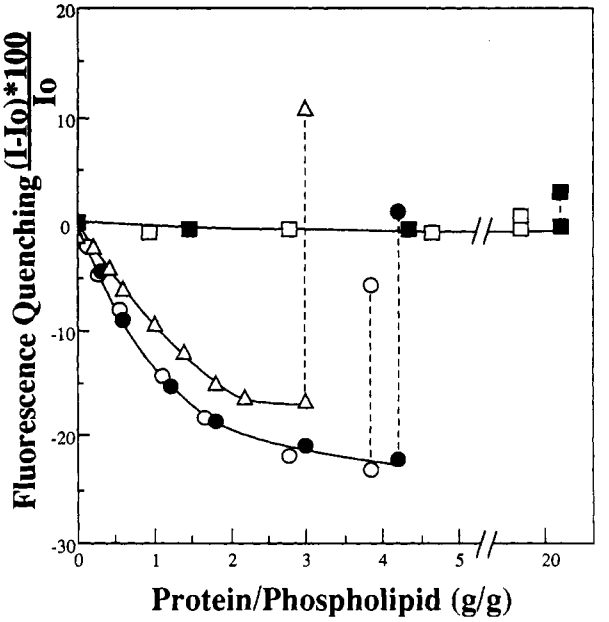

FIGURE 9: Fluorescence changes with NBD-PG. Phospholipid vesicles containing 15% NBD-PG and 85% PC were prepared by sonication and centrifugation. The vesicles (25 μg) were mixed in 1.6 mL of buffer containing 10 mM calcium, and the fluorescence changes were monitored as a function of protein concentration. The results show changes observed with the 64-kDa protein (●), PKC (△), the 32-kDa protein (○), protein Z (□), or factor X (■). The dashed lines show the effect of adding excess EGTA.

factors other than clustering of the fluorophore. This question was not pursued further. The important feature was that PKC–membrane binding caused much larger quenching of NBD-PA and was therefore distinctive.

Clustering Detected with Other Phospholipids. The binding of protein kinase C, the 64-kDa, and 32-kDa proteins to phospholipid vesicles does not display strong selectivity among acidic phospholipids. For example, protein kinase C could bind phospholipid vesicles containing PS, PG, or PI (Bazzi & Nelsestuen, 1987b). Changing the phospholipid head groups appeared to only alter the calcium requirements for binding (unpublished data). The ability of these proteins to induce fluorescence quenching was examined by using phospholipid vesicles containing 15% NBD-PG as the fluorescent probe. Once again, there were minor changes in fluorescence due to bovine factor X or protein Z (Figure 9). However, PKC and the 64-kDa and 32-kDa proteins all caused extensive quenching of the fluorescence of NBD-PG (Figure 9). The magnitude of this fluorescence quenching was comparable among these proteins and approached that obtained with vesicles containing NBD-PA.

Discussion

Earlier studies showed that the interaction of protein kinase C with calcium and phospholipids displayed several unique features (Bazzi & Nelsestuen, 1990). For example, calcium supports the association of PKC with phospholipid vesicles. In addition, phospholipid was needed to allow interaction of PKC with a large number (eight to nine) of calcium ions. These unusual features appear to be characteristic of a class of proteins that includes a 64-kDa protein and two others with molecular masses of about 32 ± 1 kDa (Bazzi & Nelsestuen, 1991a). These and other properties suggested that protein–phospholipid binding involved the formation of a large number of calcium "bridges" between the protein and the phospholipid. This study suggested that binding of these proteins to vesicles showed additional properties consistent with extensive clus-

tering of acidic phospholipids in membranes. It should be emphasized that the interactions described in this report refer to the calcium-dependent form of membrane-bound proteins. PKC also forms a calcium-independent complex that has properties of an integral membrane protein (Bazzi & Nelsestuen, 1988).

While changes in NBD-PA fluorescence can arise by several mechanisms, the observed properties strongly suggested that clustering of the fluorophores was the dominant mechanism of quenching. For example, fluorescence quenching arising from changing the conformation, orientation, or the environment of the probe are probably unidirectional and independent of vesicle composition. Changes in fluorescence due to condensation of the phospholipid acyl chains (MacDonald, 1990) or restricted lateral mobility of the probe should also be unidirectional. However, the observed fluorescence changes were highly dependent on vesicle composition. Maximum fluorescence quenching was observed with vesicles containing 15% NBD-PA, and actual dequenching of the fluorescence was observed with vesicles containing 50% NBD-PA. In addition, vesicles containing fluorescent phospholipids plus a large excess of nonfluorescent acidic phospholipids did not show significant changes in fluorescence upon protein binding. All of these characteristics could be accounted for by a relatively simple model based on phospholipid clustering due to protein binding (Figure 6).

While intended as a first approximation, the quantitative model could account for the direction as well as the magnitude of fluorescence changes. It should be emphasized that this model suggested clusters of acidic phospholipid associated with each protein molecule and did not imply the formation of extended regions of pure phospholipids. In fact, the phospholipid-binding properties of PKC and other related proteins aruged against extended regions of pure phospholipids. Increasing the phosphatidylserine content of the membrane beyond 25% did not allow binding of additional PKC (Bazzi & Nelsestuen, 1987b), suggesting that a defined number of acidic phospholipids were involved per protein molecule. While the exact number of phospholipids in each cluster could not be accurately determined, several observations indicated a relatively large number.

Since each protein bound eight to nine calcium ions (Bazzi & Nelsestuen, 1990, 1991a), a related number of acidic phospholipids may be involved (e.g., one or two phospholipids per calcium ion). The parameters needed to best fit the experimental data in Figure 6 were consistent with a large number of acidic phospholipids per protein. The following paper in this issue gives a best fit with a cooperativity coefficient of ≥ 10 for calcium (Bazzi & Nelsestuen, 1991b). Maximum protein–membrane binding at 20% phosphatidylserine (Bazzi & Nelsestuen, 1987b) would correspond to 12.8 phosphatidylserines per PKC molecule (assuming that 1 g of protein was bound per gram of phospholipid and that 60% of the phospholipid was on the outer layer of the vesicles). These lines of evidence suggest that large numbers of acidic phospholipids and calcium ions (8–13) are involved in binding each protein molecule to the membrane. This large number of contact points may explain the unusually high affinity of these proteins for membranes (Bazzi & Nelsestuen, 1991a,b).

The biological role of these findings is not yet known. However, PKC is implicated in a larger number of regulatory events (Nishizuka, 1986). Virtually all of these events concentrate on the enzymatic properties of PKC and the effect of protein–membrane binding on this parameter. No function for the 64- or 32-kDa proteins has been documented. However, it is interesting to consider the possibility that, among other roles, these proteins may serve to regulate the availability of acidic phospholipids in the cell. The extreme degree of segregation and apparently tight binding affinity might allow these proteins to decrease the availability of acidic phospholipids. This, in turn, would influence the many structures and enzymes in the cell that rely on acidic phospholipids. If such a function exists for these proteins, calcium could result in wide-ranging effects on the cell.

Relationship to Other Protein Systems. The large fluorescence changes of NBD-PA observed with these proteins appeared remarkable when compared with other classes of proteins. To our knowledge, these proteins may constitute the most extreme protein-dependent phospholipid clustering process that thas been documented. For example, vitamin K dependent proteins have been reported to induce segregation of phospholipids in membranes of certain compositions (Mayer & Nelsestuen, 1981). The degree of this segregation has been the subject of several studies, and some reports have failed to detect extended regions of phase separated lipids (Jones & Lentz, 1986; Tendian & Lentz, 1990). The results of the current study revealed two properties that may have an impact on the earlier results. First, the binding of vitamin K dependent proteins to phospholipids containing bulky substituents such as NBD was considerably different from that observed with normal phospholipids. Secondly, although factor X and protein Z bound to these fluorescent vesicles, they produced only small changes in the fluorescence signal. These small changes could be the result of either clustering a small number of acidic phospholipids per protein molecule or other modes of quenching such as condensation of acyl chains of the phospholipids. No attempts were made to accurately determine the basis for the fluorescence quenching of NBD-PA by the vitamin K dependent proteins.

Other proteins and polycations have been reported to induce segregation or lateral phase separation of phospholipids but these appear to be of a very different type. For example, poly(L-lysine) (Hartmann & Galla, 1978; Carrier & Pezolet, 1986), cytochrome *c* (Birrel & Griffith, 1976), myelin basic protein (Boggs et al., 1977), and certain biologically active polycations (Ikeda et al., 1990) have been reported to induce lateral phase separation or major reorganization of phospholipids in vesicles. These polycationic molecules probably interact electrostatically with the ionic phospholipid and may also cause vesicle aggregation. The degree of phospholipid reorganization would probably be dependent on the molecules involved [see, for example, Carrier and Pezolet (1986) and Ikeda et al. (1990)] as well as on the composition of the vesicles. Aggregation of vesicles by these molecules may also cause irreversible changes in the structure of the vesicles. In contrast, the binding of PKC to membranes induced reversible clustering of phospholipid molecules without irreversible structural changes.

Vesicular stomatitis virus matrix protein (M protein) has also been reported to induce lateral organization of phospholipids in vesicles (Wiener et al., 1985). Incorporation of M protein into vesicles containing NBD-labeled phospholipid substantially enhanced self-quenching. This protein-enhanced quenching occurred only at the phase transition or lower temperatures. At temperatures above the phase transition, the addition of this protein did not significantly change the fluorescence. The fluorescence changes observed with the proteins described here were not subjected to this restriction. Thus, these proteins must have adequate affinity to maintain a cluster of phospholipids in a fluid membrane. Extremely

high affinity is a characteristic of these proteins (Bazzi & Nelsestuen, 1991b).

ACKNOWLEDGMENTS

We are indebted to Ruth Schwalbe for providing the bovine protein Z and bovine factor X used in this study.

REFERENCES

Bazzi, M. D., & Nelsestuen, G. L. (1987a) *Biochemistry 26*, 1974.
Bazzi, M. D., & Nelsestuen, G. L. (1987b) *Biochemistry 26*, 115.
Bazzi, M. D., & Nelsestuen, G. L. (1988) *Biochemistry 27*, 7589.
Bazzi, M. D., & Nelsestuen, G. L. (1990) *Biochemistry 29*, 7624.
Bazzi, M. D., & Nelsestuen, G. L. (1991a) *Biochemistry 30*, 971.
Bazzi, M. D, & Nelsestuen, G. L. (1991b) *Biochemistry* (following paper in this issue).
Birrell, G. B., & Griffith, O. H. (1976) *Biochemistry 15*, 2925.
Boggs, J. M., Moscarello, M. A., & Papahadjopoulos, D. (1977) *Biochemistry 16*, 5420.
Bradford, M. M. (1976) *Anal. Biochem. 72*, 248.
Carrier, D, & Pezolet, M. (1986) *Biochemistry 25*, 4167.
Chattopadhyay, A., & London, E. (1987) *Biochemistry 26*, 39.
Chen, P. S., Toribara, T. Y., & Warner, H. (1956) *Anal. Chem. 28*, 1756.
Creutz, C. E., Zaks, W. J., Hamman, H. C., Crane, S., Martin, W. H., Gould, D. L., Oddie, K. M., & Parsons, S. J. (1987) *J. Biol. Chem. 262*, 1860.
Glenney, J. (1986) *J. Biol. Chem. 261*, 7247.
Glenney, J. R., Jr., Tack, B., & Powell, M. A. (1987) *J. Cell Biol. 104*, 503.
Graham, I., Gagné, J., & Silvius, J. R. (1985) *Biochemistry 24*, 7123.
Hartmann, W., & Galla, H. J. (1978) *Biochim. Biophys. Acta 509*, 474.
Hoekstra, D. (1982) *Biochemistry 21*, 1055.
Hope, M. J., Bally, M. B., Webb, G., & Cullis, P. R. (1985) *Biochim. Biophys. Acta 812*, 55.
Huang, C. (1969) *Biochemistry 8*, 344.
Huang, C., & Mason, J. T. (1978) *Proc. Natl. Acad. Sci. U.S.A. 75*, 308.
Huber, R., Schneider, M., Mayr, I., Romisch, J., & Paques, E. P. (1990) *FEBS Lett. 275*, 15.
Ikeda, T., Yamaguch, H., & Tazuke, S. (1990) *Biochim. Biophys. Acta 1026*, 105.
Jones, M. E., & Lentz, B. R. (1986) *Biochemistry 25*, 567.
Khanna, N. C., Helwig, E. D., Ikebuchi, N. W., Fitspatrick, S., Gajwa, R., & Waisman, D. M. (1990) *Biochemistry 29*, 4852.
Klee, C. B. (1988) *Biochemistry 27*, 6645.
MacDonald, R. I. (1990) *J. Biol. Chem. 265*, 13533.
Mayer, L. D., & Nelsestuen, G. L. (1981) *Biochemistry 20*, 2457.
Mayer, L. D., & Nelsestuen, G. L. (1983) *Biochim. Biophys. Acta 734*, 48.
Nelsestuen, G. L. (1984) *Methods Enzymol. 107*, 507.
Nelsestuen, G. L., & Lim, T. K. (1977) *Biochemistry 16*, 4164.
Nelsestuen, G. L., & Bazzi, M. D. (1991) *J. Bioenerg. Biomembr. 23*, 43.
Newton, A. C., & Koshland, D. E., Jr. (1990) *Biochemistry 29*, 6656.
Nichols, J. W., & Pagano, R. E. (1981) *Biochemistry 20*, 2783.
Nishizuka, Y. (1986) *Science (Washington D.C.) 233*, 305.
Paphadjopoulos, D., Poste, G., & Vail, W. J. (1979) *Methods Membr. Biol. 465*, 579.
Schlaepfer, D. D., & Haigler, H. T. (1987) *J. Biol. Chem. 262*, 6931.
Schwalbe, R. A., Ryan, J., Stern, D. M., Kisiel, W., Dahlbäck, B., & Nelsestuen, G. L. (1989) *J. Biol. Chem. 264*, 20288.
Schwalbe, R. A., Dahlbäck, B., & Nelsestuen, G. L. (1990) *J. Biol. Chem. 265*, 21749.
Sklar, L. A., Doody, M. C., Gotto, A. M., & Pownall, H. J. (1980) *Biochemistry 19*, 1294.
Tendian, S. W., & Lentz, B. R. (1990) *Biochemistry 29*, 6720.
Tilcock, C. P. S., Cullis, P. R., & Gruner, S. M. (1988) *Biochemistry 27*, 1415.
Wiener, J. R., Pal, R., Barenholz, Y., & Wagner, R. R. (1985) *Biochemistry 24*, 7651.