Importance of Phosphatidylethanolamine for Association of Protein Kinase C and Other Cytoplasmic Proteins with Membranes†

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Received August 12, 1991; Revised Manuscript Received October 24, 1991

ABSTRACT: Biological membranes exhibit an asymmetric distribution of phospholipids. Phosphatidylserine (PS) is an acidic phospholipid that is found almost entirely on the interior of the cell where it is important for interaction with many cellular components. A less well understood phenomenon is the asymmetry of the neutral phospholipids, where phosphatidylcholine (PC) is located primarily on exterior membranes while phosphatidylethanolamine (PE) is located primarily on interior membranes. The effect of these neutral phospholipids on protein–phospholipid associations was examined using four cytoplasmic proteins that bind to membranes in a calcium-dependent manner. With membranes containing PS at a charge density characteristic of cytosolic membranes, protein kinase C and three other proteins with molecular masses of 64, 32, and 22 kDa all showed great selectively for membranes containing PE rather than PC as the neutral phospholipid; the calcium requirements for membrane–protein association of the 64- and 32-kDa proteins were about 10-fold lower with membranes containing PE; binding of the 22-kDa protein to membranes required the presence of PE and could not even be detected with membranes containing PC. Variation of the PS/PE ratio showed that membranes containing about 20% PS/60% PE provided optimum conditions for binding and were as effective as membranes composed of 100% PS. Thus, PE, as a phospholipid matrix, eliminated the need for membranes with high charge density and/or reduced the calcium concentrations needed for protein–membrane association. A surprising result was that PKC and the 64- and 32-kDa proteins were capable of binding to neutral membranes composed entirely of PE/PC or PC only. The different phospholipid headgroups altered only the calcium required for membrane–protein association. For example, calcium concentrations at the midpoint for association of the 64-kDa protein with membranes containing PS, PE/PC, or PC occurred at 6, 100, and 20000 μM, respectively. Thus, biological probes detected major differences in the surface properties of membranes containing PE versus PC, despite the fact that both of these neutral phospholipids are often thought to provide "inert" matrices for the acidic phospholipids. The selectivity for membranes containing PE could be a general phenomenon that is applicable to many cytoplasmic proteins. The present study suggested that the strategic location of PE on the interior of the membranes may be necessary to allow some membrane–protein associations to occur at physiological levels of calcium and PS.

It is well established that biological membranes display asymmetrical organization such as that shown by plasma membranes (Berdelson & Barsukov, 1977; Rothman & Lenard, 1977; Devaux, 1991). The majority of phosphatidylethanolamine (PE)[1] and phosphatidylserine (PS) are located on the inner leaflet, while phosphatidylcholine (PC) is located mainly on the exterior side of the membrane. A few functions for this asymmetry have been proposed. For example, this asymmetry may facilitate and enhance membrane–membrane interactions so that biological processes involved in exocytosis and endocytosis are achieved more readily (Cullis et al., 1985; Devaux, 1991). In addition, it is well established that asymmetry with respect to acidic phospholipids helps to regulate the blood clotting system where exposure of PS from the interior of the cell is necessary to provide an appropriate membrane surface to support coagulation (Nelsestuen & Lim, 1977; Zwaal et al., 1977). However, the influence of neutral phospholipids on the association of various cytoplasmic proteins with membranes remained largely unexplored.

Often, in vitro experiments employ PC as the neutral phospholipid since it produces bilayers with greater stability. Biological membrane contain PS in a matrix composed largely of PE rather than PC. PE and PC are both neutral phospholipids that exhibit similar electrostatic properties and intrinsic binding constants for divalent ions such as calcium or magnesium (McLaughlin et al., 1981). However, they differ in their hydration properties (McIntosh & Simon, 1986; Rand & Parsegian, 1988), lamellar organization and packing, and headgroup interactions (Cullis et al., 1985; Hauser et al., 1981). These and other factors might create differences between PE and PC membranes that could be detected by biological probes.

A large number of cytoplasmic proteins that show calcium-dependent binding to membranes have been identified (Pollard et al., 1990; Smith et al., 1990; Burgoyne & Geisow, 1989; Klee, 1988). Among these are protein kinase C (PKC) and three proteins with molecular masses of 64, 32, and 22 kDa (Bazzi & Nelsestuen, 1991). These proteins displayed a variety of striking properties. For example, PKC and the 64- and 32-kDa proteins bound a large number of calcium ions, but only in the presence of phospholipids (Bazzi & Nelsestuen, 1990, 1991a). These three proteins also induced extensive clustering of acidic phospholipids in the membrane (Bazzi &

† 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; PE, phosphatidylethanolamine; PS, phosphatidylserine; dansyl-PE, *N*-dansyl-L-α-dipalmitoylphosphatidylethanolamine; BSA, bovine serum albumin; PKC, protein kinase C.

0006-2960/92/0431-1125$03.00/0

Nelsestuen, 1991b) and showed multiequilibrium, highly sequential binding to membrane surfaces (Bazzi & Nelsestuen, 1991c). The 22-kDa protein was different. It associated only with membranes of high PS content. In addition, membranes had little effect on the calium-binding properties of this protein. PKC is an important regulatory enzyme believed to be involved in many cell functions, acting as a family of functionally related isozymes (Nishizuka, 1988, 1986). Its activity is regulated by calcium, phospholipids, and diacylglycerols (Nelsestuen & Bazzi, 1991; Bell & Burns, 1991; Rando, 1988). The functions of the other proteins are unknown. However, this diverse group of proteins appear to offer suitable biological probes for detection of membrane properties that might enhance protein interactions with membranes containing the physiological level of charge density.

To date, most biochemical studies with these proteins have used phospholipid preparations consisting of acidic phospholipids alone or dispersed in PC. Both of these situations can present difficulties. Membranes of pure PS as well as a medium that has a low ionic strength are often used. Under these conditions, calcium required for association and/or activation is within the physiological range. However, membranes of pure PS do not correspond to any physiological composition and will, for example, inactivate PKC unless calcium is present (Huang & Huang, 1990). If PS is diluted with PC in order to approximate the charge density of physiological membranes, PKC is no longer inactivated (Huang & Huang, 1990), but the calcium required for protein–membrane binding or enzyme activation is often high (Bazzi & Nelsestuen, 1987a). The calcium required for interaction of the 32- and 64-kDa proteins was also high relative to intracellular calcium levels (Bazzi & Nelsestuen, 1991a). It is possible that the asymmetric distribution of phospholipids may generate conditions that allow binding at physiological levels of calcium.

In this study, we examined the interaction of four proteins with membranes of various compositions. Among other observations, it was found that use of PE as the neutral phospholipid was very important for enhancing protein–membrane interactions. The unique properties of PE are readily detected by these biological probes.

Experimental Procedures

Materials. Highly purified bovine brain phosphatidylserine (PS), phosphatidylethanolamine (PE), and egg yolk phosphatidylcholine (PC) were purchased from the Sigma Chemical Co. or from Avanti Polar Lipids, Inc. Dipalmitoyl-*N*-dansyl-L-α-phosphatidylethanolamine (dansyl-PE) was purchased from the Sigma Chemical Co. Polycarbonate filters (0.1-μm diameter) were purchased from Nucleopore Corp. Other chemicals and reagents were from the Sigma Chemical Co. and were of the highest grade available. Protein kinase C and the 64-, 32-, and 22-KDa proteins were purified from bovine brain as described previously (Bazzi & Nelsestuen, 1987a, 1991a). Blood clotting factor X, purified from bovine blood, was generously provided by Ruth Schwalbe (University of Minnesota).

Protein–Phospholipid Binding. The association of various proteins with phospholipid vesicles was measured by light-scattering intensity, flourescence energy transfer, and gel filtration chromatography. Fluorescence energy transfer measurements were performed with phospholipid vesicles that contained 10% dansyl-PE. Excitation and emission wavelengths were 284 and 520 nm, respectively. A 500-nm cutoff filter was placed in front of the emission monochromater. Direct excitation of the dansyl group with 284-nm light produced a fluorescence signal (I_o) which was used as an internal standard. An increase in the fluorescence of the dansyl group was obtained upon protein–phospholipid association (Bazzi & Nelsestuen, 1991a). The results were reported as $(I - I_o) \times 100/I_o$, where I is the fluorescence of the protein–phospholipid complex and I_o is the fluoresence of the vesicles alone.

Light-scattering intensity measurements provide quantitative estimates of the amount of protein bound to 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. The M_2/M_1 ratios were estimated from light-scattering intensity as described in detail previously (Bazzi & Nelsestuen, 1987a; Nelsestuen & Lim, 1977). In most cases, this technique was used with small unilamellar vesicles (diameter ca. 30 nm) with excitation and emission wavelengths set at 320 nm. In cases where large vesicles prepared by extrusion (diameter ca. 100 nm) were employed, the excitation and emission wavelengths were set at 650 nm. Although the particle size is significant compared to the wavelength, the latter M_2/M_1 ratios proved useful for comparison of titrations with other vesicles of similar size.

Light-scattering intensity and fluorescence energy transfer measurements were performed with a Perkin-Elmer Spectrofluorometer (model MPF 44 A) at 25 °C. In both measurements, vesicles and protein (12–25 μg of each component) were added to 1.6 mL of buffer (50 mM Tris, pH 7.5, and 100 mM NaCl), and binding was monitored upon successive additions of calcium. For each calcium addition, the solution rapidly reached a new and constant level of membrane–protein binding. Furthermore, similar levels of binding were obtained at similar calcium concentrations regardless of the history of the sample (i.e., the number of calcium additions employed).

Gel filtration chromatography was used to measure the association of PKC with vesicles of various compositions. Mixtures containing PKC, phospholipid vesicles, 1.0 mg/mL BSA, 0.5 mM DTT, and calcium at the desired concentration were applied on Sephacryl S300 columns (1.0 × 30 cm). The columns were equilibrated and eluted (0.81 mL/fraction) with a buffer containing 20 mM Tris, pH 7.9, 0.5 mM DTT, 1.0 mg/mL BSA, 10% glycerol, and calcium at either 1, 4, or 10 mM. In order to prevent prolonged exposure of PKC to high concentrations of calcium, a concentrated EGTA solution was present in the collection tubes so that each fraction would contain 1 mM EGTA in excess of calcium in the buffer. The elution position of PKC was located by enzyme activity toward protamine sulfate.

PKC Assay. The activity of PKC toward histones was measured by a procedure similar to that described by Kikkawa (1983). The assay mixture (0.25 mL) contained 20 mM Tris pH 7.9, 10 mM Mg^{+2}, 0.2 mg/mL histone (type III-S), 20 μM ATP, 100 μM EGTA, 320 μg/mL phospholipids, and calcium at the indicated concentration. The apparent free Ca^{2+} concentration in the assay solutions was calculated as described by Storer and Cornish-Bowden (1976). The K_a values for various complexes involving Ca^{2+}, Mg^{2+}, EGTA, EDTA, and ATP were taken from Fabiato and Fabiato (1979). The phospholipid vesicles were composed of PS/PE/PC/DAG at either 40:0:50:10, 0:40:50:10, or 0:0:90:10. Activity toward protamine sulfate involved an assay that omitted histone and calcium.

Vesicle Preparations. Most of the measurements were conducted with large unilamellar vesicles. These vesicles were prepared by the extrusion method (Hope et al., 1985) using 0.1-μm polycarbonate filters. Small unilamellar vesicles were prepared by either sonication and gel filtration as described

previously (Bazzi & Nelsestuen, 1987a; Huang, 1969) or by ethanol injection (Kremer et al., 1977) with carefully controlled addition of phospholipid (Lampe & Nelsestuen, 1982). These various preparation methods allowed determination of the effect of surface curvature as well as any influence that a particular preparation method may have on the surface properties of the vesicles. The extrusion method produced very homogeneous vesicles of about 100-nm diameter with low surface curvature. This technique is mild and causes minimal chemical degradation. However, larger size presents some difficulties for quantitative interpretation of light-scattering experiments. Small unilamellar vesicles produced by sonication and gel filtration are ideal for light scattering but have highly curved surfaces and may contain some degraded materials. Small vesicles prepared by ethanol injection have high surface curvature, are ideal for light scattering, and have minimal phospholipid degradation. However, this method is not easily applied to preparations containing PS due to solubility problems.

Other Methods. The average size of phospholipid vesicles containing PE was estimated using quasielastic light scattering. These measurements were performed as described by Pletcher et al. (1980) except that a Langley-Ford LSA 2 particle-sizing instrument was used. Protein concentration was determined according to the method of Bradford (1976) using BSA as the standard. Phospholipid concentrations were determined from organic phosphate (Chen et al., 1956) using a phosphorus to phospholipid weight ratio of 1:25. Unless indicated, the buffer consisted of 50 mM Tris, pH 7.5, containing 0.1 M NaCl plus calcium or EDTA at the indicated concentrations.

Results

Effect of PE on the Calcium Required for Protein–Membrane Association. The calcium concentration required for interaction of the 64-kDa protein with phospholipid bilayers was strongly dependent on the content of acidic phospholipids; vesicles containing higher amounts of PS required lower concentrations of calcium (Bazzi & Nelsestuen, 1991a). This property is illustrated in Figure 1A by comparison of membranes of 90% PS with those of 20% PS/70% PC. Titrations of membranes containing 20% PS with 70% of either PC or PE also showed a large difference (Figure 1A). The PS/PC vesicles required approximately 60 μM calcium for half binding, in agreement with previous results (Bazzi & Nelsestuen, 1991a). In contrast, with PS/PE membranes, the midpoint of protein–membrane association occurred at approximately 6 μM calcium. In fact, the calcium requirement for the PS/PE membranes was similar to that of membranes containing 90% PS (Figure 1A). This was a very surprising result since the neutral phospholipids, PC and PE, were thought to serve as dispersion media for the important anionic phospholipids, PS. All of these titrations showed rapid response to calcium so that the new level of protein–membrane binding was achieved within seconds after addition of calcium.

The 32-kDa protein displayed similar properties (Figure 1B) except that, in all cases, the 32-kDa protein required higher concentrations of calcium. The midpoint for membranes containing 20% PS in PC was approximately 300 μM calcium. This was reduced by nearly 10-fold when PE was substituted for PC (Figure 1B). Again, the binding characteristics of the 32-kDa protein to membranes containing 20% PS in PE were similar to the calcium requirements of membranes containing 90% PS. Consequently, the neutral phospholipid played an important role in protein–membrane association.

Another protein from bovine brain that associated with membranes in a calcium-dependent manner had a mass of about 22 kDa and bound only to membranes with high charge density (Bazzi & Nelsestuen, 1991a; see also Figure 1C). Association of the 22-kDa protein with membranes containing 20% PS dispersed in PC was barely detectable, regardless of the calcium concentration (Figure 1C). However, membranes containing 20% PS dispersed in PE supported the binding of this protein to nearly the same extent as membranes containing 90% PS. Membrane-binding properties of this protein illustrated a possible oversight that can occur if PC is used as the neutral phospholipid; at physiological charge density, the 22-kDa protein would not be identified as a calcium-dependent membrane-associated protein if PC were used as the neutral phospholipid.

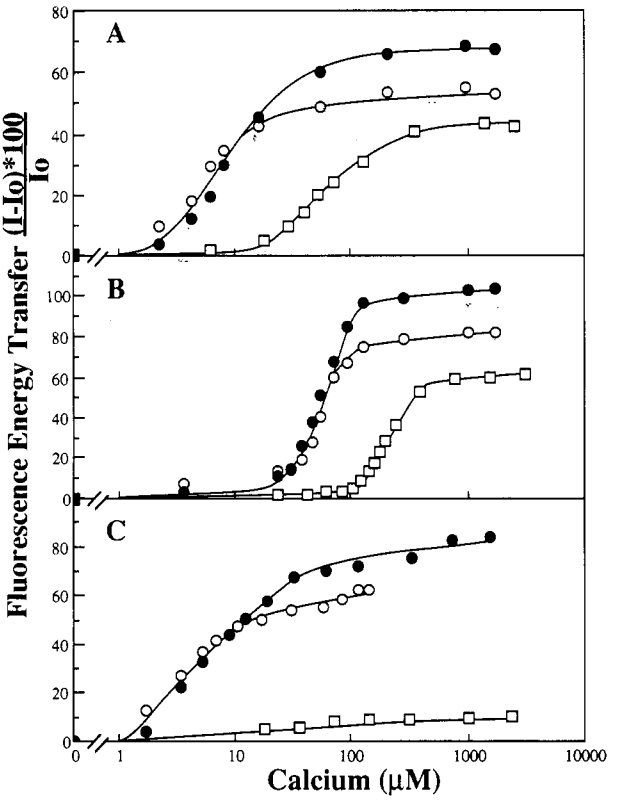

FIGURE 1: Effect of PE on protein–membrane association. Association of the 64-kDa (panel A), the 32-kDa (panel B), and the 22-kDa (panel C) proteins with membranes was examined by fluorescence energy transfer. In all panels, large unilamellar vesicles composed of 20% PS/70% PC (□), 20% PS/70% PE (●), or 90% PS (○) were prepared by extrusion. All vesicles contained 10% dansyl-PE. The measurements were performed in 1.6 mL of buffer containing 50 mM Tris, pH 7.9, 100 mM NaCl, with 25 μg of phospholipid vesicles and protein (12.5 μg of either the 64- or 32-kDa proteins or 125 μg of the 22-kDa protein). Signal intensity from the sample ($I - I_o$) is expressed relative to the intensity of the vesicles alone (I_o).

These results suggested that membranes containing PE as the inert phospholipid were substantially different from those containing PC. There appeared to be at least two manifestations of this effect: PE reduced the calcium required for some protein–membrane associations and was essential for membrane association by other proteins.

Membranes containing high amounts of PE may segregate or form nonbilayer structures, especially in the presence of calcium (Cullis et al., 1985; Tilcock & Cullis, 1981; Szoka & Papahadjopoulos, 1980; Lentz & Litman, 1978). Therefore, association of the 32-kDa protein with membranes containing 20% PS and variable amounts of PE was examined. Low percentages of PE do not alter the bilayer structure of the vesicles (Cullis et al., 1985; Szoka & Papahadjopoulos, 1980). The results showed that increasing the content of PE resulted in systematic reduction in the calcium required for protein–

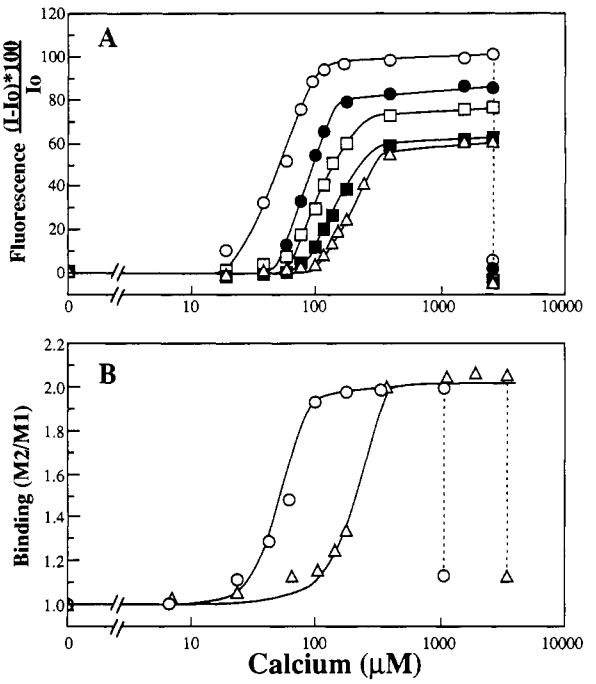

FIGURE 2: Effect of varying levels of PE. Calcium titrations for association of the 32-kDa protein with membranes were conducted using fluorescence energy transfer (panel A) and light-scattering intensity measurements (panel B). In both panels, 25 μg of vesicles was mixed with 12.5 μg of protein in 1.6 mL of buffer containing 50 mM Tris, pH 7.9, and 100 mM NaCl. Protein–phospholipid association was measured with large unilamellar vesicles composed of 20% PS, 10% dansyl-PE, and 70% PE/PC at ratios of 0:70 (△), 10:60 (■), 30:40 (□), 50:20 (●), or 70:0 (○). Fluorescence energy transfer is expressed as described in the legend to Figure 1. Light-scattering changes (excitation and emission wavelengths were set at 650 nm) were converted to the ratio of the molecular weight of the protein–vesicle complex (M_2) to that of the vesicles alone (M_1).

membrane association (Figure 2A). In all cases, binding was rapid and was reversed by addition of EGTA at the end of the titration (dashed lines in Figure 2A). Consequently, the effect of PE on protein–membrane association was related to biochemical properties of PE as a lipid matrix rather than the ability of PE to form nonbilayer structures.

For all of these proteins, membranes containing PE showed greater fluorescence energy transfer upon protein binding (Figures 1A, C and 2A). However, the enhanced signal appeared to result from improved energy transfer rather than from a greater amount of membrane-bound protein. Membranes containing 20% PS dispersed in PC or PE bound similar amounts of the 32-kDa protein as detected by light-scattering intensity measurements (Figure 2B). Light scattering is sensitive to mass changes in the particles. Apparently, the presence of PE in the vesicles altered the environment or the orientation of the fluorescent probe so that greater transfer of fluorescence energy was possible upon protein binding. However, for each membrane composition, fluorescence energy transfer appeared proportional to the amount of protein bound.

In agreement with the fluorescence measurements, light scattering showed that PS/PE membranes required lower concentrations of calcium for protein binding (Figure 2B). An important property evident in Figure 2B was that neither membrane showed evidence of secondary events such as aggregation or fusion. Light-scattering changes occurred in a controlled manner, indicative of protein–membrane association rather than uncontrolled aggregation or fusion. Furthermore, the changes were fully reversed by the addition of EGTA at the end of the experiment. Finally, the fact that both membranes bound similar amounts of protein suggested that

Table I: Stability of Membranes Containing PE[a]

composition PE/PS/PC	storage time (weeks) 0	2	4	6
	Average Vesicle Diameter (nm)			
80:20:0	138	143	150	197
70:30:0	127	138	agg[b]	
60:30:10	120	124	139	194
50:30:20	108	111	197	agg[b]
10:20:70	93	92	93	92

[a] All phospholipid preparations were extruded several times through 100-nm polycarbonate filters. The average diameter of each preparation was determined by quasielastic light-scattering measurements as described under Experimental Procedures. In all cases, phospholipid preparations (5 mg/mL) were stored in ice and were diluted and equilibrated at 25 °C prior to quasielastic light-scattering measurements. [b] agg indicates precipitation or aggregation of the stock vesicle solution.

phospholipid segregation did not occur in the PS/PE membranes. Had this been the case, the region of PS would constitute only 20% of the surface area and these membranes should bind less protein.

Factors That Might Contribute to the PE Effect. The biochemical basis for the effect of PE on protein–membrane association is unknown. The stability of phospholipid vesicles containing up to 80% PE was not a critical factor (Table I). Although vesicles with higher amounts of PE showed progressively larger diameters, all vesicles were relatively stable for periods of weeks. This result was consistent with a report that 15% acidic phospholipid is sufficient to stabilize the bilayer structure of vesicles containing PE (Cullis et al., 1985). In contrast, vesicles of 100% PE could not be detected at all (data not shown), a result that was consistent with the reported instability of pure PE bilayers.

The majority of the experiments were conducted at pH 7.9, which is considerably lower than the pK_a of the amine group of PE. Nevertheless, partial ionization of the amine group might contribute to the negative charge of membranes containing PE and thereby may contribute to the observed results. To test this possibility, interaction of the 32-kDa protein with PS/PE was measured at pH 6 and 8 (Figure 3A). Protein–membrane binding was not greatly influenced by this pH range. Association of this protein with vesicles containing 90% PS was not significantly influenced by this range of pH, either (Figure 3B). A minor inconsistency was observed among different batches of phospholipids obtained from two different commercial sources. Experiments similar to those shown in Figure 3 sometimes (two batches out of four tested) showed a small pH-dependent shift in the calcium requirement (30% higher calcium requirement at the lower pH). However, a similar shift was observed when PS from the same commercial source was used to produce vesicles containing 90% PS. The reason for this source-dependent inconsistency was unknown. However, both of these results supported the same conclusion, which was that protein–membrane association did not become more pH sensitive when PE was present. This suggested that ionization of PE was not a major contributor to enhanced protein binding to membranes containing PE.

The fluorescence measurements involved inclusion of dansyl-PE in the vesicles. The results in Figure 4 suggested that, although negatively charged, dansyl-PE did not contribute to the protein–membrane interaction. Light-scattering measurements were conducted with two populations of vesicles, one containing dansyl-PE and the other without this component. No difference in the calcium required for protein–phospholipid association was detected (Figure 4). This was similar to earlier results obtained with PKC (Bazzi & Nel-

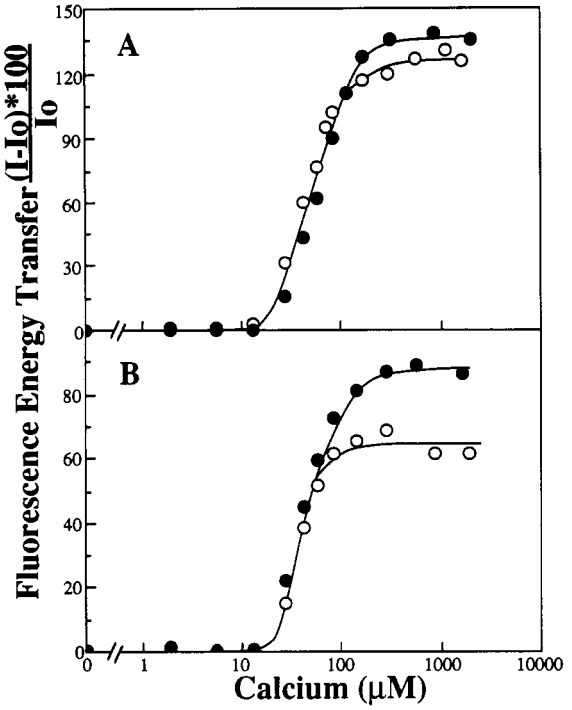

FIGURE 3: Effect of pH on protein–membrane association. Calcium titrations for association of the 32-kDa protein with membranes were conducted at pH 6.0 (○) or 8.0 (●). Large unilamellar vesicles containing 10% dansyl-PE and PS/PE at either 20:70 (panel A) or 90:0 (panel B) were prepared by extrusion. Vesicles (25 μg) and protein (12.5 μg) were diluted to 1.6 mL with buffer containing 100 mM NaCl and either 50 mM Tris, pH 8.0, or 50 mM PIPES, pH 6.0. The vesicles had been equilibrated for at least 24 h with the corresponding buffers. Fluorescence intensity due to energy transfer is expressed as described in the legend to Figure 1.

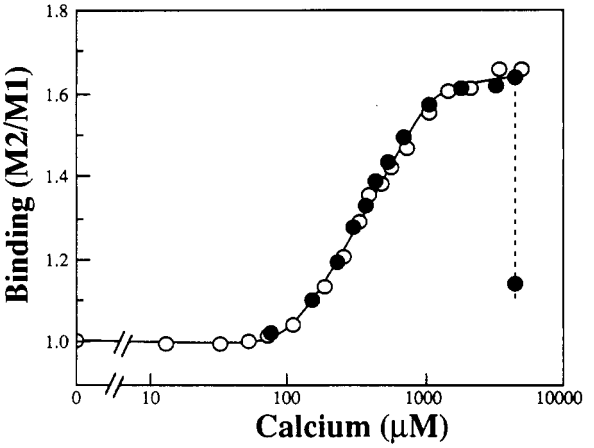

FIGURE 4: Effect of dansyl-PE on protein–membrane association. Small unilamellar vesicles containing PE/dansyl-PE/PC at either 50:10:40 (●) or 60:0:40 (○) were prepared by sonication and gel filtration as described under Experimental Procedures. Light-scattering intensity measurements (excitation and emission wavelengths were set at 320 nm) were used to measure the association of the 32-kDa protein (12.5 μg) with vesicles (25 μg) as a function of calcium concentration. Measurements were conducted as described for Figure 2, panel B.

sestuen, 1987a). These results indicated that negative charge alone was insufficient to support protein–membrane interaction; proteins may have selectivity that extends beyond ionic charge. For comparison, the presence of an additional 10% PS in these membranes would have a large impact on the calcium titration (see below). Thus, the anion character of dansyl-PE did not appear to contribute to protein–membrane binding.

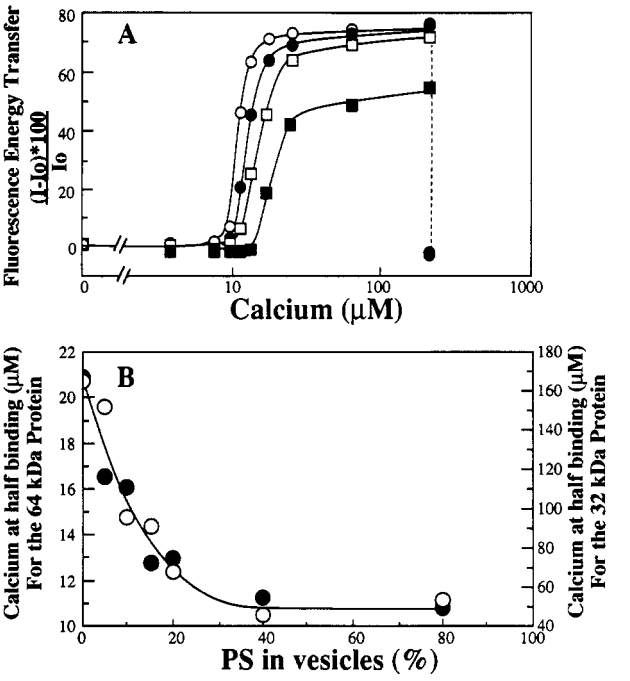

FIGURE 5: Determination of optimum PS in PE membranes. Association of the 64- and 32-kDa proteins with membranes containing various ratios of PE and PS was examined by fluorescence energy transfer. Panel A shows complete titration curves for association of the 64-kDa protein with membranes containing 10% dansyl-PE, 20% PC, and 70% PS/PE at ratios of 0:70 (■), 10:60 (□), 20:50 (●), 40:30 (○). In each case, binding was measured as a function of calcium in 1.6 mL of buffer containing 50 mM Tris, pH 7.9, and 100 mM NaCl plus 25 μg of protein and 20 μg of vesicles. Panel B shows calcium concentrations at half-maximum binding plotted as a function of PS content of the vesicles. Titrations similar to those shown in panel A were conducted for each composition. The panel shows the midpoints obtained with the 64-kDa protein (●, left-side scale) and the 32-kDa protein (○, right-side scale). All vesicle preparations were large unilamellar vesicles prepared by extrusion.

Binding of Proteins to Neutral Phospholipids. Protein–membrane association was examined using vesicles containing various amounts of PS and PE (Figure 5). In order to assure stability, membrane compositions were chosen so that these two phospholipids accounted for only 80% of the membrane. Calcium titrations for the 64-kDa protein, measured by fluorescence energy transfer, are shown in Figure 5A. In all cases, association of the 64-kDa protein was rapid, saturable, and reversed by EGTA (dashed line in Figure 5A). However, the calcium concentration needed for binding was different. The calcium concentrations at half-binding for the 64- and 32-kDa proteins are plotted as a function of the PS content (Figure 5B). The pattern for both of these proteins was similar. Between 0 and about 20% PS, the calcium requirement decreased with PS content. However, further increases in PS content resulted in only minor changes in calcium requirement (Figure 5B). A plateau above 20% PS was interesting since this closely correlated with the charge density of cytoplasmic membranes.

Negatively charged phospholipids have often been considered to be essential for the association of many proteins with membranes. Among others, this apparent requirement has been reported for the 64- and 32-kDa proteins (Bazzi & Nelsestuen, 1991a), PKC (Bazzi & Nelsestuen, 1987a; Hannun et al., 1986), and the lipocortin family of proteins (Smith et al., 1990; Klee, 1988). Therefore, an unexpected result was that the 64-kDa protein bound to membranes composed of only PE and PC (solid squares, Figure 5A). This suggested that net charge was not essential for protein–mem-

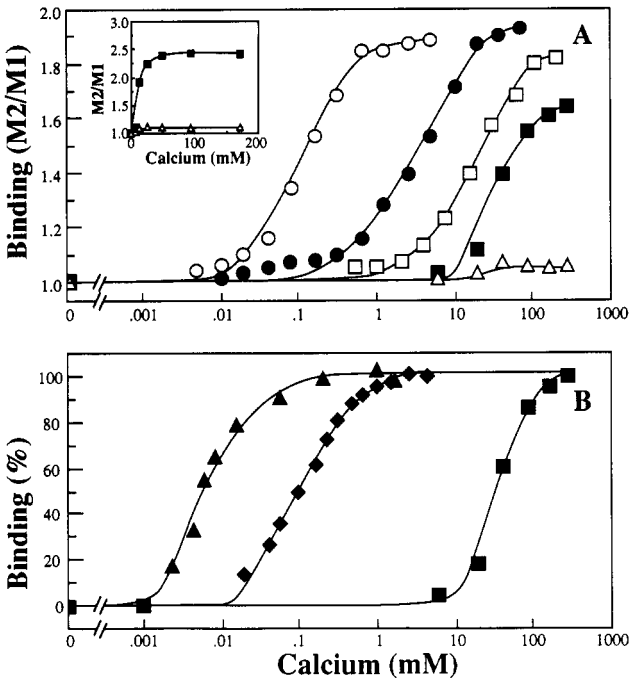

FIGURE 6: Binding of protein to PC membranes. Panel A shows the association of the 64-kDa protein (25 μg) with membranes (25 μg) containing PS/PC at ratios of 25:75 (○), 5:95 (●), 2:98 (□), or 0:100 (■). Panel A also shows a calcium titration of the PC vesicles in the absence of protein (△). These measurements were performed using light-scattering intensity (320-nm wavelength) and small unilamellar vesicles prepared by sonication and gel filtration. The inset to panel A shows results obtained with PC vesicles (25 μg) prepared by the ethanol injection method in the presence (■) or the absence (△) of the 64-kDa protein (25 μg). Panel B shows the effect of the phospholipid headgroup on protein–membrane association. Binding of the 64-kDa protein to phospholipid vesicles containing 100% PC (■), 60% PE/40% PC (◆), and 90% PS/10% dansyl-PE (▲) are shown. Binding was measured using light-scattering intensity (■, ◆) or fluorescence energy transfer measurements (▲). The signal is expressed as 0–100% of maximum in order to provide a more direct comparison.

brane interaction. The possibility that these interactions were the result of acidic phospholipid contaminants in the PE preparations was examined. Direct protein–PE interaction was supported by light-scattering experiments conducted with vesicles containing 20%, 40%, or 60% PE in PC (none containing dansyl-PE). In all cases, the total signal change, M_2/M_1 of about 1.8, was similar to that obtained with membranes containing at least 20% PS (see Figure 6A). This suggested that similar amounts of the 64-kDa protein bound to all of these vesicle preparations. If contaminating acidic phospholipids were responsible for protein binding, they would have to account for more than 50% of the PE preparations. The absence of contaminating acidic phospholipids was also supported by a lack of interaction between histones and the PE/PC membranes (data not shown). Histone, a cationic substrate of PKC, strongly interacts with membranes containing acidic phospholipids and causes vesicle aggregation that is easily detected by light scattering (Bazzi & Nelsestuen, 1987c).

Figure 6A shows calcium titrations for association of the 64-kDa protein with vesicles containing PS and PC. Vesicles containing 25% PS bound the 64-kDa protein with a midpoint of approximatley 100 μM calcium. The binding appeared to reach saturation at an M_2/M_1 value of approximately 1.8. This indicated that 1 g of phospholipid could bind approximately 0.8 g of protein. Phospholipid vesicles containing 5% or 2% PS bound similar amounts of protein but with midpoints at approximately 2 or 10 mM calcium, respectively (Figure 6A).

A surprising result was that vesicles composed entirely of PC bound approximately the same amount of protein as vesicles containing PS, but with a difference in the calcium requirement (Figure 6A). Controls showed that the light-scattering changes were due to protein–phospholipid association, not to structural changes of the vesicles. For example, light-scattering intensity changes of PC vesicles were minimal, even in 200 mM calcium (open triangles in Figure 6A). Again, histone failed to interact with these vesicles, so that contamination by acidic phospholipids was minimal. The ability of this protein to associate with membranes of pure PC corroborated the results obtained with PE/PC membranes (Figure 5).

Since phospholipid vesicles prepared by sonication might contain small amounts of degradation product (Szoka & Papahadjopoulos, 1980), the interaction of the 64-kDa protein with small unilamellar PC membranes prepared by the ethanol injection method was examined (Figure 6A, inset). The results showed calcium-dependent binding of the 64-kDa protein to these vesicles, with a midpoint of titration at approximately 20 mM calcium. Once again, the light-scattering changes were associated with protein–phospholipid binding and were not due to structural changes of the vesicles (Figure 6A, inset). Binding at 10 or 20 mM calcium was completely reversed by addition of excess EGTA (data not shown). Demonstration of EGTA reversal at the highest calcium concentrations was not possible because of solubility and the ionic strength of the medium.

The results suggested that these proteins associated with a variety of acidic or neutral phospholipids. Changing the phospholipid headgroup altered only the calcium required for binding (Figure 6B). Midpoints for binding the 64-kDa protein to membranes spanned a 3000-fold range, from 6 μM calcium for membranes of 90% PS to approximately 20 000 μM calcium for membranes of 100% PC (Figure 6B). Use of the higher calcium concentration is not a common practice so that binding of these proteins to PC has previously been undetected.

Calcium-dependent binding to neutral membranes was also observed with the 32-kDa protein (Figure 5B) and PKC (see below). However, the 22-kDa protein failed to bind to PE/PC or PC membranes, regardless of the calcium concentration (data not shown). Separate experiments also failed to detect significant interaction between blood clotting factor X and vesicles containing only PC (concentrations of up to 150 mM calcium). The latter protein, however, could associate with PE/PC membranes (Nelsestuen & Broderius, 1977).

Binding of Protein Kinase C to PE/PC Membranes. PKC displayed calcium- and phospholipid-binding properties similar to those of the 64- and 32-kDa proteins. Association of PKC with neutral membranes required relatively high calcium, a condition which presents a concern about the functional stability of PKC (Bazzi & Nelsestuen, 1989). Therefore, the association of PKC with PE/PC membranes was examined by gel filtration chromatography and enzyme activity (Figure 7).

At 1.0 mM calcium, PKC associated with phospholipid vesicles containing 40% PS and eluted near the exclusion volume of the column (Figure 7A). This was consistent with the known phospholipid-binding properties of PKC. With PE/PC membranes, the kinase activity eluted with nearly equal distribution between free and membrane-bound states (closed circles, Figure 7A). In contrast, PKC eluted pre-

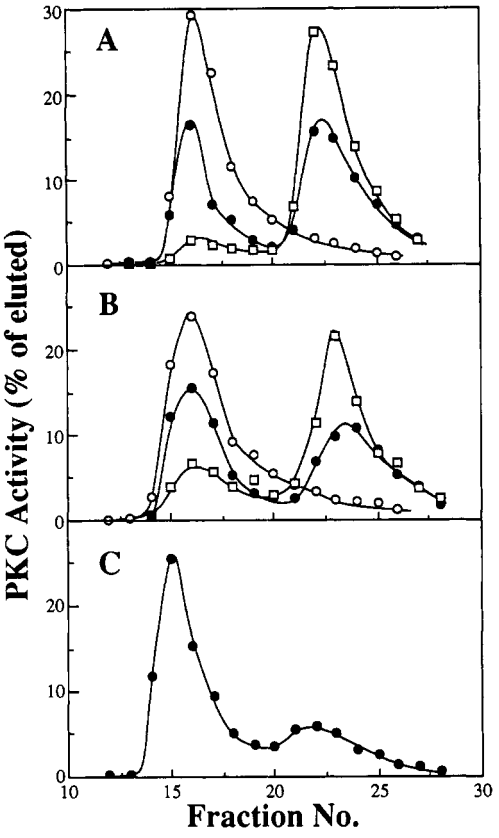

FIGURE 7: Association of PKC with neutral membranes. Association of PKC with membranes containing 50% PC, 10% DAG, and 40% of either PS (○), PE (●), or PC (□) was examined by gel filtration chromatography. Mixtures containing PKC (10 μg) and phospholipids (0.75 mg) at the appropriate calcium concentration (total volume of 0.5 mL) were applied on Sephacryl S-300 columns (1 × 30 cm) that had been equilibrated with a buffer containing 20 mM Tris, pH 7.9, 10% glycerol, 0.5 mM DTT, bovine serum albumin (0.1%), and calcium at either 1.0 mM (panel A), 4.0 mM (panel B), or 10 mM (panel C). Columns were eluted as described under Experimental Procedures. All panels show PKC activity as determined with protamine sulfate as the substrate. Recovery of enzyme activity was similar for all experiments at an individual calcium concentration. Phospholipids were large unilamellar vesicles prepared by extrusion. Elution of PKC in the absence of added phospholipid (not shown) was very similar to that obtained with PC vesicles.

dominately as the free protein in the absence of membrane or in the presence of PC membranes. These results suggested that the kinase activity eluting with PE/PC vesicles was the result of binding to PE/PC and was not due to aggregation of PKC. More quantitative binding to neutral membranes was obtained at higher calcium concentrations (Figure 7B,C). Consequently, PKC appeared similar to the 32- and 64-kDa proteins in its ability to bind to neutral membranes where it retained functional integrity.

The ability of PKC to bind to uncharged membranes raised the question of whether they could also support PKC activity (Figure 8). As expected, membranes containing PS and DAG activated PKC in a calcium-dependent manner with a midpoint of activation at approximately 1 μM and maximum activation at about 20 μM calcium. It was surprising to find that neutral membranes containing PE and DAG could also activate PKC (Figure 8), but at relatively higher calcium concentrations. Calcium in excess of 1.0 mM inhibited PKC activity under all conditions so that maximum activation of PKC by PE/PC was not achieved. Membranes containing PC only, or samples without phospholipids, failed to support significant levels of PKC activity (Figure 8). Therefore, neutral membranes containing PE appeared to specifically activate PKC if the

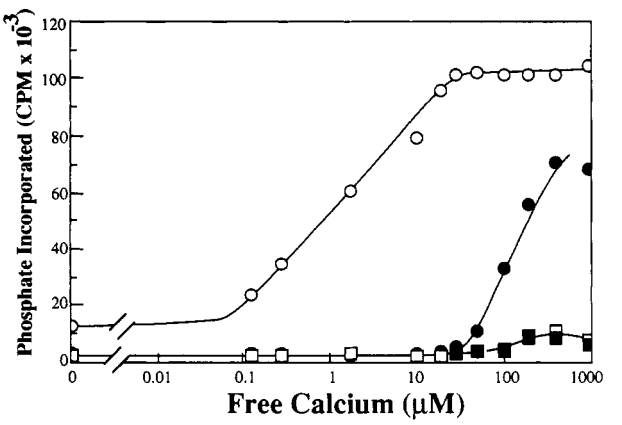

FIGURE 8: Activation of PKC by neutral membranes. PKC activity was measured in the presence of phospholipid vesicles containing 10% DAG and either 40% PS/50% PC (○), 40% PE/50% PC (●), or 90% PC (□). Also shown are results obtained in the presence of 0.5 mg/mL bovine serum albumin but in the absence of phospholipids (■). The kinase activity was measured in a mixture (0.25-mL total volume) containing 20 mM Tris, pH 7.9, 0.1 μg of PKC, 320 μg/mL phospholipids (or 0.5 mg/mL BSA), 0.2 mg/mL histone, 10 mM Mg^{+2}, 20 μM ATP, 10 μM EGTA, and variable amounts of calcium. The free calcium concentration was calculated as described under Experimental Procedures.

calcium concentration is high enough. Kaibuchi et al. (1981) also reported that PE plus DAG could activate PKC. Others failed to detect activation by PE (Wise et al., 1982; Schatzman et al., 1983). The differences in these reports may arise from a number of factor such as the assay conditions, the calcium concentration selected for the assay, or attempts to use unstable membranes of pure PE.

Detailed evaluation of the kinetic mechanism of PKC is difficult due to the extensive aggregation of anionic phospholipids by histones and other in vitro PKC substrates (Bazzi & Nelsestuen, 1987c). In fact, the calcium and phospholipid requirements of PKC appeared to be dominated by this aggregation (Bazzi & Nelsestuen, 1987b). The observation that histone did not aggregate PE/PC membranes raised the possibility that PKC activity in this system had occurred without aggregation. An in vitro assay that does not involve substrate–phospholipid aggregation would be very useful because of the many adverse effects that aggregate presents to interpretation of kinetic results (Bazzi & Nelsestuen, 1987b, 1988b). Unfortunately, these activity measurements occurred with severely aggregated components. While histones alone did not cause aggregation of the PE/PC vesicles, light-scattering intensity showed that a combination of the high levels of magnesium used in the assay (10 mM), together with histones and calcium, did cause aggregation (data not shown). Thus, virtually all of the in vitro assays for PKC activity, including those with PE/PC membranes, involve aggregated enzyme and substrate.

Discussion

Physiological membranes display an asymmetric distributions of phospholipids. The inner cytoplasmic membrane is composed primarily of negatively charged phospholipids embedded in PE, while PC is located primarily on the outer surface. Maintaining this asymmetry could be important for facilitation of membrane–membrane interactions [for a recent review, see Devaux (1991)] and for regulation of blood coagulation (Nelsestuen & Broderius, 1977). The present study suggested another fundamental role for the asymmetric distribution of the neutral phospholipids. PE provided a better surface for interaction with cytoplasmic proteins. Three of

the four cytoplasmic proteins examined showed as much as a 10-fold reduction in the calcium requirement when PE was used instead of PC. Substitution of PE for PC was necessary to even detect interaction of a fourth protein (22 kDa) with membranes containing physiological levels of PS. Therefore, the location of PE on cytoplasmic membranes is an important factor that contributes to protein–membrane interactions at physiological levels of calcium or PS.

The selectivity for PE may help solve previous difficulties in understanding the physiological relevance of certain membrane–protein associations or activations. For example, demonstration of PKC activity often required either membranes with nonphysiological high charge density (often 100% acidic phospholipid) or membranes with physiological charge density but nonphysiological calcium concentrations. Other proteins of this type display similar problems (Smith et al., 1990; Bazzi & Nelsestuen, 1991a). The present study suggested that membranes containing about 20% PS in PE, similar to the charge density of biological membranes, provided optimum conditions for binding and were as effective as membranes composed entirely of acidic phospholipids.

Several other mechanisms have been proposed to explain the interaction of these proteins with membranes at physiological calcium levels. However, it appears that few mechanisms have the potential impact of the PE effect. For example, covalent modifications such as protein phosphorylation might reduce the calcium requirement for individual proteins (Ando et al., 1989; Schlaepfer & Haigler, 1987). A conceptually different mechanism involves the highly sequential binding of certain proteins to membranes (Bazzi & Nelsestuen, 1991c). The latter mechanism emphasizes that the important characteristic is the calcium concentration at which the first protein binds to the membrane rather than the calcium concentration where the membrane is half-saturated with protein. However, the PE effect may represent a more general phenomenon that is applicable to a wider variety of membrane-interacting proteins.

The mechanism for the PE effect is not known. Trivial mechanisms, such as segregation of phospholipids or the presence of contaminating acidic phospholipids, could not account for the results. Segregation of phospholipids into separate domains would produce a small area of acidic phospholipids. Contamination could account for only a small amount of the total phospholipid. In either case, the resulting membranes could support the binding of only a small amount of protein. Instead, it was found that bilayers containing neutral phospholipids could bind the same amount of protein as PS/PC or PS membranes, although at much higher calcium concentrations.

The differences between PE and PC that are detected by the biological probes must be reflected in biochemical and/or biophysical properties of the membrane. Simple charge or the intrinsic calcium binding constant show little difference between PC and PE (McLaughlin et al., 1981). However, PE is known to have a smaller hydration shell (Rand & Parsegian, 1988), which might allow closer protein–membrane contact or better accessibility to neighboring acidic phospholipids. Alternatively, PE, which could form an extensive hydrogen-bonding network (Hauser et al., 1981), might provide membranes with partial negative charge that could enhance protein–membrane association. These and other factors, such as molecular packing and headgroup spacing, might be the important features detected by the biological probes.

The proteins used in this study appeared to represent two extremely different types of calcium-dependent membrane-binding proteins. The 22-kDa protein represented one type which was a true calcium-binding protein. Phospholipid had little effect on calcium bound to this protein (Bazzi & Nelsestuen, 1991a). The 22-kDa protein also had low affinity for phospholipid, which may be inadequate to stabilize its interaction with neutral phospholipids or with bilayers containing PC. Calcium binding to the 22-kDa protein may induce conformational changes that allow interaction with other components, such as membranes. PKC and the 64- and 32-kDa proteins represented another extreme type of behavior. They did not appear to bind any calcium unless they were simultaneously associated with membranes. The calcium- as well as the phospholipid-binding properties of these proteins were therefore highly dependent on membrane composition. These general properties may be fairly common and may apply to lipocortin I and II (Glenney, 1986; Glenney et al., 1987; Schlaepfer & Haigler, 1987) as well.

Interaction of these proteins with membranes probably consists of an initial low-affinity calcium interaction with one component, probably the phospholipid. Recruitment of the second component then generates high-affinity calcium-binding sites at the interface. This would generate high cooperativity with respect to calcium (Bazzi & Nelsestuen, 1991c) and would appear as "simultaneous" assembly. This type of protein–membrane association is possible with a variety of phospholipids, since calcium interacts with neutral as well as acidic phospholipids [for a review, see Tocanne and Teissie (1990)]. In what may be related observations, annexin IV and VI (Edwards & Crumpton, 1991) and the vitamin K-dependent blood clotting proteins (Nelsestuen & Broderius, 1977) have been shown to bind to PE-coated surfaces or to neutral PE/PC membranes, respectively.

An important feature of biological membranes appears to be their heterogeneity. The importance of this heterogeneity for interactions and/or functions of proteins has not been thoroughly investigated, probably due to experimental difficulties. For example, PE tends to form the hexagonal phase and may destabilize membrane bilayers. However, addition of as little as 15–20% of another phospholipid stabilized large unilamellar vesicles (Tilcock & Cullis, 1981; Cullis et al., 1985). Vesicles containing up to 70% PE could also produce stable membranes with high curvature (Lentz & Litman, 1978). A poorly understood basis for natural selection of PE, together with questionable stability of bilayers containing this component, have probably contributed to the tendency to use PC as the neutral phospholipid for many in vitro studies. Thus, the contribution of PE to interaction with biological systems has not been fully appreciated.

Some approaches have tried to eliminate the problems of bilayers by presenting acidic phospholipids in dispersions with detergents such as Triton X100 (Hannun et al., 1985). Comparisons of PE, PC, and detergent show some qualitative similarities such as retention of calcium-dependent interaction and reversibility. However, the current study suggested that the "inert" matrix component had a large impact on many properties of PKC–phospholipid interaction. For example, it is widely accepted that PKC interacts with acidic phospholipids, most notably PS, in a calcium-dependent manner (Bazzi & Nelsestuen, 1987a; Hannun et al., 1986). The degree of specificity for the phospholipid headgroup seems highly influenced by the choice of the "inert" component. When examined with Triton–phospholipid mixed micelles, PKC displays a high specificity for PS with strict alignment of essential residues in the serine and glycerol moieties (Lee & Bell, 1989). In contrast, PKC showed low selectivity for the acidic phos-

pholipid headgroup when it was dispersed in PC (Bazzi & Nelsestuen, 1987a,b). This study showed that, at appropriate calcium concentrations, even neutral membranes composed of PE/DAG/PC supported the binding as well as the activation of PKC. Properties that are apparent only with the detergent matrix probably do not reflect fundamental aspects of a protein–membrane interaction.

Other properties of PKC–membrane interactions could not be observed with detergent dispersions (Bazzi & Nelsestuen, 1988a, 1991a,b). For example, formation of membrane-inserted PKC, clustering of acidic phospholipids in membranes, and the sequential binding mechanism would not be observed with Triton–phospholipid micelles due to their dynamic properties and small size. Phospholipid bilayers, which present acidic phospholipids dispersed in a neutral phospholipid component, offer a more appropriate model system that mimics the biological situation more closely.

There are several reports suggesting that other proteins show preference for PE. Examples include phospholipase C (Hoffman & Majerus, 1982), prothrombin (Nelsestuen & Broderius, 1977), and PKC (Kaibuchi et al., 1981). Newton and Koshland (1989) also reported that PE enhanced the efficacy of PS dispersed in Triton micelles. Recent observations suggest that another plasma protein, serum amyloid P component (Schwalbe et al., 1990), also shows high selectivity for interaction with membranes containing PE rather than PC as the neutral phospholipid.[2] PE may function by either or both of two mechanisms: directly, by support of membrane–protein interactions, or indirectly, by providing a better surface for protein interaction with acidic phospholipids. In both cases, it is apparent that biological selection of PE rather than PC for intracellular membranes is not a coincidence.

Added in Proof

Recent evidence (R. Bianchi, I. Giambanco, G. Pula, R. Donato, M. D. Bazzi, and G. L. Nelsestuen, unpublished data) shows that the 64-kDa protein described in this paper cross-reacts with antibodies to annexin VI and also shows the identity of the 32-kDa proteins with CaBP33 (annexin V) and CaBP37 described by Donato et al. (1990).

Registry No. PK, 9026-43-1; Ca, 7440-70-2; Mg, 7439-95-4.

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Characterization of a Recombinant Extracellular Domain of the Type 1 Tumor Necrosis Factor Receptor: Evidence for Tumor Necrosis Factor-α Induced Receptor Aggregation

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Received September 11, 1991; Revised Manuscript Received October 25, 1991

ABSTRACT: An expression plasmid encoding the extracellular portion of the human tumor necrosis factor (TNF) type 1 receptor (TNF-R1) was constructed and used to generate a stable cell line secreting soluble TNF-R1 (sTNF-R1). The sTNF-R1 was purified, and its biochemical properties and its interactions with human TNF-α were examined. SDS-PAGE resolved the purified sTNF-R1 into three bands of approximate M_r 24 200, 28 200, and 32 800. Sedimentation equilibrium analysis gave a molecular weight of 25 000 for sTNF-R1 whereas the molecular weight obtained by gel filtration chromatography was approximately 55 000–60 000. Scatchard analysis of [^{125}I]TNF-α binding to sTNF-R1 revealed high-affinity binding (K_d = 93 pM), comparable to that observed for the intact receptor on whole cells. Competitive binding experiments showed that sTNF-R1 has a 50–60-fold higher affinity for TNF-α than for TNF-β, in contrast to the equal affinities of TNF-α and TNF-β for the full-length TNF-R1 transiently expressed in mammalian cells. The sTNF-R1 was found to block the cytotoxicity of TNF-α and TNF-β on a murine L-M cell assay. The sizes of the sTNF-R1·TNF-α complex determined by gel filtration chromatography and sedimentation equilibrium were approximately 141 and 115 kDa, respectively. The stoichiometry of the complex was examined by Scatchard analysis, size-exclusion chromatography, HPLC separation, amino acid composition, sequence analysis, and sedimentation equilibrium. The data from these studies suggest that at least two molecules of sTNF-R1 can bind to a single TNF-α trimer. We propose that the initiation of signaling by TNF-R1 involves TNF-α-induced receptor oligomerization.

TNF-α and TNF-β are related cytokines produced by activated macrophages and lymphocytes, respectively (Beutler & Cerami, 1989). Originally described for their ability to induce hemorrhagic necrosis of certain murine tumors and for their cytotoxicity to some tumor cell lines (Carswell et al., 1975), they have now been shown to mediate a wide variety of biological responses both in vivo and in vitro (Goeddel et al., 1986; Beutler & Cerami, 1988a,b). Binding studies with [^{125}I]-labeled TNF-α and TNF-β have revealed the presence of specific TNF receptors on the majority of somatic cell types examined (Rubin et al., 1985; Kull et al., 1985; Baglioni et al., 1985; Aggarwal et al., 1985; Tsujimoto et al., 1985; Yoshie et al., 1986). Although the biochemical events underlying TNF action have not yet been elucidated, it appears that binding of TNF to these receptors initiates the biological responses of TNF. However, the molecular forms of active TNF-α and TNF-β are still somewhat unclear. It has been shown by crystallization studies and sedimentation equilibrium that TNF-α exists as a closely packed trimer (Wingfield et al., 1987; Eck et al., 1988; Lewit-Bently et al., 1988; Jones et al., 1989); however, there are many conflicting reports in the literature as to whether the active form of TNF-α, that

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0006-2960/92/0431-1134$03.00/0