Proteins That Bind Calcium in a Phospholipid-Dependent Manner†

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ABSTRACT: Three proteins (M_r = 64K, 32K, and 22K) that bind to phospholipids in a calcium-dependent manner were purified from bovine brain. The calcium-binding properties of these proteins were investigated by equilibrium dialysis and by gel filtration chromatography. The 64- and 32-kDa proteins were found to have calcium- and phospholipid-binding properties strikingly similar to those of protein kinase C [Bazzi, M. D., & Nelsestuen, G. L. (1990) *Biochemistry 29*, 7624]. The free proteins bound limited divalent metal ion even at 200 μM calcium. However, they bound eight to nine calcium ions per protein in the presence of membranes containing acidic phospholipids. The calcium concentrations needed for protein–phospholipid binding were different for these two proteins and were strongly influenced by the phospholipid composition of the vesicles; vesicles of higher phosphatidylserine content required lower concentrations of calcium for protein–membrane association. These properties described a general type of calcium-interacting system where simultaneous interaction of all three components (protein, phospholipids, and calcium) is required. The free proteins may provide only partial coordinate bonds to each calcium ion, but complete calcium-binding sites could be generated at the protein–phospholipid interface. In contrast to the 64- and 32-kDa proteins, the 22-kDa protein bound similar amounts of calcium (two to three ions/protein) in the presence or the absence of phospholipids. The 22-kDa protein had the lowest affinity for phospholipid and the highest affinity for calcium of the three proteins tested. Thus, calcium-dependent phospholipid-binding proteins consist of several types. Calcium binding in a phospholipid-dependent manner may constitute a major type of calcium-response element in the cell. For example, the 64- and 32-kDa proteins appear to be quite abundant and may even function as a calcium buffer to modulate signaling events.

Calcium is a major second messenger in virtually all cell systems that have been studied. The most well-documented form of calcium-response elements includes the modulation or the activation of a variety of proteins (Kretsinger, 1976) such as the calmodulin-dependent enzymes (Cheung, 1980; Klee et al., 1980). Calmodulin and similar calcium-binding proteins share several biochemical properties. For example, many of these proteins are cytosolic and bind one to four calciums (Kretsinger, 1980). The calcium-binding sites exhibit a sequence homology that is referred to as the E-F hand. This calcium binding structure consists of two helix regions (the E and F helices of parvalbumin) and an intervening loop that satisfies the calcium coordination geometry.

More recently, a number of proteins that bind phospholipid in a calcium-dependent manner have been identified [for a review, see Klee (1988)]. Many members of this protein family lack the E-F hand structure, but are clearly identified as calcium-associating proteins. Geisow et al. (1986) identified a 17 amino acid consensus sequence with a conserved pattern of hydrophobic and hydrophilic residues. This sequence, termed the endonexin fold, is present in multiple copies in several calcium-dependent phospholipid-binding proteins and is proposed to form part of the calcium- or phospholipid-binding domain(s). However, the number of these repeat structures does not always correlate with the number of calcium ions bound. For example, p68, a membrane-associated Ca^{2+}-binding protein contains eight copies of this sequence (Crompton et al., 1988) but binds only one calcium ion (Owen & Crumpton, 1984). Also, protein II contains four homologous segments (Weber et al., 1987) but binds only one calcium ion (Shadle et al., 1985). Thus, prediction of the number of calcium-binding sites from protein sequence appears difficult.

The calcium-binding properties of lipocortin I and II have been reported (Glenney, 1986; Glenney et al., 1987; Schaelpfer & Haigler, 1987). Both of these proteins contain four copies of the endonexin fold and bind four or two calcium ions (lipocortin I and II, respectively). An interesting feature was that these proteins required the presence of phospholipids for efficient calcium binding. This suggested that the phospholipid acted to enhance the calcium binding affinity of the protein (Glenney, 1986; Glenney et al., 1987; Schaelpfer & Haigler, 1987).

More recently, protein kinase C (PKC)[1] was found to interact with calcium in a rather unique manner (Bazzi & Nelsestuen, 1990). This enzyme displays calcium-dependent phospholipid binding but contains no primary sequences homologous to either the E-F hand or the endonexin fold (Parker et al., 1986; Ohno et al., 1987). Direct calcium-binding measurements showed that PKC itself bound virtually no calcium while its complex with phospholipids bound a large number (eight to nine) of calcium ions. This seems a unique situation where PKC could not be accurately described as a calcium-binding protein but rather as a part of a calcium-interacting system or calcium-response element. Free PKC need not contain complete calcium-binding sites but may provide coordination bonds to calcium ions bound at the protein–phospholipid interface.

The unusual nature of the calcium–PKC interaction raised the question of whether this was a unique property of PKC or whether it was characteristic of a more abundant family

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[1] Abbreviations: EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; PC, phosphatidylcholine; PS, phosphatidylserine; dansyl-PE, *N*-dansyl-L-α-dipalmitoylphosphatidylethanolamine; PIPES, piperazine-*N*,*N*′-bis(2-ethanesulfonic acid); PKC, phospholipid- and calcium-dependent protein kinase C. 32-kDa proteins refers to a mixture of two proteins of M_r = 31K and 33 K which eluted from the gel filtration column as a single peak.

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of proteins. This study reports the isolation of three proteins (M_r = 64K, 32K, and 22K) that bound to phospholipids in a calcium-dependent manner. Two of these proteins (64- and 32-kDa proteins) displayed calcium-binding properties similar to those of PKC; the free proteins bound virtually no calcium while the membrane-associated forms bound a large (eight to nine) number of calcium ions. The 22-kDa protein was of a different type and bound calcium in the presence or absence of phospholipids. These results suggested major differences among the large number of proteins which bind phospholipid in the presence of calcium. Those proteins which bind calcium in the presence of phospholipid appear to be a relatively abundant type of calcium-response element.

Experimental Procedures

Materials. Bovine brain phosphatidylserine (PS), egg yolk phosphatidylcholine (PC), and dansyl-PE were purchased from Sigma Chemical Co. 1,2-Dipalmitoyl-L-3-*sn*-phosphatidyl-[*N*-*methyl*-^{3}H]choline (73 Ci/mmol) was purchased from Amersham Corp. $^{45}CaCl_2$ (32.89 mCi/mg) was purchased from New England Nuclear. 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. Proteins were purified to apparent homogeneity from bovine brain by a procedure similar to that published for protein kinase C (Bazzi & Nelsestuen, 1987a). Briefly, fresh brains were homogenized in a buffer (about 2 L/bovine brain) containing 20 mM Tris, pH 7.9, 0.3 M sucrose, 5 mM EGTA, and 5 mM EDTA. The homogenate was centrifuged at 13000*g* for 45 min, and the supernatant was mixed with DEAE-cellulose (250 mL of packed resin/L of extract buffer) that had been previously equilibrated with the standard purification buffer composed of 20 mM Tris, pH 7.9, 1 mM EGTA, 1 mM EDTA, and 30 mM β-mercaptoethanol. The resin was collected in a large Büchner funnel and washed with the same buffer (about 4 L/L of packed resin). The resin was packed into columns (60 × 6 cm), and each column was washed with an additional 4 L of buffer. Proteins were eluted isocratically with buffer containing 150 mM NaCl.

The major protein-containing fractions eluted by 150 mM NaCl were dialyzed against the standard buffer (two changes), the solution was made 10% with respect to glycerol, and this was loaded onto a DEAE-cellulose column (40 × 6 cm) that had been previously equilibrated with the standard buffer containing 10% glycerol. PKC was eluted isocratically with a buffer containing 20 mM PIPES, 30 mM β-mercaptoethanol, 1 mM EGTA, 1 mM EDTA, and 10% glycerol, pH 6.5 (Parker et al., 1984). The other proteins of interest remained bound to DEAE-cellulose under these conditions and were subsequently eluted by the same buffer that contained 150 mM NaCl. Calcium was added to the eluted proteins to a net concentration of 1 mM in excess over the EGTA and EDTA present in the buffer, and the resulting solution was loaded onto a phospholipid affinity column (see below). The column was extensively washed with a buffer containing 20 mM Tris, pH 7.8, 30 mM β-mercaptoethanol, 150 mM NaCl, and 1 mM Ca^{2+}. The affinity-bound proteins were eluted with a buffer of the same composition except that calcium was replaced by 2 mM EGTA. These proteins were concentrated by pressure dialysis and gel filtered on a Sephacryl S-300 column (2.5 × 120 cm). Final purification of the individual proteins consisted of a second chromatography of each peak on the same column.

Preparation of Phospholipid Affinity Column. Controlled pore glass beads (PG 1000–200, Sigma Chemical Co.) were coated with phospholipids essentially as described by Ando et al. (1989). Brain extract type 1, contained mostly PS (about 60%) and PI (10–20%) as well as other phospholipids (estimation by the Sigma Chemical Co.), were used in the preparation of the affinity column. The phospholipids (1 g) were dissolved in ethanol to give a concentration of 5 mg/mL. Siliconized glass beads (25 cm^3) were added to the solution, which was heated at 70 °C. Coating of the beads was achieved by successive additions of H_2O as described by Ando et al. (1989). The beads were then washed extensively with cold ethanol and H_2O and then equilibrated with a buffer containing 20 mM Tris, pH 7.8, 150 mM NaCl, 30 mM β-mercaptoethanol, and 1 mM Ca^{2+}.

Calcium-Binding Measurements. Calcium binding was measured by equilibrium dialysis according to a procedure similar to that employed with PKC (Bazzi & Nelsestuen, 1990). Prior to calcium-dialysis measurements, calcium chelators and β-mercaptoethanol, which were present during protein purification, were removed by dialysis against a buffer containing 20 mM Tris, pH 7.8, 100 mM NaCl, 10% glycerol, and 0.5 mM DTT (three changes). Radioactive PC (2 mg/mL, containing 0.5 μCi of ^{3}H/mL and provided as vesicles prepared by extrusion) was added to the samples to detect sample dilution that occurred during dialysis; protein and phospholipid concentrations at various stages of dialysis were estimated from the ^{3}H content of the sample assuming that the ratio of PC to other macromolecules remained the same throughout the experiment. Dialysis was conducted against large volumes of buffer (>500:1 buffer:sample) and was continued for at least 5 h at 4 °C prior to calcium measurements. The dialysis buffer consisted of 20 mM Tris, pH 7.5, 100 mM NaCl, 0.5 mM dithiothreitol, 10% glycerol, and various concentrations of $^{45}CaCl_2$. Tritium and ^{45}Ca were measured simultaneously by dual-label counting in a Beckman scintillation counter. Bound calcium corresponded to the calcium present in the sample minus calcium in the dialysis buffer.

Estimation of calcium that was bound to each protein was determined in the presence or the absence of phospholipid vesicles containing phosphatidylserine. Calcium binding to a sample of phospholipid vesicles was determined under the same experimental conditions (see below). The calcium binding to phospholipid vesicles was relatively low but was consistent with published apparent K_D values for calcium–PS interaction (Nelsestuen & Lim, 1977; Portis et al., 1979). The calcium bound to phospholipids alone was considered to be nonspecific binding and was subtracted as a background from samples containing protein plus phospholipids.

Calcium binding was also measured by the method of Hummel and Dreyer (1962). In this case, protein was mixed with 0.6–0.75 mL of the equilibration buffer in the presence or the absence of phospholipid vesicles. This mixture was incubated for 20 min prior to chromatography on a Sephacryl S-300 column (1.0 × 30 cm). The columns were equilibrated and eluted (0.75-mL fraction) with buffer consisting of 20 mM Tris, pH 7.5, 0.5 mM dithiothreitol, 10% glycerol, 100 mM NaCl, and either 20 or 50 μM calcium. The concentration of calcium in each fraction was estimated by sampling the radioactivity, and the protein content was estimated by the method of Bradford (1976). Phospholipids gave a small positive reaction in the protein assay (≤20% of the protein signal in these same samples) which was subtracted as background from samples containing protein. Nonspecific binding of calcium to phospholipid vesicles was determined under the same experimental conditions, and this was subtracted to

obtain specific calcium binding to the protein–phospholipid complex.

Estimation of Contaminating Calcium in the Dialysis Buffer. Several efforts (described previously; Bazzi & Nelsestuen, 1990) were made to assure low levels of contaminating calcium in the dialysis buffer. Remaining levels of calcium in the dialysis buffer were estimated with indo-1, a fluorescent probe that is sensitive to low concentrations of calcium (Grykiewics et al., 1985). When used at 1 μM, the concentration of contaminating calcium in the buffer was found to be about 1 μM as well (Bazzi & Nelsestuen, 1990). However, it was found that most of the calcium detected in this manner arose from calcium in the indo-1 preparation used. Consequently, the actual level of contaminating calcium was substantially less than 1 μM. This was considered insignificant relative to the concentrations needed for calcium binding to the proteins, and its influence on total calcium was ignored in subsequent calculations. The calcium concentrations reported represent calcium added to the buffer.

Protein–Phospholipid Binding. The binding of the various proteins (the 64-kDa, the 32-kDa, and the 22-kDa proteins) to phospholipid vesicles was measured by light scattering intensity and by fluorescence energy transfer methods as described in detail previously (Bazzi & Nelsestuen, 1987a). Fluorescence energy transfer from tryptophan in the protein to dansyl groups in the phospholipid was used to monitor the protein–phospholipid complex as a function of protein concentration in the medium. Phospholipid vesicles were added to 1.6 mL of buffer containing 20 mM Tris, pH 7.5, 100 mM NaCl, and calcium at the indicated concentrations. The excitation and the emission wavelengths were 284 and 520 nm, respectively. A 500-nm cutoff filter was placed in the front of the emission monochromator. Protein was added until the signal due to fluorescence energy transfer was saturated. Direct excitation of the dansyl group at 284 nm produced a reference emission intensity (I_0) and fluorescence energy transfer was expressed as a percentage change in emission intensity, $[(I-I_0) \times 100]/I_0$, where I is the fluorescence intensity of the protein–lipid complex and I_0 is the intensity of the phospholipid alone.

Fluorescence energy transfer was also used to measure protein–phospholipid binding as a function of calcium in the medium. In these experiments, protein and phospholipid vesicles were initially mixed in 1.6 mL of buffer (same as above), and the calcium concentration was increased by successive additions of calcium. All binding interactions were reversed by addition of excess EGTA.

Light scattering intensity measurements at 90° (320-nm light) were also used to measure the binding of protein to small unilamellar vesicles. This method allows quantitative estimation of the amount of protein bound to vesicles (Nelsestuen & Lim, 1977). The data 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

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

where I_2 is the light scattering intensity of the protein–lipid complex, I_1 is the light scattering intensity of the phospholipid, and $\partial n/\partial c$ is the refractive index increment of each species (Nelsestuen & Lim, 1977).

Fluorescence and light scattering measurements were made at 25 °C in a Hitachi Perkin-Elmer Model MPF 44A fluorescence spectrophotometer.

Other Methods. Large unilamellar vesicles were prepared by the extrusion method (Hope et al., 1985) with 0.1-μm polycarbonate filters. This is a very mild technique which produces very homogeneous vesicles of about 100-nm diameter. Small unilamellar vesicles were prepared by sonication and gel filtration as described previously (Huang, 1969; Bazzi & Nelsestuen, 1987a). Phospholipid concentrations were determined from organic phosphate (Chen et al., 1956) by use of a phosphorus to phospholipid weight ratio of 1:25. Gel electrophoresis was carried out by the method of Laemmli (1970). Protein bands were visualized by staining with Coomassie Blue. Standard calcium solutions were prepared as described previously (Bazzi & Nelsestuen, 1990). Protein concentration was determined according to Bradford (1976) with BSA as a standard.

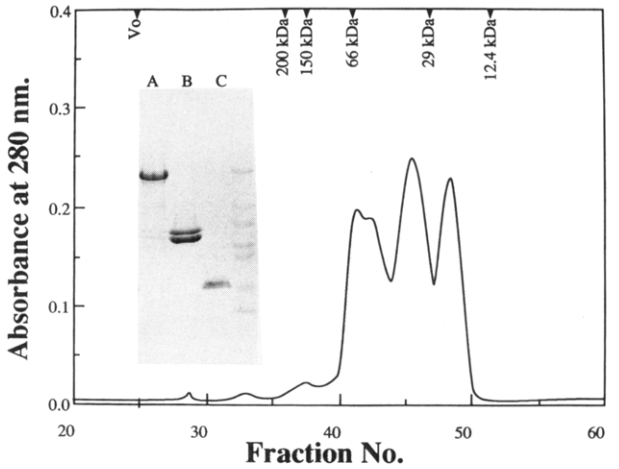

FIGURE 1: Separation of proteins by gel filtration chromatography. Proteins which bound to the phospholipid affinity column were isolated as described under Experimental Procedures. These were gel filtered on a Sephacryl S-300 column (2.5 × 120 cm). The three major peaks in the elution shown above were pooled separately and rechromatographed on the same column to obtain the final protein preparations. The elution positions of standard proteins (molecular weights given at the top) run on the same column are shown. The inset shows SDS gel electrophoresis of the 64-kDa protein (lane A), the 32-kDa proteins (lane B), and the 22-kDa protein (lane C). The molecular weights of the standards shown, beginning with the upper band, are 66 000, 45 000, 36 000, 29 000, 24 000, 20 100, and 12 400.

Results

Protein Purification. A detailed description of PKC purification from bovine brain has been outlined previously (Bazzi & Nelsestuen, 1987a). A critical step in that purification procedure involved a pH shift on DEAE-cellulose, which selectively released PKC from the resin (Parker et al., 1984). This step separated PKC from other proteins which bound to phospholipids in a calcium-dependent manner. These protins were retained on the DEAE-cellulose and were eluted with 150 mM NaCl. The latter protein fraction was further purified by calcium-dependent binding and EDTA elution from the phospholipid affinity column described under Experimental Procedures.

Separation of the various phospholipid-binding proteins was possible by chromatography on a Sephacryl S-300 column (Figure 1). The three major peaks in Figure 1 were pooled and rechromatographed separately on a Sephacryl S-300 column to give highly purified preparations of each protein. The elution positions corresponded to proteins with molecular weights of 64K, 32K, and 22K. The proteins were subsequently identified by these apparent molecular weights. Polyacrylamide gel electrophoresis in the presence of SDS under reducing conditions (Figure 1, inset) showed a single major band with the anticipated molecular weight for the 64- and 22-kDa proteins. The protein peak eluting at 32 kDa gave

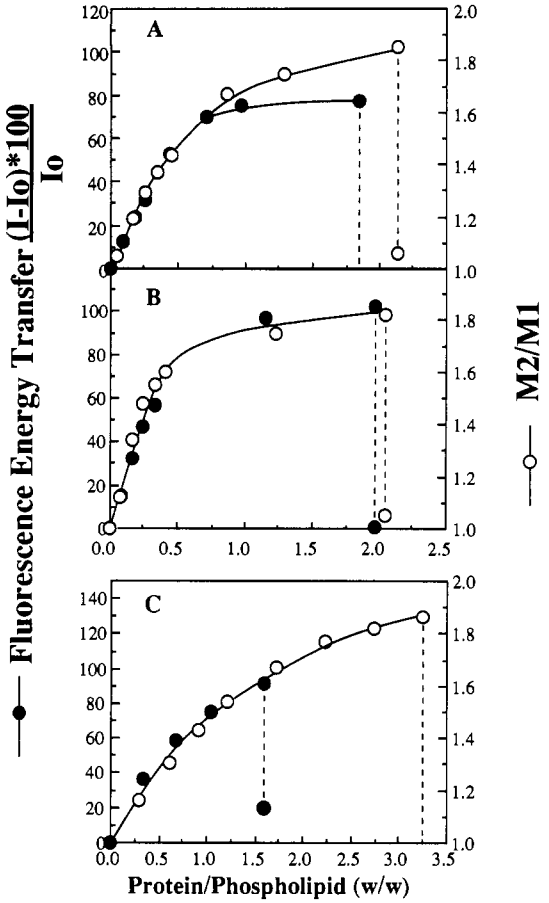

FIGURE 2: Protein–phospholipid binding. Binding of the 64-kDa protein (panel A), the 32-kDa proteins (panel B), and the 22-kDa protein (panel C) was measured by light scattering intensity (○) and fluorescence energy transfer (●) with small unilamellar vesicles prepared by methods described under Experimental Procedures. Light scattering intensities used vesicles containing PS/PC (25:75, 75 μg/1.6 mL, panels A and B) or brain extract type I (11 μg/1.6 mL, panel C). For the fluorescence energy transfer measurements, vesicles were of similar composition except that they contained 10% dansyl-PE. Light scattering intensity and fluorescence energy transfer changes were monitored after successive additions of protein to phospholipid vesicles in 1.6 mL of buffer containing 20 mM Tris (pH 7.5), 100 mM NaCl, and calcium at either 1 mM (panel A), 5 mM (panel B), or 0.31 mM (panel C) calcium. The dashed lines show the effect of adding EGTA in excess over calcium.

two bands on SDS gels of approximately equal intensity at 31 and 33 kDa. These materials were used for subsequent calcium- and membrane-binding studies.

Protein–Phospholipid Binding. Association of proteins with membranes might induce secondary events such as aggregation or fusion of phospholipid vesicles. If such events occurred, the protein–membrane complexes could be nonhomogeneous, and determination of calcium-binding stoichiometry in such a system could be inaccurate. Protein-membrane binding was therefore detected by both fluorescence energy transfer and by light scattering intensity measurements. Fluorescence energy transfer detects the close proximity of protein and phospholipids while light scattering detects mass changes and is especially sensitive to aggregation or fusion events (Bazzi & Nelsestuen, 1987b).

The binding of the 64- (Figure 2A) and 32-kDa proteins (Figure 2B) to phospholipid vesicles containing 25% PS was measured as a function of increasing protein concentration. In the presence of calcium, both the 64- and the 32-kDa proteins bound to phospholipid vesicles and produced large and saturable signals due to fluorescence energy transfer. Light scattering changes due to protein–membrane binding also

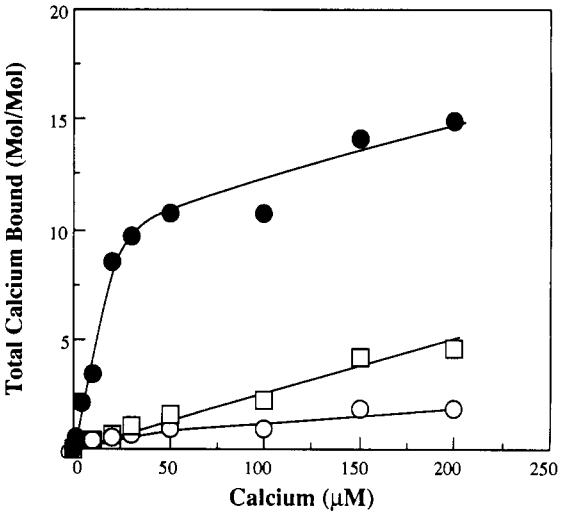

FIGURE 3: Total calcium binding measured by equilibrium dialysis. Calcium binding to each protein was determined in the presence or the absence of phospholipid vesicles. The data shown represent a typical experiment obtained with the 64-kDa protein. The total calcium bound to 0.75 mg/mL protein (○), 0.75 mg/mL protein plus 2.5 mg/mL phospholipid vesicles (●), or 2.5 mg/mL phospholipid vesicles alone (□) is plotted as a function calcium concentration. The vesicles were small unilamellar vesicles of PS/PC (25:75).

occurred in a controlled and systematic manner. This indicated that binding of these proteins to membranes occurred without secondary events such as aggregation or fusion of vesicles. The interaction of both the 64- and the 32-kDa proteins with phospholipid was of very high affinity as indicated by the nearly linear increases in molecular weight as the proteins were added. Reducing protein and phospholipid to the lowest level that was easily detected by this procedure (5×10^{-9} M protein) also failed to detect significant dissociation of the complexes (data not shown). Similar high-affinity interaction was observed for protein kinase C (estimated K_d of ≤5 nM; Bazzi & Nelsestuen, 1987a). As expected, calcium chelation resulted in dissociation of the protein–phospholipid complexes (dotted lines in Figure 2).

The 22-kDa protein exhibited somewhat different phospholipid-binding properties. Neither light scattering intensity nor fluorescence energy transfer measurements were able to detect binding between this protein and phospholipid vesicles of PS/PC (25:75; data not shown). Since the affinity column had been prepared with brain extract type I phospholipids, the interaction of the 22-kDa protein with vesicles of this composition was tested. The results in Figure 2C showed that the 22-kDa protein bound to these vesicles as indicated by both light scattering intensity and fluorescence energy transfer measurements. This binding occurred without causing aggregation or fusion of the vesicles, and calcium chelation resulted in dissociation of the protein–phospholipid complex (dashed lines in Figure 2C).

These results showed that, while all three proteins bound to phospholipid vesicles in the presence of calcium, the phospholipid-binding properties of the 22-kDa protein differed considerably from those of the 64- and 32-kDa proteins. The 22-kDa protein had a lower affinity for phospholipid than either the 64- or 32-kDa proteins and required vesicles with high acidic phospholipid content. Other results (not shown) indicated that this protein would bind to phospholipid vesicles composed of PS/PC (50:50).

Phospholipid-Dependent Calcium Binding. Calcium binding to the 64-kDa protein, to phospholipid, and to a mixture of these components is shown in Figure 3. As expected, the phospholipid alone bound low levels of calcium

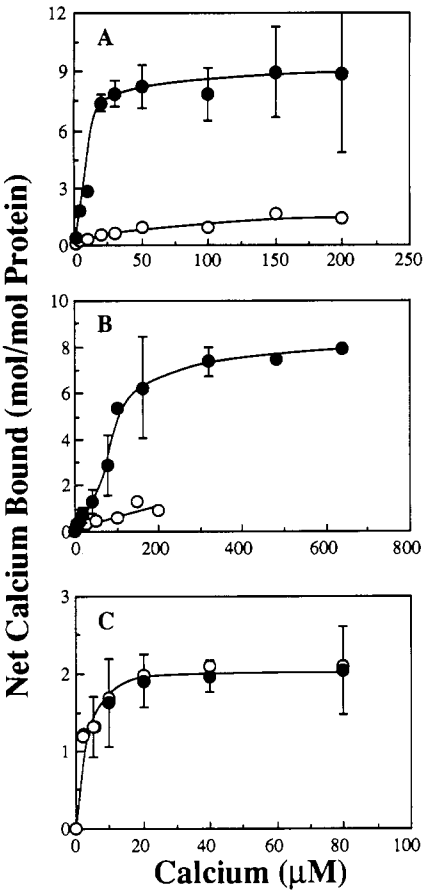

FIGURE 4: Net calcium binding measured by equilibrium dialysis. This binding of calcium to the 64-kDa protein (panel A), the 32-kDa proteins (panel B), and the 22-kDa protein (panel C) was measured in the presence (solid symbols) or the absence (open symbols) of phospholipid vesicles. The dialysis buffer contained 20 mM Tris, pH 7.5, 100 mM NaCl, 10% glycerol, 0.5 mM dithiothreitol, and the indicated concentration of calcium. Panel A shows the average and the standard deviation of three measurements of 11.7 μM 64-kDa protein with (●) or without (○) 2.5 mg/mL phospholipid vesicles. Panel B shows the average of two measurements of 18.7 μM 32-kDa proteins with (●) or without (○) 2.5 mg/mL phospholipid vesicles. In both panels, the phospholipid vesicles were composed of PS/PC (25:75) and were prepared by extrusion. Panel C shows the average and the standard deviation of three measurements of 19 μM 22-kDa protein in the absence (●) or the presence (○) of small unilamellar phospholipid vesicles composed of brain extract type I (1.14 mg/mL).

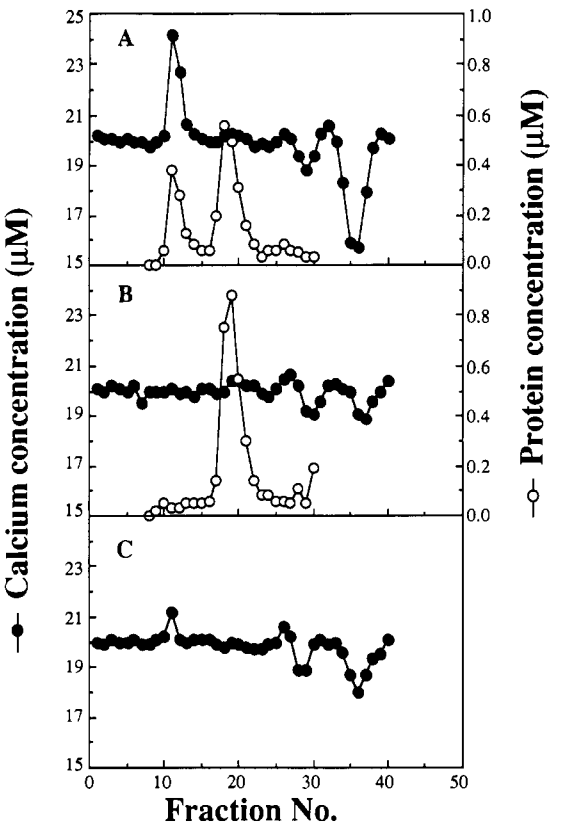

FIGURE 5: Determination of calcium binding to the 64-kDa protein by the Hummel–Dreyer technique. Samples containing either 150 μg of protein plus 250 μg of phospholipids (panel A), 150 μg of protein only (panel B), or 250 μg of phospholipid vesicles only (panel C) were incubated in 0.6 mL of equilibration buffer for 20 min. The samples were applied on a Sephacryl S-300 column (1.0 × 30 cm) equilibrated and eluted (0.75 mL/fraction) with a buffer containing 20 mM Tris, pH 7.5, 100 mM NaCl, 10% glycerol, 0.5 mM dithiothreitol, and 20 μM ^{45}Ca. Protein content (○) and radioactivity (●) were measured in each fraction. The small unilamellar phospholipid vesicles were composed of PS/PC (25:75).

(Nelsestuen & Lim, 1977; Portis et al., 1979; Bazzi & Nelsestuen, 1990). This was considered to be nonspecific calcium binding and was subtracted from the calcium bound to the protein–phospholipid complex to obtain specific calcium bound to the protein–phospholipid complex. This method of background subtraction actually overestimated the nonspecific binding. Protein–PS interaction would render some of the PS unavailable for nonspecific calcium binding. An earlier estimate for protein kinase C suggested that this could produce an error of about one calcium ion per protein molecule (Bazzi & Nelsestuen, 1990). Consequently, the amounts of specific calcium bound to the protein–membrane complex reported below constitute minimum values. Unfortunately, more accurate corrections for background would appear difficult to estimate.

Net calcium bound to the 64- (Figure 4A), 32- (Figure 4B), and 22-kDa proteins (Figure 4C) in the presence or the absence of vesicles containing acidic phospholipids was determined. In the absence of phospholipid vesicles, the 64- and 32-kDa proteins bound little or no calcium at intracellular calcium concentrations (Figure 4A,B) and showed very little interaction with calcium at much higher concentrations (200 μM). However, the corresponding protein–phospholipid complexes bound eight to nine calcium ions. A major differences between these proteins was the midpoint of the titrations which occurred at about 15 μM calcium for the 64-kDa protein (Figure 4A) or about 100 μM for the 32-kDa protein (Figure 4B).

The calcium-binding properties of the 22-kDa protein (Figure 4C) were substantially different from those of the 64- and 32-kDa proteins. In the absence of phospholipid vesicles, the 22-kDa protein bound about two calcium ions/mol of protein with a midpoint of less than 5 μM free calcium. Neither the affinity of this protein for calcium nor the apparent number of calcium ions bound was significantly influenced by the presence of phospholipids. This suggested that the 22-kDa protein represented a much different type of true calcium-binding protein which also bound to phospholipids.

Calcium binding was also examined by the gel filtration chromatography method (Hummel & Dreyer, 1962). Typical results using the 64-kDa protein at 20 μM calcium are shown in Figure 5. These conditions were insufficient to bind all of the protein to the membrane. The elution pattern showed an increase in calcium in those fractions containing the protein–membrane complex and a corresponding trough in calcium levels at the inclusion volume (Figure 5A). In contrast, there was no detectable calcium associated with the free protein (Figure 5A,B). The average stoichiometry of the peak associated with the protein–phospholipid complex was 10 calciums

per protein (fractions 11, 12, and 13 in Figure 5A). The free protein bound less than 0.2 calcium ion per protein (Figure 5B). Under the same experimental conditions, calcium binding to the phospholipid alone was very low (Figure 5C), and this was subtracted as background before calculation of calcium bound to the protein–membrane complex (Figure 5A). Other experiments conducted with 50 μM calcium showed similar properties except that all of the 64-kDa protein eluted as its complex with phospholipid (experiment as in Figure 5A; data not shown). Once again, the free protein (experiment as in Figure 5B; data not shown) showed no detected calcium binding.

An irregularity occurred in the calcium-elution profile near the inclusion volume of the column. This irregularity was observed in all three elution profiles in Figure 5 and was not associated specifically with either the protein or the phospholipid samples. While this difficulty may have been due to the column or materials used, its source was not determined. The major effect of this irregularity was to prevent accurate estimation of bound calcium from the trough of calcium that normally occurs at the inclusion volume of the column. However, this irregularity did not interfere with determination of bound calcium in the phospholipid- or protein-containing fractions. Bound calcium was therefore determined from these peaks.

The calcium-binding properties of the 32-kDa protein were also examined by gel filtration (Figure 6A,B). In this case, phospholipid vesicles composed of PS/PC (50:50) and 50 μM calcium were used. Under these conditions, a substantial portion of the 32-kDa protein was membrane associated, and a smaller portion eluted as free protein (Figure 6A). The calcium concentration increased only in those fractions containing membrane-associated 32-kDa proteins. There was no peak of calcium in the fractions containing free protein (Figure 6A and, especially, Figure 6B). This result agreed with dialysis measurements (Figure 4) and showed that the 32-kDa proteins bound calcium in a phospholipid-dependent manner.

Both techniques, equilibrium dialysis and gel filtration, suggested a large effect of phospholipid on calcium binding to the 64- and 32-kDa proteins. The only difference was the magnitude of calcium binding to the free protein. Equilibrium dialysis showed a low level of calcium binding whereas the gel filtration showed no detectable calcium binding. This was similar to the results obtained with PKC (Bazzi & Nelsestuen, 1990). The results obtained by the gel filtration technique were considered to be more reliable. These determinations did not include radiolabeled PC in the sample, and gel filtration separates the free proteins from both high and low molecular weight contaminants. If present, the latter materials may provide a small amount of surface appropriate for protein and calcium binding. Thus, the results with the gel filtration method were probably superior and indicated $\leq$0.2 calcium ion bound per protein for both the 64- and the 32-kDa proteins at 50 μM calcium.

The results in Figure 6C showed that the 22-kDa protein associated with phospholipid vesicles, and there was an increase in the calcium concentration in the fractions containing membrane-associated protein. However, elevated calcium also occurred in the fractions containing free protein (Figure 6C,D). These results substantiated the equilibrium dialysis experiments and suggested that the 22-kDa protein differed considerably from the 32- and the 64-kDa protein in that the free protein bound calcium and that phospholipid had relatively little effect on this interaction.

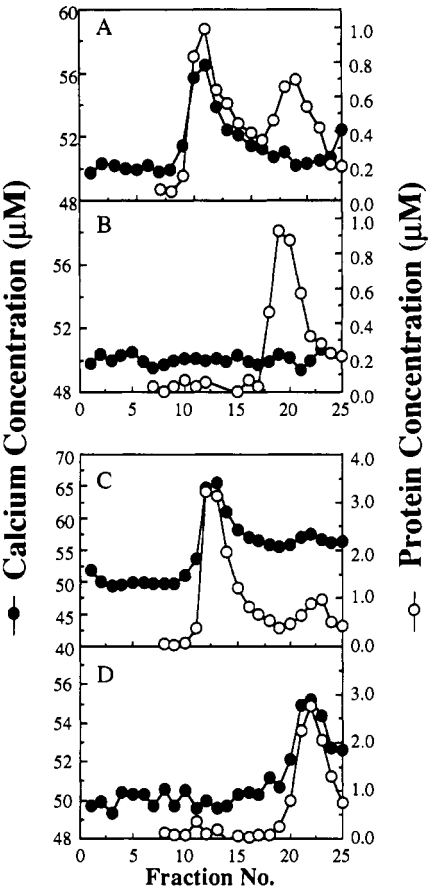

FIGURE 6: Determination of calcium binding to the 32- and 22-kDa proteins by the Hummel–Dreyer technique. Calcium binding to the 32-kDa proteins (panels A and B) and the 22-kDa protein (panels C and D) was determined with samples containing either 146 μg of protein plus 1.2 mg of phospholipids (panels A and C) or 146 μg of protein only (panels B and D). The samples were mixed with 0.75 mL of the equilibration buffer and applied on Sephacryl S-300 columns (1.0 × 30 cm) equilibrated and eluted (0.75 mL/fraction) with a buffer containing 20 mM Tris, pH 7.5, 100 mM NaCl, 10% glycerol, 0.5 mM dithiothreitol, and 50 μM ^{45}Ca. Protein content (○) and radioactivity (●) were measured in each fraction. The small unilamellar phospholipid vesicles were composed of PS/PC (50:50).

Divalent Ion Specificity. Calcium dialysis in the presence or the absence of 5 mM Mg^{2+} was conducted (Figure 7). This experiment was conducted to determine whether Mg^{2+} could partially or totally replace Ca^{2+} at any of the sites generated in the presence of phospholipids. If this were the case, Mg^{2+} should reduce the number of calcium ions bound per protein–membrane complex or the calcium concentration needed for membrane binding. Alternatively, Mg^{2+}, by ionic shielding or binding to the phospholipid, may serve as a nonproductive competitor of Ca^{2+}. In the latter case, Mg^{2+} would increase the amount of calcium needed for protein–membrane binding.

The results in Figure 7A showed that the 64-kDa protein bound the same amount of calcium (about 9 mol of calcium/mol of protein) in the presence or the absence of Mg^{2+}. This suggested that all of the calcium-binding sites generated in the presence of phospholipids were specific for calcium. However, the calcium concentrations needed to reach saturation were higher in the presence of Mg^{2+}. Under the experimental conditions used in Figure 7, the presence of Mg^{2+} shifted the midpoint of the calcium-binding curve from about 50 μM in the absence of Mg^{2+} to about 150 μM in its presence.

The 32- (Figure 7B) and 22-kDa proteins (Figure 7C) also displayed specificity for calcium; the total number of calcium ions bound (about 8 mol/mol of protein for the 32-kDa proteins or 2–3 mol/mol of protein for the 22-kDa protein) was

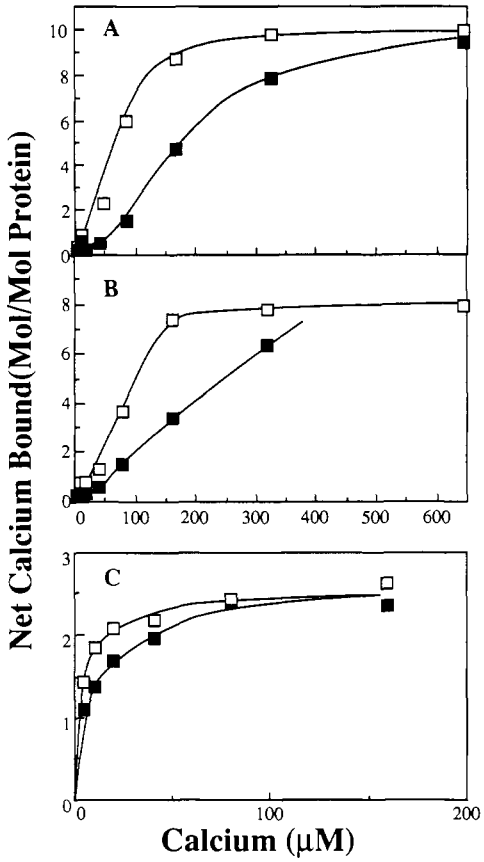

FIGURE 7: Effect of magnesium on calcium binding. Calcium binding to proteins was determined by equilibrium dialysis as described under Experimental Procedures. The dialysis buffer contained 20 mM Tris, pH 7.5, 100 mM NaCl, 10% glycerol, and 0.5 mM DTT with (■) or without (□) 5 mM Mg^{2+}. The samples in panels A and B contained 1.7 mg/mL small unilamellar phospholipid vesicles (PS/PC, 25:75) with either 11.9 μM 64-kDa protein (panel A) or 20 μM 32-kDa proteins (panel B). The experiment in panel C contained 16.6 μM 22-kDa protein.

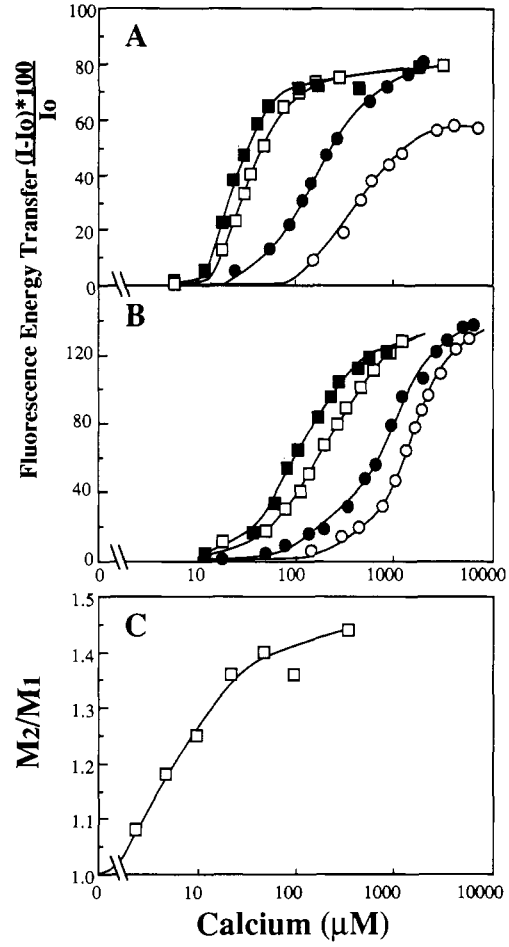

FIGURE 8: Calcium requirements for protein–membrane binding. The binding of the 64-kDa protein (panel A) or the 32-kDa proteins (panel B) was measured by fluorescence energy transfer as a function of increasing calcium concentrations. The small unilamellar phospholipid vesicles contained 10% dansyl-PE and PS at either 10% (○), 25% (●), 50% (□), or 90% (■). The remaining phospholipid was PC. These measurements were performed in 1.6 mL of buffer containing 20 mM Tris (pH 7.5), 100 mM NaCl, 48 μg of phospholipid vesicles, and 42 μg of protein. Panel C shows calcium titration of the 22-kDa protein binding to phospholipid vesicles measured by light scattering intensity. These measurements were performed in 1.6 mL of buffer containing 20 mM Tris (pH 7.5) and 100 mM NaCl with 75 μg of protein and 10 μg of phospholipid vesicles composed of brain extract type 1.

not greatly influenced by the presence of Mg^{2+}. The presence of Mg^{2+} necessitated higher calcium concentrations for calcium binding. Mg^{2+} had a less pronounced effect on calcium titration of the 22-kDa protein.

Calcium Requirements for Protein–Phospholipid Binding. The effect of membrane composition on the calcium requirement for binding the 64- (Figure 8A) and 32-kDa (Figure 8B) proteins to phospholipid vesicles was measured by fluorescence energy transfer. The results showed a strong effect of membrane composition. Membranes of higher PS content required lower concentrations of calcium. At any membrane composition, the calcium concentrations needed for binding of the 64-kDa protein were lower than those needed for binding the 32-kDa proteins. This behavior was similar to that observed with the calcium-binding measurements (Figure 4) and suggested that these two proteins might respond to different calcium levels in the cell.

For the 64- and 32-kDa proteins, the midpoints of the membrane-binding curves with vesicles containing 25% PS (closed circles in Figure 8) corresponded closely to those of the calcium-binding curves (Figures 4 and 7). This suggested that protein–membrane binding and calcium-binding measurements were associated with the same event.

The calcium requirements for association of the 22-kDa protein with phospholipid vesicles was measured by light scattering intensity (Figure 8C). The results showed calcium-dependent association with membranes composed of brain extract type 1 (see Experimental Procedures for composition), with a midpoint of approximately 10 μM. The 22-kDa protein showed very little binding to membranes containing 10% or 25% PS, regardless of the calcium concentrations (data not shown). It was interesting to note that the 22-kDa protein had the highest affinity for calcium (Figures 4C, 7C, and 8C) but required the highest charge density in the membrane for its interaction.

Discussion

Previously, we reported that protein kinase C bound calcium in a phospholipid-dependent manner (Bazzi & Nelsestuen, 1990). Free protein kinase C bound virtually no calcium at physiological levels of calcium but bound about eight calcium ions per protein in the presence of phospholipids. This paper describes two additional proteins that display similar properties. Like PKC, the isolated 64- and 32-kDa proteins bound minimal calcium even at 200 μM calcium. However, each protein bound about eight to nine calcium ions in the presence of phospholipid vesicles. This binding appeared to be specific for calcium so that magnesium appeared unable to replace calcium at any of the sites. Since the free proteins did not bind calcium to a detectable extent, it is probable that the

calcium-binding sites were generated at the protein–membrane interface. This mode of interaction, described as a calcium-bridge model, has been proposed for protein kinase C (Bazzi & Nelsestuen, 1990) and for several vitamin K dependent proteins [Schwalbe et al. (1989) and references cited therein].

Calcium-binding sites generated at the interface between two components (e.g., protein and phospholipid) should exhibit certain properties that are different from those of sites contained in a monomeric component (e.g., a protein). For example, in the former case, simultaneous interaction of all three components should generate synergistic effects; the free calcium concentrations needed to form protein–calcium–phospholipid complexes would be dependent on the concentration of each component, the ratio of the components, and the composition of a variable component such as a membrane. Calcium binding to a single entity such as a free protein molecule would be independent of these parameters. In fact, the calcium requirement for the association of the 64- or the 32-kDa proteins with phospholipids was strongly modulated by the phospholipid composition of the vesicles (Figure 8). Initial findings of a more comprehensive study suggest that certain phospholipid compositions can reduce calcium requirements for these protein–membrane interactions to intracellular calcium levels (≤ 10 μM).[2]

Calcium-binding sites generated between protein and phospholipids may serve as points of contact to maintain the protein–phospholipid complex. In fact, there may even be a relationship between the number of calcium-binding sites and the apparent affinity of various proteins for phospholipids. For example, the very high affinity interaction between phospholipids and either the 64- or the 32-kDa proteins could be the result of eight to nine attachment sites (Ca^{2+} ions) generated between protein and phospholipid. PKC also bound about eight calciums per complex and had very high affinity for phospholipids (estimated $K_d \leq 5$ nM; Bazzi & Nelsestuen, 1987a). Phospholipid-dependent calcium binding has also been observed for the vitamin K dependent proteins [reviewed in Nelsestuen (1984)]. The vitamin K dependent proteins are true calcium-binding proteins since they bind calcium in the absence of phospholipids. However, prothrombin displays up to four more calcium-binding sites when associated with membranes (Nelsestuen & Lim, 1977; Sommerville et al., 1986). The prothrombin–membrane complex has a dissociation constant of about 10^{-7} M (Nelsestuen & Lim, 1977) which would be consistent with a smaller number of calcium ions at the protein–phospholipid interface.

While the identity of the proteins isolated in this study is still unknown, their unusual calcium- and membrane-binding properties may suggest unique ways in which proteins could respond to changes in intracellular calcium. Both the 64- and the 32-kDa proteins were very abundant in brain and were isolated in amounts nearly 100-fold greater than that of PKC. This abundance, together with the large number of calcium ions bound per protein molecule, might even function as a calcium buffer in the cell. The 64- and 32-kDa proteins had different calcium requirements for binding to identical membranes. Such a property could allow different calcium concentrations to generate qualitatively different responses by involving different proteins. In addition, the synergism between phospholipid and calcium for protein–membrane binding may allow phospholipid metabolism to influence calcium responsiveness of a cell. Testing of these possibilities and others will require extensive further investigations.

[2] Bazzi and Nelsestuen, unpublished results.

At present, these proteins might all be grouped as members of a large family of proteins that are described as Ca^{2+}-dependent phospholipid-binding proteins (Klee, 1988). Several proteins with different molecular weights have been demonstrated in various tissues, and different laboratories have described these proteins with somewhat different nomenclatures (Creutz et al., 1987; Khanna et al., 1990). The various studies suggest substantial differences in the affinity for calcium and phospholipids. Among these proteins, lipocortin II (Glenney, 1986) and lipocortin I (Glenney et al., 1987; Schaelpfer & Haigler, 1987) have been shown to bind calcium in a phospholipid-dependent manner. While it seems likely that these lipocortins interact with phospholipids and calcium in a manner that is generally similar to that of the 64- and 32-kDa proteins, the lipocortins were distinct in that they bound only about two to four calcium ions per protein.

Phospholipid-dependent calcium binding was clearly not a general property of proteins that bound to phospholipid in a calcium-dependent manner. The 22-kDa protein that was isolated in this study bound similar amounts of calcium in the presence or absence of phospholipids. It seemed probable that calcium binding to the 22-kDa protein induced conformational changes that allowed interaction with phospholipids. Other members of this group of proteins might include p68, a protein isolated from lymphocytes (Owens & Crumpton, 1984), and protein II, a 32-kDa protein isolated from porcine liver (Shadle et al., 1985). Both of these proteins have been reported to contain a single calcium-binding site. However, the effect of phospholipid on the calcium-binding properties of these proteins was not reported.

Overall, this study served to expand and thereby establish the existence of a general type of phospholipid-dependent calcium-interacting proteins. This class of intracellular proteins appeared to be quite abundant and undoubtedly participates in cell calcium regulation. While it seems probable that the calcium-binding sites were generated by simultaneous interaction of protein–phospholipid–calcium, the mechanism of generating high-affinity calcium-binding sites at the interface of two low-affinity components remains to be determined.

Registry No. Ca, 7440-70-2; Mg, 7439-95-4.

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Serpin–Serine Protease Binding Kinetics: α_2-Antiplasmin as a Model Inhibitor

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ABSTRACT: We have examined in detail the kinetics of binding of the serpin α_2-antiplasmin to the serine proteases α-chymotrypsin and plasmin. These represent model systems for serpin binding. We find, in contrast to earlier published results with α_2-antiplasmin and plasmin, that binding is reversible, and slow binding kinetics can be observed, under appropriate conditions. Binding follows a two-step process with both enzymes, with the formation of an initial loose complex which then proceeds to a tightly bound complex. In the absence of lysine and analogues, equilibrium between α_2-antiplasmin and plasmin is achieved rapidly, with an overall inhibition constant (K_i') of 0.3 pM. In the presence of tranexamic acid or 6-aminohexanoic acid, lysine analogues that mimic the effects of fibrin, plasmin binding kinetics are changed such that equilibrium is reached slowly following a lag phase after mixing of enzyme and inhibitor. The K_i' is also affected, rising to 2 pM in the presence of 6-aminohexanoic acid concentrations above 15 mM. Thus extrapolation to the in vivo situation indicates that complex formation in the presence of fibrin will be delayed, allowing a burst of enzyme activity following plasmin generation, but a tight, pseudoirreversible complex will result eventually. Chymotrypsin is more weakly inhibited by α_2-antiplasmin, exhibiting an overall K_i' of 0.1 nM, after two-stage complex formation. The inhibition constant for the initial loose complex (K_i) is very similar for both enzymes. The difference in binding strength between the two enzymes is accounted for by the dissociation rate constant of the second step of complex formation. To our knowledge, this rate constant has previously not been measured for serpin interactions. The reversibility of binding argues against a complex having a covalent bond between enzyme and inhibitor. This mechanism of binding has important consequences for the measurement of serpin activity. Structure/function relationships are discussed to explain inhibitory action.

Control of serine protease activity is central to the biochemistry of blood clotting and of fibrinolysis. Unwanted proteolytic activity is prevented by the production of zymogens and, following activation, by inhibition with protein inhibitors. Serpins are a family of serine protease inhibitors present at key points in clotting and fibrinolytic pathways. Although serpins have been the subject of much research in recent years [e.g., see Huber and Carrell (1989)], there is still no generally accepted mechanism explaining how these proteins are able to act as protease inhibitors. To begin to address this question, it is necessary to carry out functional studies including kinetic analysis of binding. More complete understanding will come with detailed X-ray crystal structures of serpin complexes, but this is unavailable at present. Serpins are known to form stable 1:1 complexes with serine proteases, but the factors responsible for ensuring that the bound protein is not hydrolyzed are poorly understood. We have used the serpin α_2-antiplasmin to investigate the kinetics of inhibitor binding with two enzymes,

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