Ca^{2+}-Dependent Binding of Endonexin (Annexin IV) to Membranes: Analysis of the Effects of Membrane Lipid Composition and Development of a Predictive Model for the Binding Interaction†

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Received April 8, 1994®

ABSTRACT: Endonexin (annexin IV) is a member of the annexin family of homologous proteins that bind membranes and aggregate vesicles in a calcium-dependent fashion. This study examines the lipid modulation and mechanism of the binding of endonexin to membranes using a fluorescence energy transfer assay to measure bovine endonexin binding to well-defined large unilamellar vesicles. The calcium sensitivity for endonexin–membrane binding is observed to be highly dependent on the types of membrane lipids present. As with most annexins, negatively charged lipids best promote endonexin binding to phosphatidylcholine (PC) containing membranes. However, a comparison of 11 different types of lipids reveals that other factors such as the type of ion contributing the charge and head-group size are also important. The concentrations of calcium required for half-maximal binding of endonexin to PC vesicles containing 30% phosphatidylserine (PS) or 30% phosphatidylinositol (PI), both lipids with net charge −1, are 48 ± 6 and 114 ± 19 μM, respectively, while half-maximal binding to 30% phosphatidylinositol bisphosphate (PIP_2), with a greater net charge of −3 to −5, occurs at 65 μM calcium, similar to the calcium requirement for binding to PS. The apparent affinities of endonexin for seven different types of lipids parallel those reported for annexin V [Andree, H. A. M., Reutelingsperger, C. P. M., Hauptmann, R., Hemker, H. C., Hermans, W. T., & Willems, G. M. (1990) *J. Biol. Chem. 265*, 4923–4928], except for a greater preference of endonexin for membranes containing phosphatidic acid. Mixing PS and phosphatidylethanolamine (PE) or PS and PI in the same PC vesicle synergistically enhances endonexin–membrane binding, indicating that even lipids with no net charge such as PE may dramatically affect endonexin binding to mixed-lipid membranes. The maximum amount of endonexin able to bind to PS/PC vesicles at 1 mM calcium increases with mole % PS. A simple and general model that treats protein–membrane binding as a two-step process, with adsorption to a membrane surface followed by interaction with specific lipid molecules [Lentz, B. R., & Hermans, J. (1989) *Biochemistry 28*, 7459–7461], is extended to include the coupled binding of calcium with binding of specific lipid molecules. This extended model accurately predicts trends observed when protein and calcium titrations of endonexin binding to PS/PC vesicles are performed under a wide variety of conditions and suggests that 3–5 calcium ions and 9–18 PS molecules participate in each endonexin–membrane complex. The model also accurately predicts that the maximum binding capacity of a lipid membrane for endonexin is calcium dependent, an observation that has also been reported for other annexins.

Endonexin (annexin IV) is a member of the annexin family of homologous proteins that bind membranes and aggregate vesicles in a Ca^{2+}-dependent fashion. Annexins have been implicated in cellular processes involving membranes, such as Ca^{2+}-regulated exocytosis, Ca^{2+}-regulated cytoskeleton–membrane changes, phospholipase A_2 regulation, and blood coagulation (Geisow & Walker, 1986; Klee, 1988; Creutz, 1992). Ca^{2+}-dependent binding of annexins to pure lipid membranes *in vitro* suggests that membrane lipids are the targets of annexins in cellular membranes.

This study addresses the fundamental mechanism underlying annexin–membrane lipid interactions, using endonexin as a "model" annexin. The endonexin sequence is essentially limited to that presumably responsible for the Ca^{2+}-dependent membrane binding behavior of annexins since it has only a short N-terminal domain (Hamman et al., 1988). A carefully controlled membrane system of large unilamellar vesicles (1000-Å mean diameter) enabled us to dissect the effects of lipid composition and charge on the binding of endonexin to membranes, as well as to quantitatively analyze binding data. Vesicles comprised mostly phosphatidylcholine (PC[1]) to maintain unilamellar vesicle structure and to reduce Ca^{2+}-mediated vesicle aggregation, especially with vesicles containing PS and PA. Natural source lipids were used whenever possible so that acyl chain unsaturation and inhomogeneity would promote ideal lipid mixing.

We show that endonexin binding to membranes is modulated not just by negative membrane surface charge but also by the types of charges and other structural features of lipid head groups, such that even lipids with no net charge affect endonexin binding. A synergistic enhancement of endonexin binding to vesicles containing complex mixtures of lipids is

† This study was supported by a research grant (DK33151) and a training grant (GM07055) from the National Institutes of Health.

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® Abstract published in *Advance ACS Abstracts,* July 1, 1994.

[1] Abbreviations: DAG, diacylglycerol; dansyl-PE or dPE, *N*-[(5-(dimethylamino)naphthyl)-1-sulfonyl]dipalmitoyl-L-α-phosphatidylethanolamine; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N,N,N′,N′*-tetraacetic acid; LUV, large unilamellar vesicle; MOPS, 3-morpholinopropanesulfonic acid; PA, phosphatidic acid; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PG, phosphatidylglycerol; PI, phosphatidylinositol; PIP_2, phosphatidylinositol bisphosphate; PS, phosphatidylserine.

0006-2960/94/0433-8930$04.50/0

seen, indicating that membrane association is very sensitive to lipid composition. The complex modulation of endonexin–membrane binding is consistent with the preferential binding of certain types of lipids to endonexin. We present an explicit model for coupled lipid and Ca^{2+} binding that quantitatively predicts overall endonexin behavior. The model reproduces the details of endonexin–membrane binding and suggests a mechanistic explanation for some previously reported observations with other annexin proteins.

MATERIALS AND METHODS

Materials. PC (bovine liver), PS (bovine brain), PE (transphosphatidylation from egg PC), PI (bovine liver), PA (dioleoyl), cardiolipin (bovine heart), PG (dioleoyl), DAG (dioleoyl), and N-methylated PE derivatives (phosphatidyl-*N*-monomethylethanolamine and phosphatidyl-*N*,*N*-dimethylethanolamine, by transphosphatidylation from egg PC) were purchased from Avanti Polar Lipids and used without further purification. PIP_2 (bovine brain) was from Calbiochem, oleic acid was from Sigma, and dansyl-PE was from Molecular Probes.

Endonexin Purification. Endonexin was purified from bovine liver as described by Creutz et al. (1987). In some cases, it was purified further using an FPLC Superose 12 column before dialysis into buffer (described below) for experiments.

Vesicle and Ca^{2+} Buffer Preparation. Pure lipid vesicles were prepared by the method of extrusion (Mayer et al., 1986) using 0.1-μm polycarbonate filters (Nucleopore) and a Lipex Biomembrane extruder. Size distributions of vesicles were obtained by quasielastic light scattering using a Model 170 Nicomp autocorrelator with a helium neon laser. Vesicle preparations made with 1 mM lipid typically yielded mean diameters of approximately 1000 Å, with standard deviations of less than 30%. Size distributions were independent of lipid composition. After sizing, vesicles were passed through small columns (0.25–0.5-mL bed volume) of Chelex resin (Bio-Rad) to remove contaminating Ca^{2+}. Lipid concentrations were quantitated by the method of Bartlett (1959).

Ca^{2+}-EGTA buffers were used to buffer Ca^{2+} at concentrations below 1 mM for experiments performed at pH 7.0. Ca^{2+} buffer stocks contained 125 mM EGTA and were diluted 50-fold upon addition to experimental samples. Ca^{2+} buffers were standardized against a 100 mM Ca^{2+} standard solution (Orion) using an Orion Ca^{2+} electrode.

Endonexin Quantitation. Endonexin was quantitated by UV absorption in guanidine hydrochloride using the method of Edelhoch (1967). All absorbance spectra for endonexin, in the presence and absence of guanidine hydrochloride, were corrected for apparent background light scattering by subtracting scattering blanks, created from a suspension of nondairy creamer. Similar relative concentrations between two endonexin stocks were obtained using either corrected UV absorbance or a Bradford protein dye assay (Bradford, 1976). The extinction coefficient for native endonexin at 278.5 nm was determined to be 25 100 M^{-1} cm^{-1} (26 900 M^{-1} cm^{-1} before the correction for light scattering).

Fluorescence Energy Transfer Assay. Endonexin–membrane association was monitored using a fluorescence energy transfer assay. Dansylated PE molecules, containing a dansyl fluorophore covalently linked to the primary amine of the ethanolamine of PE, were incorporated in small (usually 5 mol %) proportions into lipid vesicles. Endonexin–membrane binding was then detected by the distance-dependent energy transfer between excited endonexin aromatic residues and the dansyl fluorophores localized to the vesicles (Bazzi & Nelsestuen, 1987; Vaz et al., 1977). Fluorescence measurements were performed on a SPEX Fluorolog 2 fluorometer with excitation at 280 nm and emission monitored at 510 nm. A 400-nm cutoff filter was placed between the sample and the emission monochrometer, and band passes of either 9.2 or 18.5 nm were used for the excitation beam and 18.5 nm was used for the emission beam. Experiments were performed at ambient temperature.

Samples were prepared in chelexed buffers of 25 mM MOPS and 100 mM NaCl at pH 7.0 (experiments performed at pH 9 used 25 mM Tris instead of MOPS). Sample volumes were 300 μL and contained 4 μM total lipid. Data were collected for buffer alone, then for vesicles and Ca^{2+}, and then after the addition of endonexin. Intensity measurements were averaged for 31 s, and fresh samples were prepared for individual data points in titrations. Increases in fluorescence were normalized to the fluorescence of vesicles alone or of vesicles with Ca^{2+} to give percent increases in dansyl fluorescence. Upon the addition of endonexin, the maximum fluorescence change was found to occur as quickly as could be measured (ca. 5–10 s). Dansyl-PE intensities exhibited slow decays (typically a 1–2% decrease in intensity over 31-s intervals) that may reflect a slow photobleaching.

Model for Endonexin–Membrane Binding. The data were fit with a model for protein–membrane association developed by Lentz and Hermans (1989) that was extended to include Ca^{2+} binding. In the Lentz and Hermans model, protein–membrane association proceeds as a two-step reaction with initial membrane surface adsorption (affinity k_M), followed by the binding of lipid molecules of type A with affinity k_A in two-dimensional surface reactions. The partition function for this model is $Q = 1 + k_M[M](1 + k_A X_A)^L$, where [M] is the concentration of membrane binding sites, and X_A is the mole fraction of free lipid A in the membrane that binds to L independent and identical sites on the protein (Lentz & Hermans, 1989).

The model was extended to include the coupled binding of Ca^{2+} with lipid molecules in the two-dimensional surface reactions using the allosteric model of Monod, Wyman, and Changeux (Monod et al., 1965; Cantor & Schimmel, 1980). For a protein with m lipid binding sites and one Ca^{2+} binding site, the partition function becomes $Q = 1 + k_M[M]\{(1 + k_L X_A)^m + k_C[Ca^{2+}](1 + k_{CL} X_A)^m\}$, where k_C is the microscopic binding constant for Ca^{2+} in the absence of lipid A, and the binding of Ca^{2+} to the protein converts the m lipid binding sites from microscopic affinity k_L to microscopic affinity k_{CL}. Generalization to a protein with n Ca^{2+} binding sites, each coupled with m lipid binding sites, produces the partition function

$$Q = 1 + k_M[M]\{(1 + k_L X_A)^m + k_C[Ca^{2+}](1 + k_{CL} X_A)^m\}^n$$

The fraction of total protein bound to membranes is then $(Q-1)/Q$. For fits of Ca^{2+} titrations, calculated curves were normalized by dividing the fraction of total protein bound to membranes by the fraction in the limit that $[Ca^{2+}]$ goes to infinity: $k_M[M](1 + k_{CL} X_A)^{nm}/\{1 + k_M[M](1 + k_{CL} X_A)^{nm}\}$. For fits of protein titrations, the calculated concentration of membrane-bound protein, $[protein]_{total}[(Q-1)/Q]$, was scaled to the experimentally observed change in dansyl fluorescence. Since endonexin bound membranes with an extremely high affinity in protein titrations (Results), the change in dansyl fluorescence per mole of endonexin bound to membrane was

taken as the slope, determined by linear regression, of increased dansyl fluorescence with added total endonexin below vesicle saturation.

Data fitting was performed using a nonlinear least-squares algorithm developed by Johnson and colleagues (Johnson, 1983; Johnson & Frasier, 1985; Johnson & Faunt, 1992). Fitting proceeded until the fractional change in variance between the calculated curve and the data decreased to 1 part in 10^4. The free lipid A concentration was evaluated as the root of a conservation of mass equation using an interval division algorithm.

RESULTS

Dansyl-PE Fluorescence as a Measure of Endonexin–Membrane Binding. Endonexin–membrane association was monitored in this study primarily by Ca^{2+}-dependent endonexin enhancement of dansyl-PE fluorescence in vesicles containing 5 mol % dansyl-PE. This assay relies on the distance-dependent energy transfer from protein aromatic residues to dansyl-PE that occurs when protein molecules are localized to vesicle surfaces (Vaz et al., 1977; Bazzi & Nelsestuen, 1987). The accuracy of this assay in reporting endonexin–membrane association was tested by examining changes in dansyl-PE excitation and emission spectra, detecting quenching of endonexin intrinsic fluorescence, and comparing dansyl-PE fluorescence data with light scattering data.

Endonexin causes Ca^{2+}-dependent increases in dansyl-PE fluorescence (Figure 1A) along with a blue shift in the dansyl-PE emission maximum from 519 to 508 nm (not shown). Increases are reversible upon the addition of EGTA, and Ca^{2+} alone does not significantly affect dansyl fluorescence. Background dansyl-PE fluorescence triggered by the 280-nm excitation beam (Figure 1A) is not affected by endonexin, as tested by adding endonexin at Ca^{2+} concentrations too low to support membrane binding.

The Ca^{2+}-stimulated binding of endonexin to membranes increased dansyl-PE fluorescence both by increasing the dansyl intrinsic quantum yield and by energy transfer. Increases over a broad range of excitation wavelengths (Figure 1A) indicate increased dansyl intrinsic fluorescence (increased intrinsic quantum yield), while the greatest percent increase at 283 nm (Figure 1B) suggests energy transfer from aromatic amino acid residues. The increased intrinsic dansyl fluorescence and the blue shift in dansyl emisssion may result from an alteration of the membrane surface environment upon endonexin binding, especially since the total endonexin concentration was sufficient to nearly saturate vesicle surfaces. Since enhanced intrinsic dansyl fluorescence and energy transfer both titrated together, the total dansyl fluorescence enhancement was used to monitor protein–membrane association.

Fluorescence energy transfer from endonexin to dansyl-PE was also evident from the quenching of the intrinsic fluorescence of endonexin molecules bound to membranes containing dansyl-PE. Endonexin intrinsic fluorescence increased upon binding to 30%/70% PS/PC vesicles containing no dansyl-PE, similar to an observation reported for annexins I and V (Meers, 1990; Meers & Mealy, 1993). However, the intrinsic fluorescence actually decreased upon binding to similar vesicles containing 5% dansyl-PE (endonexin bound equally well to both types of vesicles; will be discussed later). Endonexin emissions at 315 and 330 nm increased by 15% and 44%, respectively, when endonexin bound to vesicles containing no dansyl-PE, but decreased by 16% and 9% at these same wavelengths when endonexin bound to vesicles containing 5% dansyl-PE.

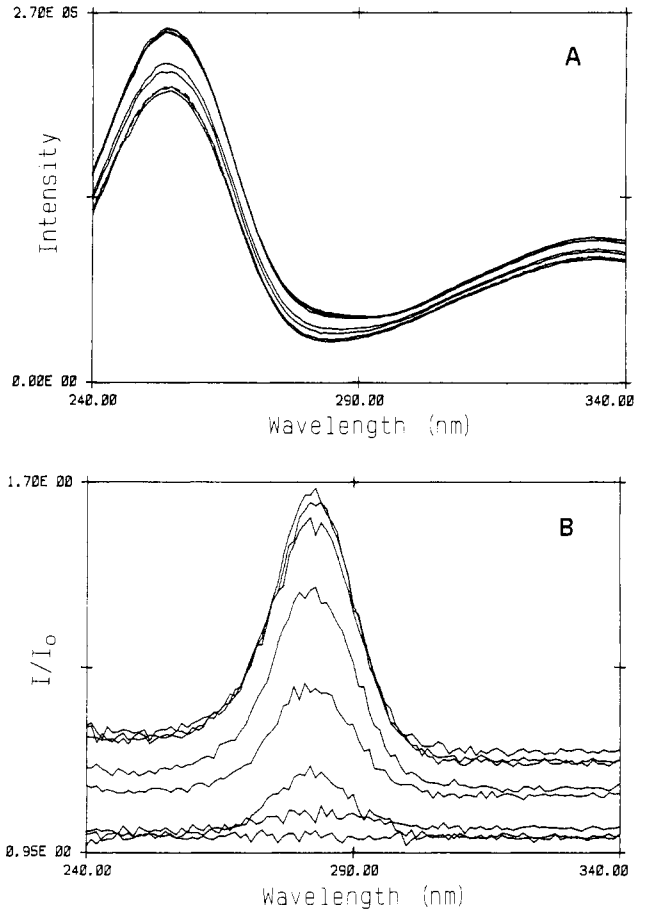

FIGURE 1: (A) Dansyl-PE excitation spectra after the addition of endonexin (33 nM) to 30% PIP_2/PC/5% dansyl-PE vesicles at different Ca^{2+} concentrations. Curves increase from 18 μM to 1 mM Ca^{2+}, with the average spectrum for vesicles and Ca^{2+} before the addition of endonexin represented by a dashed curve (emission monitored at 510 nm). (B) Ratio excitation spectra (+endonexin/−endonexin) for data in A.

Ninety-degree light scattering provided additional evidence that the endonexin-mediated increase in dansyl fluorescence reflected protein–membrane association. Rapid increases in 355-nm light scattered at 90° (up to 120% for 43 nM protein and 4 μM lipid) were observed under the same conditions where endonexin enhanced dansyl-PE fluorescence. A Ca^{2+} titration of increased 90° light scattering with 30% PS/65% PC/5% dansyl-PE vesicles exactly coincided with that of dansyl fluorescence enhancement. Increases in light scattering were rapidly reversible upon the addition of EGTA. The increases in 90° light scattering were not simply due to vesicle aggregation: quasielastic light scattering measurements detected no increase in vesicle size distribution under conditions of increased 90° light scattering.

Endonexin Binding Capacities of PC-Containing Vesicles Increase with Mole % Negatively Charged Phospholipid but Do Not Depend Solely on Membrane Surface Charge. Binding of annexins to PC-containing membranes below 1–2 mM Ca^{2+} generally requires the presence of negatively charged phospholipids. A correlation between the amount of negatively charged lipid present and the amount of endonexin able to bind to PC-containing membranes was tested by performing protein titrations of endonexin binding to PC/5% dansyl-PE vesicles containing different values of mole % PS. In the presence of 1 mM Ca^{2+}, an increase in the mole % PS from 25% to 95% resulted in an increased maximum binding of endonexin (Figure 2). The relatively small increase in binding

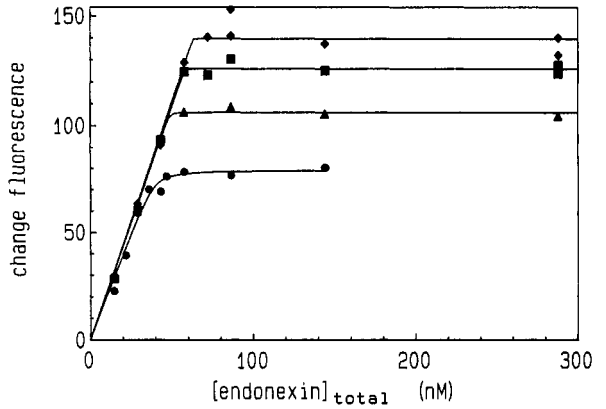

FIGURE 2: Protein titration data for endonexin binding to PC/5% dansyl-PE vesicles containing 25% (●), 39% (▲), 54% (■), or 95% (◆) PS at 1 mM Ca^{2+} and best fits of Langmuir binding isotherms to individual protein titrations. The fitted values of K and total membrane site concentrations are (bottom to top) 2×10^9 M^{-1}, 39 nM; 1×10^{11} M^{-1}, 49 nM; 8×10^{11} M^{-1}, 58 nM; and 5×10^{12} M^{-1}, 63 nM.

between 54% and 95% PS might result from steric hindrance on the limited vesicle surface area.

Saturation of the 95% PS/5% dansyl-PE vesicles probably reflects the maximum amount of endonexin that can be accommodated on the 1000-Å extruded vesicles. A Langmuir binding isotherm ($[protein]_{bound} = K[protein]_{free}[sites]_{free}$) fit the 95% PS titration with a membrane binding site concentration ($[sites]_{total}$) of 63 nM and an apparent minimum binding constant K of 2.5×10^9 M^{-1} (lower limit for best fit: 5×10^{12} M^{-1}). If we ssume that half of the 4 μM lipid present is on the outer monolayer of vesicles and available for endonexin binding, the derived 63 nM concentration of membrane binding sites corresponds to 32 outer monolayer lipids for each membrane-bound endonexin molecule. Annexin V has been reported to bind 20% PS/PC vesicles with an affinity of 10^{10} M^{-1} or greater while covering 42 outer monolayer lipid molecules (Andree et al., 1990) and to bind 95% PS/5% pyrenyl-PC vesicles with an affinity of 1.1×10^8 M^{-1} while covering 59 outer monolayer lipid molecules (Meers et al., 1991). One significant difference between endonexin and annexin V is that while less endonexin can bind to 25% PS membranes than to 100% PS membranes, the same amount of annexin V binds to both 20% PS membranes and 100% PS membranes (Andree et al., 1990). This may reflect a greater affinity of annexin V than endonexin for PC membranes when PS is present or cooperative binding of annexin V molecules and not endonexin molecules through protein–protein interactions (Andree et al., 1990).

PC/5% dansyl-PE vesicles containing 30% of most types of negatively charged phospholipids exhibited similar protein titrations at 1–2 mM Ca^{2+} (e.g., Figure 3A). Even vesicles containing 30% PI or 30% PIP_2, lipids with very different charge (PI −1, PIP_2 −3 to −5 at pH 7; van Paridon et al., 1986), exhibited similar protein titrations (data not shown), suggesting that total membrane surface charge alone does not determine the maximum amount of endonexin able to bind to vesicles. Vesicles containing 30% cardiolipin were an exception in that they exhibited an almost 2-fold greater endonexin binding capacity (Figure 3B). While protein titrations to 30% PS, PA, PI, and PG (65% PC/5% dPE) vesicles saturated at 35–40 nM (Figure 3), a protein titration to 30% cardiolipin/PC/dPE vesicles saturated at approximately 95 nM endonexin (Figure 3B). Since cardiolipin is equivalent to two molecules of PA linked through their respective phosphates by a glycerol molecule, the binding

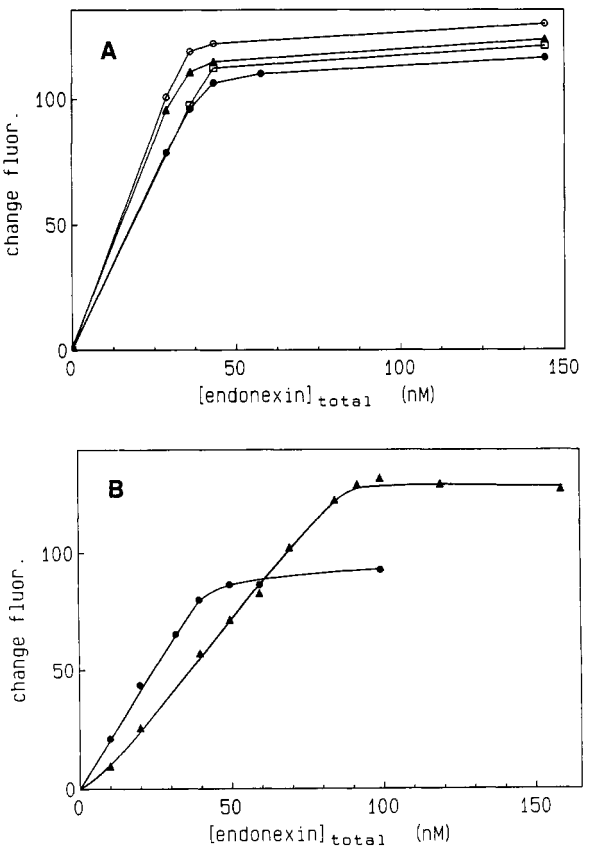

FIGURE 3: (A) Protein titrations for endonexin binding to PC/5% dPE vesicles containing 30% PS (●), 30% PG (□), 30% PA (▲), or 30% PI (○) at 2 mM Ca^{2+}. (B) Protein titrations of endonexin binding to PC/5% dPE vesicles containing 30% PI (●) and 30% cardiolipin (▲) at 1 mM Ca^{2+}.

capacity of PC vesicles containing negatively charged phospholipids would appear to depend on the number of *sn*-3-phosphates or the fractional surface area of vesicles composed by the negatively charged lipid. A minimum number of negatively charged lipids simply may be required to augment endonexin interactions with PC molecules in stabilizing an endonexin–membrane complex (see Discussion).

Ca^{2+} Titrations of Endonexin–Membrane Binding Depend on the Types of Lipids Present. We wished to compare the relative abilities of different lipids to enhance endonexin binding to PC-containing membranes. Protein titrations of endonexin binding to most types of vesicles at high Ca^{2+} concentrations (1–2 mM) were so similar that relative affinities for binding to different vesicles could not be distinguished. However, Ca^{2+} sensitivities for endonexin binding to vesicles can be used as a relative measure of endonexin–membrane stability since membrane binding is Ca^{2+}-dependent. The less stable an endonexin–Ca^{2+}–membrane complex, the more Ca^{2+} required to form it. As seen in Figure 4, Ca^{2+} titrations of endonexin–membrane binding could distinguish endonexin affinity for binding different types of vesicles. These comparisons are all given in terms of the bulk Ca^{2+} concentrations of the medium and not boundary layer Ca^{2+} concentrations at vesicle surfaces (see Discussion).

Table 1 summarizes the results of Ca^{2+} titrations of endonexin binding to several different types of vesicles. Maximum absolute fluorescence increases in these titrations for different vesicles prepared and measured at the same time differed by less than 20%, except for vesicles containing DAG, which exhibited a 60% greater maximum intensity at the same endonexin concentration. Although, as seen in the table,

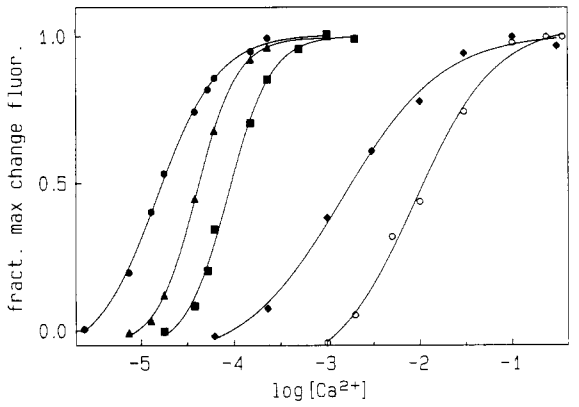

FIGURE 4: Ca^{2+} titrations of endonexin binding to PC/5% dPE vesicles containing 30% cardiolipin (●), 30% PS (▲), 30% PI (■), or 30% PE (◆) and to 95% PC/5% dPE vesicles (○).

Table 1: Comparisons of Endonexin Binding to PC/5% Dansyl-PE Vesicles Containing Different Types of Lipids

charge	vesicle	$[Ca^{2+}]$ for half-maximal binding of endonexin to vesicles[a]
0	30% PE	1.95 ± 0.07 nM (2)
	25% DAG	9.1 mM (1)
	95% PC	10.5 ± 2.0 mM (5)
	30% PE(Me)	>10 mM[b]
	30% $PE(Me)_2$	>10 mM[b]
	100% PC	≫10 mM[b]
−1	30% PA	48.3 ± 6.2 μM (2)
	30% PG	54.8 μM (1)
	30% PS	48.2 ± 7.9 μM (7)
	30% PI	114.0 ± 19.1 μM (7)
	30% oleic acid	1.1 mM (1)
−2	30% cardiolipin	17.7 μM (1)
−3 to −5	30% PIP_2	64.9 μM (1)

[a] Half-maximal $[Ca^{2+}]$ (±standard deviation) was determined from fits of the Hill equation to data using the GraphPAD graphing package. Parentheses give the number of determinations. [b] Relative Ca^{2+} sensitivities were determined by inhibition of endonexin binding to 95% PC/5% dansyl-PE vesicles by using respective vesicles containing no dansyl-PE (detailed in the text).

negatively charged lipids generally promote greater endonexin–membrane stability, a simple correlation was not observed among lipids of the same charge. For example, endonexin required more Ca^{2+} to bind to 30% PI/65% PC/5% dPE (half-maximum, 114 μM Ca^{2+}) vesicles than to 30% PA, PS, or PG/PC/dPE vesicles (half-maximum, 50 μM Ca^{2+}). The increased negative charge of PIP_2 (−3 to −5 compared with −1) enhanced endonexin binding relative to PI (half-maximum, 65 μM Ca^{2+}) but not relative to the other lipids with net charge −1 (PA, PS, and PG). Also, an increase in the number of methyl groups on N-methylated derivatives of PE resulted in diminished endonexin–membrane stability (all of the derivatives of PE should be zwitterionic at pH 7).

The binding of endonexin to some of the vesicles listed in Table 1 (e.g., 25% DAG/PC/dPE, 30% PE/PC/dPE) occurred at Ca^{2+} concentrations approaching those that promoted endonexin binding to 95% PC/5% dansyl-PC vesicles (Figure 4). To verify that binding of endonexin to these vesicles was independent of the presence of dansyl-PE, identical vesicles containing no dansyl-PE were used to compete with endonexin binding to 95% PC/5% dansyl-PE vesicles. Enhanced dansyl fluorescence observed with endonexin binding to 95% PC/5% dansyl-PE vesicles at 100 mM Ca^{2+} was reduced by 80% with 10-fold excess 30% DAG/70% PC (no dansyl-PE) vesicles, but only by 26% with 10-fold excess pure (100%) PC vesicles (vesicles premixed before the addition of endonexin and Ca^{2+}). This indicates that DAG enhances the binding of endonexin relative to pure PC vesicles. This also indicates that 5% dansyl-PE stabilizes endonexin binding to PC membranes (the 26% inhibition by pure PC vesicles was attained following a slow decay from an initial inhibition of 59%; presumably, endonexin was slowly exchanging from the 10-fold excess vesicles to the PC/dansyl-PE vesicles). The Ca^{2+} threshold for binding pure PC vesicles appears to be greater than 10 mM since no inhibition of endonexin binding to PC/5% dansyl-PE vesicles was observed at this Ca^{2+} concentration. Ten-fold greater concentrations of vesicles containing 30% PE derivatives [PE, PE(Me), or $PE(ME)_2$] reduced the enhanced dansyl fluorescence of endonexin binding to PC/5% dansyl-PE vesicles by 85%, <15%, and <15%, respectively, at 10 mM Ca^{2+} and by 85%, 80%, and 75%, respectively, at 100 mM Ca^{2+}.

While 5 mol % dansyl-PE alone stabilized endonexin binding to PC membranes (relative to pure PC membranes), its presence does not affect comparisons of endonexin binding to other vesicles. First, a constant 5% dansyl-PE was used in all vesicles. Second, 5% dansyl-PE exhibited no perturbation of endonexin binding to vesicles at less than 1 mM Ca^{2+}. For endonexin binding to 30% PS/PC vesicles containing 1%, 5%, or 15% dansyl-PE, a shifted Ca^{2+} titration was apparent only with vesicles containing 15% dansyl-PE (half-maximal Ca^{2+} from approximately 50 to 20 μM). In addition, Ca^{2+} titrations of endonexin binding to 30% PS vesicles containing no dansyl-PE, measured by 90° light scattering or by increased endonexin intrinsic fluorescence, were essentially identical to titrations of endonexin binding to similar vesicles containing 5% dansyl-PE (90° light scattering) or 3% dansyl-PE (endonexin intrinsic fluorescence), as measured by enhanced dansyl fluorescence.

Negative Lipid Charge Enhances Endonexin Binding to Vesicles Independent of Lipid Structure. In assessing the role of negative lipid charge in stabilizing the endonexin–membrane interaction, lipids with different head-group structures, as well as with different charges, were compared. Comparisons of endonexin binding to membranes of identical lipid composition but different charge were achieved using lipids whose charge was sensitive to pH. Binding of endonexin to 30% PI/65% PC/5% dPE vesicles was used as a control, as the charge of PI does not change over the pH range 6–9 (Abramson et al., 1968). Increasing the pH from 6 to 9 did not affect endonexin binding to 30% PI/PC/dPE vesicles (Figure 5A) nor to 95% PC/5% dPE vesicles (data not shown), indicating that the endonexin protein itself is not functionally altered over this pH range.

As seen in Figure 5B, increasing the pH from 6 to 9 shifted Ca^{2+} titrations for endonexin binding to 7.5% PA/87.5% PC/5% dPE vesicles to lower Ca^{2+} concentrations. Vesicle protein capacities were not significantly altered, as determined by protein titrations. The pK_a for the second deprotonation of PA in membranes is near 8 (Trauble & Eibl, 1974; Van Dijck et al., 1978), so that increasing the pH from 6 to 9 increased the net charge of PA molecules from −1 to −2. The shifted Ca^{2+} titrations suggest an increased negative lipid charge stabilizes endonexin–membrane binding.

Similar results were observed with vesicles containing either 4.4% PIP_2 or 25% oleic acid. The pK_a's for the second deprotonation of the monoester phosphates of PIP_2 are near 7 (van Paridon et al., 1986; Toner et al., 1988). At pH 6, the half-maximal Ca^{2+} for endonexin binding to 4.4% PIP_2/PC/dPE vesicles was approximately 250 μM. At pH 7 it decreased to 120 μM, and at pH 9 it decreased to 90 μM. The pK_a of oleic acid in PC membranes has been determined to be 7.6 (Hamilton & Cistola, 1986). Half-maximal Ca^{2+} for en-

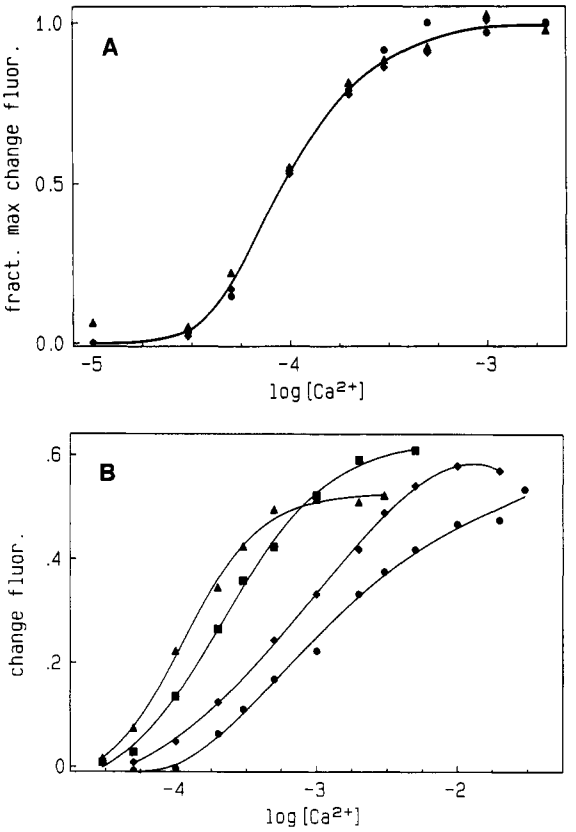

FIGURE 5: (A) Ca^{2+} titrations of endonexin binding to 30% PI/PC/dPE vesicles at pH 6 (●), 7 (◆), and 9 (▲). (B) Ca^{2+} titrations of endonexin binding to 7.5% PA/PC/dPE vesicles at pH 6 (●), 7 (◆), 8 (■), and 9 (▲).

donexin binding to 25% oleic acid/PC/dPE vesicles decreased from 5.0 mM at pH 6 to 1.2 mM at pH 9. The enhanced binding of endonexin to oleic acid/PC/dPE vesicles at increased pH was also observed by competing endonexin binding to PC/dansyl-PE vesicles with 10-fold excess vesicles containing 25% oleic acid and no dansyl-PE. Endonexin-induced increases in the dansyl fluorescence of PC/dPE vesicles at 100 mM Ca^{2+} were reduced by 91% at pH 9 but only by 66% at pH 6, by the presence of 25% oleic acid/75% PC vesicles.

Increasing the Mole % of Certain Types of Lipids Mixed with PC Increases the Apparent Affinity of Endonexin Binding to Vesicles. Increasing the mole % of certain lipids such as PS and PE in PC vesicles shifted Ca^{2+} titrations of endonexin–membrane binding to lower Ca^{2+} concentrations. Half-maximal binding of endonexin to PS/PC/dPE vesicles was reduced from 235 μM Ca^{2+} for 15% PS/80% PC/5% dPE vesicles to 45 μM for 30% PS/65% PC/5% dPE vesicles (see Figure 7A). For PA/PC/dPE vesicles at pH 6, increasing the mole % PA from 7.5% to 15% reduced the half-maximal Ca^{2+} concentration for endonexin–membrane binding from at least 1.26 to 0.18 mM. Half-maximal Ca^{2+} concentrations for endonexin binding to 0%, 15%, 30%, and 60% PE in PE/PC/dPE vesicles were 8.9, 2.8, 1.8, and 0.5 mM, respectively (Figure 6). Since a change in the mole % PE did not alter the membrane surface charge, the modulation of endonexin–membrane binding by the mole % lipid present was not solely due to alterations of membrane surface charge.

Mixing Two Different Types of Lipids together in PC Vesicles Synergistically Enhances Endonexin–Membrane Binding. The effects of mixing two different types of lipids that each individually promote endonexin binding to PC/dPE membranes were examined using PC vesicles containing

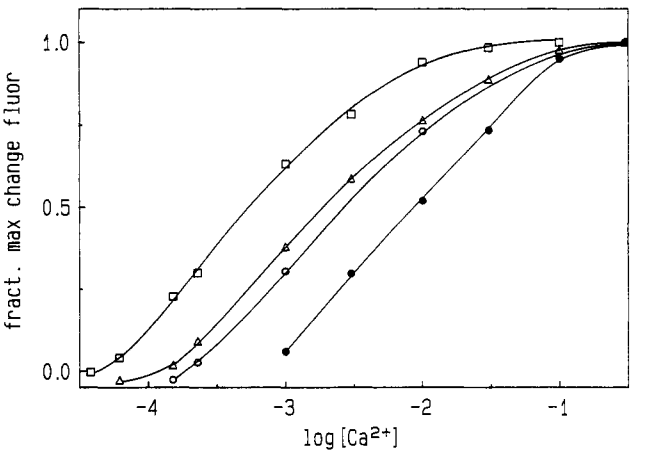

FIGURE 6: Ca^{2+} titrations of endonexin (38 nM) binding to PC/5% dPE vesicles containing 0% (●), 15% (○), 30% (△), and 60% (□) PE.

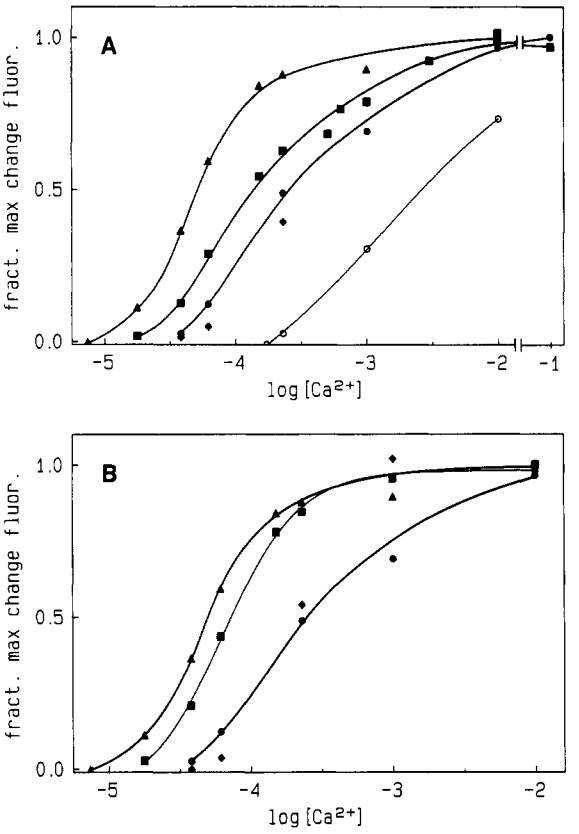

FIGURE 7: (A) Ca^{2+} titrations of endonexin binding to PC/5% dPE vesicles containing 15% PE (○), 15% PS (●), 15% PS/15% PE (■), and 30% PS/PC/dPE (▲) and of endonexin binding to a mixture of 15% PS/PC/dPE vesicles and 15% PE/PC/dPE vesicles (◆). (B) Ca^{2+} titrations of endonexin binding to PC/dPE vesicles containing 15% PS (●), 15% PS/15% PI (■), and 30% PS (▲) and of endonexin binding to a mixture of 15% PS/PC/dPE vesicles and 15% vesicles PI/PC/dPE (◆). Concentrations were 40 nM endonexin and 4 μM total lipid for each type of vesicle.

mixtures of either PE and PS or PI and PS. As seen in Figure 7A,B, mixing these lipids in the same vesicle enhanced endonexin–membrane binding. The shift in Ca^{2+} sensitivity induced by the presence of 15% PE in 15% PE/15% PS/65% PC/5% dPE vesicles, compared to 15% PS/80% PC/5% dPE vesicles, was especially dramatic since it occurred at Ca^{2+} concentrations where endonexin did not bind to 15% PE/80% PC/5% dPE vesicles (Figure 7A) and since it could not be due to changes in the membrane surface charge. Importantly, the shifts in Ca^{2+} sensitivity depended on the lipids being in

the same vesicle. Mixing either 15% PE/PC/dPE vesicles (Figure 7A) or 15% PI/PC/dPE vesicles (Figure 7B) with 15% PS/PC/dPE vesicles resulted in no shift in the Ca^{2+} threshold (the "toe" of the Ca^{2+} titration curve) from 15% PS/PC/dPE vesicles alone.

Ca^{2+} titrations of endonexin–membrane binding progressively shift to higher Ca^{2+} concentrations (progressively weaker binding) for endonexin binding to PS/PC/dPE, PI/PC/dPE, and PE/PC/dPE vesicles (e.g., Figure 4 and Table 1). This order parallels the shifts in Ca^{2+} titrations incurred when these lipids were incorporated into the 15% PS/PC/dPE vesicles: additional PS caused the greatest shift, PI caused a slightly smaller shift, and PE caused the smallest shift (Figure 7A,B). The ability of a type of lipid to enhance endonexin binding to a membrane containing other types of lipids appears to depend on how well the lipid alone enhances endonexin binding to PC/dPE membranes.

Binding of Endonexin to Membranes Was Not Inhibited by Soluble Lipid Head-Group Analogues. To test whether lipid head groups alone provide the specificity and stability for endonexin–membrane association, soluble head-group analogues were used to attempt the inhibition of endonexin–membrane binding. The presence of 1 mM phosphate or phosphoserine did not inhibit endonexin binding to 30% PS/PC/dPE vesicles at a saturating 2 mM Ca^{2+}. Some inhibition was seen at lower Ca^{2+} concentrations, but this resulted entirely from the depletion of free Ca^{2+} by Ca^{2+} binding to phosphate or phosphoserine, as measured by a Ca^{2+} electrode. Similarly, some inhibition of endonexin binding to PI/PC/dPE vesicles was seen in the presence of inositol hexaphosphate, but this also correlated with the depletion of free Ca^{2+}. The ability of glycerophosphorylserine (GPS) to inhibit endonexin–membrane binding was tested by monitoring endonexin intrinsic fluorescence changes, since GPS altered the dansyl-PE fluorescence. GPS (1 mM) did not significantly reduce the enhanced endonexin intrinsic fluorescence observed in the presence of 2 mM Ca^{2+} and 30% PS/PC vesicles containing no dansyl-PE, and it did not promote a Ca^{2+}-dependent increase in endonexin intrinsic fluorescence in the absence of vesicles. High-affinity binding of endonexin to lipids must depend on structural aspects other than the lipid head group and/or depend on the high local concentrations of lipid head groups that would be found on membrane surfaces.

Ca^{2+} Binds to Endonexin in the Absence of Membranes. Ca^{2+} alone caused increases in endonexin intrinsic fluorescence (Figure 8) similar to those reported for annexins I and V (Meers, 1990). Similar difference emission spectra ($\pm Ca^{2+}$) were obtained with excitation at either 280 or 295 nm, suggesting that the increases were mostly due to enhanced and red-shifted tryptophan (not tyrosine) emission. The increases titrated with a half-maximum of 25–30 mM Ca^{2+}. Binding appeared specific for Ca^{2+} since 100 mM Mg^{2+} caused less than a 2% increase in intensity, as opposed to 50–60% for Ca^{2+}, and did not reduce the increase in fluorescence observed with 30 mM Ca^{2+} when Ca^{2+} and Mg^{2+} were added together.

Ca^{2+} titrations of endonexin intrinsic fluorescence changes at these high (>1 mM) Ca^{2+} concentrations were also performed in the presence of 30% PS/70% PC vesicles. If Ca^{2+} is binding to sites on endonexin that regulate endonexin–membrane binding, then increases in endonexin intrinsic fluorescence in the presence of 30% PS/70% PC vesicles should saturate at 1 mM Ca^{2+}, where membrane binding saturates. Although endonexin fluorescence increases in the presence of vesicles did correlate with membrane binding (Figure 8 and above), a second phase of increased fluorescence was apparent

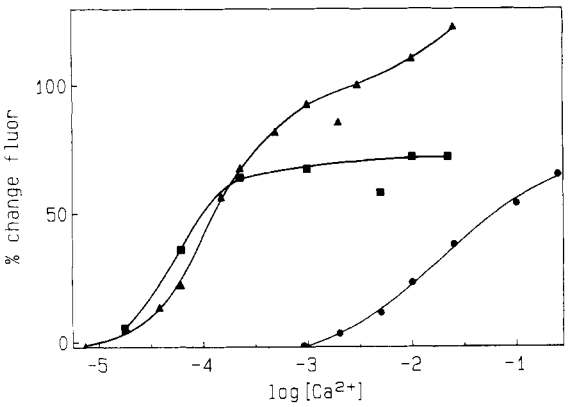

FIGURE 8: Ca^{2+} titrations of endonexin intrinsic fluorescence in the absence (●) and presence (▲) of 30% PS/70% PC (no dansyl-PE) vesicles and of the endonexin-enhanced dansyl fluorescence of 30% PS/PC/dPE vesicles (■). Conditions: 8 μM total lipid; 58 nM endonexin; excitation wavelength, 280nm; emmission was integrated between 334 and 375 nm for intrinsic fluorescence and between 470 and 550 nm for dansyl-PE.

at greater Ca^{2+} concentrations, where membrane binding had saturated (Figure 8; the total endonexin in this experiment was approximately 20% less than vesicle capacity). This additional Ca^{2+} binding could be to sites that regulate endonexin–vesicle aggregation, which has been reported to occur at Ca^{2+} concentrations greater than those required for membrane binding (Sudhof et al., 1984).

DISCUSSION

Modulation of Ca^{2+} Sensitivity for Endonexin–Membrane Binding. Membrane charge, phospholipid head-group structure, and membrane composition all contribute to the complex modulation of Ca^{2+}-dependent binding of endonexin to membranes. The stabilization of endonexin–membrane binding by negative membrane charge is evident in comparisons of endonexin binding to vesicles containing lipids with similar head-group structure but different charge, such as DAG and PA, PE and PS, and PI and PIP_2 (Table 1). In addition, increasing the negative charge of PA, PIP_2, and oleic acid in PC vesicles by increasing the pH enhanced endonexin–membrane binding. This latter enhancement may reflect a nonspecific charge effect since the types and intramolecular locations of the titrated charges differ among the three types of lipids. Negative lipid charge can stabilize lipid binding to positive regions of endonexin and/or create a local boundary layer of increased Ca^{2+} concentration at the membrane surface. In fact, shifted Ca^{2+} titrations of annexin V binding to different mole % PS/PC membranes (Andree et al., 1990), along with titrations of annexin V binding to oleic acid/PC vesicles at different pH (Meers & Mealy, 1993), have been shown to coincide when normalized for calculated surface Ca^{2+} concentrations (Meers & Mealy, 1993).

Shifts in the Ca^{2+} titrations of endonexin binding to well-defined PC vesicles containing different types of lipids indicate that the types of charged groups and other structural motifs of membrane lipid molecules also affect endonexin–membrane association. For example, 30% PA/PC/dPE (30% PA/65% PC/5% dansyl-PE) and 30% PIP_2/PC/dPE vesicles were bound at similar Ca^{2+} concentrations, despite the 3–4 times greater negative surface charge of the PIP_2/PC vesicles (Table 1). The *sn*-3-phosphate of phospholipids may be especially important. Half-maximal Ca^{2+} for endonexin binding to PA/PC/dPE vesicles, where *sn*-3-PA phosphates provide a net negative membrane charge, is 20-fold less than for endonexin

binding to oleic acid/PC/dPE vesicles at pH 9, where oleic acid carboxylates provide a net negative membrane charge. Similarly, PE-containing membranes were bound much more tightly than DAG-containing membranes. Both lipids have no net charge, but the zwitterionic PE contains a charged *sn*-3-phosphate group.

Besides the *sn*-3-phosphate of phospholipids, lipid headgroup size may affect endonexin–lipid interactions. Endonexin affinity for membranes containing PI, with its large inositol ring, is weaker than for membranes containing PA, PS, or PG, in spite of having the same negative charge. The progressively weaker affinity of endonexin binding to vesicles containing progressively *n*-methylated derivatives of PE [PE, PE(Me), $PE(Me)_2$, $PE(Me)_3$ = PC] correlates with increased steric hindrance or diminished capacities for either hydrogen bonding or positive charge neutralization by deprotonation. The influence of lipid structure on endonexin–membrane association may reflect specific interactions between endonexin and membrane lipids or differences in lipid–Ca^{2+} binding or lipid–lipid interactions. Unfortunately, not enough is known presently about these two latter types of interactions to draw meaningful correlations.

The relative proportions of different lipids present in vesicles modulate endonexin–membrane binding. As observed with annexin V binding to PS/PC membranes (Andree et al., 1990), increasing the mole % PS or PA shifted Ca^{2+} titrations of endonexin binding to PS/PC/dPE or PA/PC/dPE membranes to lower Ca^{2+} concentrations. However, endonexin also exhibits similar shifts with increased mole % zwitterionic PE in PE/PC/dPE membranes, indicating that shifts are not solely due to changes in membrane surface charge. Mixing two different types of lipids in the same PC membrane also resulted in enhanced endonexin–membrane binding. Even PE, which alone did not support endonexin binding well, dramatically enhanced endonexin binding to PS/PC/dPE vesicles at low Ca^{2+} concentrations. Thus, lipids that only weakly stimulate endonexin binding to PC membranes may dramatically affect endonexin binding to mixed-lipid cellular membranes.

The synergism exhibited by mixing PE and PS or PI and PS in the same PC/dPE membranes could reflect different types of lipids interacting together to create favorable conditions for endonexin–membrane binding. However, there seems little reason to expect that negatively charged PI and zwitterionic PE would both independently interact with PS in a similar manner to enhance endonexin binding to PS/PC/dPE membranes. Another possibility is that the synergism reflects an apparent cooperative binding of certain types of lipids to endonexin, with multiple, different types of lipids able to bind to each membrane-bound endonexin molecule. In this way, the presence of PS, for example, could allow endonexin to bind PE at Ca^{2+} concentrations where endonexin otherwise does not bind PE (Figure 7A). This might simply result from PS localizing endonexin to membrane surfaces where endonexin experiences a greater local concentration of PE. The preferential binding of certain types of lipid molecules to endonexin is consistent with the observed lateral segregation of PG induced by endonexin in PG/PC membranes (Junker & Creutz, 1993). Such preferential binding does not preclude the binding of PC molecules that are also present in vesicles. Instead, a stable endonexin–membrane complex at low Ca^{2+} (where endonexin does not bind pure PC membranes) may require a minimum number of a certain type of lipid molecule, in addition to available PC molecules. The mechanism for endonexin–membrane binding will be explored in more detail in following sections.

Comparison of the Lipid Specificity of Endonexin with That of Other Annexins. The lipid specificity of endonexin determined here parallels that determined for annexin V. In a study that employed planar bilayers of PC mixed with different lipids, the relative order for preferential annexin V–membrane binding was cardiolipin > PG > PS > PI > PA > PE >> PC (Andree et al., 1990). The major distinction between endonexin and annexin V is the relatively weaker binding of annexin V to PA/PC membranes. This difference could reflect amino acid sequence differences within the endonexin and annexin V conserved "core" regions that are believed to be responsible for the Ca^{2+}-dependent membrane binding activity of all annexins. Other annexins also exhibit preferred binding to PS and PI over PC (Glenney, 1986; Schlaepfer & Haigler, 1987; Schlaepfer et al., 1987; Andree et al., 1990; Meers & Mealy, 1993). More comprehensive studies with other annexins should reveal any correlations between subtle differences in lipid specificity and amino acid sequence, providing a greater understanding of the molecular mechanism of annexin–membrane binding.

Quantitative Analysis and Modeling of the Endonexin–Membrane Interaction. The molecular mechanism for endonexin–membrane binding was further analyzed by modeling membrane binding data. The features of endonexin–membrane binding data, such as shifts and broadening of Ca^{2+} titrations with changes in endonexin and lipid concentrations and alterations of protein titrations with changes in membrane lipid composition, provide particular insights into the nature of the endonexin–membrane interaction. A simple model, described in the Materials and Methods section and section c here, was found to accurately predict these features.

(a) The Shapes of Ca^{2+} Titrations Reflect Coupled Binding of Lipids and Ca^{2+} to Endonexin and Apparent Cooperative Ca^{2+} Binding. The Ca^{2+} titrations of endonexin–membrane binding reported here were generally performed using near-saturating concentrations of endonexin to allow the greatest sensitivity in detecting increases in dansyl fluorescence above background dansyl fluorescence. However, either decreasing the endonexin concentration (e.g., Figure 9) or increasing the lipid concentration (below) resulted in steeper Ca^{2+} titrations. This could reflect endonexin binding to a subpopulation of vesicles containing a greater mole % PS. However, in our model, this change in the Ca^{2+} titration shape was readily accounted for by the coupling between Ca^{2+} and lipid in promoting endonexin–membrane association. High ratios of endonexin to lipid result in a significant depletion of available lipid with endonexin–membrane binding. This means that as Ca^{2+} is increased, a greater fraction of total lipid is depleted and apparent endonexin–membrane binding becomes weaker. We found experimentally that keeping the ratio of endonexin to lipid constant resulted in identical normalized titrations. For example, doubling the concentration of 30% PE/PC/dPE vesicles resulted in a steeper Ca^{2+} titration, but doubling both the lipid and protein concentrations resulted in identical normalized binding curves (data not shown).

The dependence of the breadth of Ca^{2+} titrations on the ratio of protein to lipid explains previously contradictory reports in the literature. Ca^{2+} titrations of annexin V (endonexin II, vascular anticoagulant protein α) binding to PS/PC membranes were fit in one study with a Hill coefficient of 3.1 (Schlaepfer et al., 1987), but in another study with a coefficient of 1.6 (Andree et al., 1990). The latter study with the smaller coefficient and broader Ca^{2+} titration used a total protein concentration in excess of the available membrane binding sites (Andree et al., 1990).

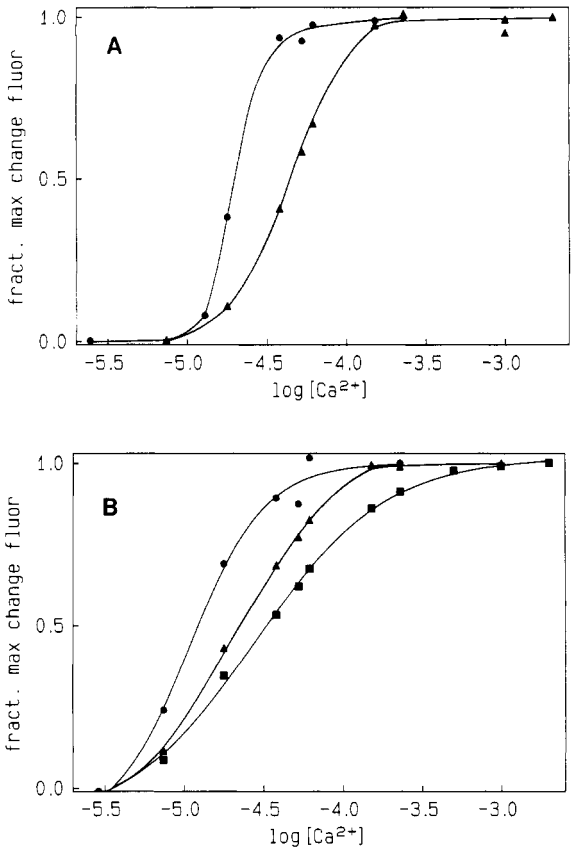

FIGURE 9: Ca^{2+} titrations of endonexin binding to vesicles at different ratios of total protein to total lipid: (A) 30% PS/PC/dPE vesicles (4 μM total lipid) with 2.9 (●) and 29 nM (▲) endonexin; (B) 36% PA/PC/dPE vesicles (4 μM total lipid) with 22 (●), 36 (▲), and 50 nM (■) endonexin.

In the presence of 10-fold excess membrane sites to endonexin, Ca^{2+} titrations of endonexin–membrane binding are quite steep. Ca^{2+} titrations of endonexin binding to 10-fold excess 25%PS/PC/dPE and 30% PS/PC/dPE vesicles were fit with Hill coefficients of 5.6 ± 1.2 and 5.3 ± 0.9, respectively. Ca^{2+} titrations of endonexin binding to 10-fold excess 30% PE and 60% PE vesicles were fit with Hill coefficients of 3.9 ± 0.4 and 3.7 ± 0.6, respectively. Such large Hill coefficients suggest cooperative binding of multiple Ca^{2+} ions in the endonexin–membrane interaction.

(b) Protein Titrations Reflect the Preferential and Cooperative Binding of Certain Types of Lipids to Endonexin. The protein titration data indicate that certain types of lipids may preferentially bind to endonexin. For example, increasing the mole % PS in PS/PC/dPE membranes increased the maximum amount of endonexin able to bind to these vesicles (Figure 2). If endonexin–membrane binding were the result of simple adsorption to charged surfaces, high endonexin concentrations would have resulted in vesicle surface saturation for all titrations. The similar endonexin binding capacities of 30% PI, PA, and PG and the more highly charged 30% PIP_2/PC/dPE vesicles further suggest that the number of mobile charges in a membrane, and not the total membrane surface charge, determines how much endonexin can bind to vesicles. Preferential binding of certain types of lipids to endonexin is also consistent with enhanced endonexin binding, when increasing the mole % lipid (including zwitterionic PE) in PC membranes, and with the lateral segregation of PG induced by endonexin in PG/PC membranes (Junker & Creutz, 1993).

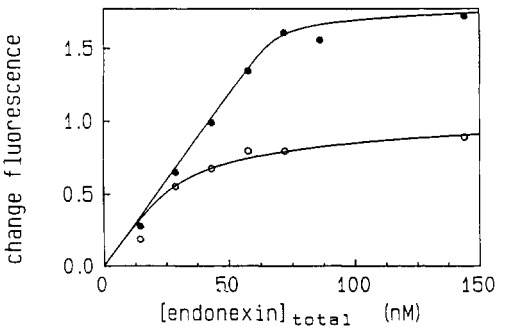

FIGURE 10: Simultaneous fits of model to protein titrations of endonexin binding to 36% PA/PC/dPE vesicles at 1 mM (●) and 38 μM (○) Ca^{2+}. Fit parameters are $k_C = 1.2 \times 10^{-7}$ M, $k_{CL} = 215$, $n = 4.3$, and $nm = 8.9$. k_M was set to 1 M^{-1}, and all parameters were constrained to yield a 17 μM Ca^{2+} half-maximum for a Ca^{2+} titration of endonexin binding to excess PS/PC/dPE vesicles (see text).

If certain types of lipids do preferentially bind to endonexin, that binding is cooperative. The 25% PS/PC/dPE and 39% PS/PC/dPE protein titration data (where vesicle surface area did not limit binding) in Figure 2 were fit by Langmuir binding isotherms with available membrane site concentrations of 39 and 49 nM, respectively. If we ssume that half of the PS is exposed on the outer membrane leaflet, this translates into 13 and 16 PS molecules per endonexin–membrane binding site, respectively. That multiple PS (or other negatively charged lipid) molecules interact with each membrane-bound endonexin molecule instead of becoming diluted among competing excess endonexin molecules indicates cooperativity in the binding of PS to endonexin.

Cooperativity in lipid binding is also evident in protein titrations of endonexin binding to vesicles at less than saturating Ca^{2+} concentrations. A protein titration of endonexin binding to 36% PA/PC/dPE vesicles at 38 μM Ca^{2+} could not achieve as great an increase in dansyl fluorescence as that observed with a saturating 1 mM Ca^{2+}, even with excess total endonexin (Figure 10). If lipid (PA) molecules are bound cooperatively, a titration of the fraction of endonexin bound vs *free* lipid will be sigmoidal and steep, with the fraction of protein bound increasing dramatically over a small increase in the free lipid concentration. Weaker binding shifts the titration to greater free lipid concentrations, but still produces a sigmoidal curve. In protein titrations, the fraction of endonexin bound decreases at vesicle saturation as more added endonexin remains free in solution. For the fraction of endonexin bound to decrease dramatically over a small decrease in free lipid (a small increase in endonexin binding), this saturation must be sharp. Decreasing Ca^{2+} results in apparently weaker but still cooperative binding, so that the sharp saturation still occurs, but at a greater free lipid (smaller bound protein) concentration. This effect is predicted by a model that incorporates cooperativity in lipid binding (below) and may explain similar observations reported elsewhere for porcine liver endonexin (Shaldle & Weber, 1987), synexin (annexin VII), bovine endonexin, and p68 (annexin VI; Zaks & Creutz, 1990), as well as for other proteins that may be annexins (Bazzi & Nelsestuen, 1991).

(c) Model for Endonexin–Membrane Binding. Endonexin–membrane binding data were analyzed using a simple and general model for Ca^{2+}-dependent protein–lipid binding (see Materials and Methods). The model is based on that developed by Lentz and Hermans (Cutsforth et al., 1989; Lentz et al., 1989) in which protein–membrane binding is treated as a two-step process where initial protein–membrane adsorption (affinity k_M) is followed by the binding of certain types of lipid molecules in two-dimensional surface reactions (intrinsic

affinity k_L). Depending on the relative magnitudes of k_M and k_L, the model represents a simple Langmuir binding isotherm (large k_M) or a more complex specific protein–lipid equilibrium (small k_M, large k_L). In our model, we include coupled binding of Ca^{2+} with the binding of individual lipid molecules to the protein, thus producing the partition function $Q = 1 + k_M[M]\{(1 + k_L X_A)^m + k_C[Ca^{2+}](1 + k_{CL}X_A)^m\}^n$. The affinity constants are k_M, membrane adsorption; k_C, microscopic Ca^{2+} binding; k_{CL}, microscopic lipid binding to a site coupled with an occupied Ca^{2+} binding site; and k_L, microscopic lipid binding to a site coupled with an unoccupied Ca^{2+} binding site. n is the number of Ca^{2+} sites on each protein molecule, and m is the number of specific lipid binding sites coupled with each Ca^{2+} binding site.

The high-affinity protein titrations that do not saturate vesicle surfaces (e.g., 25% PS/PC/dPE and 39% PS/PC/dPE, Figure 2) can only be fit with small values of the adsorption constant k_M (e.g., $<10^{-2}$ M^{-1} for 39% PS/PC/dPE in Figure 2) and a compensating high-affinity binding of specific lipids (large $k_C k_{CL}$). A decrease in k_M below 10^{-2} M^{-1} (with a compensating increased $k_C k_{CL}$) produced curves that approached a limiting sharp titration, so that only an upper limit of k_M can be determined. Eight titrations of endonexin binding to 24–54% PS/PC/dPE vesicles were best fit with PS stoichiometries (nm) of 9–18, the variation reflecting, in part, the sensitivity of fits to uncertanties in $[endonexin]_{total}$, $[lipid]_{total}$, and mole % PS. These stoichiometries are within the range of 3–17 PG molecules per endonexin–membrane complex estimated from the lateral segregation of PG in PG/PC vesicles (Junker & Creutz, 1993).

The Ca^{2+} stoichiometry n was better determined by fitting Ca^{2+} titrations of endonexin–membrane binding. The model was fit to multiple Ca^{2+} titrations simultaneously rather than to individual Ca^{2+} titrations to better determine parameters and test overall consistency with the data. k_M was kept small (<1 M^{-1}) to be consistent with protein titrations. The total concentration of membrane sites was taken as the total lipid concentration divided by 64 (±10 did not affect the fits) inner and outer monolayer lipids covered by each membrane-bound endonexin molecule, as determined by the protein titration of endonexin binding to 95% PS vesicles. An upper limit for k_C of 33 M^{-1} was used for fits, since the threshold for endonexin binding to pure PC vesicles is greater than 10 mM Ca^{2+} and the half-maximal Ca^{2+} concentration for altered endonexin intrinsic fluorescence in the absence of membranes is approximately 30 mM (Results).

Figure 11A illustrates a simultaneous fit of five Ca^{2+} titrations of endonexin binding to PS/PC/dPE vesicles, where the titrations differ in total lipid concentration, total endonexin concentration, ratio of lipid to endonexin, and mole % PS. The quality of fits (residuals) and determinations of Ca^{2+} (n) and PS (nm) stoichiometries was essentially identical for values of k_M between 1 and 10^{-10} M^{-1} and values of k_C between 1 and 33 M^{-1}. Over these ranges of k_M and k_C, best fits of n and nm ranged from 4.8 to 5.3 and from 10.4 to 11.1, respectively (confidence ranges for individual fits were less than 25% for n and less than 20% for nm). Thus, Ca^{2+} and lipid (PS) stoichiometries were well-defined, despite the uncertainties in the affinity constants. As seen in Figure 11B, the parameters from these fits to the Ca^{2+} titrations were generally consistent with the protein titrations.

Observed shifts in the Ca^{2+} sensitivity of endonexin binding to 30% PS versus 60% PS and 30% PE versus 60% PE vesicles (not shown) were not as great as predicted from fitting the five Ca^{2+} titrations of endonexin binding to 15% and 30% PS

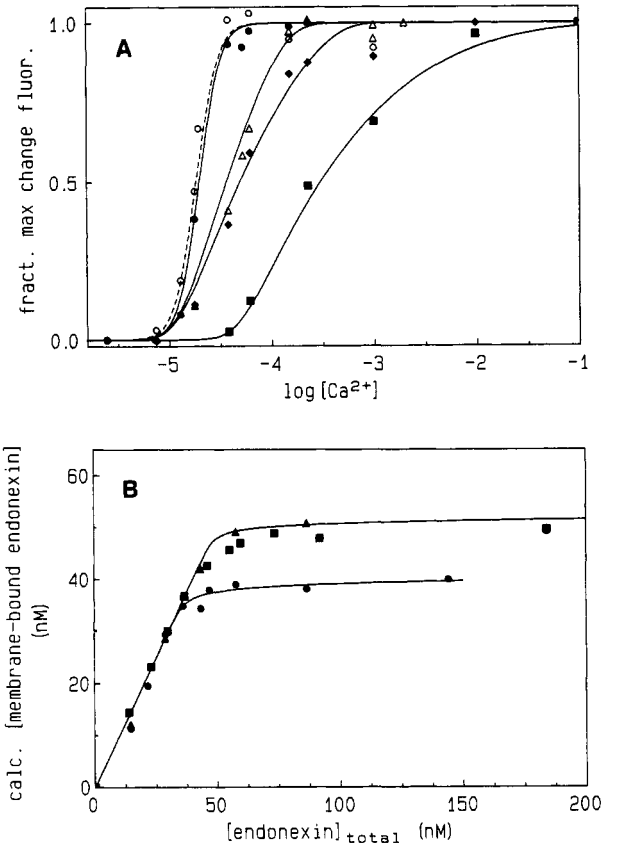

FIGURE 11: (A) Simultaneous fits of model to Ca^{2+} titrations of endonexin binding to 15% PS/PC/dPE vesicles (■, 4 μM lipid, 40 nM endonexin), 30% PS/PC/dPE vesicles (◆, 4 μM lipid, 40 nM endonexin), 30% PS/PC/dPE vesicles (△, 4 μM lipid, 29 nM endonexin), 30% PS/PC/dPE vesicles (●, 4 μM lipid, 2.9 nM endonexin), and 25% PS/PC/dPE vesicles (○, 40 μM lipid, 32 nM endonexin). Parameters are $k_C = 33$ M^{-1}, $k_M = 10^{-10}$ M^{-1}, $k_{CL} = 5 \times 10^3$, $n = 4.8$, and $nm = 10.3$. (B) Experimental data and predicted protein titrations based on parameters used in panel A for endonexin binding to 25% PS (●, same data as Figure 2) and to two different preparations of 30% PS vesicles (▲, ■). Calculated [membrane-bound protein] was derived from the linear change in fluorescence with $[endonexin]_{total}$ (see Materials and Methods).

vesicles. Ca^{2+} and lipid stoichiometries were 3.5 for the PS/PC data and 2 and 3, respectively, for the PE/PC data. The inconsistency probably reflects the oversimplification of the model. Ideal lipid behavior (uniform lateral mixing and homogeneous charge distribution) may not hold at high mole % lipid in PC (Macdonald & Seelig, 1987a,b; Seelig & Macdonald, 1989). In addition, the model ignores the influence of membrane surface charge, which contributes to the shifts in Ca^{2+} titrations with altered mole % PS and must be compensated in the fitting of k_{CL}. Finally, the model also ignores binding of PC to endonexin, which instead must be compensated for in part in the fitting of k_M.

Despite its limitations, however, the model accurately predicts the trends observed in endonexin–membrane binding and specifies a mechanism consistent with the observed data. The small k_M required to fit protein titrations indicates that endonexin–membrane stability arises primarily from interactions between endonexin and certain types of lipids and not from general surface absorption. The apparent cooperativity in lipid binding evident in protein titrations results from multiple lipids being required to stabilize each membrane-bound endonexin molecule. This cooperativity can also be interpreted as arising from a reduction in dimensionality, since the local concentration of lipid A ($X_{A,total}$) on the membrane surface is much greater than the bulk concentration of lipid

in solution ($[M]_{total}$, $X_{A,total} \gg [M]_{total}$; Mosior & MacLaughlin, 1992). Models that explicitly assume a local concentration effect (Mosior & MacLaughlin, 1992) are mathematically very similar to the model of Lentz and Hermans (1989) when k_M is small. With the apparent cooperativity in lipid binding, the model can simultaneously fit protein titrations of endonexin binding to PA/PC vesicles at different Ca^{2+} concentrations (Figure 10). For this latter fit, parameters were constrained to satisfy a 17 μM half-maximal Ca^{2+} concentration for a Ca^{2+} titration of endonexin binding to excess PS/PC vesicles (e.g., Figure 9; endonexin binds PA/PC/dPE and PS/PC/dPE vesicles with similar Ca^{2+} sensitivities).

The model predicts the steep and apparently cooperative Ca^{2+} titrations of endonexin binding to 10-fold excess vesicles (Figure 11A). This is because lipids bind cooperatively to endonexin, and Ca^{2+} binding and lipid binding are coupled. The coupling between Ca^{2+} and lipids also results in the accurately predicted broadening of Ca^{2+} titrations when the total endonexin concentration is increased to levels where available lipids become depleted (Figure 11A). Additionally, since membrane binding is more heavily weighted by specific lipid binding than by surface adsorption ($k_C k_{CL} X_A \gg k_M[M]$), Ca^{2+} titrations are shifted more by changes in X_{total} (Figure 11A) than by changes in $[M]_{total}$ (Figure 11A). This latter shift with increased mole fraction PS, unlike similar shifts with increased PE, is likely to have also resulted in part from increases in the membrane surface charge not accounted for by the model.

The coupled binding of Ca^{2+} and certain types of lipid molecules to endonexin can explain observations with other annexin proteins. Ca^{2+} titrations of annexin–membrane binding are often cooperative with Hill coefficients of 3–5, even when Ca^{2+} concentrations are corrected for increased surface concentrations resulting from membrane surface charge (Sudhof et al., 1984; Schlaepfer & Haigler, 1987; Schlaepfer et al., 1987; Meers & Mealy, 1993). Consistent with fits of endonexin–membrane binding Ca^{2+} titrations, multiple (3–6) Ca^{2+} binding sites have been identified in X-ray crystal structures of annexin V (Huber et al., 1990; Concha et al., 1993). If these sites do regulate protein–membrane binding, they could be coupled with lipid binding either allosterically (Concha et al., 1993) or by Ca^{2+} bridging lipid molecules to the protein (Huber et al., 1990).

ACKNOWLEDGMENT

Many thanks to Dr. Michael L. Johnson for the use of and help in implementing his nonlinear regression program and to Dr. Julia L. Kibelbek for critical reading and discussion of this manuscript.

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