Calcium-Dependent Annexin V Binding to Phospholipids: Stoichiometry, Specificity, and the Role of Negative Charge†

Paul Meers* and Tanya Mealy

Departments of Pathology and Biophysics, Boston University School of Medicine, 80 East Concord Street, Boston, Massachusetts 02118-2394

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ABSTRACT: Annexin V is a Ca^{2+}-dependent, phospholipid-binding protein that may have one or more membrane-related functions. The binding of annexin V to phospholipids in a detergent micelle matrix was studied to attempt to determine directly the stoichiometry of specific phospholipid-binding sites and the importance of negative charge. When annexin V binds to phospholipids, a large increase (severalfold) of the emission intensity of tryptophan 187 is observed. This intensity change was used to monitor the binding to phosphatidylcholine (PC) or phosphatidylserine (PS) at varying ratios with the detergent, octaethylene glycol monododecyl ether ($C_{12}E_8$). No binding to PC alone in these micelles could be observed, while approximately 10 PS molecules per micelle were required to observe binding. However, inclusion of negatively charged amphiphiles in the micelles, such as oleic acid or dodecyl sulfate, allowed the observation of binding to PC and decreased the number of phospholipids per micelle necessary for binding to both PS and PC. By including increasing proportions of dodecyl sulfate in the $C_{12}E_8$ micelles, a minimum average number of PS or PC per micelle of approximately 3–4 was required for complete binding. Labeling with photoreactive phospholipids under similar conditions led to an average of approximately 4–5 phospholipids covalently bound per annexin V monomer. Since annexin V has four similar domains, it is reasonable to suggest that one phospholipid binding site is associated with each domain, although as few as three functional domains may be sufficient for binding. Efficient binding required certain structural features of the phospholipid, including a phosphate group, an *sn*-2 acyl chain, and at least a few carbons on the *sn*-2 chain. Phospholipid headgroups were almost irrelevant, except for important surface charge effects on the interfacial ionic double layer. A negative surface charge on the micellar aggregate nonspecifically increases the Ca^{2+} concentration near the micelle surface and may also directly enhance the affinity of annexin V for phospholipids, as shown by the decreased two-dimensional phospholipid concentration necessary for binding. The ability to bind to zwitterionic phospholipids in the presence of a nonspecific negative surface charge may be relevant to the extracellular functions of this protein. Relatively weak individual phospholipid-binding sites that easily exchange were observed, suggesting rapid exchange of phospholipids between the sites on membrane-bound annexin V. These data suggest a working hypothesis that includes approximately four binding sites specific for phospholipid phosphate groups and *sn*-2 acyl chains. In this hypothesis, high affinity for phospholipid membranes is attained by the multisite nature of binding, the ability of the annexin to utilize most or all of the phospholipids in the membrane, and the high local Ca^{2+} and phospholipid concentrations that occur only near the membrane surface.

The annexins are a group of Ca^{2+}-dependent, membrane-binding proteins. Annexin V currently serves as a structural prototype for the annexin family as the first member for which a crystal structure has been solved (Huber et al., 1990, 1992; Concha et al., 1993). A number of possible functions have been suggested for the annexins, which are mainly related to the phospholipid-binding activities of these proteins. Annexin V may function in phospholipid-dependent inhibition of blood coagulation (Reutelingsperger et al., 1985; Funakoshi et al., 1987), in intracellular inhibition of protein kinase C (Schlaepfer et al., 1992), or as a voltage-dependent Ca^{2+} channel (Rojas et al., 1990). Other annexins have been implicated in various aspects of signal transduction or in the mediation of membrane–membrane contact as part of intracellular membrane fusion processes [see Meers (1990) for references].

The sequences of most annexins comprise a unique N-terminal region followed by four 60–70 amino acid repeated sequences [see Pepinsky et al. (1988)]. Removal of a short part of the N-terminus from some of the annexins has shown that Ca^{2+} and phospholipid binding resides in the repeated regions [see the discussion in Meers (1990)]. A highly conserved consensus sequence in each repeat has been suggested to be the Ca^{2+}- and/or phospholipid-binding site (Geisow et al., 1986). Contact of consensus sequence tryptophans of annexins IV and V with membrane phospholipids was demonstrated by fluorescence studies (Meers, 1990). The proximity of the annexin II tryptophan to a Tb^{3+}-binding site was also observed (Marriott et al., 1990). Structural characterization of annexin V, crystallized in the absence of phospholipids (Huber et al., 1990, 1992), confirmed the four-domain structure, showing several incompletely ligand-bound Ca^{2+} ions along the same surface ("convex") as the consensus sequence tryptophan. It has been speculated that these Ca^{2+} ions, complexed by ligands contributed by consensus sequence residues, may participate in binding to phospholipids because of structural similarity to the phospholipase A_2-binding site, where Ca^{2+} directly interacts with phospholipids (Huber et al., 1990). A positive electrostatic potential may also be concentrated on the same side of the Ca^{2+}-bound protein

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* Address correspondence to this author.

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(Karshikov et al., 1992). The demonstration of contact of the consensus sequence tryptophan of annexin V with membrane phosholipids (Meers, 1990) and the subsequent observation of Ca^{2+}-binding sites (with potential for phospholipid binding) along the same side of the protein (Huber et al., 1990) strongly suggest that this surface of annexin V binds to membrane phospholipids.

However, the structure of the Ca^{2+}-bound form of human annexin V from the original crystal structure (Huber et al., 1990, 1992) appears to differ in aqueous solution and when it is membrane-bound (Meers, 1990; Meers & Mealy, 1993), particularly within the third domain. Recent crystal structures of the Ca^{2+}-bound form of native rat annexin V (Seaton et al., 1990; Concha et al., 1993) and mutants of human annexin V (A. Burger and R. Huber, personal communication) are consistent with structural aspects of the third domain inferred from fluorescence studies (Meers & Mealy, 1993). More recently, structures have been solved for chicken annexin V (Bewley et al., 1993) and human des 1–32 annexin I (Weng et al., 1993). Bound phospholipids were not observed in any of the structures, and there is still relatively little information on annexin phospholipid-binding sites. A low-resolution structure of a two-dimensional array of annexin V on a phospholipid monolayer has been presented (Brisson et al., 1991), but no direct measurement of the structure or number of phospholipids bound to annexins has been made.

Other types of phospholipid–annexin stoichiometries have been measured. The number of membrane phospholipids required per monomer at binding saturation ranges from 42 to 59 (Andree et al., 1990; Meers et al., 1991), reflecting the packing of the annexin on the membrane surface. A second stoichiometry is the number of phospholipids physically covered by the bound protein, which is different from the above number because the irregular shapes of proteins can leave open patches of phospholipid, even at binding saturation. The area from the crystal structure of annexin V suggests that it can cover approximately 26 phospholipids (Huber et al., 1992). The remaining stoichiometry to be determined is whether the annexin V molecule has a defined number of specific phospholipid binding sites. Because of the weak binding of short-chain monomeric phospholipids, it has been difficult to define binding sites by crystallography (Huber et al., 1992) or by fluorometric methods (Tait et al., 1989). Binding to phospholipid membranes is complicated by the fact that all phospholipids bind to annexins, even phospholipid probes with modified headgroups (Meers et al., 1992; Meers & Mealy, 1993). Therefore, phospholipid ligands cannot be diluted in the membrane by other phospholipids, and a membrane-bound annexin will necessarily make nonspecific contact with many phospholipids.

Another unresolved aspect of annexin binding is the role of negative surface charge. It has been stated or assumed previously that annexins are specific for negatively charged phospholipids and that there may even be a specific interaction of the protein with negatively charged headgroups (Huber et al., 1992). However, it was shown recently that annexin V does bind the zwitterionic phospholipid, phosphatidylcholine (Andree et al., 1990; Meers & Mealy, 1993), and that the intrinsic Ca^{2+} dependence for binding to various phospholipids is nearly the same in most situations when the Guoy–Chapman double layer is taken into account (Meers & Mealy, 1993). These studies suggest that the negatively charged phospholipid headgroups do not have a specific interaction with annexin V, but they have not addressed the possibility that negative surface charge may enhance the avidity of the Ca^{2+}-dependent interaction of annexins with phospholipids in some other manner.

The problems of binding site stoichiometry and the role of negative charge are addressed in this study by monitoring the change in intrinsic fluorescence of annexin V specifically associated with binding to phospholipids (Meers & Mealy, 1993) and by using detergent micelles to dilute the specific ligands for annexin V. Using these systems, the role of negative charge and specific phospholipid-binding sites can be studied separately. Data are also presented to begin to clarify the structural specificity of the interaction of annexin V with phospholipids as opposed to negative charge.

MATERIALS AND METHODS

3-Palmitoyl-2-(1-pyrenyldecanoyl)-L-α-phosphatidylcholine (pyrene-PC or pPC),[1] 5-doxylstearic acid (5-DSA), and succinimido-*p*-azidobenzoate (SAB) were obtained from Molecular Probes (Eugene, OR). Bovine brain phosphatidylserine (PS), egg phosphatidylcholine (PC), phosphatidate (PA, derived from egg PC), phosphatidylethanolamine (PE, derived from egg PC), *N*-(lissamine-rhodamine B-sulfonyl)-PE (Rh-PE), 2-[12-[(7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino]dodecanoyl]-1-hexadecanoyl-*sn*-glycero-3-phosphocholine (acyl-NBD-PC), 1-palmitoyl-2-(5-doxylstearoyl)-*sn*-glycero-3-phosphocholine (5-PC), dilauroylphosphatidylcholine (DLPC), dinonanoylphosphatidylcholine (DNPC), dihexanoylphosphatidylcholine (DHPC), and 1-oleoyl-*sn*-glycero-3-phosphocholine (lyso-PC) (all >99%) were purchased from Avanti Polar Lipids (Birmingham, AL). EDTA (99.5%) and L-α-glycerophosphate were from Sigma (St. Louis, MO). Oleic acid (OA) and 1,2-dioleoylglycerol (DOG) were from NuChek Prep (Elysian, MN). Octaethylene glycol monododecyl ether ($C_{12}E_8$)was from Fluka (Ronkonkona, NY). $CaCl_2$ (>99%), KCl (>99%), NaCl (>99%), and TES (98%) were from Fisher (Pittsburgh, PA). Bicinchoninic acid and octyl β-D-glucopyranoside (octyl glucoside)were from Pierce (Rockford, IL).

Micelle Preparation. All experiments, except for detergent titrations, were performed by adding the indicated amount of $C_{12}E_8$ to a sample first, followed by any negatively charged amphiphiles and then any phospholipids (usually in the form of vesicles initially). Annexin V was added to samples last. Most experiments, except where indicated, were performed in 80 mM NaCl, 50 mM TES, and 0.1 mM EDTA (pH 7.4) (buffer A).

Phospholipid concentrations were determined using a phosphate assay as described in Kingsley and Feigenson (1979) modified from Chen et al. (1956), Bartlett (1959), and Morrison (1964).

Protein Preparation. Recombinant human annexin V (PAP-I, endonexin II) was prepared from a JA-221 *Escher-*

[1] Abbreviations: pyrene-PC or pPC, 3-palmitoyl-2-(1-pyrenyldecanoyl)-L-α-phosphatidylcholine; PS, bovine brain phosphatidylserine; PC, egg phosphatidylcholine; PA, phosphatidate derived from egg PC; PE, phosphatidylethanolamine derived from egg PC; Rh-PE, *N*-(lissamine-rhodamine B-sulfonyl)-PE; acyl-NBD-PC, 2-[12-[(7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino]dodecanoyl]-1-hexadecanoyl-*sn*-glycero-3-phosphocholine; 5-PC, 1-palmitoyl-2-(5-doxylstearoyl)-*sn*-glycero-3-phosphocholine; DLPC, 1,2-dilauroyl-*sn*-glycero-3-phosphocholine; DNPC, 1,2-dinonanoyl-*sn*-glycero-3-phosphocholine; DHPC, 1,2-dihexanoyl-*sn*-glycero-3-phosphocholine; lyso-PC, 1-oleoyl-*sn*-glycero-3-phosphocholine; OA, oleic acid; DOG, 1,2-dioleoylglycerol; dicaprylin, 1,2-dihexanoylglycerol; AB-PE, *N*-azidobenzoyl-PE; 5-DSA, 5-doxylstearic acid; SDS, sodium dodecyl sulfate; EDTA, ethylenediaminetetraacetate; TES, *N*-[tris(hydroxymethyl)methyl]-2-aminoethanesulfonic acid; $C_{12}E_8$, octaethylene glycol monododecyl ether; octyl glucoside, octyl β-D-glucopyranoside; SAB, succinimido-*p*-azidobenoate; cmc, critical micelle concentration.

ichia coli expression system kindly provided by Dr. R. Blake Pepinsky of Biogen Corporation (Cambridge, MA). Bacterial cells were collected and lysed in the presence of 1 mM toluenesulfonyl fluoride and a cocktail of protease inhibitors (final concentration of 1 μg/mL pepstatin A, leupeptin, aprotinin, chymostatin, and antipain; Sigma). Lysis was achieved by 20 rounds of 30 s of sonication followed by 30 s without sonication on ice, using a Heat Systems-Ultrasonics, Inc., Model W-225R sonicator with a titanium microtip. This was followed by three rounds of freezing and thawing. After the removal of debris by sedimentation and dialysis into the appropriate buffer, purification was by affinity chromatography as in Meers et al. (1987). This final product was at least 99% pure by single-dimension gel electrophoresis of 0.3-μL samples on a Phast gel system (Pharmacia, Piscataway, NJ) with detection by Coomassie Blue (1.5 mg/mL protein in sample buffer) or silver stain (0.7 mg/mL protein in sample buffer). The maximal increases in annexin V tryptophan fluorescence for several preparations used in this report were approximately the same, but were only about 70% of those observed previously (Meers, 1990; Meers & Mealy, 1993). In all other respects, the preparations studied here were essentially identical to those used previously. One possible explanation for the fluorescence difference is the presence in previous or present prepartions of a material that slightly quenches fluorescence in either the aqueous or micellar environment.

Protein concentrations for most experiments were determined by the bicinchoninic acid assay (Smith et al., 1985) and then converted to concentrations based on tryptophan absorption. The conversion factor was determined by comparing the absorption at 295 nm of aliquots of a stock of annexin V to a known concentration (by weight) of lysyltryptophyllysine.

Fluorescence Measurements. Fluorescence measurements were made using an SLM 8000C fluorometer (Urbana, IL). Tryptophan excitation was at 295 nm, with full emission spectra monitored from 300 to 400 nm for most experiments. Excitation and emission wavelengths for other experiments are given in the figure captions. Fluorescence changes were induced by the addition of a concentrated stock of $CaCl_2$, and corrections for dilution were made, if necessary. Cuvettes were thermostated to 25 °C and continually stirred during all fluorescence measurements.

Photolabeling Experiments. *N*-(Azidobenzoyl)phosphatidylethanolamine (AB-PE) was synthesized by suspending 10 μmol of PE and 20 μmol of $C_{12}E_8$ in 1 mL of 90 mM NaCl/20 mM sodium borate at pH 9.0. Succinimido-*p*-azidobenzoate (SAB) (25 μmol) in DMSO was added to this mixture (final DMSO concentration of 3%). After the mixture was stirred for 1 h, 1 mL of 100 mM Tris (pH 8.0) was added and allowed to react for 1 h with the remaining unreacted SAB. The preparation was then diluted approximately 50-fold and dialyzed extensively against several changes of buffer A to remove detergent. All steps were performed under dark or dim conditions. The final yield was approximately 50%. Thin-layer chromatography was performed on silica gel plates (Fisher, Pittsburgh, PA) using the solvent system 65:25:5 chloroform/methanol/concentrated ammonium hydroxide. Purity was assessed as greater than 95%; no $C_{12}E_8$ was detectable. AB-PE was stored frozen as a suspension in buffer. Photoaffinity cross-linking was accomplished by the placement of samples in quartz cuvettes approximately 25 cm from the center of a 450-W xenon lamp and exposure for 15 s (this resulted in minimal sample heating). Subsequent extraction of the detergent and phospholipid not linked to the protein was accomplished as described (Wessel & Flügge, 1984). One step was added to the described extractions, which involved washing the final protein pellet with 0.5 mL of 1:1 chloroform/methanol. Phospholipid and protein concentrations were determined as described above.

RESULTS

Tryptophan Fluorescence Emission Intensity Increase as a Binding Assay. It was previously shown that Ca^{2+}-dependent binding of annexin V to phospholipid membranes results in contact of tryptophan 187 with the bilayer phospholipids (Meers, 1990). At a surface Ca^{2+} concentration above approximately 3 mM, a conformational change of the protein occurs that exposes tryptophan 187 in a position where it can contact membrane bilayers as the protein binds (Meers & Mealy, 1993). This contact results in an environment where the increased viscosity is probably responsible for the observation of a large increase in emission intensity, while the maximum wavelength remains fairly high (i.e., red-shifted; Meers, 1990; Meers & Mealy, 1993). Correlation between binding to bilayers and intensity increases has been shown in several ways. First, the increase in tryptophan fluorescence is always dependent on the presence of both Ca^{2+} and phospholipids (Meers, 1990; Meers & Mealy, 1993). Second, collisional quenching of the tryptophan residue, when annexin V binds to membranes containing quenching phospholipid derivatives, correlates with the increased intensity when it binds to membranes without quenchers (Meers, 1990). Third, Ca^{2+} concentrations that lead to annexin V binding, detected by a membrane-inserted excimer-forming phospholipid probe (Meers et al., 1991), also lead to increased tryptophan fluorescence (Meers & Mealy, 1993). Fourth, the expected decreased exposure of the annexin V tryptophan to aqueous soluble quenchers upon binding to membranes also correlates with the increase in tryptophan fluorescence (Meers & Mealy, 1993). Fifth, we have observed that qualitative estimation of the amount of annexin V that pellets with large PS/PC liposomes at various Ca^{2+} concentrations correlates well with the increase in tryptophan fluorescence (data not shown). Sixth, we also titrated the Ca^{2+} dependence of the fluorescence increase of the tryptophan of annexin V for binding to vesicles of various mol % of PS in PC (data not shown, but similar to that shown in Figure 2). The Ca^{2+} dependence of the binding is expected to be the same as or somewhat higher than (Andree et al., 1992) that measured for the same headgroup compositions by ellipsometry on planar bilayers (Andree et al., 1990). The Ca^{2+} concentrations for half-maximal binding to 20, 5, and 1 mol % PS in PC were 0.16 ± 0.1, 3.2 ± 0.45, and 15.3 ± 3.4 mM, respectively, by tryptophan fluorescence (± sd, $n = 3$) and 0.22, 1.5, and 8.6 mM, respectively, by ellipsometry. Therefore, although there are Ca^{2+}-independent mechanisms of binding that do not involve tryptophan fluorescence changes (Meers & Mealy, 1993), increased tryptophan fluorescence is equivalent to Ca^{2+}-dependent binding to membrane phospholipids, and the assumption is made that the percentage of the maximal increase is proportional to the percentage of the protein bound. Application to micelles is discussed below.

Effect of Negative Charge on Phospholipid Binding. Annexin V had also been previously shown to bind in a Ca^{2+}-dependent manner to phosphatidylserine (PS) dispersed in micelles of the detergent $C_{12}E_8$ by tryptophan fluorescence (Meers and & Mealy, 1993). The requirements for observation of binding to micelles were studied further in experiments shown in Figure 1. It is clear that binding to phosphatidate (PA) is also observed, while no increase in

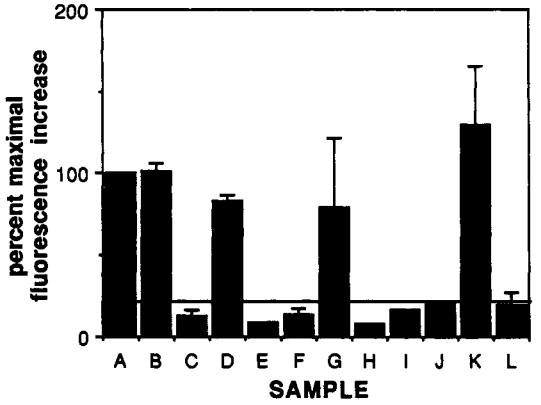

FIGURE 1: Binding of annexin V to phospholipids in micelles as measured by tryptophan fluorescence. Sample A represents the fluorescence increase in tryptophan upon annexin V binding to vesicles composed of PS/PC (1:3) induced by 100 μM or higher Ca^{2+} (normalized to 100%). Other samples contained 600 μM $C_{12}E_8$ (B–D) or 1.1 mM $C_{12}E_8$ (E–J) and 100 μM PS (B), 100 μM PC (C), 100 μM PA (D), 200 μM PC plus 50 μM OA (E), 200 μM PC plus 100 μM OA (F), 200 μM PC plus 250 μM OA (G), 250 μM OA alone (H), 50 μM PC plus 250 μM OA (I), 100 μM PC plus 250 μM OA (J), 200 μM PC plus 250 μM SDS (K), and 250 μM SDS alone (L). The annexin V concentration in all experiments was approximately 0.8 μM. All experiments were performed at 25 °C in 80 mM NaCl, 50 mM TES, and 0.1 mM EDTA (pH 8.0). Error bars, where they are shown, represent ± standard deviations for triplicate samples. The line on the graph represents the approximate percent increase due to Ca^{2+} addition in the absence of lipids, which generates a red shift only. Samples with OA, although measured as rapidly as possible, showed a slow but significant Ca^{2+}-dependent aggregation of micelles to larger light-scattering structures.

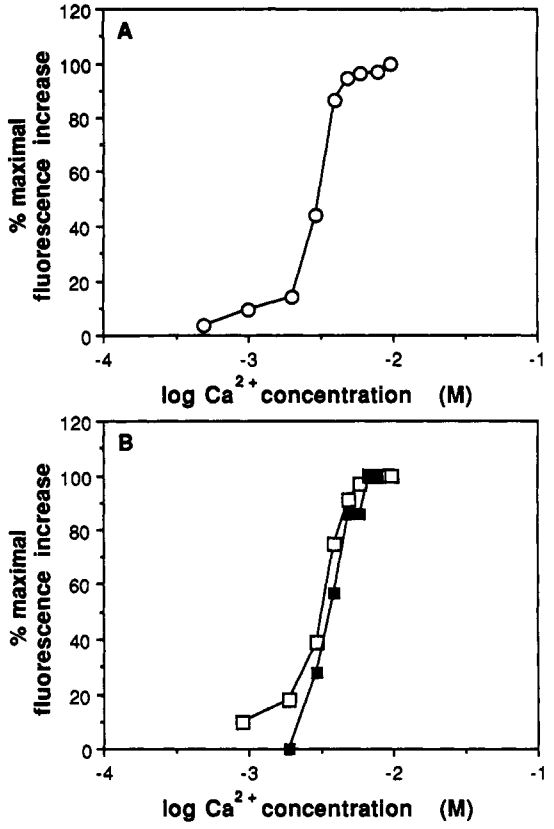

FIGURE 2: (A) Calcium dependence of annexin V binding to micelles. Samples contained 1.1 mM $C_{12}E_8$, 200 μM SDS, and 200 μM PC in buffer A. Annexin V was 0.8 μM. The percentage of the maximal increase in tryptophan fluorescence was plotted verus Ca^{2+} concentration. (B) Correlation of tryptophan fluorescence increase to binding as measured by fluorescence resonance energy transfer. Samples both contained 0.4 mM $C_{12}E_8$, 233 μM SDS, and 0.3 μM annexin V. Samples also contained either 30 μM PC (□) or 20 μM PC and 10 μM acyl-NBD-PC (■). The sample with PC alone was monitored by tryptophan fluorescence. The sample containing PC and acyl-NBD-PC was monitored by the observation of fluorescence resonance energy transfer from tryptophan to NBD (excitation 280 nm, emission 530 nm). The percentage of the maximal observed increase in emission intensity is plotted in each case. Experiments were performed in buffer A at 25 °C.

tryptophan fluorescence is observed for micelles containing phosphatidylcholine (PC) (Figure 1B–D). The latter result was the case even at hundreds of millimolar Ca^{2+} (data not shown). However, if sufficient oleic acid (OA) was included in the micelles with PC, a similar increase in tryptophan fluorescence was observed (Figure 1G). The fluorescence increase was dependent on both the OA (Figure 1E–G) and PC (Figure 1F,H–J) concentrations in the micelles, suggesting that a minimum number of each component per micelle may be necessary for binding. The requirement of negative charge in the micelles was nonspecific, as even sodium dodecyl sulfate (SDS) was able to elicit apparent binding to PC (Figure 1K,L). SDS was well below its critical micelle concentration in these experiments, and previously observed Ca^{2+}-independent changes in tryptophan fluorescence associated with denaturation (Meers, 1990) were not observed. Therefore, negative charge can be supplied by the phospholipid itself, a fatty acid, or an alkyl sulfate with similar binding results. In each case, the charge appears to nonspecifically enhance the affinity of annexin V for phospholipids.

Two types of experiments are shown in Figure 2 to further verify correspondence between binding to micelles (as opposed to membranes, discussed above) and the increased tryptophan emission intensity. In Figure 2A, the Ca^{2+} dependence of binding to a 1:1 combination of SDS and PC in $C_{12}E_8$ micelles at pH 7.4 is shown. A half-maximal increase in fluorescence required Ca^{2+} in the millimolar range, which is only slightly higher than that required for the same amount of PS alone in $C_{12}E_8$ (Meers & Mealy, 1993), possibly because of the slightly lower pH. After binding to the PC-containing micelles, acrylamide quenching was monitored by titration. A Stern–Volmer quenching constant of 6.9 M^{-1} was observed, consistent with the low aqueous exposure of tryptophan in previously studied annexin V–phospholipid membrane complexes (Meers & Mealy, 1993). Therefore, SDS substitutes well for the negative charge on the PS molecule in terms of Ca^{2+} dependence and mediates a PC–annexin complex in micelles similar to PS–annexin complexes and membrane complexes previously studied (Meers & Mealy, 1993).

In a second experiment, the fluorescence energy transfer from tryptophan to an NBD derivative of PC was compared to the increase in tryptophan emission intensity upon binding (Figure 2B). Since energy transfer depends on the close approach of tryptophan to the NBD group in the micelle, it reports binding directly. The Ca^{2+} dependence of binding measured by the two methods is identical. Therefore, this experiment also indicates that binding to micelles is measured accurately by tryptophan fluorescence alone.

Since the large increase in tryptophan fluorescence intensity used to monitor binding required the presence of negative surface charge in every case, it was still possible that binding in the absence of negative charge might occur without any change in the tryptophan fluorescence, i.e., false negatives would occur. In order to rule out this possibility, tryptophan fluorescence quenching experiments were performed. Because the annexin V tryptophan is exposed at the phospholipid-binding surface of the protein at the Ca^{2+} concentrations used (i.e., above 3 mM; Meers & Mealy, 1993), it is reasonable to expect that micelle-bound quenchers would decrease the

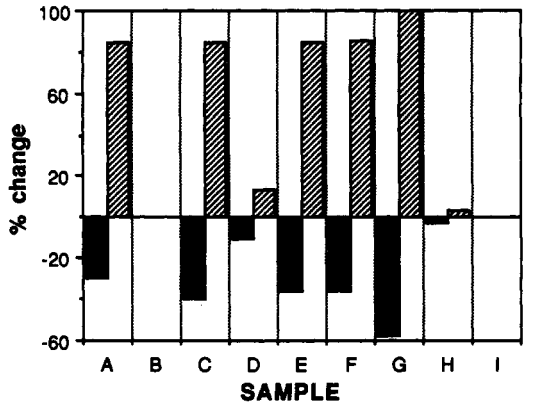

FIGURE 3: Binding to micelles as measured by quenchers of tryptophan fluorescence compared to binding measured by the fluorescence increase in the absence of quenchers. Quenching (filled bars) is expressed as the percentage decrease in fluorescence intensity compared to a nonquenching sample. All samples were in buffer A with 1.1 mM $C_{12}E_8$ and 0.8 μM annexin V. Quenching samples also contained (A) 700 μM SDS, 40 μM PC, and 10 μM acyl-NBD-PC; (B) 40 μM PC and 10 μM acyl-NBD-PC; (C) 700 μM SDS, 25 μM PC, and 25 μM acyl-NBD-PC; (D) 700 μM SDS and 25 μM NBD-PC; (E) 700 μM SDS and 50 μM 5-PC; (F) 700 μM SDS and 75 μM 5-PC; (G) 700 μM SDS and 100 μM 5-PC; (H) 700 μM SDS and 100 μM 5-DSA; and (I) 700 μM octyl glucoside, 40 μM PC, and 10 μM acyl-NBD-PC. Ca^{2+} was added to each sample (14 mM) and changes in the tryptophan fluorescence intensity at 345 nm were measured. In the nonquenching samples, acyl-NBD-PC or 5-PC was replaced by PC; 5-DSA was replaced by OA. The increase in tryptophan fluorescence in the nonquenching samples (hatched) was normalized by setting the maximal increase to 100 (about 2-fold in this system) and the minimal increase to 0 (about 1.16-fold in this system). The minimal 16% increase is solely due to a red shift not associated with annexin V binding to phospholipids (Meers & Mealy, 1993).

fluorescence of micelle-bound annexin V under any circumstances. Therefore, the quenching of annexin V fluorescence by micelle-bound quenchers was compared to the fluorescence increase observed in similar nonquenching systems. Quenching experiments, using an NBD derivative of PC that quenches through energy transfer and a nitroxide derivative of PC that quenches through a collisional mechanism, were compared to the fluorescence response of annexin V when binding to unlabeled lipids. When annexin V bound to micelles containing acyl-NBD-PC in the presence of sufficient SDS, a significant quenching of the tryptophan fluorescence was observed (Figure 3A,C). However, in the absence of SDS, the tryptophan fluorescence was not quenched (Figure 3B).

When the amount of total PC in the micelle was too low, lower binding was reflected by both less quenching and a smaller fluorescence increase in the nonquenching system (Figure 3D). This correlation also held for quenching by a spin-labeled derivative of PC with a doxyl group at the 5-position of the *sn*-2 stearic acid acyl chain (Figure 3E–G). When a nonionic detergent, octyl glucoside, was substituted for SDS, no binding was observed (Figure 3I), suggesting that dilution of the large hydrophilic poly(oxyethylene) groups of $C_{12}E_8$ is not a factor that, by itself, allows annexin binding. In every case, the magnitude of the increase in tryptophan fluorescence of the nonquenching system correlated with the magnitude of quenching in the corresponding system with quenchers. Therefore, this constitutes further evidence that the large increase in annexin V tryptophan fluorescence, upon binding to nonquenching micelles, does indeed reflect binding and, more importantly, that an absence of an intensity increase reflects a lack of binding. This also confirms the fact that annexin V binds to PC in $C_{12}E_8$ micelles only in the presence of negatively charged amphiphiles.

Another question of interest, at this point, was whether Ca^{2+} induces annexin V to bind nonspecifically to negatively charged surfaces in the absence of phospholipids. It appears that this is not the case in the systems studied here. No Ca^{2+}-dependent increase in tryptophan fluorescence was observed in controls for Figure 1 (H and L) involving oleic acid/$C_{12}E_8$ micelles or SDS/$C_{12}E_8$ micelles without phospholipids. Also, when 5-doxylstearic acid (5-DSA) was incorporated into $C_{12}E_8$ micelles, no Ca^{2+}-dependent quenching of tryptophan fluorescence was observed (Figure 3H). Furthermore, little or no quenching of tryptophan fluorescence by acyl-NBD-PC was observed below a certain threshold number of phospholipids per micelles (see below), despite the presence of a large amount of negatively charged amphiphile and sufficient quencher to efficiently quench any bound annexin V (compare Figure 3C and 3D). Therefore, annexin V does not appear to bind directly to SDS or OA alone under these conditions and requires a minimal number of phospholipid molecules per micelle.

Stoichiometry of Phospholipid Binding. The fact that a minimal number of phospholipids per micelle appeared to be required for binding suggested that a minimal stoichiometry for annexin V could be measured. Since mixed micelles of SDS and $C_{12}E_8$ were to be used for these experiments, the effect on critical micelle concentration (cmc) and micelle size was examined in Figure 4. $C_{12}E_8$ alone forms micelles of approximately 120–125 molecules (Tanford et al., 1977) with a cmc of approximately 50–100 μM. The cmc's for the mixed phospholipid/detergent micelles used in this study were estimated using the excimer-to-momomer ratio of an acyl chain pyrene derivative of phosphatidylcholine, designated pPC. In Figure 4A, it is clear that the cmc for this system occurs at approximately 80 μM detergent at the given phospholipid/detergent ratios (approximately five phospholipids per micelle). This cmc value was not changed significantly in the presence of a large amount of SDS. A small shift of the curve to the left in the presence of SDS above the cmc is a reflection of the incorporation of SDS acyl chains into the micelles, decreasing the number of pPC molecules per micelle.

A mixture of a rhodamine-labeled derivative of phosphatidylethanolamine and an NBD acyl-chain derivative of PC was used to assess effects on micelle size. The NBD and rhodamine probes form a fluorescence resonance energy transfer pair, such that the rhodamine probe quenches the NBD fluorescence under these conditions. When the number of rhodamine phospholipids drops below 1–2 per micelle, an increase in NBD fluorescence is expected. In Figure 4B, the NBD fluorescence is shown as a function of detergent concentration. In the case of $C_{12}E_8$ alone, NBD fluorescence begins to increase at a threshold concentration of detergent well above the cmc. The arrows show the concentration at which the average micelles contain only two or one molecule(s) of Rh-PE, with the assumption that there are 120 acyl chains per micelle and that the cmc is 80 μM. There is a relatively linear increase in NBD fluorescence between two and one Rh-PE per micelle and an apparent change in the slope at about one Rh-PE per micelle, suggesting that quenching is complete only above an average of two or more Rh-PE per micelle. Although the exact micelle size cannot be calculated from this experiment, the observed behavior is reasonable because it is likely that, at only one quencher per micelle, a significant proportion of the micelles will have no quenchers, due to the inhomogeneity of the distribution. Furthermore, even if all of the micelles were identical in size and composition, a single Rh-PE may not be sufficient to quench completely from all relative positions within the micelle.

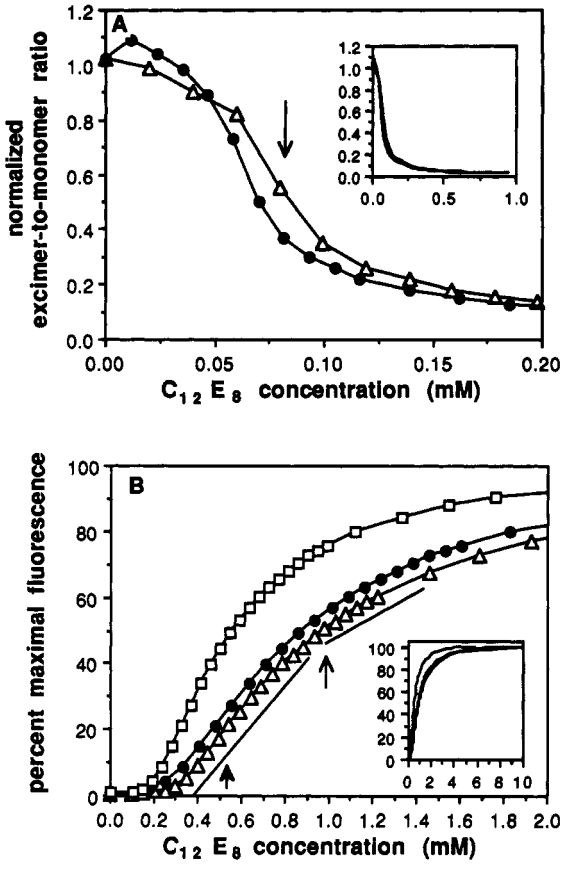

FIGURE 4: Effect of SDS on critical micelle concentration and micelle size. (A) Vesicles composed of pyrene-PC/PC at a ratio of 1:4 and a total phospholipid concentration of 20 μM were titrated with $C_{12}E_8$ (△) or a stock of $C_{12}E_8$ mixed with SDS at a ratio of 1:0.7 (●). Samples were excited at 344 nm. When titrated with detergent, the sample was allowed to mix for 2 min, after which time emission was immediately measured at 377 nm for monomer intensity and 480 nm for excimer intensity. Experiments were performed at 25 °C in buffer A under ambient O_2 conditions. Data are plotted in terms of the total $C_{12}E_8$ concentration and are normalized to 1.0 for the maximum excimer-to-monomer ratio observed in the vesicles before detergent addition. All of the data taken are plotted on a larger scale in the inset. The arrow designates the critical micelle concentration. (B) Vesicles were prepared by sonication (bath type, Laboratory Supplies Co., Hicksville, NY) of acyl-NBD-PC, Rh-PE, and PC in a ratio of 1:5:20, respectively, at a total phospholipid concentration of 260 μM in buffer A. This stock was diluted to 40 μM total phosholipid and titrated with $C_{12}E_8$ (△), or 100 μM $C_{12}E_8$ was added and then the sample was titrated with a stock of $C_{12}E_8$ and SDS in a ratio of 1:0.7. The latter titration was plotted in terms of the total $C_{12}E_8$ concentration (□) or the total hydrocarbon chain concentration of SDS and $C_{12}E_8$ combined (●). Excitation of NBD was at 450 nm, and emission intensity was monitored at 530 nm. The data are normalized to the maximal observed fluorescence intensity at the highest detergent concentrations. These data can be observed in the inset, which is plotted on a larger scale. The arrow on the left indicates the point at which micelles contain an average of 2 Rh-PE per micelle; the arrow on the right indicates 1 Rh-PE per micelle calculated on the basis of an aggregation number of 120 and a cmc of 80 μM $C_{12}E_8$.

The data with SDS differed from those of $C_{12}E_8$ alone if the curves were both plotted in terms of the total $C_{12}E_8$ concentration. This was due to dilution of the micelles by SDS. However, the curves are identical when plotted in terms of the total hydrocarbon chain concentration, assuming that all of the SDS contributes to the micelles. This strongly suggests that the micelle size stays approximately the same in the presence of SDS and that each SDS molecule contributes one alkyl chain to the micelles. This behavior is not completely unexpected, as SDS has the same alkyl chain length as $C_{12}E_8$. Its aggregation number under these conditions is approximately 90 (Mysels & Princen, 1959), which is similar to $C_{12}E_8$. In all experiments, $C_{12}E_8$ is in excess of SDS, SDS is well below its critical micelle concentration, and SDS is added to samples after the formation of $C_{12}E_8$ micelles. Therefore, the assumptions are made in subsequent experiments that all micelles have an aggregation number of 120 total hydrocarbon chains (Tanford et al., 1977), including all SDS or phospholipid molecules, and that there is a constant $C_{12}E_8$ monomer concentration of 80 μM.

Using these numbers, it is possible to estimate the average number of phospholipids per micelle necessary for annexin V binding. This was accomplished by measuring the Ca^{2+}-dependent change in the tryptophan fluorescence intensity as a function of the phospholipid content of the micelles. Since the observation of binding to PC required the presence of a negatively charged amphiphile, SDS was used in all of the experiments shown in Figure 5A. The amount of SDS per micelle was varied as well as the PC concentration. These experiments show that increases in the percentages of SDS in the micelles minimized the number of PC molecules per micelle necessary for annexin V binding. By approximately 40 mol % SDS, the minimum requirement for phospholipids per micelles appears to have been reached. At the highest mole percentages of SDS used, the required average number of PC molecules per micelle appeared to reach a minimum of approximately three (see data in Figure 6A). Binding in all cases was highly cooperative. The estimated Hill coefficient at the highest SDS concentration was also 3 (Figure 6A) when plotted in terms of the estimated PC per micelle.

Figure 5B shows that, in the case of phosphatidylserine (PS) alone in $C_{12}E_8$ micelles, approximately 10 phospholipid molecules per micelle are sufficient for binding. SDS also minimized the PS per micelle required for annexin V binding (Figure 5B). As for PC, an average of approximately 3–4 phospholipids per micelle was required for complete binding (see data in Figure 6B), although there may be a slightly greater requirement for PS. In Figure 5A,B, the micelles are in a greater than 6-fold excess over the protein. Therefore, it is possible that a portion of the micelles, significant with respect to the protein concentration, may have a larger than average number of phospholipids, suggesting that the average may be regarded as a minimum binding stoichiometry. Therefore, annexin V binding appears to require at least approximately three phospholipids with little or no headgroup specificity. Negative surface charge modulates the avidity of the annexin V–phospholipid interaction, decreasing the two-dimensional concentration of phospholipid necessary for binding. This is at least partly the result of an increase in the effective Ca^{2+} concentration at the micelle surface due to negative charge (Meers & Mealy, 1993).

The stoichiometry of binding was also studied by labeling with a photoreactive phospholipid derivative (Figure 7). Micelles of $C_{12}E_8$, SDS, and *N*-(azidobenzoyl)phosphatidylethanolamine (AB-PE) were induced to bind to annexin V with Ca^{2+} under conditions similar to those used in the tryptophan fluorescence experiments of Figures 5 and 6, where all of the protein is bound to the micelles. The assumption is made that all of the annexin V molecules are dynamically bound to phospholipids via specific binding sites under these conditions. After exposure to high-intensity UV light, the annexin V was extracted from the detergent/phospholipid mixtures, and the amount of covalently bound phospholipid was assessed by protein and phosphate assays. The number of total phospholipid molecules per annexin V appeared to have a constant value of approximately 4–5 at an increasing total AB-PE concentration or number of AB-PE per micelle.

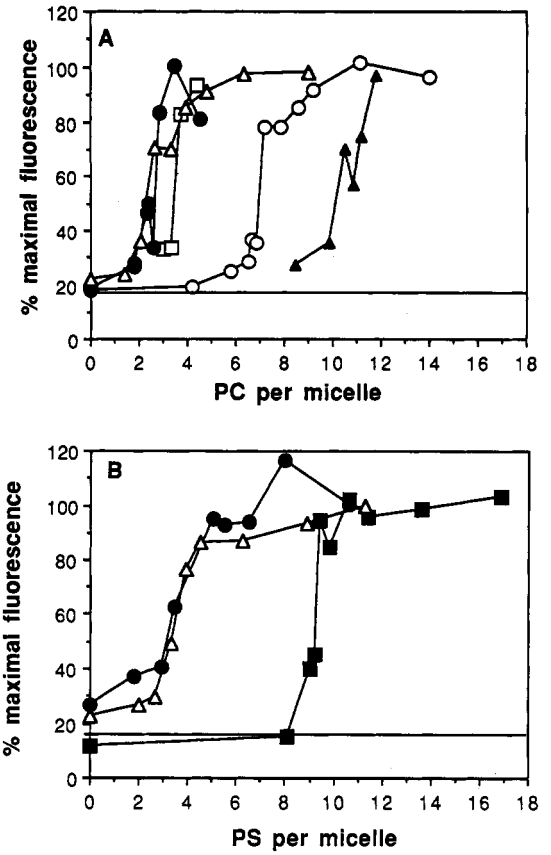

FIGURE 5: Effect of negative charge on the number of phospholipid molecules per micelle required for annexin V binding. (A) All samples contained 1.1 mM $C_{12}E_8$ except for one (●), which contained 400 μM. All samples contained 0.8 μM annexin V and were in buffer A at 25 °C. Samples also contained 200 (16 mol % of total detergent, ▲), 300 (23 mol %, ○), 500 (33 mol %, □), 700 (41 mol %, △), or 270 μM (46 mol %, ●) SDS. Tryptophan fluorescence emission spectra were taken for buffer only, after the addition of detergents and lipids, after the addition of annexin V, and after the addition of 14 mM Ca^{2+}. The intensity at 345 nm was measured after Ca^{2+} addition and compared to that before Ca^{2+} addition (corrected for any detergent or lipid background). For comparison of the curves, the Ca^{2+}-induced increase in tryptophan fluorescence was normalized separately for each SDS concentration, setting the approximate maximal observed intensity increase to 100%. The actual maximal intensity increases at the highest SDS concentrations were approximately two-thirds of those at the lowest concentrations, probably due to partial quenching by the micellar SDS when the protein binds. The average number of phospholipids per micelle was calculated by assuming that each micelle was composed of 120 acyl chains contributed proportionally by the various detergents and phospholipids. It was also assumed that 80 μM $C_{12}E_8$ remained in monomer form. Estimated Hill coefficients, plotted in terms of phospholipid/micelle, were 3 (46 mol % SDS), 8 (23 mol % SDS), and 11 (16 mol % SDS). (B) Experiments were performed as above with the following exceptions. PS was the phospholipid instead of PC. Samples contained 1.1 mM $C_{12}E_8$ (■, △) or 400 μM $C_{12}E_8$ (●). SDS concentrations were 0 (■), 700 (41 mol % of total detergent, △), or 270 μM (46 mol %, ●). Estimated Hill coefficients were 11, 3, and 3, respectively.

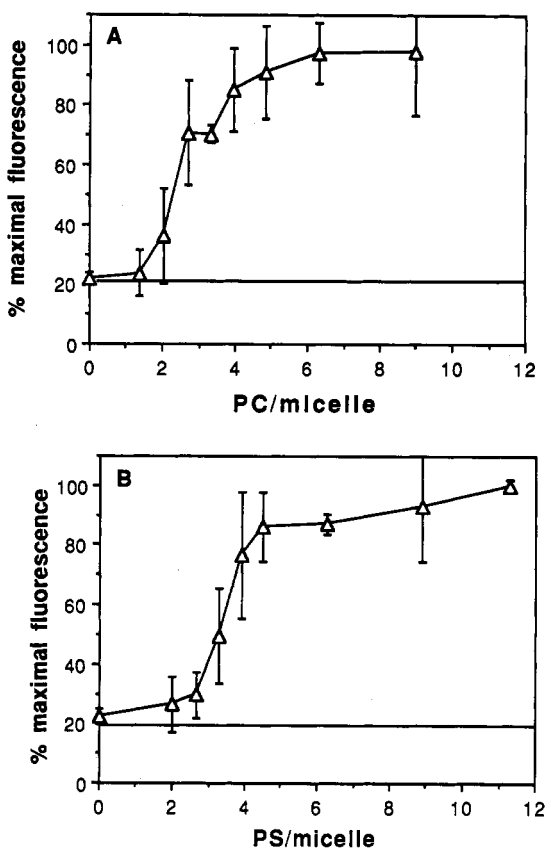

FIGURE 6: Minimal average number of PC (A) or PS (B) molecules per micelle required for annexin V binding. Conditions and data are as described for Figure 5. Only the 41 mol % SDS curves are shown. Error bars represent ± sd, n = 3 or 4.

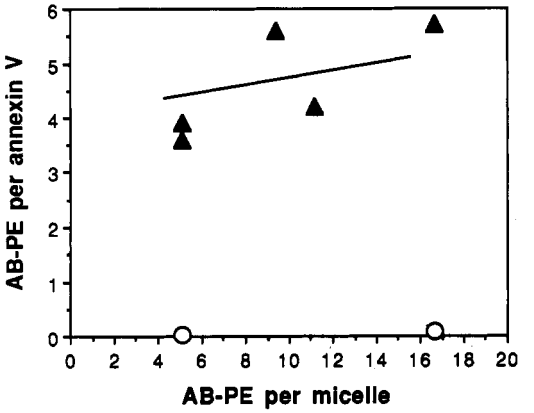

FIGURE 7: Number of photoreactive phospholipids covalently bound to annexin V as a function of the total phospholipid concentration. Samples contained 4.2 mM $C_{12}E_8$, 2.8 mM SDS, AB-PE ranging from 0.28 to 1.12 mM, and 23 μM annexin V in a total of either 0.3 or 0.6 mL. Ca^{2+} was added to a final concentration of 15 mM, and the samples were allowed to incubate for at least 1 min. Samples were then exposed to high-intensity UV light. After exposure, samples were extracted to remove detergent and unbound phospholipid as described in Materials and Methods. The ratio of phosphate to protein concentrations is plotted versus the estimated average number of phospholipids per micelle during the photoreaction (▲). Some samples were extracted without Ca^{2+} and without exposure to UV light (○). The line is drawn arbitrarily.

Controls without Ca^{2+} and without UV exposure showed negligible amounts of phospholipid. The fact that nearly all of the phospholipids in the micelle label the protein when there are approximately four per micelle suggest that annexin V may sequester the phospholipids, originally randomly distributed around the micelle, into specific binding sites. If all of the phospholipids within a micelle could react nonspecifically with the bound annexin, one would expect higher labeling, at 16 per micelle for instance (Figure 7). Therefore, this probe may specifically label the phospholipid-binding sites of annexin V in a stoichiometry not much higher than the minimal number of phospholipids required per micelle for binding. Futher studies to determine the sites of labeling by proteolytic fragmentation are the only way to be certain about the specificity of the labeling for definite sites on the protein. However, the fairly good agreement between the photolabeling and micelle-binding results as a function of phospholipids per micelle suggests that a limited number of specific sites is labeled.

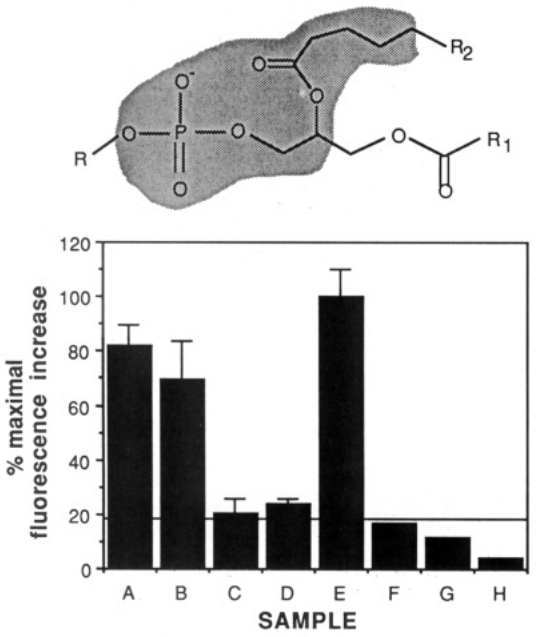

FIGURE 8: Effect of structural modifications of phospholipid ligands on binding to annexin V. All samples contained 0.8 μM annexin V. $C_{12}E_8$ was 1.1 mM (A–G) or 0 (H). SDS was 700 (A–E), 200 (F, G), or 0 μM (H). Samples also contained 100 μM DLPC (A), 100 μM DNPC (B), 100 μM lyso-PC (C), 100 μM DOG (D), 100 μM PC (E), 200 μM 1,2-dihexanoylglycerol (F), 200 μM dihexanoyl-PC (G), or 500 mM L-α-glycerophosphate (H). Ca^{2+} (14 mM) was added to induce binding. The increase of fluorescence in sample E (PC in SDS/$C_{12}E_8$ micelles) was defined as 100%. The line on the graph denotes the approximate increase in fluorescence obtained by inducing a red shift in the tryptophan fluorescence by adding Ca^{2+} in the absence of phospholipids (Meers & Mealy, 1993). Error bars, where shown, denote ± sd, $n = 3$. A model of a phospholipid is shown with some of the putative areas of interaction with annexin V (R = headgroup, R_1 = *sn*-1 acyl chain, R_2 = part of the *sn*-2 acyl chain).

Specificity of the Phospholipid-Binding Sites. Further experiments were performed to begin to elucidate details of the structural specificity of the phospholipid-binding sites. Figure 3 has already indicated a lack of binding to free fatty acids and efficient binding to a derivative of PC with a large modification at the 5-position of the *sn*-2 acyl chain. In Figure 8, the efficiency of binding to some other phospholipid derivatives is shown. The absence of the *sn*-2 acyl chain inhibited binding, as did an acyl chain that was too short. Annexin V was able to bind to an intermediate chain length derivative, dinonanoyl-PC, but not to dihexanoyl-PC, under the conditions of the experiment. It was somewhat surprising that binding by dihexanoyl-PC was not observed, despite the fact that a doxyl group at the 5-position was accommodated. A nonspecific hydrophobic interaction may be necessary at this location. It is also possible that the dihexanoyl-PC was less than totally partitioned into the micelles. The phosphate group also appears to be required for binding, as 1,2-dioleoylglycerol did not seem to bind. No evidence of glycerophosphate binding was observed at the concentration used, but micelles were absent, and immobilization of tryptophan at the surface of an aggregate is needed for the fluorescence increase to be observed (Meers & Mealy, 1993). The observations in Figure 1 and previous papers (Andree et al., 1990; Meers & Mealy, 1993) demonstrate that the phospholipid headgroup is relatively unimportant in terms of the annexin phospholipid-binding site. Therefore, some of the relevant parts of the phospholipid molecule for annexin V binding range from the phosphate group to the ester linkage, and possibly the first few carbons of the *sn*-2 acyl chain (Figure 8).

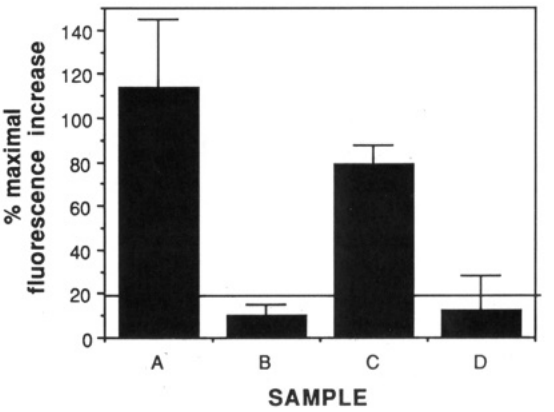

FIGURE 9: Effect of dilution of phospholipids after binding to annexin V. Samples contained 600 μM $C_{12}E_8$ and 100 μM PS (A, B) or 100 μM PA (C, D). The increase in fluorescence intensity due to the addition of 14 mM Ca^{2+} was normalized as in Figure 1. The excess tryptophan fluorescence (over initial, before Ca^{2+}) is shown after the addition of Ca^{2+} alone (A, C) and upon the subsequent addition of 11 mM $C_{12}E_8$ (B, D). Data are shown ± sd, $n = 3$. The line represents the Ca^{2+}-dependent increase in tryptophan fluorescence in the absence of lipids due to a red shift (Meers & Mealy, 1993).

Exchange of Phospholipids from Annexin V-Binding Sites. The exchange of an annexin molecule from one vesicle membrane to another has been shown to be quite slow (Meers et al., 1991). However, the exchange of individual phospholipids from annexin-binding sites has not been studied. Rapid exchange was demonstrated by first binding annexin V to phospholipids in micelles and then adding more detergent to dilute the phospholipid to below the threshold number of phospholipids per micelle. If the annexin-phospholipid complexes dissociate, the tryptophan fluorescence (corrected for background from the added detergent) would drop to its initial level. In Figure 9, it is clear that this is the case for both PS and PA. After the addition of excess $C_{12}E_8$, the tryptophan fluorescence returned to the level found in the absence of phospholipids. This dissociation of the annexin from phospholipids was faster than a few seconds. Therefore, the binding of phospholipids to individual sites on annexin V is relatively weak, and exchange occurs rapidly under these conditions.

DISCUSSION

Relationship between Domains and Phospholipid-Binding Sites. The number of phospholipid-binding sites on the annexins has been difficult to determine by conventional crystallographic methods because of the apparently low affinity of annexins for monomeric short-chain phospholipids (Tait et al., 1989; Huber et al., 1992). Two-dimensional crystals of annexin V on phospholipid surfaces give only low-resolution structural data (Brisson et al., 1991). The fluorescence studies presented here can give detailed information on the structural specificity and stoichiometry of annexin V phospholipid-binding sites. By diluting the phospholipid ligands in detergent micelles, it was possible to obtain a relatively direct estimation of the minimum number of phospholipid-binding sites necessary for annexin V binding. The direct observation of approximately 3–5 binding sites per annexin V monomer is logical in terms of what is known about the structure of the protein. Annexin V consists of four similar domains that comprise five helices each: four approximately perpendicular to the surface of the membrane and one parallel. Together they form the "endonexin fold" thought to be responsible for annexin binding to Ca^{2+} and (less clearly) for binding to phospholipids (Huber et al., 1990). Our data suggest that each domain binds specifically to a single phospholipid

molecule. It has also been suggested that phospholipid binding may reside in, or require, only three domains (Ernst et al., 1991). Our data are not sufficiently accurate to distinguish between three and four binding sites per annexin monomer and are consistent with both suggestions. The existence of four sites (though only three may be necessary) is particularly appealing based on the general structural similarity of the four consensus sequence regions (Concha et al., 1993). The relatively high apparent cooperativity of binding (Figure 5) suggests that annexin V does not bind to a membrane or a micelle until all of the binding sites can be filled.

Nonspecific Interactions with Negative Charge. One of the most important facts revealed by these studies is the distinction between nonspecific and specific binding factors. From the data presented, it is clear that the binding of annexin V involves several phospholipid-specific binding sites as well as a nonspecific interaction with negative charge. Therefore, maximally efficient annexin V membrane binding nonspecifically requires negative surface charge to increase the local Ca^{2+} concentration at the micelle surface (to several millimolar; Meers & Mealy, 1993) and probably to interact directly with the annexin V molecule itself. The apparently inconsistent ability of annexin V to bind to pure PC membranes (Andree et al., 1990; Meers & Mealy, 1993), as opposed to the lack of binding to PC in micelles, is probably the result of a higher surface concentration of phospholipid and possibly a small amount of contaminating negatively charged fatty acid in the bilayer studies.

While the data presented contribute information on the specificity and mechanism of binding to phospholipids, the interaction with negative charge is more nebulous. Though it is possible that the effect of negative charge is exclusively due to an increased surface concentration of Ca^{2+} (Meers & Mealy, 1993), the fact that binding to PC in micelles was observed only in the presence of negatively charged amphiphiles (see Figure 1 data), even at very high Ca^{2+} concentrations, suggests a direct interaction between annexin V and the negative surface charge. There are several ways in which the surface charge could modulate affinity. Annexin V appears to have several Ca^{2+}-binding sites localized to one surface of the molecule (Huber et al., 1990). It has also been observed that at least one crystal form of annexin V (Huber et al., 1990) has a large associated dipole moment. It is possible that the positively charged Ca^{2+} sites and/or the dipole moment are involved in attracting and/or orienting the annexin V molecule as it approaches the membrane surface in such a way as to optimize binding to phospholipids. This would be consistent with the suggestion of Karshikov et al. (1992) that the positively charged Ca^{2+}-binding surface of the protein would be important for this interaction. However, a negatively charged phospholipid headgroup probably is not necessary, contrary to the suggestions of these authors. The structure of the membrane-bound form of annexin V is at least somewhat different (Meers & Mealy, 1993; Concha et al., 1993) from the structure analyzed (Karshikov et al., 1992), especially in the third repeated domain. Therefore, some caution must be exercised, at this point, in interpreting the electrostatic potential of the protein. Nonetheless, an attracting or orienting role for the negative charge would be consistent with the data presented here.

Although no evidence of nonspecific Ca^{2+}-dependent binding to negatively charged surfaces alone was observed in our studies, it would not be unreasonable to expect low-affinity binding to such sites. This may explain data suggesting the direct binding of annexin IV to fatty acids (Edwards & Crumpton, 1991). If there is, in fact, a nonspecific electrostatic attraction (as opposed to orientation alone) of annexin V to negatively charged surfaces, then induced clustering of negatively charged amphiphiles under the protein would also be expected. Besides attraction or orientation of the annexin as it approaches the membrane or micelle surface, it is also possible that interaction with a negatively charged surface structurally modifies the specific phospholipid binding sites in the protein by conformational change, so as to enhance affinity. This would seem to be a less likely factor, because phospholipid ligands clearly dissociate easily from the protein on dilution with detergent (Figure 9 data). In conclusion, annexin V appears to utilize negative surface charge to increase the surface concentration of Ca^{2+}, and it also probably has a nonspecific interaction that enhances affinity at any Ca^{2+} concentration.

Affinity of Individual Phospholipid-Binding Sites Is Low. We have previously shown that the intrinsic affinity of annexin V for Ca^{2+} is relatively low, requiring approximately 3 mM Ca^{2+} for half-maximal binding (Meers & Mealy, 1993). The fact that annexin V does not bind to micelles with less than 3–4 phospholipids suggests that the intrinsic binding affinity for phospholipids is also quite low on an individual binding site basis. The bulk concentration of phospholipid in the experiments above was generally in the range of 10 μM or greater. Therefore, the intrinsic binding constants for individual phospholipid-binding sites on the annexin V molecule cannot be greater than 10^5 M^{-1}, in agreement with previous results (Tait et al., 1989). Annexin V binding does occur at a phospholipid surface concentration of as little as approximately 100 mM, as calculated by treating the micellar surface as a region of a finite thickness of 1 nm [as in Mosior and McLaughlin (1992)] and determining the phospholipid concentration in a shell of that thickness surrounding micellar oblate ellipsoids with principal axes of 2.7 and 1.3 nm (Tanford et al., 1977). Therefore, the binding constants for individual sites are greater than 10 M^{-1}. The low affinity of individual binding sites as compared to multiple sites [e.g., Tait et al. (1989) and Andree et al. (1990)] may be explained by the fact that, after binding of the first site, other sites experience an effectively very high ligand concentration at the membrane surface. Therefore, despite the relatively low affinity of individual sites, the combination of several sites can greatly enhance the apparent affinity for membranes or surfaces by reduction of dimensionality as discussed in a number of papers [e.g., Lentz and Hermans in Cutsforth et al. (1989), Kim et al. (1991), and Mosior and McLaughlin (1992)].

If an apparent association constant is defined as

$$K_{app} = [\text{bound protein}]/[\text{free protein}][\text{free phospholipid}]$$

one can then make a simple calculation using standard methods for analyzing multiple site binding [e.g., see Mosior and McLaughlin (1991)], a surface phospholipid concentration for all but the first binding event, and assuming four equivalent phospholipid binding sites per annexin V. This gives

$$K_{app} = k(4 + 6k[L]_s + 4k^2[L]_s^2 + k^3[L]_s^3)$$

with k equal to the equivalent intrinsic binding constants for each site and $[L]_s$ equal to the surface concentration of phospholipid (100 mM), one obtains K_{app} equal to 2.9×10^4, 10^9, and 10^{13} M^{-1} for intrinsic individual binding constants of 10^2, 10^3, and 10^4 M^{-1}, respectively. Electrostatic effects may also increase apparent affinity and give apparent cooperativity. These effects have been saturated by including an excess of

negatively charged amphiphile in most of these experiments. A precise modeling of binding will clearly require much more data. However, a high-affinity multisite binding is consistent with the previously observed high-affinity binding (Andree et al., 1990; Tait et al., 1989).

High individual association constants would actually be detrimental to the action of annexin V as a membrane-binding protein. For instance, Andree et al. (1990) estimated that the overall association constant for annexin V binding to phospholipid membranes is approximately 10^{10} M^{-1}. If *individual* binding sites had such a strong affinity for phospholipids, one would expect that the protein would bind to phospholipid monomers, since the critical micelle concentration of long-chain phospholipids is in this range. This could actually inhibit binding to membranes if the vesicle concentration were lower than the monomer concentration, i.e., if the total phospholipid concentration were less than about 10 μM for large unilamellar vesicles (approximately 0.1–0.2 μm in diameter). Annexins are designed to bind not to phospholipid monomers, but to membranes.

A working hypothesis for annexin V binding, based on these data, should now include a nonspecific enhancement of binding by negatively charged surfaces, approximately four phospholipid-specific binding sites with relatively weak individual binding constants and rapid exchange between bound and free phospholipids of various headgroups in the membrane. This latter point would be consistent with the observation of annexin V binding to PC, but only at very high Ca^{2+} concentrations or in the presence of acidic phospholipids (Andree et al., 1990; Meers et al., 1991; Meers & Mealy, 1993).

Specificity of the Phospholipid-Binding Site. The data presented here suggest some of the characteristics of the specific phospholipid binding sites. On the basis of the structure of the Ca^{2+}-binding sites, a similarity to phospholipase A_2 has been suggested (Huber et al., 1992). In the phospholipase A_2-binding site, a Ca^{2+} ion binds to protein ligands as well as coordinating a phosphate oxygen and *sn*-2 acyl chain ester carbonyl oxygen (Scott et al., 1990). The direct measurements of annexin V binding to various phospholipids and phospholipid analogs reported here are consistent with such a binding site. Clearly the headgroup of the phospholipid makes little difference in the intrinsic binding parameters. Differences in acyl chains also make little difference up to a certain point. A few carbons at the *sn*-2 acyl chain appear to be helpful for binding, but the phosphate group and glycerol backbone are necessary. Therefore, the characteristics of annexin V binding, as studied thus far, closely imitate those of phospholipase A_2. It is still unknown whether other annexins have similar phospholipid-binding sites, but the strong homology in these regions of the sequences suggests that they do. Preliminary data (P. Meers and T. Mealy, unpublished results) show that annexin I can also bind to pure PC vesicles in a Ca^{2+}-dependent manner, suggesting the same lack of headgroup specificity for this annexin. Further analysis of other phospholipid analogs, particularly other *sn*-1 and *sn*-2 acyl chain lengths, phosphate group analogs, and ester analogs, will give a clearer insight into the detailed structure of the annexin V phospholipid-binding site.

Physiological Significance. The lack of headgroup specificity for annexin V phospholipid-binding sites may have an important physiological consequence. If the annexin were specific for acidic phospholipids, it would be expected that the cytoplasmic faces of cellular membranes would be the exclusive membrane-binding domain for this protein, since most acidic phospholipids are concentrated on the cytoplasmic side of membranes. However, the fact that binding to PC is quite avid in the presence of negative surface charge suggests that the extracellular faces of biological membranes may serve as sites for annexin binding as well. Negative surface charge may be provided by acidic carbohydrates or polypeptides on the exterior cell surface. The avidity of annexin binding may also be modulated by regulated changes in the effective surface charge, such as the regulated exposure of negatively charged phospholipids. Further studies on localization and physiological functions of the annexins are clearly warranted.

Thus, annexins are exquisitely designed for binding at membrane surfaces, with binding sites concentrated on one side of the molecule to take advantage of the two-dimensional nature of the membrane. Membrane binding is high-affinity because (1) binding is nonspecific enough to take advantage of most or all of the phospholipids in the membrane; (2) individual binding sites are designed to be of low affinity to allow rapid exchange between all the phospholipids in the membrane; (3) multiple binding sites on the protein, however, greatly increase the overall affinity; and (4) annexins take advantage of the locally high concentrations of Ca^{2+} induced by negative surface charge and the locally high concentration of phospholipids that occurs only near membranes.

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