Phospholipid Determinants for Annexin V Binding Sites and the Role of Tryptophan 187†

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ABSTRACT: Annexin V is part of a family of Ca^{2+}-dependent phospholipid-binding proteins, whose purported functions are related to their interactions with biological membranes. While Ca^{2+}-dependent binding to phospholipids is well-established, the specific structural interactions within the phospholipid-binding sites have only been inferred to resemble those of phospholipase A_2, with no direct structural evidence. In this study, the binding avidity of various phospholipid analogs, with variations at the headgroup or *sn*-2 acyl chain, was monitored in a $C_{12}E_8$ detergent micelle system using the increase in fluorescence of tryptophan 187. Micelles also contained excess negative surface charge to saturate a nonspecific component of the binding. The Ca^{2+} and phospholipid concentrations required for the binding of annexin V to various phospholipid headgroups were very similar, except for the relatively weak binding to phosphatidylinositol (PI). The unique close proximity of the PI sugar ring to the phosphate group may lead to steric hindrance in this case. Binding was also strongly dependent on the presence of an *sn*-3 phosphate group and an *sn*-2 acyl chain, as previously observed. The relatively shallow nature of the annexin V phospholipid-binding sites was reflected by the nearly equivalent binding of D and L versions of phospholipids, i.e., a large shift in the position of the *sn*-1 acyl chain is accommodated in this case. Binding of annexin V does not specifically require an ester carbonyl oxygen, as it occurs with ether-linked, amide-linked, and phosphonate-linked *sn*-2 hydrocarbon chains, under these conditions. Binding to an analog without an electronegative group at the *sn*-2 position (hexadecylphosphocholine) was observed to be of intermediate avidity, indicating the importance of the hydrophobic nature of the *sn*-2 acyl chain as well as electronegative groups at the linkage position. Further support for hydrophobic interactions came from the dependence of binding on the lengths of the *sn*-2 acyl chains of other analogs. Consistent with such limited hydrophobic interactions is the observation that the maximal fluorescence of tryptophan 187 was greatly affected by the nature of the *sn*-2 acyl chain. Competitive binding between known specific ligands and a phospholipid derivative with an *sn*-2 quenching group further suggests that tryptophan 187 makes contact with specifically bound phospholipids, probably at the *sn*-2 acyl chain. Thus, hydrophobic interactions at the *sn*-2 acyl chain, along with interactions at the phosphate group and possibly the ester carbonyl oxygen, appear to account for the major phospholipid determinants for annexin V binding.

Annexin V is a Ca^{2+}-dependent membrane-binding protein that may function in the phospholipid-dependent inhibition of blood coagulation (Reutelingsperger et al., 1985; Funakoshi et al., 1987), in the 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 other references and Creutz (1992) for a review]. These putative physiological functions all depend on the Ca^{2+}-induced interaction of annexins with phospholipids.

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+}-dependent phospholipid binding resides in the repeated regions [see references 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).

Structural characterization of annexin V, crystallized in the absence of phospholipids (Huber et al., 1990, 1992; Concha et al., 1993; Sopkova et al., 1993), showed a four-domain structure with several Ca^{2+} ions along the same ("convex") surface as the consensus sequence tryptophan (located in the third domain). These results, taken together with the previous demonstration of contact of the consensus sequence tryptophan (Trp 187) of annexin V with membrane phospholipids (Meers, 1990), strongly suggested that this convex surface of annexin V binds to the membrane. Ca^{2+} binding to annexin V alone results in a conformational change that exposes Trp 187 (Meers & Mealy, 1993a; Concha et al., 1993; Sopkova et al., 1993) in a position where it can make contact with membrane phospholipids. The large change in tryptophan fluorescence that is observed when this side of annexin V binds to the bilayer has been a useful method to monitor Ca^{2+}-dependent binding to phospholipids (Meers, 1990; Meers & Mealy, 1993a,b). It has been shown that the Ca^{2+} ions on this convex surface are complexed by ligands contributed by consensus sequence residues (Huber et al., 1990; Concha et al., 1993; Sopkova et al., 1993), and it has been speculated that these ions may directly participate in binding to phospholipids because of structural similarity to the phospholipase A_2-binding site (Huber et al., 1990).

A general characteristic of several membrane-binding proteins that has emerged in recent studies is the coexistence

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of relatively chemically nonspecific interactions that target membranes generally, as well as more chemically specific interactions involving specific binding pockets, i.e., sites for a specific substrate. This behavior is observed for protein kinase C (Bazzi & Nelsestuen, 1991; Orr & Newton, 1992a,b; Lee & Bell, 1992; Newton, 1993), as well as for pancreatic and venom phospholipase A_2 (van Dam-Mieras et al., 1975; Jain et al., 1986, 1989). Similarly, a nonspecific interaction of annexin V with negatively charged surfaces has been predicted from the original crystal structure (Karshikov et al., 1992) and observed experimentally (Meers & Mealy, 1993b). Annexins IV (Junker & Creutz, 1993) and VI (Bazzi & Nelsestuen, 1991) also appear to interact with negatively charged phospholipids or other negatively charged lipids (Edwards & Crumpton, 1991).

In the case of annexin V, the effect of negative surface potential plays two roles: it increases the local Ca^{2+} concentration near the surface and enhances the affinity of the annexin for the membrane or micelle surface (Meers & Mealy, 1993a,b). This effect has been separated from specific binding to phospholipids by using $C_{12}E_8$[1] micelles with a high negative surface charge (from dodecyl sulfate). These micelles provide an inert detergent matrix in which annexin affinity for various phospholipid derivatives can be assessed by dilution of their surface concentration within the micelles. Such systems, along with tryptophan fluorescence measurements, have been used to demonstrate apparently specific phospholipid-binding sites with a binding stoichiometry that may be consistent with one site per domain (Meers & Mealy, 1993b). These sites act together in a very cooperative manner, at least partially because of the aggregated nature of the substrate.

Since the crystal structures of phospholipid-bound annexin V have not been solved, the nature of the specific contacts between phospholipids and annexin V that are necessary for binding is still a matter of extrapolation from the phospholipid-free structure. In the study presented here, phospholipids or derivatives thereof are incorporated into the mixed micelles, discussed above, to test the importance of the structure of various parts of the phospholipid molecule. The results reported here allow the determination of the general characteristics of the phospholipid determinants necessary for binding to the specific sites of annexin V, particularly the site or sites within the third domain. Furthermore, an experiment is presented to begin to ascertain the relationship between the location of tryptophan 187 and the specifically bound phospholipids.

[1] Abbreviations: PC, egg phosphatidylcholine; PE, phosphatidylethanolamine derived from egg PC; PI, phosphatidylinositol from bovine liver; SM, sphingomyelin from bovine brain; PM, brain phosphatidylethanolamine containing 50% plasmalogens; Rh-PE, *N*-(lissamine rhodamine B sulfonyl)-PE; acyl-NBD-PC, 2-[12-[(7-nitro-2,1,3-benzoxadiazol-4-yl)amino]dodecanoyl]-1-hexadecanoyl-*sn*-glycero-3-phosphocholine; DPPC, 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine; D-DPPC, 2,3-dipalmitoyl-*sn*-glycero-1-phosphocholine; lyso-PC, 1-oleoyl-*sn*-glycero-3-phosphocholine; OA, oleic acid; DOG, 1,2-dioleoylglycerol; DOPG, 1,2-dioleoyl-*sn*-glycero-3-phospho-*rac*-1-glycerol; POPC, 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine; POPS, 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphoserine; POPA, 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphate; POPE, 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphoethanolamine; POPG, 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phospho-*rac*-1-glycerol; OAPC, 1-oleoyl-2-acetyl-*sn*-glycero-3-phosphocholine; PAF, 1-alkyl-2-acetyl-*sn*-glycero-3-phosphocholine or platelet-activating factor; D,L-di-*O*-C_{16}-PC, racemic mixture of 1,2-dihexadecyl-*sn*-glycero-3-phosphocholine and 2,3-dihexadecyl-*sn*-glycero-1-phosphocholine; C_{18},C_1-PC, 1-octadecyl-2-methyl-*sn*-glycero-3-phosphocholine; SDS, sodium dodecyl sulfate; EDTA, ethylenediaminetetraacetate; TES, *N*-tris(hydroxylmethyl)methyl-2-aminoethanesulfonic acid; $C_{12}E_8$, octa(ethylene glycol) monododecyl ether; cmc, critical micelle concentration.

MATERIALS AND METHODS

N-(Lissamine rhodamine B sulfonyl)-PE (Rh-PE), 2-[12-[(7-nitro-2,1,3-benzoxadiazol-4-yl)amino]dodecanoyl]-1-hexadecanoyl-*sn*-glycero-3-phosphocholine (acyl-NBD-PC), *N*-(7-nitro-2,1,3-benzoxadiazol-4-yl)phosphatidylethanolamine (NBD-PE, derived from transesterified egg PE), 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC), 2,3-dipalmitoyl-*sn*-glycero-1-phosphocholine (D-DPPC), 1-oleoyl-*sn*-glycero-3-phosphocholine (lyso-PC), 1,2-dioleoyl-*sn*-glycero-3-phospho-*rac*-1-glycerol (DOPG), 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC), 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphoserine (POPS), 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphate (POPA), 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphoethanolamine (POPE), 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phospho-*rac*-1-glycerol (POPG), phosphatidylinositol from bovine liver (PI), egg phosphatidylethanolamine transesterified from phosphatidylcholine (PE), sphingomyelin from bovine brain (SM), brain phosphatidylethanolamine containing 50% plasmalogens (PM), 1-oleoyl-2-acetyl-*sn*-glycero-3-phosphocholine (OAPC), 1-alkyl-2-acetyl-*sn*-glycero-3-phosphocholine (PAF) (all >99%) were purchased from Avanti Polar Lipids (Birmingham, AL). 1,2-Dioleoylglycerol (DOG) was from Nu-Chek Prep (Elysian, MN).

The racemic mixture of 1,2-dihexadecyl-*sn*-glycero-3-phosphocholine and 2,3-dihexadecyl-*sn*-glycero-1-phosphocholine (D,L-di-*O*-C_{16}-PC), 1-octadecyl-2-methyl-*sn*-glycero-3-phosphocholine (C_{18},C_1-PC), hexadecylphosphocholine, and EDTA (99.5%) were from Sigma (St. Louis, MO). Octa(ethylene glycol) monododecyl ether ($C_{12}E_8$, >98%) was from Fluka (Ronkonkona, NY). $CaCl_2$ (>99%), KCl (>99%), NaCl (>99%), and TES (98%) were from Fisher (Pittsburgh, PA). Bicichoninic acid was from Pierce (Rockford, IL).

All phospholipids were checked for purity by silica gel thin-layer chromatography in 65/25/5 chloroform/methanol/concentrated NH_4OH. No significant impurities could be detected (ca. >99%).

3-*O*-Hexadecyl-2-*O*-[hexadecyl-(*S*)-(thiophosphonyl)]-*sn*-glycero-1-phosphoethanolamine (D-C_{16},SPC_{16}-PE) and 1-*O*-hexadecyl-2-*O*-(hexadecylphosphonyl)]-*sn*-glycero-3-phosphate were kindly supplied by Dr. Michael Gelb (University of Washington) and were used as received.

Micelle Preparation and Characterization. All experiments, except for detergent titrations, were performed [also see Meers and Mealy (1993b)] by first adding the indicated amount of $C_{12}E_8$ to a sample, followed by any negatively charged amphiphiles and then any phospholipid analogs (usually in the form of vesicles initially). Dissolution of phospholipid vesicles into micelles was ascertained by observation of the turbidity. Annexin V was added to samples last, before Ca^{2+} addition. Most experiments, except where indicated, were performed in 80 mM NaCl, 50 mM TES, and 0.1 mM EDTA (pH 7.4) (buffer A).

In the $C_{12}E_8$/SDS (1.1 mM/700 μM) micelles used in these studies, the strongest binding was to phospholipids such as PC, whereas lyso-PC, one of the most polar phospholipid derivatives tested, showed the weakest binding (see Results). Therefore, the characteristics of micelles containing PC or lyso-PC were investigated. Samples with 100 μM PC (approximately 5–6 per micelle), 300 μM PC, or 300 μM lyso-PC (approximately 15 per micelle) were tested. Samples were applied to a Sephacryl S-200 column and eluted with 100 μM $C_{12}E_8$ and 700 μM SDS in buffer A. All phospholipid phosphate eluted in a single peak with an approximate distribution coefficient (K_{av}) of 0.2 for all samples, which is approximately the size expected for $C_{12}E_8$ micelles. Fur-

thermore, similar samples of lyso-PC or PC were filtered partially through centrifugal filters with a 10 000 molecular weight cutoff. No phosphate was detected in the filtrate, indicating that all of the PC or lyso-PC was in the form of micelles. Therefore, we assume that all micelles are of the same size and that all phospholipid derivatives completely or nearly completely incorporate into the micelles. Complete partitioning of long-chain lysophospholipids into similar micelles (Triton X-100) is commonly assumed (Oishi et al., 1988; Lee & Bell, 1992). Another single long-chain amphiphile, hexadecylphosphocholine, also showed characteristics of complete partitioning into micelles, as evidenced by its ability to bind to annexin V even near the lowest concentration per micelle, at which binding to double-chain phospholipids is observed.

Vesicles of lyso-PC with oleic acid in a 1/1 ratio were prepared by drying a film of these components from stock solutions in solvents. The dried film was rehydrated with buffer A, with gentle shaking and a few seconds of vortexing. The vesicular nature of dispersions of equimolar lyso-PC and oleic acid was assessed by turbidity. At 100 μM lyso-PC, the turbidity of the 1/1 mixture was at least 10-fold greater than that for lyso-PC alone. OA (100 μM) also showed no significant turbidity. Other vesicles were prepared in buffer A by extrusion 10 times through 0.1-μm polycarbonate membranes, as previously described (Mayer et al., 1986).

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 (≥97%) (PAP-I, endonexin II) was prepared as previously described (Meers & Mealy, 1993b). Annexin I (recombinant human, ≥92%) was prepared from BL21 *Escherichia coli* as previously described (Meers & Mealy, 1993b). Protein concentrations were measured by the bicinchoninic acid method (Smith et al., 1985).

Fluorescence Measurements. Fluorescence measurements were made using an SLM 8000C fluorometer (Urbana, IL). Tryptophan excitation was at 295 nm, with full emission monitored at 345 nm for most experiments. 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. Additional Ca^{2+} was always added after the initial amount to determine whether larger increases in fluorescence were possible at higher Ca^{2+} concentrations.

The results of binding experiments are expressed as the percentage increase in fluorescence (i.e., 100% represents a 2-fold increase in fluorescence). The horizontal line in some figures represents the background lipid-independent increase in fluorescence intensity.

RESULTS

Basic Requirements for Binding. Binding of annexin V was monitored by the increase in tryptophan fluorescence that occurs when tryptophan 187 is immersed into the viscous environment at the interfacial region of the membrane or micelle and/or when it makes contact with a specifically bound phospholipid molecule (see below). While there are some significant differences in the maximal tryptophan fluorescence in micelles of various compositions, an increase of approximately 100% or greater is generally observed, as opposed to a phospholipid-independent increase of approximately 25–35% due to a red shift that results from Ca^{2+} binding alone (Meers & Mealy, 1993a). Previous data using this method suggested that only the phosphate group and *sn*-2 acyl chains of phospholipids are important for binding to annexin V (Meers & Mealy, 1993b). Therefore, it was of interest to study further the structural requirements for efficient binding of phospholipids to specific annexin V-binding sites. The positions of various groups modified on the basic glycerol backbone structure of phospholipids are shown in Figure 1. In normal biological phospholipids (see bottom structure, Figure 1), the phosphate group corresponds to R_c, the *sn*-2 acyl chain (plus the glycerol oxygen) to R_b and the *sn*-1 acyl chain (plus the *sn*-1 carbon and oxygen of glycerol) to R_a. The various phospholipid derivatives tested and the locations of the corresponding data in the figures are listed in Table 1. Many derivatives displayed maximal binding at 10 mM Ca^{2+} and six molecules per micelle. Comparison between tryptophan response at 6 and 16 molecules per micelle induced by 10 mM Ca^{2+} was used as a benchmark for comparing the binding of many of the phospholipid derivatives, with three major categories resulting: binding at both concentrations, binding at only the highest concentration, or binding at neither concentration. In some cases, Ca^{2+} dependencies were measured as well.

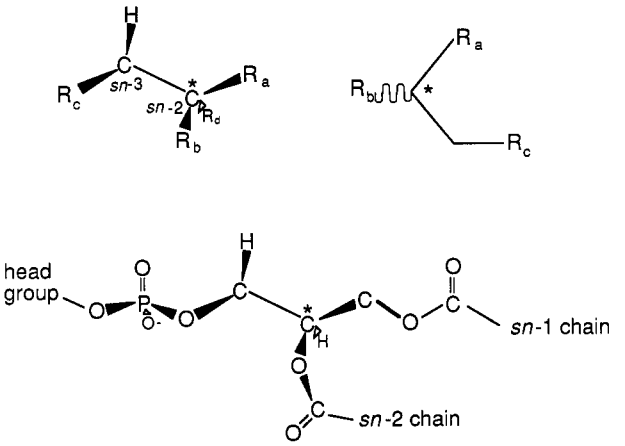
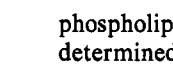
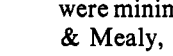


FIGURE 1: Generalized structure of phospholipids used in this study. In a normal L-α- phospholipid, R_a represents the *sn*-1 acyl chain plus the *sn*-1 carbon and oxygen of glycerol, R_b represents the *sn*-2 acyl chain plus the glycerol oxygen, R_c represents the phosphate group, and R_d represents a proton, as shown in the lower structure. These groups have been changed as indicated in Table 1 for the various derivatives studied.

Data for binding to dioleoylglycerol (DOG) and lyso-PC are shown in Figure 2A. The previously observed lack of binding to DOG and lyso-PC is confirmed here by the lack of an increase in tryptophan fluorescence (beyond the phospholipid-independent level), even at higher surface concentrations (approximately 16 per micelle, 300 μM in this case) than previously tested. Binding to egg PC, by contrast, was observed at less than six phospholipids per micelle (100 μM PC, in this case) at 10 mM Ca^{2+}.

Lack of binding to lyso-PC and DOG was also confirmed by long-range fluorescence resonance energy transfer between the tryptophan and a micelle-localized energy-transfer acceptor, as was previously done for other micellar mixtures (Meers & Mealy, 1993b). The rationale was that this method would detect binding, even if the annexin V tryptophan were not in position to undergo the large intensity increase that normally occurs. In Figure 2B, the results of such an experiment are shown. The binding of annexin I, which orients its N-terminal region with its single tryptophan away from

Table 1: Structures of Phospholipid Analogs Tested and Location of Data

figure	lipid name (abbrev.)	R_a	R_b	R_c	R_d
2A, 3A	POPC	(CH_2O)-palmitoyl chain	*O*-oleoyl chain	phosphocholine	H
2B, 4A, 4B, 5A, 5B	DPPC	(CH_2O)-palmitoyl chain	*O*-palmitoyl chain	phosphocholine	H
2C, 6A	lyso-PC	(CH_2O)-oleoyl chain	OH	phosphocholine	H
2D	DOG	(CH_2O)-oleoyl chain	*O*-oleoyl chain	OH	H
3B	POPS	(CH_2O)-palmitoyl chain	*O*-oleoyl chain	phosphoserine	H
3C	POPA	(CH_2O)-palmitoyl chain	*O*-oleoyl chain	phosphate	H
3D	POPE	(CH_2O)-palmitoyl chain	*O*-oleoyl chain	phosphoethanolamine	H
3E	POPG	(CH_2O)-palmitoyl chain	*O*-oleoyl chain	phosphoglycerol	H
3F–H	PI	(CH_2O)-long acyl chain	*O*-long acyl chain	phosphoinositol	H
3I	PE	(CH_2O)-long acyl chain	*O*-long acyl chain	phosphoethanolamine	H
3J	DOPG	(CH_2O)-oleoyl chain	*O*-oleoyl chain	phosphoglycerol	H
4C,D	D-DPPC	H	*O*-palmitoyl chain	phosphocholine	(CH_2O)palmitoyl chain
5C–E	SM	$(CHOH)C{=}C$-long alkyl chain	NH-long acyl chain	phosphocholine	H
5F,G	PM	$(CH_2O)C{=}C$-long alkyl chain	*O*-long acyl chain	mostly phosphoethanolamine	H
5H,I	D,L-1,2-di-*O*-C_{16}PC	(CH_2O)-hexadecyl chain (50%), H (50%)	*O*-hexadecyl chain	phosphocholine	H (50%), (CH_2O)-hexadecyl chain (50%)
5J,K	hexadecylphosphocholine	H	$C_{14}H_{29}$	phosphocholine	H
5L	D-C_{16},SPC_{16}-PE	H		phosphoethanolamine	(CH_2O)-hexadecyl
5M	C_{16},PC_{16}-PA	(CH_2O)-hexadecyl		phosphate	H
6B–D	OAPC	(CH_2O)-oleoyl chain	*O*-acetyl chain	phosphocholine	H
6E–G	PAF	(CH_2O)-long alkyl chain	*O*-acetyl chain	phosphocholine	H
6H,I	C_{18},C_1-PC	(CH_2O)-octadecyl	OCH_3	phosphocholine	H

the binding surface of the protein (Glenney, 1986; Huang et al., 1987; Meers et al., 1992), was first monitored by this method. PS was chosen as a positive control for binding, under conditions where no micellar aggregation occurs (Meers & Mealy, 1993b). When PS and a small amount of the acceptor, NBD-PE (approximately 2 per micelle), were present in the micelles, Ca^{2+}-dependent binding resulted in substantial quenching of the tryptophan fluorescence, despite the fact that the annexin I tryptophan does not make contact with phospholipids (Meers, 1990) and is probably located some distance away from the micelle surface. Therefore, if annexin V binds to the membrane in any manner, it is expected that its tryptophan should also be well-quenched by this probe through energy transfer. This was in fact the case, as seen by the binding of annexin V to PS-containing micelles in Figure 2B. When the micelles lacked PS or when there was a substitution of lyso-PC or DOG for PS, no significant quenching of tryptophan fluorescence was observed, indicating no binding to the micelles by any means. Therefore, the phospholipid phosphate group and the *sn*-2 acyl chain are required for annexin V binding under the conditions of these experiments. Furthermore, on the basis of these experiments and similar ones presented previously (Meers & Mealy, 1993b), it is assumed that the increase in tryptophan fluorescence alone, in the absence of added quenching probes, is sufficient to monitor binding. Although the maximal increase in tryptophan fluorescence depends on the particular lipid to which it binds, the avidity of binding can be assessed by its dependence on the two-dimensional concentration of ligands within the micelles or by its Ca^{2+} dependence (as discussed above).

Effects of Various Headgroups on Annexin V Binding. Previous results suggested a relatively unimportant role for the phospholipid headgroup as a determinant for the annexin V phospholipid-binding sites based on the similarities of PS and PC binding (Meers & Mealy, 1993b). We have further studied various phospholipid headgroups in negatively charged micelles to qualitatively determine whether any significant differences exist. The avidity of binding of annexin V to micelles can be modulated either by changing Ca^{2+} concentration or by changing the two-dimensional concentration of phospholipids in the micelles. Since it was previously determined that approximately 3–4 phospholipids per micelle were minimally required for the binding of annexin V (Meers & Mealy, 1993b) under these conditions, the various headgroups were initially compared at a slightly higher concentration (six phospholipids per micelle, 100 μM phospholipid in most experiments). The data (Figure 3) show that, for POPS, POPC, POPA, POPE, and POPG at excess Ca^{2+} (10 mM), maximal binding was observed and did not increase at higher Ca^{2+} concentrations. One exception was PI (Figure 3, samples F–H), which required more than 16 phospholipids per micelle to reach maximal binding. Note that no binding was observed at 100 μM PI, where binding to the other phospholipids could be observed.

The dependence on phospholipid concentration was also reflected in the Ca^{2+} dependence for binding (Table 2). The Ca^{2+} concentration for a half-maximal tryptophan fluorescence increase was close to the same for all of the phospholipids tested above, except for PI. No binding to PI could be observed for up to 20 mM Ca^{2+}, when it was incorporated at approximately six per micelle, as were the other phospholipids. Approximately 3–10 times more Ca^{2+} was required for PI binding, even when the PI concentration in the micelles was three times higher than the concentrations of the other phospholipids. Therefore, the suggestion that headgroups are of relatively little importance is confirmed, with the exception of PI, which has a bulky sugar ring directly attached to the phosphate group of the phospholipid. Since the phosphate group is a required binding determinant, one possible interpretation is that direct binding of annexin V to the phosphate group is hindered by this proximal ring structure, which is not present in any of the other phospholipids. Note also that the phospholipids with generally the bulkiest groups (PC, PS) require more Ca^{2+} than those with smaller headgroups (PE, PA). Therefore, except for some possible inhibition of binding by bulky groups close to the phosphate group, no strong binding determinants appear to exist in the phospholipid headgroups for interaction with specific annexin V-binding sites.

Phospholipid Stereochemistry. The putative phospholipid-binding sites in annexin V, at the site of bound Ca^{2+} ions

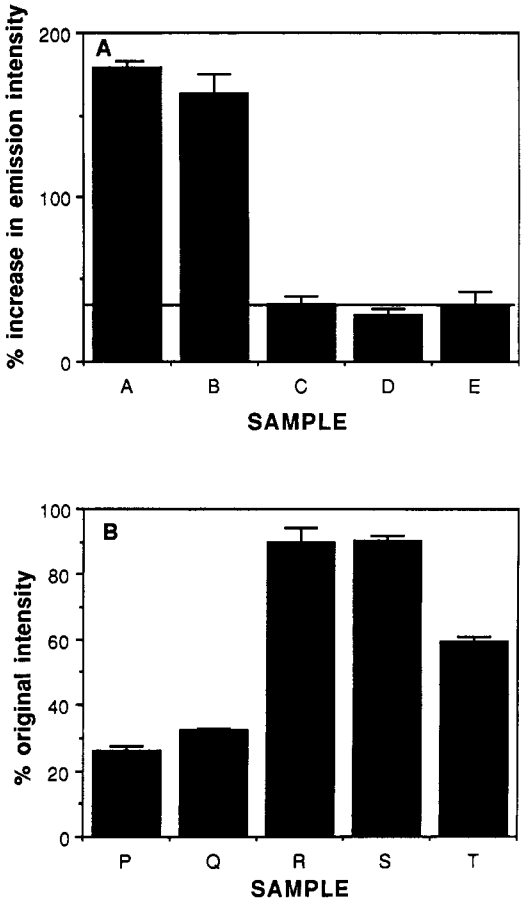


FIGURE 2: Panel A: Effect of phosphate group and *sn*-2 acyl chain on the binding of annexin V to phospholipid-containing micelles. Samples contained 10 μg/mL annexin V and the lipids indicated in Table 1 at 100 (A), 300 (B–D), or 0 μM (E). All samples contained 1.1 mM $C_{12}E_8$ and 700 μM SDS. Lipids added to the micelles were POPC (A), DPPC (B), lyso-PC (C), DOG (D), or none (E). Experiments were performed in buffer A at 25 °C. 10 mM Ca^{2+} was added, and the percentage increase in tryptophan fluorescence was measured as described in Materials and Methods. Panel B: Annexin V binding to micelles as measured by NBD quenching of tryptophan fluorescence. Quenching was measured in the presence of 10 mM Ca^{2+} as the percentage of the original emission intensity of annexin V tryptophan (without NBD quenchers). Annexin V samples (10 μg/mL) contained 300 μM $C_{12}E_8$, 140 μM SDS, 6 μM NBD-PE, and 20 μM PS (P), 40 μM PS (Q), 20 μM DOG (R), or 20 μM lyso-PC (S). Annexin I samples (20 μg/mL) contained 300 μM $C_{12}E_8$, 140 μM SDS, 6 μM NBD-PE, and 40 μM PS (T). For all data n = 3, ±sd shown.

along the "convex" face of the protein, do not appear to be deeply set in clefts in the crystal structure of the protein (Concha et al., 1993; Sopkova et al., 1993). This suggests that the annexin may accommodate large groups in various positions distal to the specific phospholipid-binding determinants. One test of the accommodation of large groups is to invert the stereochemistry at the *sn*-2 carbon of the glycerol moiety of the phospholipid. In Figure 4, binding to the D and L versions of dipalmitoyl-PC (DPPC) was studied (A and B vs C and D). The relative avidity for the two stereoisomers was assessed (as in the case of PI above) by the difference between the tryptophan response at 6 phospholipids per micelle and 16 phospholipids per micelle. It is clear that binding is nearly complete at six per micelle (100 μM) for both stereoisomers under the conditions of the experiments, although there may be a slightly weaker binding to the D isomer. Since the *sn*-2 acyl chain is important for the binding of annexin V (as shown in this article), the position of its carbonyl oxygen relative to the phosphate group can be

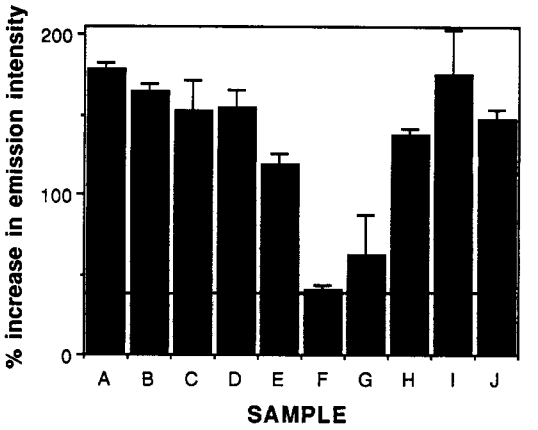


FIGURE 3: Effect of phospholipid headgroup on annexin V binding to micelles. All samples contained 10 μg/mL annexin V, 1.1 mM $C_{12}E_8$, and 700 μM SDS. Experiments were performed in buffer A at 25 °C. 10 mM Ca^{2+} was added and the percentage increase in tryptophan fluorescence was measured as described in Materials and Methods. Phospholipids incorporated into the samples are POPC (A), POPS (B), POPA (C), POPE (D), POPG (E), PI (F, G, H), PE (I), and DOPG (J). Samples contained 100 (A–F, I, J), 200 μM (G), or 300 μM (H) total phospholipid. For all experiments n = 3, ±sd shown.

Table 2: Ca^{2+} Dependence of Binding to Phospholipids with Varying Headgroups

phospholipid[a]	Ca^{2+} concentration (mM) for half-maximal binding ● sd[b]	n[c]
POPC	1.8 ± 0.31	3
POPS	1.6 ± 0.28	3
POPA	0.64 ± 0.03	3
POPE	0.53 ± 0.06	3
POPG	2.0 ± 0.40	4
PI	>20[d]	6
PI, 300 μM	4.9 ± 0.73	3

[a] Samples consisted of 1.1 mM $C_{12}E_8$, 700 μM SDS, and 100 μM of the phospholipid designated, except for one of the PI samples, which was 300 μM. All samples contained 10 μg/mL annexin V. [b] From half-maximal increase in annexin V tryptophan fluorescence. [c] Number of experiments. [d] No binding was observed under these conditions up to 20 mM Ca^{2+}.

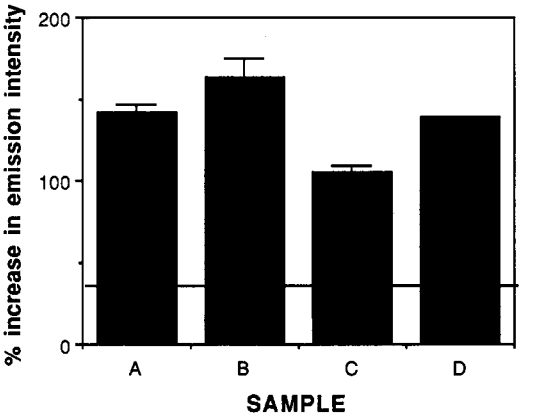


FIGURE 4: Effect of stereochemistry of the glycerol moiety on annexin V binding to DPPC in detergent micelles. Conditions are identical to Figure 3, except for the phospholipids used. The phospholipids incorporated were L-DPPC (A, B) and D-DPPC (C, D). Total phospholipid concentrations were 100 (A, C) or 300 μM (B, D). For all data n = 3, ±sd shown.

maintained in both stereoisomers, but only if a proton on the glycerol *sn*-2 carbon and the *sn*-1 carbon with its acyl chain exchange positions (Figure 1). Therefore, the area surrounding the *sn*-2 carbon of the glycerol must be relatively open to accommodate the *sn*-1 acyl chain on either side.

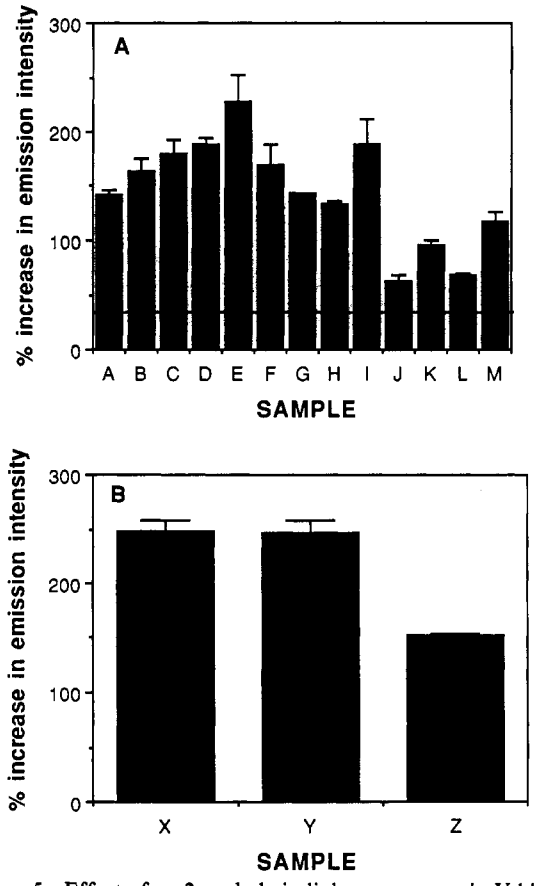


FIGURE 5: Effect of *sn*-2 acyl chain linkage on annexin V binding to mixed micelles. Panel A: Binding to micelles. Conditions are identical to Figure 3, except for the phospholipids used. The phospholipids incorporated were DPPC (A, B), SM (C–E), PM (F, G), D,L-di-*O*-C_{16}-PC (H, I), hexacedylphosphocholine (J, K), D-C_{16}, SPC_{16}-PE (L), and C_{16},PC_{16}-PA (M). Total phospholipid concentrations were 100 (A, C, F, H, J, L, M), 200 (D), or 300 μM (B, E, G, I, K). For data A–K, $n = 3$; for L and M $n = 2$. Panel B: Binding to vesicles. Samples contained 100 μM total phospholipid and 10 μg/mL annexin V in buffer A at pH 8.0. Compositions were 1/1 PS/PC (X), 3/1 PC/OA (Y), and 1/1 lyso-PC/OA (Z). 400 μM Ca^{2+} was added to each sample and gave the maximal fluorescence increase. For all data, $n = 3$, ±sd shown.

Importance of the sn-2 Acyl Chain Linkage. The importance of the linkage of the *sn*-2 hydrocarbon chain to the glycerol phosphate moiety was also studied by measuring binding to derivatives with this chain linked in various ways. The avidity of binding was assessed in a qualitative manner, similar to that used to compare PI binding to other phospholipids (above). Binding at 6 phospholipids per micelle and 16 phospholipids per micelle was compared. Strong binding was indicated by a large and comparable increase in tryptophan fluorescence at both concentrations. Weak binding was indicated by a magnitude of tryptophan fluorescence that increased progressively (above background) with increasing phospholipid concentration (from 6 to 16 per micelle). In Figure 5A, ether linkages, phosphonate linkages, and amide linkages were studied. Binding to DPPC (samples A and B), sphingomyelin (samples C and D), plasmalogens (samples F and G), and D,L-di-*O*-C_{16}PC (H and I) was at or near maximal at 6 phospholipids per micelle (A, C, F, H). Experiments at the lowest phospholipid concentration also showed some binding to phosphonate-linked hydrocarbon chains (L and M). Therefore, within the resolution of these experiments, the mere presence of an *sn*-2 chain linked in any way was sufficient to elicit at least some binding of annexin V. In all of the derivatives tested, except the one used in J and K, there is potential for an interaction with the annexin-bound Ca^{2+} ion involving a nucleophilic phospholipid moiety such as occurs in phospholipase A_2. The experiments shown for samples J and K are of particular interest because they involve hexadecylphosphocholine, a derivative that does not contain any glycerol *sn*-2 oxygen. There is only a hydrocarbon chain directly esterified to the phosphate group. This derivative would allow only hydrophobic interaction at a position equivalent to the *sn*-2 chain of phospholipids. As the data show, there was apparently some degree of binding even at only six lipids per micelle. The maximal increase in fluorescence was observed at approximately 16 per micelle (as determined by further Ca^{2+} additions), although the magnitude is much smaller than that for other lipids. Therefore, hydrophobic interactions at this position are likely to contribute to annexin V binding and may be related to the tryptophan response.

It was also possible to study the binding of annexin V to phospholipids that completely lack an *sn*-2 chain linkage. Since equimolar ratios of long-chain fatty acids and lyso-phospholipids spontaneously form vesicles (Jain et al., 1980), it was desirable to depart from the micellar format to monitor annexin V binding. The presence of the fatty acids, in this case, allowed annexin V binding to lyso-PC (Figure 5B, sample Z) with a Ca^{2+} dependence for a half-maximal fluorescence increase of 0.63 ± 0.01 mM ($n = 3$). The maximal increase in annexin V tryptophan fluorescence for this binding was much lower than that for binding to OA/PC vesicles (Figure 5B, sample Y). Therefore, the spatial relationship between the fatty acid and lyso-PC, which is implied by the fact that bilayers are formed, is important for annexin V binding to lyso-PC. Apparently, the colocalization of the fatty acid at the *sn*-2 position along with the negative surface charge imparted by some of the fatty acids in the OA/lyso-PC membranes is sufficient to allow binding. When oleic acid and lyso-PC are colocalized into micelles, where the fatty acid may not be interacting directly with the lyso-PC molecule and where the local concentration of the lyso-PC is lower than that in the vesicles, no apparent binding of annexin V was observed (not shown).

Therefore, it appears that an *sn*-2 ester carbonyl oxygen is not specifically required for binding. This interaction may exist, but in a relatively flexible form in the annexin. The results also suggested a possible hydrophobic interaction between part of the *sn*-2 acyl chain and the annexin.

Role of the Length of the sn-2 Acyl Chain. The importance of the length of the acyl chain is shown in Figure 6. Here, various derivatives of ether- or ester-linked phospholipids with short or long *sn*-2 acyl chains are compared. It is clear that the length of this acyl is critical for binding. No binding at all was observed to lyso-PC, under the conditions of these experiments, at approximately 16 per micelle (Figure 2). When the acyl chain at the *sn*-2 position was intermediate in length (1–2 carbons), binding of low to intermediate affinity was observed, requiring >16 phospholipids per micelle for a maximal increase in tryptophan fluorescence for the PAF analog, OAPC (Figure 6, samples B–D), and for C_{18},C_1-PC (Figure 6, samples H and I). PAF itself exhibited maximal binding at only approximately 12 per micelle (Figure 6, samples E–G). Binding to all of these analogs is of lower affinity than binding to long-chain phospholipids. These data further support a hydrophobic interaction with annexin V involving the first few carbons of the *sn*-2 acyl chain. The fact that the maximal increase in tryptophan fluorescence is also much

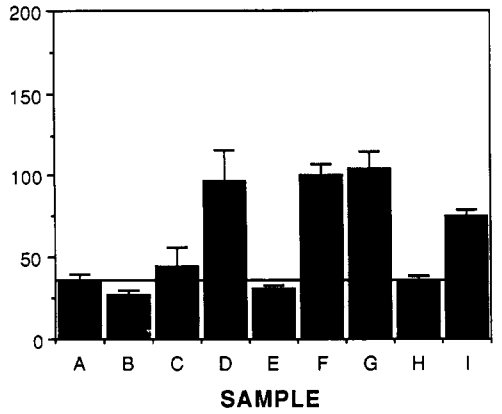


FIGURE 6: Effect of *sn*-2 acyl chain length on annexin V binding to mixed micelles. Conditions are identical to Figure 3, except for the phospholipids used. The phospholipids incorporated were lyso-PC (A), OAPC (B–D), PAF (E–G), and C_{18},C_1-PC (H, I). Total phospholipid concentrations were 100 (B, E, H), 200 (C, F), or 300 μM (A, D, G, I).

Table 3: Effect of Competition by Nonquenching Phospholipids on the 5-PC-Mediated Quenching of Tryptophan 187

lipid additions to micelles[a]	% maximal fluorescence	n^b
30 μM 5-PC	52.5 ± 0.7	2
30 μM 5-PC, 60 μM POPC	66.5 ± 3.5	2
30 μM 5-PC, 70.5 μM $C_{12}E_8$, 49.5 μM SDS	54 ± 1.4	2

[a] Micelles consisted of 400 μM $C_{12}E_8$ and 210 μM SDS plus the designated additional lipids. All samples contained 10 μg/mL annexin V. Fluorescence changes were measured after the addition of 10 mM Ca^{2+}. [b] Number of experiments.

lower than that for the long-chain phospholipids (e.g., see Figure 6, samples E–G) also suggests a possible direct interaction between the tryptophan and the *sn*-2 acyl chain of the specifically bound phospholipid.

Direct Interaction of Trp 187 with Specifically Bound Phospholipids. Since hydrophobic interactions of the *sn*-2 acyl chains with annexin V appear to be important for binding, it was of interest to investigate whether tryptophan 187 may make direct contact with the *sn*-2 chain of the phospholipid directly bound to the annexin V site(s) in the third domain. This was tested using 5-PC, a derivative of PC with a quenching nitroxide moiety at the 5-position of the *sn*-2 acyl chain. It was reasoned that if 5-PC directly binds to the specific annexin V-binding sites and if it quenches tryptophan 187 when located in those sites, then nonquenching phospholipids that are known to bind to the same sites would competitively inhibit the quenching of Trp 187 by 5-PC. If quenching were due to nonspecific contact of the quenching phospholipids with the tryptophan, then the main mechanism by which quenching could be relieved is by the dilution of quenchers. In Table 3, the quenching of tryptophan fluorescence was monitored as a function of 5-PC and POPC incorporated into mixed micelles. Incorporation of POPC substantially decreased the quenching of tryptophan fluorescence by 5-PC. Control experiments showed that the decrease in quenching was not solely due to dilution of the quenching probe as a result of the addition of the POPC hydrocarbon chains into the micelles. When an equal number of $C_{12}E_8$ hydrocarbon chains was added to the 5-PC-containing micelles, their effects on quenching were much smaller than those of the POPC. Thus, either part of the tryptophan quenching results from direct contact with the *sn*-2 chain nitroxide moiety of the specifically bound 5-PC, and/or binding of the nonquenching phospholipids shields the tryptophan from nonspecific contact quenching. In the former case, a direct interaction with the *sn*-2 chain is indicated. In the latter, such an interaction is possible, but other phospholipid moieties could be responsible.

Although these data strongly indicate direct interaction between specifically bound phospholipids and tryptophan 187, probably through the *sn*-2 acyl chain, there is also clearly some nonspecific contact of tryptophan 187 with the bulk of the micelle lipids. For instance, if it is assumed that 5-PC and POPC bind equally well to annexin V, then it would be expected that the quenching would be reduced by 67% (giving 84% maximal fluorescence) in the micelles containing 2/1 POPC/5-PC, if specifically bound phospholipids were solely responsible for quenching. Actually, the effect of POPC is only about one-half as large as expected for this situation. Therefore, this tryptophan is probably also exposed to quenching by lipids in the micelle not bound to the specific annexin sites, within the accuracy of our assumption. Increased quenching at higher 5-PC concentrations (beyond the minimal number of phospholipids per micelle necessary for binding; not shown) is consistent with this suggestion. Therefore, Trp 187 appears to contact both the specifically bound phospholipid as well as the general lipid environment of the micelle.

DISCUSSION

Headgroups Do Not Contain Determinants for Annexin V-Binding Sites. By studying annexin V binding in a micellar system with a large negative surface charge, it has been possible to separate the intrinsic affinities of the annexin V phospholipid-binding sites (taken together) from effects on binding that are the result of the charge or structure of the phospholipid aggregates (i.e., micelles or membranes) to which the protein binds. The effect of charge is eliminated by including saturating amounts of negative surface charge on the micelles. Since micelles are very dynamic structures, the structural constraints on the phospholipids within the micelles are probably relatively small, so that the properties of the annexin V-binding sites can be probed in terms of the covalent structure of the phospholipids tested. Binding under physiological conditions could depend on properties of the protein, covalent structure of the lipids, and properties of the phospholipid aggregates, such as geometric constraints on phospholipid conformations, surface potential of the bilayer (Meers & Mealy, 1993a), and overall curvature of the binding surface (Andree et al., 1992). The purpose here is strictly to study the intrinsic properties of the phospholipid-specific sites of the protein in isolation. In this defined setting, the only major effect of headgroup structure appears to be inhibitory by a steric mechanism.

Since most biological membranes do have substantial negative surface potential contributed by various lipid and nonlipid components, it is possible that studies in negatively charged micelles also reveal important properties of binding under physiological conditions. Therefore, for instance, despite previous results demonstrating relatively large headgroup-related differences in avidity of annexin V binding to model membranes (Schlaepfer et al., 1987; Andree et al., 1990; Blackwood & Ernst, 1990), annexin V may be able to utilize a relatively broad mixture of phospholipid types for binding to complex, negatively charged, mixed-phospholipid biological membranes. PC, for instance, would probably be an important binding site for annexin V in membranes that contain sufficient negative surface charge. However, binding to pure PC vesicles is not observed at physiological Ca^{2+} concentrations (Schlaepfer et al., 1987; Blackwood & Ernst, 1990; Meers & Mealy,

1993a). On the other hand, the relatively weak binding to PI that has been previously observed in vesicular systems (Schlaepfer et al., 1987; Blackwood & Ernst, 1990) is also observed in the micellar systems used here. Regulation of the expression of negative charge on the membrane surface and/or local Ca^{2+} concentrations could be an important means of regulation of annexin V binding to phospholipids and could be used to impart more or less headgroup specificity. Other properties of the annexin–membrane system, such as the ability to cluster negatively charged lipids near the site of annexin binding (Junker & Creutz, 1993), may also effectively confer some degree of headgroup specificity, although the intrinsic specificity of the individual sites probably remains unchanged.

Binding of Annexins to Biologically Active Lipids. Among the most interesting results from this anionic micellar system is the observation of binding of annexin V to platelet-activating factor (PAF), because of its potent biological activity. As discussed above, the physiological relevance of this effect depends on the ability of negative surface charge present on biological membranes to mediate annexin binding. Annexin I, a very abundant component of human leukocytes, has been implicated as a mediator of antiinflammatory effects (Wallner et al., 1986). One particular site of exogenous annexin I activity has been proposed to be human neutrophils. Injection of annexin I inhibits interleukin-1-induced, PAF-mediated migration of mouse neutrophils to a site of inflammation (Peretti & Flower, 1993). The fact that annexin V shows PAF-binding activity suggests that one possible mechanism by which annexin I may exhibit these antiinflammatory effects is by direct binding of PAF.

Relationship of the Annexin V Structure to Binding Determinants and Comparison to Phospholipase A_2. It has previously been suggested that annexin V-binding sites bear a structural similarity to phospholipase A_2 catalytic sites (Huber et al., 1992). Phospholipase A_2 molecules utilize relatively nonspecific interactions, probably both hydrophobic and ionic in nature (Jain et al., 1986), for binding *per se*. This binding involves the interfacial binding site(s). But the catalysis of *sn*-2 acyl chain hydrolysis involves a Ca^{2+}-bound site where the Ca^{2+} ion specifically ligates the phosphate group of the phospholipid and the *sn*-2 carbonyl oxygen (Scott et al., 1990). This catalytic site is the site thought to possess similarity to the annexin V Ca^{2+}-binding sites. Therefore, information about the specificity of the annexin V sites should be compared to the catalytic specificity or the efficacy of various specific inhibitors of catalysis by phospholipase A_2. Catalysis is specific for the cleavable *sn*-2 ester, but analogs of the ester or of the transition state can bind the catalytic site and inhibit catalysis. These analogs include phosphonate derivatives and are very sensitive to stereochemistry at the glycerol *sn*-2 carbon (Jain et al., 1989), as opposed to annexin V, which appears to bind almost equally well to D- or L-DPPC (Figure 4). The ability of annexin V to bind both stereoisomers of DPPC is probably a result of the relatively shallow nature of the annexin V-binding sites, as opposed to phospholipase A_2 where a cleft isolates the phospholipid *sn*-2 ester linkage into a solvent-free pocket for catalysis to occur.

Specific inhibitors of phospholipase A_2 catalysis can also vary as to the substituents located in the region of the *sn*-2 acyl linkage. For instance, phosphonates (Jain et al., 1989) and amides (Thunissen et al., 1990) act as inhibitors. Both have available an electronegative group analogous to the carbonyl oxygen of the ester linkage for coordination to Ca^{2+}. It appears, from the data presented here, that annexin V phospholipid-binding sites (there are probably around 4; Meers & Mealy, 1993b) also may not require a specific interaction with the carbonyl oxygen of an *sn*-2 acyl chain ester. Although there may be some direct Ca^{2+} interaction with electronegative groups in the *sn*-2 acyl chains, a relatively limited hydrophobic interaction of the protein with *sn*-2 acyl chains of the bound phospholipids also appears to be important. In the annexin V crystal structure that corresponds most closely to the characteristics of the membrane-bound form of annexin V, as determined by fluorescence measurements (Meers, 1990; Meers & Mealy, 1993a), it is clear that there is a hydrophobic residue at a roughly equivalent position in the AB loop in each domain pointing away from the bulk of the protein to the putative position of the membrane (Concha et al., 1993; Sopkova et al., 1993). This position corresponds to the second hydrophobic X residue in the conserved AB loop sequence X-K-G-X-G-T. In domain 3 of human annexin V, this residue structurally corresponds to tryptophan 187 (there is an extra amino acid in the third loop).

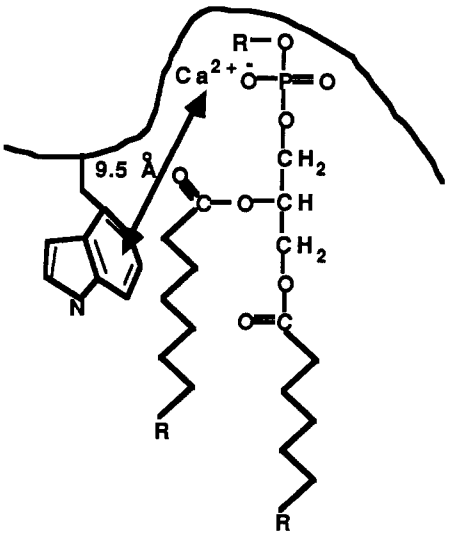


FIGURE 7: Schematic representation of possible phospholipid–tryptophan interactions in annexin V. The drawing is only approximately to scale.

Therefore, it is possible that these particular residues are responsible for the direct interaction of annexin V with the *sn*-2 acyl chains of phospholipids specifically bound to the Ca^{2+} ion residing in the AB loops (see Figure 7). The distance from the Ca^{2+} ion complexed in the AB loop of domain 3 to the center of the tryptophan ring is approximately 9.5 Å in the annexin V crystal structure (Concha et al., 1993; measurement courtesy of B. Seaton). This distance is more than adequate to allow tryptophan 187 to interact directly with the *sn*-2 chain of a phospholipid if the Ca^{2+} ion is complexed directly to a phosphate oxygen. One may further speculate that since domains 1 and 3 contain longer side chains at this position (leucine and tryptophan) than 2 and 4 (both alanine), the former two domains may be specifically responsible for the strongest interaction with the hydrocarbon part of the *sn*-2 acyl chain. Consistent with tryptophan interaction with the *sn*-2 acyl chain is the fact that the maximal tryptophan fluorescence of the micelle- or membrane-bound annexin varies with the structure of the phospholipid, with the largest differences observed when the *sn*-2 acyl chain was modified (Figures 5 and 6). Therefore, besides a general interaction of Trp 187 with the lipid aggregates, there may be a hydrophobic interaction of Trp 187 with the specifically annexin-bound phospholipid.

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REFERENCES

Andree, H. A. M., Reutelingsperger, C. P. M., Hauptmann, R., Hemker, H. C., Hermens, W. T., & Willems, G. M. (1990) *J. Biol. Chem. 265*, 4923–4928.

Andree, H. A. M., Stuart, M. C. A., Hermens, W. T., Reutelingsperger, C. P. M., Hemker, H. C., Frederik, P. M., & Willems, G. M. (1992) *J. Biol. Chem. 267*, 17907–17912.

Bartlett, G. R. (1959) *J. Biol. Chem. 234*, 466–468.

Bazzi, M. D., & Nelsestuen, G. L. (1991) *Biochemistry 30*, 7961–7969.

Blackwood, R. A., & Ernst, J. D. (1990) *Biochem. J. 266*, 195–200.

Chen, P. S., Jr., Toribara, T. Y., & Warner, H. (1956) *Anal. Chem. 28*, 1756–1758.

Concha, N. O., Head, J. F., Kaetzel, M. A., Dedman, J. R., & Seaton, B. A. (1993) *Science 261*, 1321–1324.

Creutz, C. E. (1992) *Science 258*, 924–931.

Edwards, H. C., & Crumpton, M. J. (1991) *Eur. J. Biochem. 198*, 121–129.

Funakoshi, T., Heimark, R. L., Hendrickson, L. E., McMullen, B. A., & Fujikawa, K. (1987) *Biochemistry 26*, 5572–5578.

Geisow, M. J., Fritsche, U., Hexham, J. M., Dash, B., & Johnson, T. (1986) *Nature 320*, 636–638.

Glenney, J. R., Jr. (1986) *J. Biol. Chem. 261*, 7247–7252.

Huang, K.-S., McGray, P., Mattaliano, R. J., Burne, C., Chow, E. P., Sinclair, L. K., & Pepinsky, R. B. (1987) *J. Biol. Chem. 262*, 7639–7645.

Huber, R., Schneider, M., Mayr, I., Römisch, J., & Paques, E.-P. (1990) *FEBS Lett. 275*, 15–21.

Huber, R., Berendes, R., Burger, A., Schneider, M., Karshikov, A., Luecke, H., Römisches, J., & Paques, E. (1992) *J. Mol. Biol. 223*, 683–704.

Jain, M. K., van Echteld, C. J. A., Ramirez, F., de Gier, J., de Haas, G. H., & van Deenen, L. L. M. (1980) *Nature 284*, 486–487.

Jain, M. K., Maliwal, B. P., deHaas, G. H., & Slotboom, A. J. (1986) *Biochim. Biophys. Acta 860*, 448–461.

Jain, M. K., Yuan, W., & Gelb, M. H. (1989) *Biochemistry 28*, 4135–4139.

Junker, M., & Creutz, C. E. (1993) *Biochemistry 32*, 9968–9974.

Karshikov, A., Berendes, R., Burger, A., Cavalié, A., Lux, H.-D., & Huber, R. (1992) *Eur. Biophys. J. 20*, 337–344.

Kingsley, P. B., & Feigenson, G. W. (1979) *Chem. Phys. Lipids 24*, 135–147.

Lee, M.-H., & Bell, R. M. (1992) *Biochemistry 31*, 5176–5182.

Mayer, L. D., Hope, M. J., & Cullis, P. R. (1986) *Biochim. Biophys. Acta 858*, 161–168.

Meers, P. (1990) *Biochemistry 29*, 3325–3330.

Meers, P., & Mealy, T. R. (1993a) *Biochemistry 32*, 5411–5418.

Meers, P., & Mealy, T. R. (1993b) *Biochemistry 32*, 11711–11721.

Meers, P., Mealy, T. R., Pavlotsky, N., & Tauber, A. I. (1992) *Biochemistry 31*, 6372–6382.

Morrison, W. R. (1964) *Anal. Biochem. 7*, 218–224.

Newton, A. C. (1993) *Annu. Rev. Biophys. Biomol. Struct. 22*, 1–25.

Oishi, K., Raynor, R. L., Charp, P. A., & Kuo, J. F. (1988) *J. Biol. Chem. 263*, 6865–6871.

Orr, J. W., & Newton, A. C. (1992a) *Biochemistry 31*, 4661–4667.

Orr, J. W., & Newton, A. C. (1992b) *Biochemistry 31*, 4667–4673.

Pepinsky, R. B., Tizard, R., Mattaliano, R. J., Sinclair, L. K., Miller, G. T., Browning, J. L., Chow, E. P., Burne, C., Huang, K.-S., Pratt, D., Wachter, L., Hession, C., Frey, A. Z., & Wallner, B. P. (1988) *J. Biol. Chem. 263*, 10799–10811.

Perreti, M., & Flower, R. J. (1993) *J. Immunol. 150*, 992–999.

Reutelingsperger, C. P. M., Hornstra, G., & Hemker, H. C. (1985) *Eur. J. Biochem. 151*, 625–629.

Rojas, E., Pollard, H. B., Haigler, H. T., Parra, C., & Burns, A. L. (1990) *J. Biol. Chem. 265*, 21207–21215.

Schlaepfer, D. D., Mehlman, T., Burgess, W. H., & Haigler, H. T. (1987) *Proc. Natl. Acad. Sci. U.S.A. 84*, 6078–6082.

Schlaepfer, D. D., Jones, J., & Haigler, H. T. (1992) *Biochemistry 31*, 1886–1891.

Scott, D. L., White, S. P., Otwinowski, Z., Yuan, W., Gelb, M. H., & Sigler, P. B. (1990) *Science 250*, 1541–1546.

Smith, P. K., Krohn, R. I., Hermanson, G. T., Mallia, A. K., Gartner, F. H., Provenzano, M. D., Fujimoto, E. K., Goeke, N. M., Olson, B. J., & Klenk, D. C. (1985) *Anal. Biochem. 150*, 76–85.

Sopkova, J., Renouard, M., & Lewit-Bentley, A. (1993) *J. Mol. Biol. 234*, 816–825.

Thunissen, M. M. G. M., Ab, E., Kalk, K. H., Drenth, J., Dijkstra, B. K., Kuipers, O. P., Dijkman, R., de Haas, G. H., & Verheij, H. M. (1990) *Nature 347*, 689–691.

van Dam-Mieras, M. C. E., Slotboom, A. J., Pieterson, W. A., & de Haas, G. H. (1975) *Biochemistry 14*, 5387–5393.

Wallner, B. P., Mattaliano, R. J., Hession, C., Cate, R. L., Tizard, R., Sinclair, L. K., Foeller, C., Chow, E. P., Browning, J. L., Ramachandran, K. L., & Pepinsky, R. B. (1986) *Nature 320*, 77–82.