Interactions of Annexins with Membrane Phospholipids†

Paul Meers,*,‡ David Daleke,§ Keelung Hong,∥ and Demetrios Papahadjopoulos∥

Department of Pathology, William B. Castle Hematology Laboratory, Boston University School of Medicine, Boston, Massachusetts 02118, Department of Chemistry, Indiana University, Bloomington, Indiana 47405, and Cancer Research Institute, University of California, San Francisco, California 94143

Received March 7, 1990; Revised Manuscript Received October 18, 1990

ABSTRACT: The annexins are proteins that bind to membranes and can aggregate vesicles and modulate fusion rates in a Ca^{2+}-dependent manner. In this study, experiments are presented that utilize a pyrene derivative of phosphatidylcholine to examine the Ca^{2+}-dependent membrane binding of soluble human annexin V and other annexins. When annexin V and other annexins were bound to liposomes containing 5 mol % acyl chain labeled 3-palmitoyl-2-(1-pyrenedecanoyl)-L-α-phosphatidylcholine, a decrease in the excimer-to-monomer fluorescence ratio was observed, indicating that annexin binding may decrease the lateral mobility of membrane phospholipids without inducing phase separation. The observed increases of monomer fluorescence occurred only with annexins and not with other proteins such as parvalbumin or bovine serum albumin. The extent of the increase of monomer fluorescence was dependent on the protein concentration and was completely and rapidly reversible by EDTA. Annexin V binding to phosphatidylserine liposomes was consistent with a binding surface area of 59 phospholipid molecules per protein. Binding required Ca^{2+} concentrations ranging between approximately 10 and 100 μM, where there was no significant aggregation or fusion of liposomes on the time scale of the experiments. The polycation spermine also displaced bound annexins, suggesting that binding is largely ionic in nature under these conditions.

The annexins (Geisow et al., 1987) are a recently recognized class of proteins that bind to phospholipid membranes in a Ca^{2+}-dependent manner. The intracellular functions of these proteins are as yet unknown, but Ca^{2+}-dependent binding to membranes may be an important regulatory phenomenon. One possible function is modulation of intracellular fusion in such processes as exocytosis, a hypothesis supported by the ability of many of the annexins to aggregate and mediate fusion of vesicles (Creutz et al., 1978; Hong et al., 1981, 1982a,b; Meers et al., 1987a, 1988a,b; Drust & Creutz, 1988; Ali et al., 1989).

The sequenced human annexins are highly homologous except for unique N-terminal domains (Geisow et al., 1986; Huang et al. 1986; Kristensen et al., 1986; Saris et al., 1986; Weber et al., 1987; Südhof et al., 1988). In the homologous sequences there is a repeating motif that is proposed to include the phospholipid- and Ca^{2+}-binding domains (Geisow et al., 1986). A consensus sequence within each repeat has been implicated in phospholipid binding (Ali et al., 1989; Meers, 1990), but little other structural information is currently available.

Pyrene-labeled phospholipids have been used previously to measure phase separation and changes in lateral mobility of phospholipids in response to binding of proteins and other molecules. Protein-induced phase separation leads to an isothermal increase in the excimer-to-monomer ratio [e.g., Jones and Lentz (1986)] and breaks in the temperature dependence of the ratio, corresponding to the main phase transitions of separated phospholipid phases. Above the phase transition temperature, a decrease in the excimer-to-monomer ratio has been observed in the presence of cytochrome b_5 (Friere et al., 1983), polylysine (Hartmann & Galla, 1978), or cholesterol (Galla & Hartmann, 1980). This phenomenon is attributed to a decrease in the lateral mobility of the phospholipids.

In order to elucidate the details of the Ca^{2+}-dependent interaction of annexins with membranes, we have used these properties of a pyrene derivative of phosphatidylcholine to detect annexin binding to phospholipid membranes. Parts of this work were presented previously in abstract form (Meers et al., 1987c, 1988c).

Materials and Methods

3-Palmitoyl-2-(1-pyrenedecanoyl)-L-α-phosphatidylcholine (pyrene-PC)[1] was obtained from Molecular Probes (Eugene, OR). Bovine brain phosphatidylserine (PS), phosphatidate (derived from egg phosphatidylcholine) (PA), L-α-dioleoylphosphatidylglycerol (PG), phosphatidylinositol from bovine liver (PI), egg phosphatidylcholine (PC), and phosphatidylethanolamine (transesterified from egg phosphatidylcholine) (PE) were purchased from Avanti Polar Lipids (Birmingham, AL). *N*-[Tris(hydroxymethyl)methyl]-2-aminoethanesulfonic acid (TES) and poly(L-lysine) (type 1B; average molecular weight 400 000) were obtained from Sigma (St. Louis, MO). $CaCl_2$ (>99%), $MgCl_2$ (>99%), and NaCl (>99%) were from Fisher (Pittsburgh, PA). Polycarbonate filters were from Nuclepore (Pleasanton, CA).

Vesicle Preparation. Large unilamellar vesicles encapsulating the desired material were prepared by the reverse-phase evaporation method (Szoka & Papahadjopoulos, 1978) as

† This investigation was supported by Research Grant 1570 C-1 (P.M.) from the Massachusetts Chapter of the American Cancer Society, by a postdoctoral fellowship (P.M.) from the Arthritis Foundation, and partially by Grants GM-28117 (K.H. and D.P.) and GM-41790-01A1 (P.M.) from the National Institutes of Health.

* To whom correspondence should be addressed.
‡ Department of Pathology, Boston University School of Medicine.
§ Department of Chemistry, Indiana University.
∥ Cancer Research Institute, University of California, San Francisco.

[1] Abbreviations: pyrene-PC, 3-palmitoyl-2-(1-pyrenedecanoyl)-L-α-phosphatidylcholine; PS, bovine brain phosphatidylserine; PG, L-α-dioleoylphosphatidylglycerol; PI, phosphatidylinositol from bovine liver; PC, egg yolk phosphatidylcholine; PA, phosphatidate derived from egg yolk; EDTA, ethylenediaminetetraacetate; EGTA, [ethylenebis(oxyethylenenitrilo)]tetraacetic acid; TES, *N*-[tris(hydroxylmethyl)methyl]-2-aminoethanesulfonic acid.

0006-2960/91/0430-2903$02.50/0

modified by Wilschut et al. (1980). Liposomes were extruded through 0.2-μm and then 0.1-μm polycarbonate filters during this procedure (Szoka et al., 1980). This method generates mostly unilamellar vesicles (Düzgünes et al., 1983). Phospholipid concentrations were determined by using a phosphate assay as described in Kingsley and Feigenson (1979), modified from Chen et al. (1956), Bartlett (1959), and Morrison (1964).

Protein Preparation. Synexin was purified by a modification of the technique of Creutz et al. (1978) as described in Meers et al. (1987a, 1988a).

The 67- and 32-kDa calelectrins (annexin IV and VI) were purified from bovine liver as previously described (Südhof & Stone, 1987) with small modifications. Recombinant human lipocortin I and lipocortin V (annexin I and V) were kindly provided by Dr. R. Blake Pepinsky of Biogen Corp. These proteins were at least 98% pure by single-dimension gel electrophoresis.

Fluorescence Measurements. Fluorescence measurements were made with a Spex Fluorolog or Fluorolog 2 fluorometer. For the pyrene-PC probe, the excitation wavelength was 344 nm. Emission was measured at 377 nm for time courses.

The contribution of scattered light was assessed by first measuring the change in 90° light scattering when the emission monochromators were set at the excitation wavelength (344 nm). There was a significant (typically 50%) increase in 90° light scattering upon annexin binding. When this amount of scattering change was reproduced by adding turbid unlabeled phosphatidylcholine vesicles, no change was observed at the fluorophor emission wavelengths (377 nm). Instead, a greater than 10-fold increase in light scattering at the excitation wavelength (344 nm) was required to generate an approximate 10% increase in observed intensity through the emission monochromators.

When buffers were purged of oxygen by bubbling with argon for 1–2 h, the overall pyrene fluorescence quantum yield was higher as demonstrated by Chong and Thompson (1985), but approximately the same fractional increase in fluorescence was observed upon polylysine binding whether or not the sample had been deoxygenated (not shown). Hence, all experiments were performed under ambient oxygen conditions, and all buffers were well equilibrated before experiments.

All samples were maintained at 25 °C in 100 mM NaCl, 5 mM TES, and 0.1 mM EDTA at pH 7.4 (buffer A). Additions of materials to initiate binding, such as Ca^{2+}, were made with small aliquots of a concentrated stock. All Ca^{2+} concentrations are expressed as the excess free Ca^{2+} over the initial EDTA concentration except where noted as total Ca^{2+}. When a Ca^{2+} electrode and standard solutions were used, this assumption for free Ca^{2+} concentration was found to hold for 10 μM Ca^{2+} and higher. In all experiments, Ca^{2+} was added last to a solution containing the appropriate vesicles and protein. Each protein concentration was a separate experiment rather than part of a titration to eliminate a possible effect of prebound protein on the subsequent binding of more protein or the possible effect of slow changes throughout the titration of a single sample (e.g., vesicle aggregation or fusion).

The binding of 14 or 28 nM annexin V to 1 μM pyrene-PC/PS vesicles was approximately the same in the presence or absence of 5 μg/mL ovalbumin, as monitored by pyrene probe fluorescence. Therefore, carrier protein was omitted because of the possibility of unwanted interactions with vesicles or annexins.

Lifetime measurements were performed on an SLM 4800C lifetime spectrofluorometer with a modulation frequency of 6 MHz. Samples were continuously stirred at 25 °C. Only modulation lifetimes were measured.

Calculations. Theoretical binding isotherms were calculated on the basis of a simple model of binding in which protein molecules were considered as ligands that bind independently to sites on the phospholipid vesicles with apparent association constant K'. Each site consists of n phospholipid molecules.

$$[\text{protein}] + [\text{phospholipid}_n] \Leftrightarrow [\text{protein–phospholipid}_n]$$

The maximal increase in fluorescence was taken as 17.2% on the basis of the binding of 10 μg/mL annexin V to 10 μM total phospholipid. The observed change in fluorescence divided by the maximal change was taken to be proportional to the number of sites occupied divided by the total sites.

$$[\text{protein–phospholipid}_n]/[\text{phospholipid}_n]_{\text{total}} = \Delta/\Delta_{\max}$$

A simple quadratic equation was solved to give [protein–phospholipid$_n$].

$$[\text{protein–phospholipid}_n] = (1/2)\,[(L_t + P_t + 1/K') - [(L_t + P_t + 1/K')^2 - 4P_tL_t]^{1/2}]$$

where L_t represents $[\text{phospholipid}_n]_{\text{total}}$ and P_t represents $[\text{protein}]_{\text{total}}$. Note that L_t is a variable parameter equal to $[\text{phospholipid}]_{\text{total}}/n$. Δ is obtained from

$$\Delta = \Delta_{\max}[\text{protein–phospholipid}_n]/[\text{phospholipid}_n]_{\text{total}}$$

Protein/phospholipid ratios were calculated by assuming that 50% of the total phospholipid was accessible to protein.

Data were fit (Figure 2) by using a spreadsheet program (Microsoft Excel) to determine the sum of the squares of the differences between the data and theoretical values. First one parameter was incremented until a minimum was reached; then the procedure was repeated on the other parameter and so on until a true minimum was reached (within the number of significant figures for our data).

Results

When annexins were allowed to bind to phospholipid vesicles in the presence of Ca^{2+}, we observed an increase of the fluorescence of phospholipid fluorophors that exhibit quantum yields dependent on their two-dimensional concentration (Meers et al., 1987c, 1988c). The effect was always dependent on the presence of both Ca^{2+} and an annexin. One probe of this type is 3-palmitoyl-2-(1-pyrenedecanoyl)-L-α-phosphatidylcholine (pyrene-PC). At 5 mol % in phosphatidylserine (PS) vesicles, pyrene-PC exists as monomers with emission peaks at 377 and 395 nm and as excimers with a peak at 480 nm (Galla & Sackmann, 1974). When annexin V was induced to bind to these vesicles by Ca^{2+}, a decrease in the excimer-to-monomer ratio was observed. Figure 1 shows a time course of monomer fluorescence at 377 nm, where the extent of increase of fluorescence was clearly dependent on the annexin V concentration. Conversely, if fluorescence as monitored at 480 nm, where excimers emit, a decrease in fluorescence was observed upon binding (not shown). The rate of fluorescence increase was faster than the mixing time in the cuvette under the conditions of Figure 1 but showed some dependence on protein concentration under more dilute conditions. The observed fluorescence change was not oxygen dependent, nor did it result from stray light effects (Materials and Methods). The direction of the change indicated that lateral phase separation of phospholipids is not induced by protein binding under these conditions, as segregation of the probes would lead to a decrease in monomer fluorescence and an increase in excimer fluorescence.

Synexin, endonexin (annexin IV), the 67-kDa calelectrin (annexin VI), lipocortin I (annexin I), and annexin V were

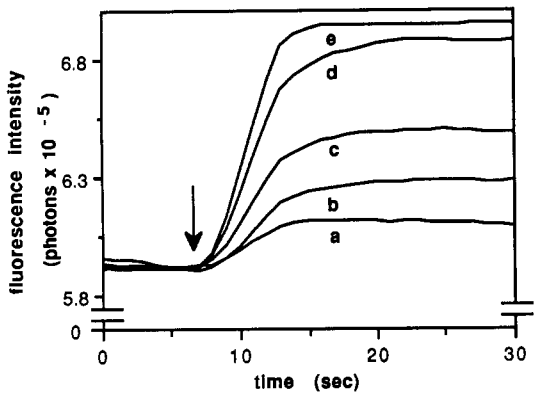

FIGURE 1: Dependence of probe response on protein concentration. Annexin V at a final concentration of (a) 11.4 nM, (b) 28.6 nM, (c) 57.2 nM, (d) 143 nM, or (e) 286 nM was added to vesicles composed of 5 mol % pyrene-PC in PS at a total phospholipid concentration of 10 μM in buffer A at 25 °C. At the arrow a final concentration of 100 μM Ca^{2+} was added to the sample as fluorescence emission at 377 nm was monitored.

Table I: Effects of Various Polypeptides on the Fluorescence of 5 mol % Pyrene-PC in PS

polypeptide[a]	polypeptide concn (μg/mL)	phospholipid concn (μM)	% increase in monomer fluorescence
human serum albumin	9	1	0
ribonuclease	5.7	1	0
polylysine	5.25	1	7.7 ± 0.4
ovalbumin	5	0.25	0
bovine serum albumin	5	0.25	0
parvalbumin	12.5	0.25	0
synexin	1	0.25	13.3 ± 2
endonexin (annexin IV)	1	0.25	13.2
	1	1	12.8 ± 0.8
67-kDa calelectrin (annexin VI)	1	0.25	10.6
	1	1	11.7 ± 0.7
lipocortin I (annexin I)	1	0.25	7.9
	1	1	6.9 ± 0.9

[a] Either the samples were preincubated with polypeptide followed by addition of 100 μM Ca^{2+} or the effect of the polypeptide alone was evaluated (human serum albumin, ribonuclease, and polylysine). All experiments were performed in buffer A at 25 °C.

all able to cause similar Ca^{2+}-dependent changes in the fluorescence of the pyrene-PC probe (see Table I). Ovalbumin, bovine serum albumin, and the Ca^{2+}-binding protein parvalbumin, did not mediate any Ca^{2+}-dependent change of the pyrene-PC fluorescence in PS vesicles. However, binding of polycationic polylysine caused an increase in fluorescence at 377 nm without any added Ca^{2+} (Table I).

The excimer-to-monomer ratio of the pyrene probe is dependent on its concentration within the membrane, its lateral diffusion coefficient, and the fluorescence lifetime of the excimer (Galla & Sackmann, 1974). In order to determine whether changes in the fluorescence lifetimes of the probes could account for the observed effect, appropriate measurements were made before and after annexin V or polylysine binding. In Table II it is shown that the lifetime of the excimer remained essentially unchanged upon polylysine binding to PS vesicles whether or not the sample had been degassed. Annexin V binding also had no significant effect on the excimer lifetime of the probe. Though a change in lifetime smaller than the limits of error cannot be ruled out, it would appear not to completely account for the observed effect on excimer-to-monomer ratio. A small change of monomer lifetime (positive for annexin V, negative or positive for polylysine) was observed. A partial contribution of these changes to monomer intensity

Table II: Effect of Binding on Pyrene-PC Fluorescence Lifetimes[a]

peaks obsd[b]	without polylysine	with polylysine[c]
monomer	60.8 ± 2.4	63.2 ± 1.9
excimer	100.4 ± 6.3	98.1 ± 9.7
monomer, degassed[d]	70.5 ± 5.1	66.6 ± 2.1
excimer, degassed	109.9 ± 5.4	111.6 ± 13.5
peaks obsd (with annexin V)[e]	without Ca^{2+}	with Ca^{2+}[f]
monomer	61.2 ± 2.7	66.6 ± 2.6
excimer	96.7 ± 3.8	98.2 ± 5.8

[a] All experiments were performed at 25 °C with 20 μM total phospholipid. Vesicles were composed of 5 mol % pyrene-PC and 95 mol % PS. All lifetimes were calculated from demodulation data and are expressed in nanoseconds. Lifetimes were averaged from ten measurements. For annexin V binding, two separate averages of ten were used, and a weighted average of these two values was taken. [b] No annexins were present. Monomer lifetimes were determined at 377 nm. Excimer lifetimes were determined at 480 nm. [c] 4.2 μg/mL polylysine. [d] Argon gas was bubbled for 2 h into the buffer used for these samples. [e] 286 nM annexin V. [f] 100 μM Ca^{2+}.

is possible but seems unlikely.

In order to rule out the possibilities of direct specific interaction between the pyrene probe and the annexin or an indirect effect of the annexins on the quantum yield of monomers only, two experiments were performed. In one experiment, the probe was kept at 5 mol %, but PS was replaced by PC, a phospholipid to which annexins do not bind under these conditions. This substitution abolished any Ca^{2+}-dependent increase (100 μM Ca^{2+}) in fluorescence of the pyrene probe induced by any of the annexins, indicating that they do not interact specifically with the probe (not shown). When the pyrene probe was incorporated into PS vesicles (1 μM total phospholipid) at decreasing percentages of the total phospholipid (5%, 2%, 1%, and 0.4%), where a progressively lower fraction of excimers is formed, the magnitude of the annexin–dependent (50 nM annexin V) change in fluorescence at 377 nm decreased monotonically. Therefore, the increase in fluorescence intensity at 377 nm requires the existence of excimers and is not due to a specific interaction between probe and protein.

On the basis of the above results, the binding of annexins to membranes was modeled by assuming two states in which monomers of annexin V interact with sites on the vesicles consisting of n phospholipids, leading to a change of the effective lipid lateral diffusion coefficient to some lower value. This change produces an increase in the fluorescence at 377 nm (Figure 1) with a maximal value characteristic of the particular annexin. With this simple model, theoretical curves were fit to the data in Figure 2A to obtain the apparent interaction association constant (K') and the apparent stoichiometry (n). The data were obtained by varying the protein concentration at three different phospholipid concentrations. When the differences between the data and three theoretical curves were minimized, a requirement for 59 phospholipid molecules (n) and an apparent association constant (K') of 1.12 × 10^8 were measured. The number n probably reflects the constraint on the maximum number of annexin V molecules that can fit on the surface of a vesicle. As such, n represents an estimated upper limit on the number of phospholipids that can directly interact with a single annexin molecule. It is clear from the data at 10 μM phospholipid in Figure 2A that a minimum interaction stoichiometry of approximately 20 phospholipids per annexin monomer is defined by the phospholipid/protein ratio at which maximal fluorescence change is reached. In order to test the predictive value of these constants, the phospholipid concentration was varied as shown

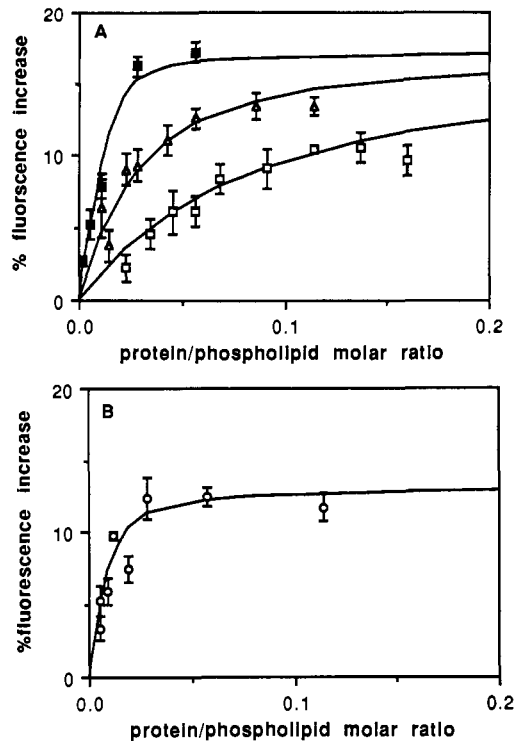

FIGURE 2: Binding isotherms for annexin V. The extent of increase of fluorescence at 377 nm was measured after addition of 100 μM Ca^{2+} to samples. (A) Samples contained vesicles of 5 mol % pyrene-PC in PS at total concentrations of (□) 0.25 μM, (△) 1 μM, and (■) 10 μM. These curves consist of 21, 18, and 10 measurements, respectively. Annexin V concentrations varied as shown. Molar ratios of protein/phospholipid (x axis) were based on half of the total phospholipid concentration. Solid lines represent curves fit to the data by using an apparent association constant (K') of 1.12×10^8 M^{-1} and an apparent lipid/protein stoichiometry (n) of 59. Data were fit by simultaneously minimizing the squares of the differences between data and theory for all three curves and all 49 measurements. The reduced χ_r^2 with this format for the fit was 0.96. All experiments were performed at 25 °C in buffer A. (B) Samples contained vesicles of 5 mol % pyrene-PC in PS at varying total phospholipid concentrations. The total annexin V concentration was held constant at 28.6 nM. The solid line was generated by using the constants from part A.

in Figure 2B, and the theoretical curve, plotted with $K' = 1.12 \times 10^8$ and $n = 59$, showed reasonable agreement with the data.

Relative binding to any other type of vesicle could be measured by competition, with binding to the pyrene-PC/PS vesicles used as a standard. In Figure 3, competition with unlabeled PS containing 5 mol % egg PC is shown. Binding of annexin V to the labeled vesicles is inhibited by the presence of unlabeled vesicles. With this method, binding to vesicles of several phospholipid compositions was measured. In Table III is shown annexin V binding to vesicles of 25% PC and 75% of an acidic phospholipid. The effectiveness of competition of the various vesicles is measured by the fluorescence increase (in percent maximal) that is observed in the presence of the competing vesicles, where 100% is the fluorescence increase in the absence of competing vesicles. Therefore, lower numbers indicate better competition. The selectivity of annexin V between phosphatidylglycerol (PG), phosphatidate (PA), and PS is relatively low under these conditions, but it does not bind to phosphatidylinositol (PI) or PC (not shown) at all.

The Ca^{2+} dependence for binding of annexin V (28.6 nM) was also monitored by using vesicles composed of 5 mol % pyrene-PC in PS (1 μM). The binding curve was sigmoidal, and approximately 50 μM Ca^{2+} was required for half-maximal binding under these conditions, in close agreement with Schlaepfer et al. (1987).

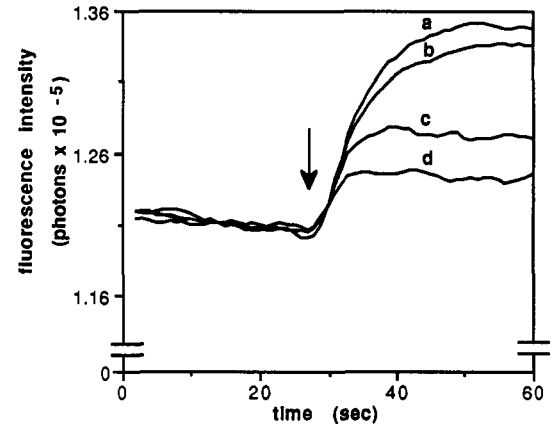

FIGURE 3: Competition of unlabeled vesicles for annexin V binding. Initial preparations contained 28.6 nM annexin V and vesicles composed of 5 mol % pyrene-PC at a total phospholipid concentration of 1 μM. Samples also contained vesicles of 5 mol % egg PC in PS at (a) 0, (b) 1, (c) 5, or (d) 10 μM total phospholipid. Free Ca^{2+} at a concentration of 100 μM was added at the arrow. The sample was in buffer A at 25 °C.

Table III: Effect of Phospholipid Composition on Annexin V Binding[a]

competing vesicles	% max fluorescence increase	competing vesicles	% max fluorescence increase
none	100	PS/PC (3/1)	72 ± 7
PG/PC (3/1)	62 ± 2	PI/PC (3/1)	115 ± 12
PA/PC (3/1)	62 ± 9		

[a] Binding of 20 nM annexin V to PS vesicles with 5 mol % pyrene-PC at 2 μM total phospholipid was measured upon addition of a free Ca^{2+} concentration of 100 μM for each sample. The increase in fluorescence at 377 nm was set to 100%. A final concentration of 200 μM EDTA was subsequently added to reverse the binding. The appropriate competing vesicles (2 μM total phospholipid) were added to the same sample, and 200 μM total Ca^{2+} was then added to give 100 μM free Ca^{2+}. The increase in fluorescence was measured and divided by the first reading to give the percentage of maximal binding to the probe-containing vesicles. The mean of four measurements for PS and two measurements for the other phospholipids is shown. All experiments were performed in buffer A at 25 °C on a Perkin-Elmer LS-5 fluorometer.

In order to gain more information about the factors that mediate binding, the reversibility of binding was also studied. Binding was completely and very rapidly reversible for all annexins when excess EGTA or EDTA was added within 10 min, though slower irreversible binding is not precluded. In Figure 4A, annexin V binding is shown to be reversible more than once by EDTA. Physiological concentrations of spermine (100–500 μM) (Figure 4B) appeared to reverse annexin V binding. At 500 μM, spermine alone caused a small drop in monomer fluorescence that is absent at 100 μM. With other annexins, 100 μM spermine is also sufficient to significantly reverse binding (not shown).

Discussion

In this report, we have demonstrated a useful fluorescence method for the estimation of the amount and rate of annexin binding to phospholipid vesicles. The rate of exchange between vesicles and competition for binding by any other membrane can potentially be measured. The method requires little protein and no modification of the protein, allowing many different annexins to be compared and possibly allowing proteins modified by site-directed mutagenesis to be studied.

Mechanism of the Fluorescence Response. Annexins could decrease the excimer-to-monomer ratio by effectively diluting the probes, changing the lifetimes, or lowering the effective

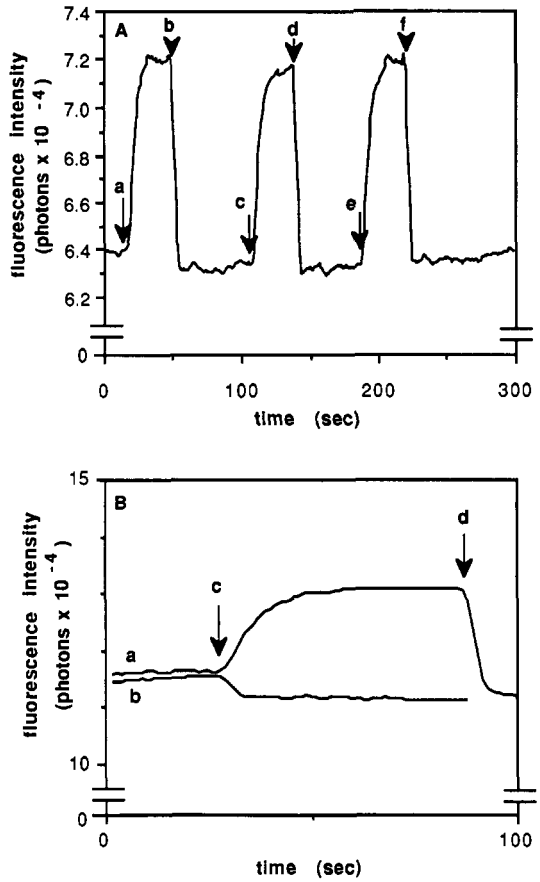

FIGURE 4: Reversibility of annexin V binding. (A) The sample contained 1 μM total phospholipid composed of 5 mol % pyrene-PC in PS and 50 nM annexin V. Each Ca^{2+} addition (points a, c, and e) gave a free Ca^{2+} concentration of approximately 100 μM as measured by the Ca^{2+} electrode. Each EDTA addition (points b, d, and f) gave approximately 100 μM excess EDTA over Ca^{2+}. Experiments were performed in buffer A at 25 °C. (B) The sample contained 1 μM total phospholipid composed of 5 mol % pyrene-PC in PS (a) with or (b) without 28.6 nM annexin V. In curve a 100 μM Ca^{2+} was added at point c and 500 μM spermine at point d. In curve b, 500 μM spermine was added at point c.

lateral mobility of the probes. Effective dilution is unlikely in that it would require the protein to act as a molecule that is exchangeable with phospholipids in the bilayer matrix. Disruption of preexisting pyrene-PC domains by annexin binding could also show the same effect, but this seems unlikely because of the relatively low concentration of probe in the membrane and the fact that similar results were seen with different probes (Meers et al., 1987c, 1988c, unpublished data). Changes in lifetimes have been ruled out by our measurements.

A decrease in the effective lateral diffusion coefficient of the lipid probes provides the most likely explanation for the change in excimer-to-monomer ratio. It is possible that the simultaneous interaction of a number of phospholipids with each large protein molecule provides a microenvironment where lateral diffusion is low. Indeed, a large polypeptide such as polylysine, which should not insert deeply into the membrane (Papahadjopoulos et al., 1975), decreased the excimer-to-monomer ratio, whereas a small polycation, spermine, did not. Annexins could also decrease effective lateral mobility by generating obstacles as concluded by Friere et al. (1983) with respect to a similar phenomenon induced by cytochrome b_5. An obstacle could be any object within the two-dimensional lattice of the membrane that has a lower lateral mobility than the bulk of the phospholipid (Eisinger et al., 1986; Saxton, 1987).

Protein–Lipid Stoichiometry. Recently, a stoichiometry of approximately 550 for binding of fluoresceinated annexin V to PS/PC (20/80) vesicles in the presence of 1 mM Ca^{2+} was reported (Tait et al., 1988, 1989). While the manuscript was being reviewed, a stoichiometry of 42 was reported when ellipsometry was used (Andree et al., 1990). This latter number is close to our determination of 59 and seems physically reasonable. A spherical 35-kDa protein with a density of 1.0 g/mL would have a radius of 2.4 nm. If half the surface area of the sphere were available for interaction with phospholipids, it would measure 36 Å^2. Assuming each phospholipid occupies 0.7 nm^2, a ratio of 52 phospholipids per annexin V is obtained. A projection of the sphere onto the membrane surface is half that area. This number could be higher if there were any flattening or elongation of the sphere. A direct comparison of the determinations is difficult because different vesicle or bilayer preparation methods, phospholipid compositions, and Ca^{2+} concentrations were used. Aggregation of vesicles and lateral phase separations at high (1 mM or more) Ca^{2+} concentrations may contribute to the differences. The avidity of binding by our measurements is apparently lower than in the previous reports, probably because of the 10–30-fold lower Ca^{2+} concentration used. At very low protein (<5 nM) and phospholipid concentrations (<100 nM), millimolar concentrations of Ca^{2+} may be required for binding.

It is important to note that our calculated values for n do not represent the number of phospholipid binding sites on an annexin molecule but the number of phospholipids whose motion is affected by a single annexin moleule, and it may represent an upper limit because it is partially governed by the shape and packing of bound annexin V on the surface of the bilayer. Multiple bilayers in vesicles would also lead to exaggerated n. Finally, though there is adequate agreement of the theoretical curves with the data taken under various conditions, it does not preclude a better description of the data by a more complex model that could include possible nonlinearity of probe response and protein-packing effects.

Binding Interactions. Rapid reversibility of binding by Ca^{2+} chelators [also observed by Maurer-Fogy et al. (1988)] indicates that ionic interactions are important for annexin V binding. Reversibility by the polycation spermine supports this conclusion, though is not clear whether spermine removes Ca^{2+} from the surface of the bilayer or from an annexin binding site or both. Ionic binding may be mediated by positively charged annexin groups, including a highly conserved arginine in the consensus sequence (Geisow et al., 1986; Pepinsky et al., 1988), and possibly by the bound Ca^{2+} ions themselves. Though binding is largely ionic in nature, it could include some hydrophobic interactions, which would explain the relative lack of headgroup specificity as observed for synexin (Meers et al., 1987b). A similar situation exists for cationic myelin basic protein (Sankaram et al., 1989a,b).

Rate of Binding. The rapidity of the increase in fluorescence (10–30 s) is important with regard to binding in that some annexins may bind more than one membrane simultaneously, leading to aggregation and/or fusion and possibly to a different mode of annexin binding. At this rate, binding must reach equilibrium long before vesicles can aggregate, even at an annexin-assisted rate constant of about 10^7 M^{-1} s^{-1} (Meers et al., 1988a). Therefore, all annexin binding to a single bilayer occurs before any effect on the rate of fusion of vesicles, an assmuption made in several previous publications (Hong et al., 1981, 1982a,b; Meers et al., 1988a,b).

ACKNOWLEDGMENTS

We thank Dr. William Flick Coleman and Dr. Nancy

Kolodny at Wellesley College for the use of a Spex Fluorolog 2 fluorometer. Use of this instrument was partially supported by the Instrumentation and Laboratory Improvement Program of the National Science Foundation. We are grateful to Dr. R. Blake Pepinsky of Biogen Corp. for supplying lipocortins I and V.

Registry No. Ca^{2+}, 7440-70-2; spermine, 71-44-3.

REFERENCES

Ali, S. M., Geisow, M. J., & Burgoyne, R. D. (1989) *Nature 340*, 313–315.

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.

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

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

Chong, P. L., & Thompson, T. E. (1985) *Biophys. J. 47*, 613–621.

Creutz, C. E., Pazoles, C. J., & Pollard, H. B. (1978) *J. Biol. Chem. 253*, 2858–2866.

Drust, D. S., & Creutz, C. E. (1988) *Nature 331*, 88–91.

Düzgünes, N., Wilschut, J., Hong, K., Fraley, R., Perry, C., Friend, D. S., James, T. L., & Papahadjopoulos, D. P. (1983) *Biochim. Biophys. Acta 732*, 289–299.

Eisinger, J., Flores, J., & Petersen, W. P. (1986) *Biophys. J. 49*, 987–1001.

Friere, E., Markello, T., Rigell, C., & Holloway, P. W. (1983) *Biochemistry 22*, 1675–1680.

Galla, H.-J., & Sackmann, E. (1974) *Biochim. Biophys. Acta 339*, 103–115.

Galla, H.-J., & Hartmann, W. (1980) *Chem. Phys. Lipids 27*, 199–219.

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

Geisow, M. J., Walker, J. H., Boustead, C., & Taylor, W. (1987) *Biosci. Rep. 7*, 291–298.

Hartmann, W., & Galla, H.-J. (1978) *Biochim. Biophys. Acta 509*, 474–490.

Hong, K., Düzgünes, N., & Papahadjopoulos, D. (1981) *J. Biol. Chem. 256*, 3641–3644.

Hong, K., Düzgünes, N., & Papahadjopoulos, D. (1982a) *Biophys. J. 37*, 297–305.

Hong, K., Düzgünes, N., Ekerdt, R., & Papahadjopoulos, D. (1982b) *Proc. Natl. Acad. Sci. U.S.A. 79*, 4642–4644.

Jones, M. E., & Lentz, B. R. (1986) *Biochemistry 25*, 567–574.

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

Maurer-Fogy, I., Reutelingsperger, C. P. M., Pieters, J., Bodo, G., Stratowa, C., & Hauptmann, R. (1988) *Eur. J. Biochem. 174*, 585–592.

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

Meers, P., Ernst, J. D., Hong, K., Düzgünes, N., Fedor, J., Goldstein, I. M., & Papahadjopoulos, D. (1987a) *J. Biol. Chem. 262*, 7850–7858.

Meers, P., Hong, K., & Papahadjopoulos, D. (1987b) in *Calcium Binding Proteins in Health and Disease* (Norman, A. W., Vanaman, T. C., & Means, A. R., Eds.) pp 388–390, Academic Press, Orlando, FL.

Meers, P., Papahadjopoulos, D., & Hong, K. (1987c) *Biophys. J. 51*, 169a.

Meers, P., Bentz, J., Alford, D., Nir, S., Papahadjopoulos, D., & Hong, K. (1988a) *Biochemistry 27*, 4430–4439.

Meers, P., Hong, K. & Papahadjopoulos, D. (1988b) *Biochemistry 27*, 6784–6794.

Meers, P., Papahadjopoulos, D., & Hong, K. (1988c) *Biophys. J. 53*, 319a.

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

Papahadjopoulos, D., Moscarello, M., Eylar, E. H., & Isac, T. (1975) *Biochim. Biophys. Acta 401*, 317–335.

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., & Wallner, B. P. (1988) *J. Biol. Chem. 263*, 10799–10811.

Sankaram, M. B., Brophy, P. J., & Marsh, D. (1989a) *Biochemistry 28*, 9685–9691.

Sankaram, M. B., Brophy, P. J., & Marsh, D. (1989b) *Biochemistry 28*, 9692–9698.

Saxton, M. J. (1987) *Biophys. J. 52*, 989–997.

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

Südhof, T. C., & Stone, D. K. (1987) *Methods Enzymol. 139*, 30–35.

Szoka, F., & Papahadjopoulos, D. (1978) *Proc. Natl. Acad. Sci. U.S.A. 75*, 4194–4198.

Szoka, F., Olson, F., Heath, T., Vail, W., Mayhew, E., & Papahadjopoulos, D. (1980) *Biochim. Biophys. Acta 601*, 559–571.

Tait, J. F., Sakata, M., McMullen, B. A., Miao, C. H., Funakoshi, T., Hendrickson, L. E., & Fujikawa, K. (1988) *Biochemistry 27*, 6268–6276.

Tait, J. F., Gibson, D., & Fujikawa, K. (1989) *J. Biol. Chem. 264*, 7944–7949.

Wilschut, J., Düzgünes, N., Fraley, R., & Papahadjopoulos, D. (1980) *Biochemistry 19*, 6011–6021.