Annexin V Binding to the Outer Leaflet of Small Unilamellar Vesicles Leads to Altered Inner-Leaflet Properties: ^{31}P- and ^{1}H-NMR Studies†

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ABSTRACT: Calcium-dependent binding to phospholipid membranes is closely associated with annexin functional properties. In these studies, ^{31}P- and ^{1}H-nuclear magnetic resonance (NMR) experiments have been performed to study the effects of binding of recombinant rat annexin V to sonicated small unilamellar vesicles (SUVs). High-resolution ^{31}P-NMR spectra of SUVs containing mixtures of synthetic phosphatidic acid (PA) and phosphatidylcholine (PC) show resolvable resonances corresponding to the inner-leaflet PA, outer-leaflet PA, and PC phosphoryl groups. When annexin binding occurs, the outer-leaflet PA ^{31}P resonance shifts while that of PC is unaffected, consistent with selective binding of the protein to the phosphoryl moiety of the PA component. Further, annexin V binding to membrane outer-leaflet phospholipids has a measurable effect on inner-leaflet phospholipids of intact vesicles. ^{1}H-NMR T_1 relaxation measurements of SUVs containing acyl-chain-perdeuterated PC show no effects on the PA hydrocarbon–chain segmental motions upon annexin binding. Circular dichroism measurements indicate that the protein does not undergo a significant conformational change upon binding to the vesicles. The observed NMR changes do not correspond to proton or calcium gradients, nor to lateral segregation of extended patches of homogeneous phospholipids. The combined evidence suggests that selective, peripheral annexin–membrane interactions influence the environment of the inner vesicular surface. The mechanism proposed is a protein-induced change in vesicle morphology that corresponds to reduced curvature.

Annexins are a family of structurally homologous proteins that interact with phospholipid membranes in a calcium-dependent manner [for a review, see Klee (1988), Crompton *et al.* (1988), Burgoyne and Geisow (1989), Smith *et al.* (1990), Creutz (1992), and Swairjo and Seaton (1994)]. Twelve annexins have been identified to date, from simple eukaryotes to higher plants and animals (Moss, 1992). Although the physiological functions of annexins have not been determined, the proteins have been implicated in numerous cell processes, including exocytosis and membrane fusion (Creutz, 1992), ion channel activity or regulation (Pollard *et al.*, 1992; Diaz-Muñoz *et al.*, 1990), and inhibition of membrane-binding enzymes such as phospholipase A_2 (Davidson *et al.*, 1987) and protein kinase C (Schlaepfer *et al.*, 1992).

Annexins possess common structural elements that suggest a shared mechanism of calcium-dependent membrane attachment. It is believed that this attachment process is a prerequisite for the cellular functions of annexins. The primary structures of all annexins are characterized by a highly conserved core region that contains the calcium- and membrane-binding apparatus. Annexin sequences also contain highly variable N-terminal regions that range greatly in length. Annexin V, a 35-kDa protein, possesses little more than the conserved annexin core, as its N-terminal region is minimal. The crystal structures of rat (Concha *et al.*, 1993), human (Huber *et al.*, 1992; Sopkova *et al.*, 1993), and chicken (Bewley *et al.*, 1993) annexin V have been determined at high resolution.

The diverse cellular roles proposed for the annexins have lead to some controversy as to the precise nature of the annexin–membrane interaction. The *in vitro* calcium channel activity of annexin V and certain other annexins [for a review, see Pollard *et al.* (1992)] suggests that if annexins behave similarly to other ion channels, they will traverse the membrane bilayer. Karshikov and co-workers (Karshikov *et al.*, 1992) offer an alternative mechanism, based on electrostatic calculations, for channel activity in which electroporation of the membrane occurs when the annexin molecule binds to the membrane surface. Most biophysical studies of annexins [for a review, see Swairjo and Seaton (1994)] support a peripheral rather than integral membrane interaction. High-resolution electron microscopic images show annexin V molecules, consistent with the annexin crystal structure, assembled on the surface of monolayers composed of acidic phospholipids (Brisson *et al.*, 1991; Mosser *et al.*, 1991). Crystal structures of annexin V show that the surfaces of the protein, including the one believed to face the membrane, are strongly hydrophilic and therefore very unlikely to penetrate the bilayer to any great extent. The contribution of the phospholipid membrane itself to *in vitro* properties of annexins is not well understood. Many different membrane properties may change as a result of annexin binding, and much remains to be learned in this area.

To investigate in detail what happens to lipid bilayers when annexin V binds, we have used ^{31}P- and ^{1}H-NMR[1] spectroscopy to study the calcium-dependent interaction of annexin V with small unilamellar vesicles (SUVs) containing synthetic phosphatidylcholine (PC) and phosphatidic acid (PA). Annexins characteristically display a very strong preference for

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anionic membrane phospholipids (Andree *et al.*, 1990; Blackwood & Ernst, 1990) and do not bind liposomes of pure PC. Characterization of our SUV model system by EM and NMR has been described previously (Swairjo *et al.*, 1994). These SUVs remain structurally intact and unaggregated under the experimental conditions used. Annexin V, unlike some other annexins, does not aggregate vesicles. These SUVs give distinct ^{31}P-NMR resonances for inner-leaflet PA, outer-leaflet PA, and PC phosphoryl groups, thus providing a sensitive probe of protein–membrane interactions on both sides of the membrane (Swairjo *et al.*, 1994). Acyl-chain-perdeuterated PC allows us to differentiate the PA and PC hydrocarbon chains, and to selectively look at the effects on PA acyl chains when annexin V interacts with the PA phosphoglycerol backbone. In the present study, circular dichroism (CD) also is used to monitor whether specific NMR phenomena are associated with a significant change in annexin V structure in the SUV-bound state. The demonstrated similarity between the molecular structure of soluble (crystal structure) and membrane-bound (on phospholipid monolayers) forms of the protein predicts that their CD spectra should show little change if the protein remains localized to the outer membrane surface.

MATERIALS AND METHODS

Preparation of Small Unilamellar Vesicles (SUVs). Synthetic 1,2-dioleoyl-*sn*-glycero-3-phosphocholine (DOPC), 1,2-dioleoyl-*sn*-glycero-3-phosphate monosodium salt (DOPA), and 1,2-dimyristoyl-d_{54}-*sn*-glycero-3-phosphocholine (perdeuterated DMPC) are purchased from Avanti Polar Lipids (Birmingham, AL) and used without further purification. SUVs are prepared as follows. Pure DOPA and mixtures of DOPA/DOPC in a molar ratio of 1:1 are dried from chloroform solutions under nitrogen, lyophilized, and then suspended in buffer (50 mM HEPES, 100 mM KCl, 0.1 mM DTT, and 0.02% NaN_3, pH 7.4) to final concentrations of 32 mM. "Calcium-free" samples are prepared in buffers containing 50 μM EGTA; the 1 mM calcium buffer contained 1.05 mM $CaCl_2$ and 50 μM EGTA. Lipids used for ^{1}H NMR experiments are suspended in ^2H_2O adjusted to pH 7.4 with NaOD and DCl. The lipid suspensions are sonicated under standard conditions for an interval of 7–9 min using a Branson W-350 sonicator with a microtip. Large lipid structures are separated by centrifugation at 17000*g* for 60 min and discarded. Samples are kept under nitrogen/argon during all steps of preparation. The purity of the phospholipids before and after sonication is assessed by extracting vesicles in organic solvent and recording ^{31}P spectra. The integrity of the acyl-chain double bond is checked with thin-layer chromatography on silica gel G (Alltech, Deerfield, IL) using a solvent of $CHCl_3/CH_3OH/H_2O$ (65:25:4, v/v) and followed by iodine staining. The final phospholipid concentrations are determined by the ashing procedure for total phosphate according to the method of Ames and Dubin (1960).

Characterization of the SUV System. The SUVs used in this study have been characterized in the presence and absence of calcium using electron microscopy, light scattering, and NMR techniques (Swairjo *et al.*, 1994). The morphology and size distribution of the SUVs in the presence or absence of calcium and protein have been evaluated by negative-stain electron microscopy. The electron micrographs show a relatively homogeneous population of round vesicles with diameters in the range 150–250 Å. The EM results reveal no significant aggregation or fusion of the SUVs into larger vesicles under our experimental conditions (Figure 1). Both in the absence and in the presence of calcium, no more than 7% of the total vesicle population was above 300 Å in diameter.

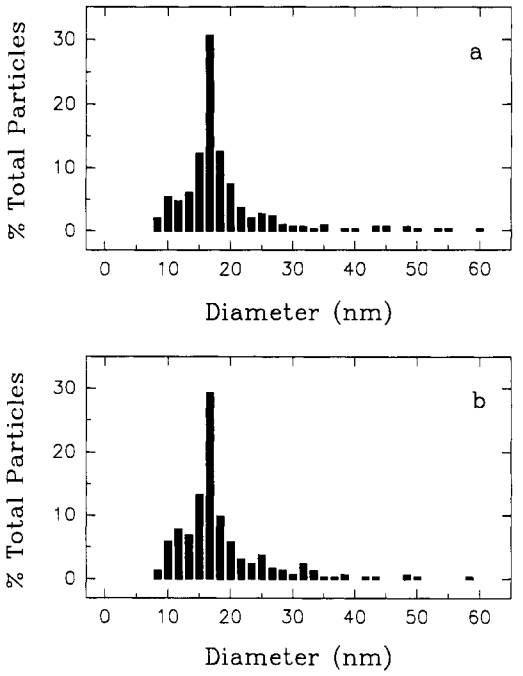

FIGURE 1: Diameter distribution of sonicated SUVs containing PA/PC (1:1), pH 7.4: (a) in the presence of 1 mM $CaCl_2$; (b) in the presence of 1 mM $CaCl_2$ and 30 μM annexin V. A total of 294 particles were sized from multiple electron micrographs.

Expression and Purification of Wild-Type Recombinant Rat Annexin V. Cultures of *Escherichia coli* strain JM105 containing annexin V cloned in the expression vector pKK233-2 (Pharmacia) are grown overnight at 37 °C in LB medium (Bio 101) with 100 μg/mL ampicillin and then diluted 10-fold into fresh LB medium with 100 μg/mL ampicillin. When the optical density at 600 nm has reached a value of 0.3–0.5, isopropyl β-D-thiogalactopyranoside (IPTG) is added to a final concentration of 3 nM and growth continued for 4 h. Bacteria are harvested by centrifugation, washed once with 100 mL of phosphate-buffered saline (PBS) and 10 mM ethylenediaminetetraacetic acid (EDTA) disodium salt, and stored at −20 °C overnight. IPTG-stimulated *E. coli* expressing recombinant annexin V are sonicated for 2–3 min on ice in sonication buffer (PBS, 10 mM EDTA, 6 M urea, 1% Triton X-100, 0.5 μg/mL leupeptin, 0.5 μg/mL pepstatin, and 0.2 mM phenylmethanesulfonyl fluoride). The sonicate is centrifuged for 15 min at 25000*g*, and the supernatant is recovered and dialyzed 3 times at 4 °C against 1 L of dialysis buffer (50 mM Tris-HCl, pH 8.0, 100 mM NaCl, 3 mM NaN_3, and 1 mM EDTA). The dialyzed sample is centrifuged for 20 min at 15000*g*, and the supernatant is filtered through a 0.22 μm nitrocellulose membrane. The annexin V is then purified as we describe for natural rat annexin V (Kaetzel *et al.*, 1990). The properties of natural and recombinant wild-type rat annexin V are comparable. Protein concentration was

[1] Abbreviations: SUV, small unilamellar vesicle; NMR, nuclear magnetic resonance; DTT, dithiothreitol; DOPC, 1,2-dioleoyl-*sn*-glycero-3-phosphocholine; DOPA, 1,2-dioleoyl-*sn*-glycero-3-phosphate; DMPC, 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine; PA, phosphatidic acid; PC, phosphatidylcholine; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; PS, phosphatidylserine; IPTG, isopropyl β-D-thiogalactopyranoside; PBS, phosphate-buffered saline; EDTA, ethylenediaminetetraacetic acid.

measured according to Lowry *et al.* (1951) using a bovine serum albumin standard.

^{31}P and ^{1}H NMR. WALTZ ^{1}H-decoupled ^{31}P (202.33 MHz) spectra are recorded on a Varian Unity 500 spectrometer using a 6 kHz sweep width, 9344 data points, 90° flip angle, 0.779 s acquisition time, 2 s relaxation delay, 15 Hz line broadening, and 200 transients. For locking purposes, vesicle stock suspensions are slightly diluted with $^{2}H_2O$ to final lipid concentrations of 28–30 mM. A capillary of phosphoric acid (80%) is used as an external reference in all experiments. All measurements are done at room temperature.

^{1}H NMR (499.84 MHz) spectra are recorded on the same spectrometer using 6 kHz sweep width, 30 272 data points, 50° flip angle, 3.3 s acquisition time, 2 s relaxation delay, 4 Hz line broadening, and 50 transients. ^{1}H chemical shifts are measured relative to residual HDO at 4.75 ppm. Spectral deconvolutions and line-width calculations are performed using the "fitspec" protocol of the Varian VNMR 4.1 software. ^{1}H and ^{31}P T_1 relaxation measurements are performed using an inversion–recovery pulse sequence with a 11-s relaxation period.

The protocol for the NMR measurements is as follows. A series of NMR spectra of 30 mM SUVs in the presence of 1 mM $CaCl_2$ is taken as annexin V is added in steps to final concentrations of 1, 2, 5, 10, 20, and 30 μM; 20 mM EGTA is then added, and a final NMR spectrum is recorded. All samples are allowed to reach equilibrium before making the NMR measurement. Stock solutions (SUVs, protein, $CaCl_2$, EGTA) are prepared in the same pH 7.4 buffer. The pH of each sample was measured before its NMR spectrum was recorded. EM characterization of the SUVs used in these experiments confirms that within the time-course of the NMR experiment, no significant time-dependent vesicle fusion occurs. Negative controls include SUVs in the presence of 1–4 mM $CaCl_2$ or SUVs in the presence of annexin and no Ca^{2+}. Samples and controls after each step are immediately assayed for annexin binding.

Phospholipid-Binding Assay. A variation on the centrifugation assay described previously (Concha *et al.*, 1992) is used to detect protein–SUV binding. NMR samples and controls, prepared and treated identically throughout the study, are transferred into Centricon-100 centrifugal microconcentrator tubes (Amicon, Beverly, MA) with filters of molecular weight cutoff of 100 000. The microconcentrators are prewashed with 0.1 N NaOH followed by three washes with buffer (50 mM HEPES, 100 mM KCl, 0.1 mM DTT, and 0.02% NaN_3, pH 7.4). The SUVs are separated by ultrafiltration at 755*g*. The resulting retentates (20–40 μL from an initial sample volume of 0.5 mL) are washed twice by suspending in buffer with the calcium concentration of the initial sample and centrifuging at 755*g*. The filtrates and washed retentates are collected and analyzed by SDS–PAGE according to Laemmli (1970) and Weber and Osborn (1969).

Circular Dichroism. Circular dichroic (CD) spectra are recorded at room temperature on an Aviv CD spectrometer, Model 62DS (Aviv, Lakewood, NJ). A 200 μL quartz cuvette with 1 mm path length is used for measurements over the wavelength region 240–205 nm. The optical activity of a sample containing 1.6 μM annexin V and 45 μM $CaCl_2$ was measured in the 240–205 nm range at 0.2 nm intervals. The average of 15 such measurements was taken to reduce the noise. SUVs containing PA/PC (1:1) are then added to a final phospholipid concentration of 1.6 mM, and a similar averaged CD spectrum is obtained. CD spectra of SUVs in the absence and presence of calcium are also taken. In the above wavelength range, the SUVs are optically inactive. The buffer conditions and relative concentrations used for the CD measurements are similar to those used for the NMR experiments. All spectra are baseline-corrected. The molar ellipticity calculation is based on the molecular weight calculated from the rat annexin V amino acid sequence (Pepinsky *et al.*, 1988).

RESULTS

^{31}P-NMR Spectra of PA/PC SUVs. The high-resolution ^{31}P NMR spectrum of a sample of sonicated SUVs containing a 30 mM DOPA/DOPC 1:1 mixture at pH 7.4 consists of a single PC resonance at –0.64 ppm (relative to 80% phosphoric acid) and a downfield doublet corresponding to the PA phosphate at 0.65 and 1.80 ppm (Figure 2, panel A). Through pH titration and other experiments using these vesicles, it has been established that the two components of the doublet correspond to PA molecules on the inner and outer leaflets of the membrane bilayer (Swairjo *et al.*, 1994). The outer- and inner-leaflet PA molecules give rise to the downfield (1.80 ppm) and upfield (0.65 ppm) ^{31}P resonances, respectively. The separation of these resonances reflects differences, caused by high vesicle curvature, in the pK_a of the inner-leaflet and outer-leaflet PA molecules.

^{31}P-NMR Analysis of the Interaction of Annexin V with Phospholipid Vesicles. Addition of 1 mM $CaCl_2$ to calcium-free SUV samples does not affect the ^{31}P resonances of the PA or PC molecules in DOPA/DOPC (1:1) SUVs (Figure 2, panel A). Samples containing protein and SUVs but no calcium give ^{31}P spectra identical to those from samples of SUVs alone (data not shown). Addition of protein to SUVs in the presence of calcium, however, results in an observed upfield shift of the outer-leaflet PA resonance and a downfield shift of the inner-leaflet PA resonance (Figure 2, panel A). The changes in the PA chemical shifts increase with increasing amounts of protein, though the rate of change differs for the resonances arising from the inner- or outer-leaflet PA molecules (Figure 2, panel B). The PC resonance remains unchanged during the protein titration. The 30 μM protein concentration corresponds to 30% coverage of the available lipid surface, by calculations that assume solid two-dimensional protein packing on the SUV surface. Results from binding assays performed on each sample containing both calcium and SUVs confirm that the protein is completely bound under these experimental conditions. Upon addition of 20 mM EGTA, the shifts of the PA resonances are partially reversed (Figure 2, both panels). Further addition of EGTA results in severe vesicle aggregation and prohibits NMR observation of complete reversibility of calcium-dependent binding of annexin V to SUVs. However, as seen in binding assays, higher concentrations of EGTA lead to little or no protein in the pellet, as expected for fully reversible binding. Since the solution pH of the sample was unchanged, these ^{31}P chemical shift changes are free of pH-dependent effects. The same annexin-dependent changes in the outer- and inner-leaflet PA ^{31}P chemical shifts were observed when SUVs containing 100% DOPA were used (Figure 2, panel B). The higher chemical shifts observed in pure PA vesicles relative to mixed PA/PC vesicles are associated with headgroup packing differences (Swairjo *et al.*, 1994).

Upon titration with annexin, neither the ^{31}P longitudinal (T_1) nor the transverse (T_2) (as measured by line width) relaxation times change significantly for PC molecules. Similarly, relaxation times for PA molecules undergo little change except for oscillating behavior, consistent with exchange broadening, in the outer-leaflet PA molecules (Figure

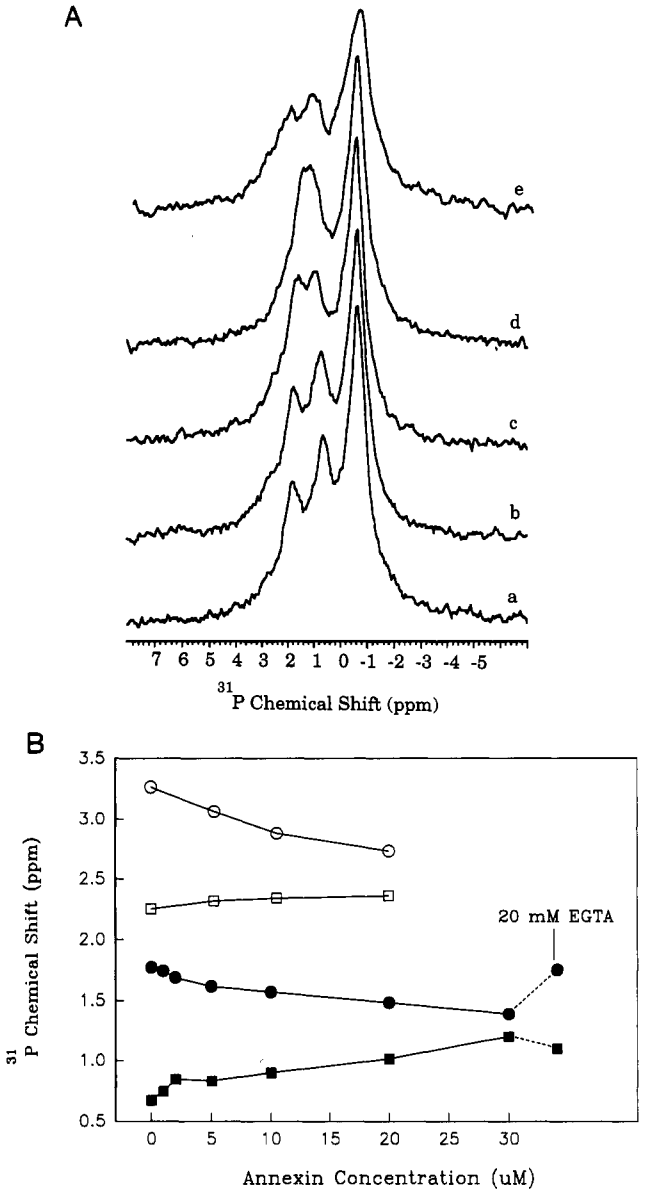

FIGURE 2: (Panel A) Effect of annexin binding on the ^{31}P-NMR (202.3 MHz) spectrum of PA/PC (1:1) SUVs: (a) 30 mM PA/PC (1:1) SUVs; (b) SUVs in the presence of 1 mM $CaCl_2$; (c, d) SUVs in the presence of 1 mM $CaCl_2$ and 10 μM (c) or 30 μM (d) annexin V; (e) the same sample as in (d) with 20 mM EGTA added. The sample was kept under constant buffer conditions (pH 7.4) throughout the entire experiment. (Panel B) Dependence of the ^{31}P chemical shifts (relative to 80% phosphoric acid) of PA molecules in the outer (●) and inner (■) surface of PA/PC (1:1) SUVs on total annexin concentration. The chemical shifts were determined from a series of ^{31}P spectra similar to those described in panel A. ^{31}P chemical shifts of the outer-leaflet PA (○) and inner-leaflet PA (□) in pure-PA SUVs measured in a similar experiment also are shown. The zero-annexin data point corresponds to samples containing 1 mM $CaCl_2$. After the addition of 30 μM annexin to the PA/PC SUVs, 20 mM EGTA was added to chelate the calcium and reverse the protein binding.

3a,b). This behavior reflects exchange between annexin-bound and annexin-free PA headgroups.

To investigate whether calcium is introduced into the inner SUV compartment via protein-associated channel activity, leading to changes in chemical shift of the inner-leaflet PA, ^{31}P NMR spectra were taken from samples of SUVs containing 1:1 PA/PC. The vesicles were either loaded with 1 mM $CaCl_2$ or prepared in calcium-free buffer. The calcium-loaded SUVs were prepared by suspending and sonicating dry lipid in calcium-containing buffer and then washing the resulting vesicles in calcium-free buffer by ultrafiltration. The two spectra were identical, eliminating the possibility that the observed shift in the inner-leaflet PA resonance upon interaction of annexin with SUVs is due to a direct interaction of calcium ions with the inner-PA phosphoryl group.

^{1}H-NMR Analysis of Annexin V Interacting with DOPA/DMPC SUVs. In order to assess the effect of annexin on PA acyl-chain behavior, ^{1}H-NMR spectra were obtained of SUVs containing a 32 mM mixture of DOPA and chain-perdeuterated DMPC in 1:1 molar ratio. The buffering capacity of DOPA was sufficient to keep a stable pH throughout the course of the experiment. Only the PA molecules contributed intensity in the region below 3.0 ppm. T_1 values of the acyl-chain CH_2 resonances were unaffected by addition of increasing amounts of protein (Figure 3c). The observed slight decrease of 7.0% in the T_1 relaxation time of the PA acyl-chain terminal methyl protons in the presence of annexin may be attributed to local and limited changes in the chain-to-chain packing upon protein binding. The line widths of the ^{1}H resonances of the DOPA acyl-chain methylenes, the terminal methyl, and the methylene groups adjacent to the double bond are generally unaffected by the presence of calcium and/or protein (Figure 3d). However, an apparent annexin-dependent increase by 40% in the line width of the PC choline CH_2N resonance is observed.

Circular Dichroism of Membrane-Bound Annexin V. Figure 4 shows a characteristic CD spectrum of annexin V (1.6 μM plus 45 μM $CaCl_2$) in the absence of SUVs. The spectrum exhibits a large negative minimum at 222 nm with molar ellipticity of –34.0. This spectral feature is indicative of α-helical secondary structure, which predominates in annexin crystal structures. Upon addition of SUVs (1.6 mM final phospholipid concentration), the molar ellipticity at 222 nm decreased only slightly to the value –31.0. The difference spectrum between Ca^{2+}–annexin samples with or without SUVs is predominantly flat, with a slight decrease in slope due to light scattering. Binding assays performed on CD samples of annexin V plus calcium and vesicles confirm that all protein is in the vesicle-bound form. CD spectra from a sample of SUVs at the same phospholipid concentration (1.6 mM) in the absence and in the presence of 45 μM $CaCl_2$ showed little or no molar ellipticity in the entire wavelength range 240–205 nm (data not shown).

DISCUSSION

The key finding in our studies is that annexin V exhibits calcium-dependent binding to the outer membrane surface of SUVs, and that this binding perturbs *both* the inner and outer membrane leaflets. This effect is associated with fully intact vesicles that have not aggregated or fused, nor exhibit other signs of reduced structural stability. Further, the annexin molecule does not appear to become embedded into the membrane bilayer under these conditions. The combined results suggest an indirect mechanism must therefore be invoked for the perturbation of the inner-leaflet environment by annexin binding to the outer-leaflet PA molecules.

In these studies, annexin binding is localized to the outer membrane surface of intact liposomes. The protocols used in these studies involve addition of annexin V to intact SUVs, and the extreme curvature of these SUVs precludes the entry of protein into the vesicle without breakdown of the liposome. Electron micrographs show that the integrity and size distribution of these vesicles are maintained in the presence of calcium (Swairjo *et al.*, 1994) and annexin (Figure 1). The binding data confirm that annexin V is fully bound, in the

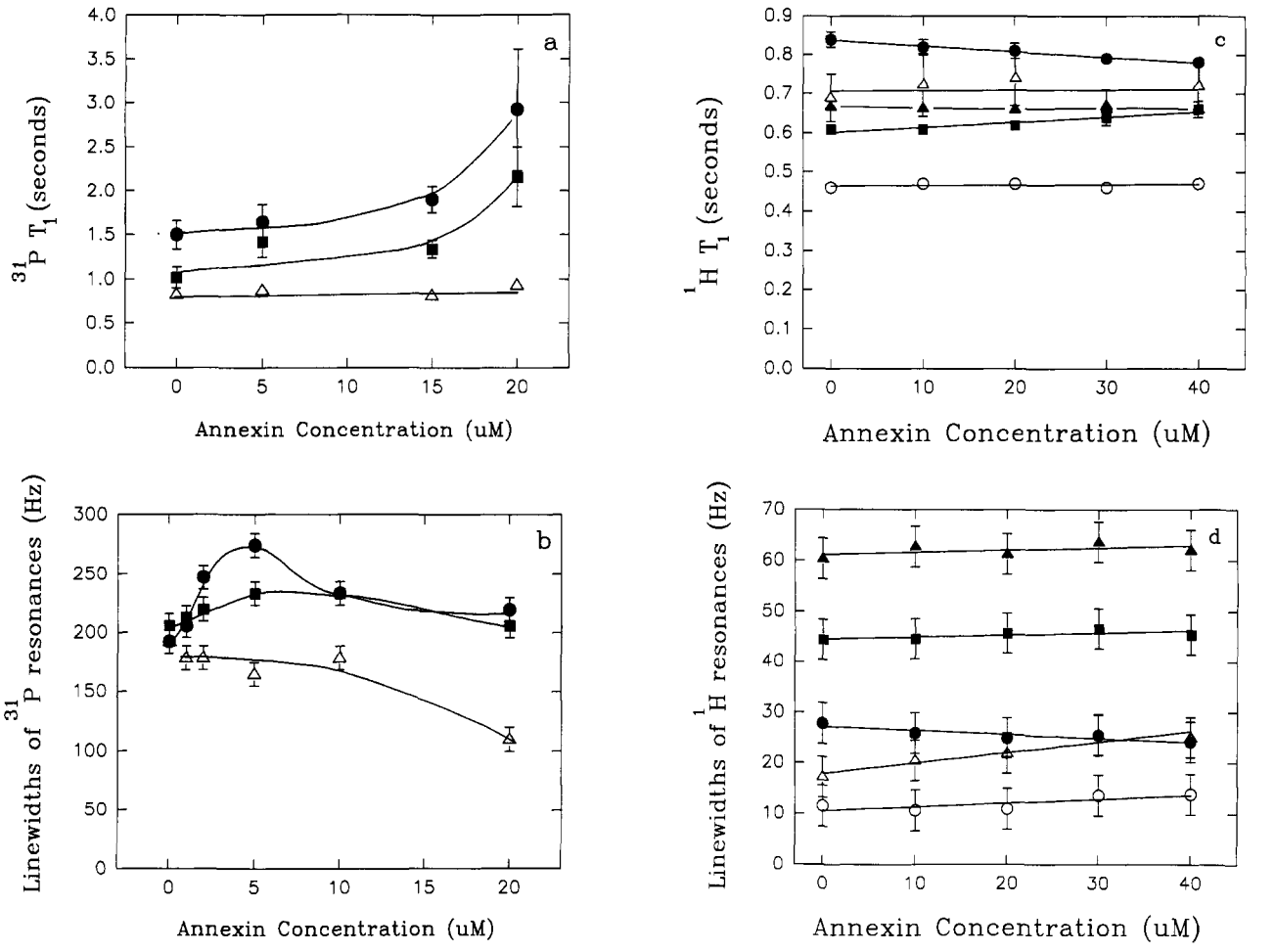

FIGURE 3: Effect of annexin V binding to SUVs containing a 1:1 mixture of DOPA and chain-perdeuterated DMPC on the ^{31}P T_1 relaxation times (a) and line widths of the ^{31}P resonances (b) from outer-leaflet PA (●), inner-leaflet PA (■), and PC molecules (▲), and the ^{1}H T_1 relaxation times (c) and line widths of the proton resonances (d) from PA acyl-chain methylenes (▲), the PA terminal methyls (●), the PA methylenes adjacent to the double-bond (■), the choline *N*-methylenes (△), and the choline *N*-methyls (○). Error bars smaller than the symbols are not shown. The zero-annexin data point corresponds to samples containing 1 mM $CaCl_2$.

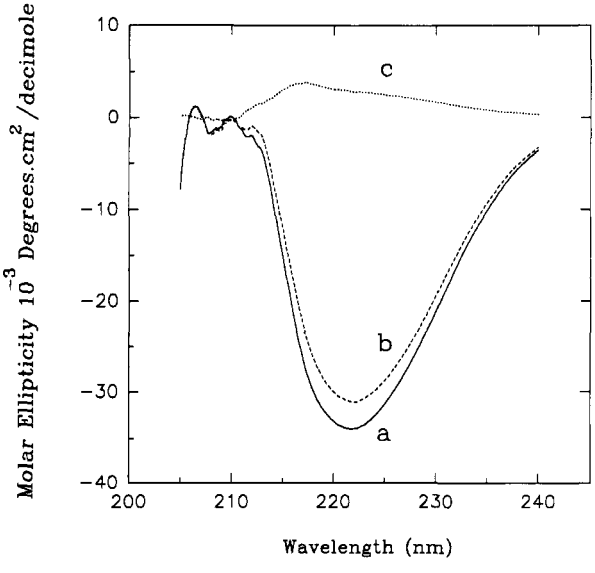

FIGURE 4: Far-UV CD spectra of annexin V in the absence (a) and presence (b) of PA/PC (1:1) SUVs; (c) is the difference (b) – (a). In (a), the sample contained 1.6 μM annexin V and 45 μM $CaCl_2$. In (b), 1.6 mM SUVs were added to the same sample in (a). Measurements were done at room temperature and in solutions buffered at pH 7.4.

presence of calcium, to PA-containing SUVs under the appropriate experimental conditions. Therefore, protein-related effects observed at the inner leaflet or lipid bilayer apparently originate from annexin binding to the SUV outer surface.

Calcium-dependent, specific binding between phospholipids and annexins is generally believed to involve the phosphoryl moiety of the phosphoglycerol backbone. Lipids lacking phosphoryl moieties do not bind annexins (Meers & Mealy, 1994). Annexins strongly prefer anionic phospholipids to PC [e.g., see Blackwood and Ernst (1990) and Concha *et al.* (1992)]. Annexins bind well to PA, which has no esterified alcohol, suggesting that a negatively charged phosphoryl group is sufficient for binding. However, PC binding is not favored, which presumably reflects the influence of the headgroup. Pigault and co-workers (1994) show that annexin V binding to phosphatidylserine (PS)-containing liposomes is unchanged in PC:PS from 1 to 400, but weakens incrementally up to PC:PS = 800. They conclude that annexin binding occurs specifically and chiefly to PS, though weak nonspecific interactions with PC cannot be ruled out. We detect no binding between annexin V and pure PC SUVs by binding assay, though binding is equally strong between pure PA SUVs and 1:1 PA/PC SUVs. The NMR data presented herein correlate well with these observations. First, the ^{31}P chemical shift changes in outer-leaflet PA but not PC resonances underscore the likely involvement of lipid phosphoryl groups in binding to annexin V. Specific binding interactions involving the phosphoryl groups would be expected to have a significant

impact on ^{31}P-NMR resonances. The ^{1}H-NMR data give some indication that the PC choline headgroup may be involved in nonspecific electrostatic interactions with the annexin while the protein is bound specifically to the PA component. However, without changes in its ^{31}P chemical shift, the PC choline ^{1}H effect is not sufficient to indicate specific annexin binding to PC.

The peripheral nature of the binding between annexin V and PA-containing SUVs is supported by NMR relaxation measurements and CD spectra. The ^{1}H-NMR measurements of PA acyl chains show no evidence of major perturbations of the lipid bilayer. Further evidence that annexin V does not embed itself into the membrane bilayer is offered by CD results. The molecular structure of annexin V is typical of a soluble protein. The membrane-facing surface has very few hydrophobic determinants (Concha *et al.*, 1993), and there are no obvious hydrophobic patches on the other surfaces. The helices in the structure are considerably shorter than known membrane-spanning helices (Huber *et al.*, 1992). Therefore, a major conformational rearrangement would be required for insertion into the membrane to become energetically allowable. The CD results show no significant conformational differences in the soluble and SUV-bound forms of annexin V. This finding is consistent with the annexin V molecule remaining at the SUV surface. Ravanat *et al.* (1992) find, in their neutron scattering study, that annexin V binds to the outer SUV surface with a thickness of approximately one protein molecule.

Since annexin V cannot access the inner-leaflet PA polar moieties for direct binding, as it can those on the outer leaflet, the ^{31}P-NMR chemical shift changes of inner-leaflet PA molecules must arise from a different, indirect mechanism. As noted above, the outer-leaflet PA spectral change contains a contribution from direct binding of the PA phosphoryl group to annexin V. However, this cannot be the case with the inner-leaflet PA molecules in intact SUVs. Moreover, the control experiments show that the altered inner environment of the SUVs is not a result of introduction of calcium or proton gradients as a consequence of annexin binding to the outer SUV surface (data not shown). The behavior of the inner-leaflet PA resonance appears to be related to pK_a. Previous characterization of this SUV system has shown that the pK_a values of PA phosphoryl groups are influenced by vesicle curvature, an effect that is observed in ^{31}P-NMR spectra. The annexin-induced coalescence of inner- and outer-leaflet PA ^{31}P resonances observed in SUVs is qualitatively similar to that observed in ^{31}P spectra of protein-free unilamellar vesicles of increasing size (Swairjo *et al.*, 1994). In the SUVs, the pK_a of the outer-leaflet PA phosphate is 7.6, and that of the inner-leaflet PA is higher than 10. Decreasing surface curvature increases the pK_a of the outer-leaflet PA molecules and decreases the pK_a of the inner-leaflet PA molecules. The NMR experiments were performed in solutions buffered at pH 7.4. Therefore, in the absence of annexin, the outer-leaflet PA is roughly a 50:50 mixture of monoanion and dianion forms, whereas the inner-leaflet PA is completely monoanionic. These ionization states are altered upon annexin binding, giving rise to changes in the ^{31}P chemical shifts.

Annexin-induced lateral segregation of phospholipids into homogeneous patches, a possible explanation for the altered inner-leaflet PA environment, is not consistent with the NMR results. In SUVs containing pure PA, the same changes in the inner-leaflet PA ^{31}P chemical shift are observed as in the mixed PA/PC SUVs. While large-scale lateral separation of PA from PC would be expected to give rise to changes in the inner-leaflet PA ^{31}P signals, such changes cannot occur in a pure PA vesicle system. In other studies, fluorescence quenching of annexin IV and V binding to vesicles containing pyrene-labeled phospholipids has shown that annexins reduce phospholipid lateral mobility but without evidence of lipid segregation (Meers *et al.*, 1991). In other reports, annexins IV and VI appear to reverse the lipid-segregating effects of calcium in large vesicles containing phosphatidylserine (PS), and disrupt the formation of the $Ca^{2+}(PS)_2$ complex that would otherwise form extended patches (Sobota *et al.*, 1993). The NMR results are therefore in agreement with studies using other model systems that annexin–membrane binding does not appear to result in formation of extended patches of pure acidic phospholipids.

The result that annexin binding induces pK_a changes on both leaflets without disruption of the hydrocarbon chain region of the bilayer or loss of the membrane integrity can be explained most straightforwardly by a mechanism in which the bound annexin exerts mechanical force on the SUV membrane, reducing the curvature that was established by sonication. Annexin-induced reduction of vesicle curvature has been noted by other investigators. Cryo EM images of large unilamellar vesicles incubated with annexin V in the presence of calcium show multiple facets and sharp edges (Andree *et al.*, 1992). The facets were interpreted as rigid sheets, as large as 100 × 100 nm, of membrane-bound protein clusters that flatten the membrane surface while keeping the overall bilayer structure intact. EM images of SUVs (Andree *et al.*, 1992; this work) were not able to resolve facets, understandably since they would be expected to exceed the resolution limits of the technique. Nor were flattened disks observed in SUVs (Andree *et al.*, 1992; this work). Given the extreme curvature of the SUVs, annexin-induced reduction of membrane curvature cannot proceed very far without encountering resistance to further distortion of vesicle shape. The reduced curvature in the SUVs need not be extensive to be detected by NMR; curvature reduced by only a few degrees may be sufficient to create observable effects. Further, the morphological changes may be dynamic, operating on a time-scale compatible with observation by NMR, but not EM.

The mechanical force capable of distorting vesicle curvature may be exerted by sheets of annexin polymers bound to the membrane surface. Calcium-dependent annexin self-association in the presence of liposomes (Zaks & Creutz, 1991; Concha *et al.*, 1992) and two-dimensional annexin crystals covering phospholipid monolayers (Newman *et al.*, 1989; Mosser *et al.*, 1991) have been reported. The functional significance of this property is implied by the high quantities of annexins found in cells, where they can comprise up to 2% of total cell protein (Schlaepfer & Haigler, 1990). Sheet formation by annexin V on membrane surfaces has been related to anticoagulant activity (Andree *et al.*, 1992). It is conceivable that protein-induced effects on membrane curvature, as demonstrated for clathrin, may lead to regulation of the budding of clathrin-coated vesicles by annexin VI observed by Lin *et al.* (1992).

In summary, our studies confirm that annexin V binds to the surface of small unilamellar vesicles and causes changes in the properties of both membrane surfaces. We have shown that this effect is not associated with aggregation, fusion, or liposome breakdown, nor with substantial bilayer insertion (e.g., translocation) of the annexin molecule, nor by lateral association of PA molecules. The best explanation consistent with our data is a morphological change in vesicle curvature, perhaps induced by sheets of annexin V on the outer membrane

surface. This peripheral binding event could compete with membrane-regulated enzymes (Andree *et al.*, 1992) such as blood coagulation factors, phospholipase A_2, and protein kinase C and alter the lipid environment around integral membrane proteins. Dynamic changes in curvature also may alter membrane permeability, thus influencing annexin-induced calcium-release properties.

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