Influence of Annexin V on the Structure and Dynamics of Phosphatidylcholine/Phosphatidylserine Bilayers: A Fluorescence and NMR Study†

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ABSTRACT: The consequences of the binding of annexin V on the structure and dynamics of PC/PS bilayers were studied by means of fluorescence polarization, ^{31}P NMR, ^{2}H NMR, and fluorescence recovery after photobleaching (FRAP). Even at complete coverage of the lipid bilayers by the protein, annexin V showed no influence on the lipid molecular packing and the acyl chain flexibility of both PC and PS. The fluorescence polarization of the probe DPH, the ^{31}P NMR spectra, and deuterium quadrupolar splittings of P(d_{31})OPS remained unchanged. However, upon binding of annexin V, two distinct populations of PC were visible in ^{2}H NMR, which were in slow exchange on the deuterium NMR time scale (microseconds). One component in the spectrum was identical to the protein-free sample, while a second, broad, component appeared. The presence of the protein induced a decrease in the transverse relaxation times (T_{2e}), indicative of the appearance of slow motions (milliseconds to microseconds), in the P(d_{31})-OPS spectrum and in the P(d_{31})OPC broad component. FRAP experiments were carried out with the probes C_{12}-NBD-PC and C_{12}-NBD-PS: at saturation, annexin V reduced the lateral diffusion rate of PC by 40% and nearly blocked the diffusion of PS. These combined experiments are consistent with a model in which annexin V enters a proteolipidic complex in the form of an extended 2D network, stabilized by specific interactions with PS. As seen from the lateral diffusion rates and the acyl chains NMR spectral parameters, two separate lipid populations appear, presumably corresponding to those interacting with annexinV (PC and PS) and protein free domains (mainly PC).

Annexins constitute a large family of structurally related proteins which have the common characteristic of binding strongly to phospholipids in a calcium-dependent manner (*1*, *2*). They are widely spread in many eukaryotic organisms and tissues, and a nomenclature based on the existence in their amino acid sequence of four highly conserved domains and a variable tail leads to their discrimination, today, among 13 different annexin subclasses (*3*). Although various potential activities have been inferred from *in vitro* investigations, many of them related to the ability of these proteins to interact with membranes (blood coagulation, signal transduction, anti-inflammatory processes, membrane trafficking, and ion channel activity), the real biological functions of annexins remain to be elucidated (see, for recent reviews, refs *4* and *5*). The structure of some of the members of the family, especially annexin V, has been elucidated at atomic level. The data were obtained either from X-ray diffraction studies on 3D protein crystals (*6*−*10*) or from electron microscopy studies on 2D protein crystals obtained at the contact of lipid monolayers (*11*, *12*). In contrast, the structure of the peptidolipid complexes formed when annexins interact with lipids is still unknown; in spite of one study dealing with the influence of annexin IV on the lateral motions of lipids (*13*), the influence of annexins on the organization and dynamics of lipids remains poorly documented. However, this point is of particular interest because the biological functions of annexins are believed to depend primarily on their interactions with membrane lipids. This may also concern the hypothesis that the voltage-dependent calcium channel activity demonstrated for annexin V implies a destabilization of those lipid molecules which are in contact with the protein (*14*).

In the present study, we asked the question of whether annexin V can alter the "fluidity" of membrane lipids. Herein, the term "fluidity" is to be understood in its largest meaning, including the conformational, rotational, and translational mobilities of lipids which were studied using ^{2}H and ^{31}P NMR spectroscopy, fluorescence polarization, and fluorescence recovery after photobleaching (FRAP)[1] experiments. Because annexin V shows high affinity for acidic phospholipids, in particular for phosphatidylserine, experiments were carried out on membrane model systems

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[1] Abbreviations: brain-PS, bovine brain L-α-phosphatidyl-L-serine; POPC, 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine; C_{12}-NBD-PC, 1-acyl-2 [12-[(7-nitro-2-1,3-benzoxadiazol-4-yl)amino]dodecanoyl]-phosphatidylcholine; C_{12}-NBD-PS, 1-palmitoyl-2-[12-[(7-nitro-2-1,3-benzoxadiazol-4-yl)amino]dodecanoyl]phosphatidylserine; CSA, chemical shift anisotropy; DM(d_{27})PC, 1-myristoyl-2-myristoyl(d_{27})-*sn*-glycero-3-phosphocholine; P(d_{31})OPC, 1-palmitoyl(d_{31})-2-oleoyl-*sn*-glycero-3-phosphocholine; P(d_{31})OPS, 1-palmitoyl(d_{31})-2-oleoyl-*sn*-glycero-3-phosphoserine; DPH, 1,6-diphenyl-1,3,5-hexatriene; *E. coli*, *Escherichia coli*; EDTA, ethylenediaminetetraacetic acid; Egg-PC, egg yolk phosphatidylcholine; FID, free induction decay; FITC, fluorescein isothiocyanate; FRAP, fluorescence recovery after photobleaching; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; IFV, interdigitated fusion vesicles; LUV, large unilamellar vesicles; NMR, nuclear

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(LUVs, IFVs, oriented bilayers and planar supported bilayers) composed of phosphatidylcholine and phosphatidylserine in the liquid crystalline state with specifically labeled PC and PS probes.

MATERIALS AND METHODS

Materials. Egg-PC, P(d_{31})OPC, P(d_{31})OPS, DM(d_{27})PC, C_{12}-NBD-PC, POPC, and C_{12}-NBD-PS were purchased from Avanti Polar Lipids (Birmingham, AL). Bovine brain-PS (fraction III), DPH, FITC isomer I, and glycine were purchased from Sigma (St. Louis). Deuterium-depleted water was obtained from Aldrich. Phospholipids showed a single spot on silica gel thin layer chromatography and were used without further purification. Salts and solvents were of analytical grade. The concentration of phospholipids in stock solutions was determined by phosphorus assay (*15*).

Expression of Annexin V in the Bacterium Escherichia coli. Annexin V was expressed in BL21DE3 *E. coli* strain electrotransformed with pRSET AV vector as described elsewhere (*16*). The purification of the human recombinant protein relied on its calcium-dependent binding to bovine brain lipids (*17*). The purity of annexin V was controlled by 15% SDS−PAGE (Blue Coomassie staining) and electrospray mass spectrometry (*16*). Concentration of annexin V in stock solutions was determined by absorption spectroscopy using a molar extinction coefficient of 22 000 M^{-1} cm^{-1} at 280 nm (*18*).

Labeling of Annexin V with Fluorescein. Purified annexin V was dialyzed against the labeling buffer (150 mM NaCl, 1 mM $CaCl_2$, and 10 mM sodium borate, pH 9.0). Annexin V (50 μmol) was added to FITC isomer I (1 mmol), and the mixture was kept 16 h at 4 °C in the dark. The reaction was stopped by addition of 10 mmol of glycine. The fluorescent protein was purified by gel filtration on a PD 10 column (Pharmacia Biotechnology). Its purity was checked by 15% SDS−PAGE experiment and electrospray mass spectrometry.

Preparation of Small Unilamellar Vesicles. The lipid vesicles, planar supported lipid bilayers, and oriented bilayers used in this study were all prepared from SUVs composed of mixtures of phosphatidylserine and phosphatidylcholine in the molar ratio 1:9 and of a given probe [DPH, C_{12}-NBD-PC, C_{12}-NBD-PS, P(d_{31})OPC and P(d_{31})OPS lipids] at the desired concentration. The desired amounts of phosphatidylcholine, phosphatidylserine and probe were taken from stock solutions in chloroform and mixed together. The mixture was dried under nitrogen at 40 °C and traces of solvent were evaporated under vacuum. Lipids were suspended in 1 mL of the appropriate buffer: 10 mM Na_2HPO_4 and 100 mM NaCl, pH 7.0 (buffer A), or 10 mM HEPES and 100 mM NaCl, pH 7.5 (buffer B). The lipid suspension was sonicated 15 min in pulsed mode (50% duty cycle) using a tip Branson B15 sonifier. Titanium particles were eliminated by centrifugation (13 400 g, 10 min) using a bench centrifuge.

Preparation of Planar Supported Lipid Bilayers for FRAP Experiments. Planar supported lipid bilayers were formed by adsorption and fusion of SUVs on cleaned glass coverslips mounted on a small Plexiglas tank adapted to the microscope stage (*19*−*22*). SUVs were prepared in 1 mL of buffer A using 2 mg of an egg-PC/brain-PS/C_{12}-NBD-PC or C_{12}-NBD-PS (9:1:0.1 mol/mol) mixture. Cleaning of the coverslip was of prime importance with respect to the formation of the bilayer. Coverslips were first rinsed with ethanol 95%, then immersed for 30 min in sulfochromic acid, and finally washed extensively (15 times in large volumes of water) with deionized water (MilliQ plus 185, Millipore). In order to protect them from dust or contamination, the clean coverslips were stored in deionized water. Just before use, one coverslip was taken out of water and placed on the top of the sample cell. The cell was filled with the SUV suspension (diluted to a concentration of 0.2 mg/mL in buffer A) to contact with the coverslip. Spontaneous adsorption and fusion of vesicles on the glass surface took place and led to the formation of a stable planar supported lipid bilayer. After 2 h, buffer A in the cell was exchanged slowly with 1 vol of buffer B. Calcium was added to the subphase and then annexin V at various concentrations. At the end of a set of FRAP experiments, EDTA was added to the subphase in order to check the reversibility of the binding of annexin V to the lipid bilayer. After each addition of calcium, annexin V, or EDTA, the cell was stabilized for 30 min before starting FRAP experiments. All experiments were carried out in triplicate.

Preparation of Interdigitated Fusion Vesicles for NMR Experiments. Large unilamellar vesicles like the IFVs are required in NMR experiments to limit artifacts coming from lateral diffusion of the lipids on a curved surface. IFVs (50 μmol) composed of a DM(d_{27})PC/bovine brain-PS (9:1 mol/mol) mixture were prepared in buffer 10 mM HEPES and 100 mM NaCl, pH 7.5, as described previously (*23*). IFVs were left to stabilize overnight after addition of $CaCl_2$ at the final concentration of 1 mM. Annexin V (0.32 μmol) in buffer A was added to the IFVs suspension. Prior to NMR experiments (before and after addition of annexin), vesicles were sedimented by centrifugation (13 400 g, 30 min using a bench centrifuge) and the pellet was resuspended in the same buffer. The final concentration of lipids (phosphorus assay) and annexin V were determined and led to a ratio of 43 phospholipids per annexin V.

Preparation of Oriented Bilayers for NMR Experiments. Oriented samples were prepared from SUV suspension (21 mM) composed of P(d_{31})OPC/brain-PS and POPC/P(d_{31})-OPS mixtures (9:1 mol/mol) as described elsewhere (*24*). Calcium and annexin V were added to the suspension at the final concentration of 0.82 mM and 0.2 mM, respectively. Left for 15 min to stabilize, the suspension was spread onto glass plates, previously cleaned by overnight immersion in fuming nitric acid and then carefully rinsed and dried. Five cycles of hydration (saturated KCl atmosphere, 80% relative humidity)−dehydration were applied to the sample. Before NMR experiments, hydrated plates were stacked in the NMR tube, hydration in the tube was controlled by blotting paper wetted with saturated KCl solution in water.

Fluorescence Polarization Experiments. Experiments were carried out with large unilamellar vesicles (LUVs) obtained from SUVs prepared in buffer B and labeled with DPH (0.5 mol %) as described above. Six cycles of freezing (−78 °C) and thawing under vigorous shaking were applied

magnetic resonance; PC, phosphatidylcholine; PG, phosphatidylglycerol; PS, phosphatidylserine; SDS-PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis; SUV, small unilamellar vesicles; T_1, longitudinal relaxation time; T_{2e}, transverse relaxation time.

to the SUV suspension (0.2 mg of lipids, 0.26 μmol). In order to prevent artifacts in fluorescence measurements due to light scattering, the LUVs obtained in this way were then diluted with buffer B to the absorbance of 0.05 at 348 nm. To this LUV suspension (0.02 mg of lipids/mL), annexin V was added to the final concentrations of 20, 40, 80, 160, 240, and 400 nM corresponding to lipid to protein molar ratios Ri of 1300, 650, 325, 162, 108, and 65, respectively.

At each step, fluorescence polarization was measured with a T-format automatic apparatus of our fabrication, which has been described elsewhere (*25*). Samples were placed in a closed thermostated housing system equipped with stirring and temperature monitoring (Peltier element). Temperature was controlled to within 0.3 °C whereas the polarization p was determined to within ±1% (relative values). The excitation and emission wavelengths of DPH were 348 and 430 nm, respectively. Temperature was raised stepwise from 4 to 40 °C at a rate of 0.7 °C/min. Fluorescence polarization is given as $p = (I_V - I_H)/(I_V + I_H)$.

FRAP Experiments. Experiments were carried out under conditions of constant incident light intensity and of "uniform disk illumination", using an apparatus based on a Leitz Ortholux II fluorescence microscope. A finite differentiation method was used for the calculation of the diffusion coefficient (D), the mobile fraction (M), and for statistical analysis of the data. Both the apparatus and the numerical method of calculation of D and M are described elsewhere (*26*). D and M values are given at a 95% confidence level. From a strict statistical point of view, D values can be considered as being different from each other only in the absence of overlap between their extreme D_{min} and D_{max} values. The excitation beam from a mercury arc lamp was focused on samples through a Leitz x63/1.3 oil-immersion objective. The radius of the illuminated area was 2.9 μm. Bleaching rates of around 40% were obtained for a bleaching time of 250 ms. This bleaching time was consistent with the 2 s recovery half-time currently found with planar supported lipid bilayers. At least 20 FRAP experiments were carried out for each experimental condition tested. All experiments were carried out in triplicate. The temperature was 20 °C.

Binding Experiments with Planar Supported Bilayers. Binding of the fluorescein-labeled protein on planar supported lipid bilayers was monitored by measuring the fluorescence signal originating from the bilayer with the FRAP apparatus. The illumination spot (5.8 μm in diameter, depth of focus 2.5 μm) was focused at the level of the bilayer. For each condition tested, 50 fluorescence intensities were measured and averaged. Fluorescence from free FITC-annexin V in the subphase was also measured by defocusing the microscope in the water phase. The fluorescence signal from the aqueous phase was less than 8% of the fluorescence signal from the bilayer. It was not affected by addition of calcium to the subphase.

NMR Experiments. ^{2}H NMR spectra were recorded on a Bruker AMX-500 at 76.77 MHz with a spectral width of 0.5 MHz using a quadrupolar echo sequence (*27*). The two 90° pulses of 5 μs each were spaced with an echo delay of 40 μs. For P(d_{31})OPC [respectively, P(d_{31})OPS] labeled bilayer experiments, a total of 2K (respectively, 16K) free induction decays (FIDs) of 4K time domain were accumulated with a repetition delay of 1 s. T_{2e} measurements were performed by varying the delay between the two 90° pulses. T_1 measurements were carried out using the standard inversion recovery quadrupolar sequence (180_x-t_1-90_x-t_2-90_y-t_2-AQ). ^{31}P spectra were recorded at 202.22 MHz with a spectral width of 0.25 MHz using a Hahn echo sequence (*28*). The two pulses (3.25 μs for $\pi/2$ pulse) were spaced with an echo delay of 40 μs. A total of 8K FIDs of 4K time domain were accumulated with a repetition delay of 1.7 s. Prior to Fourier transformation and to ensure that the first starting point for the Fourier transformation corresponded to the top of the echo, the first points were removed. Then, a squared cosine function was applied to the FID. All NMR experiments were performed at a temperature well above the gel to liquid crystalline phase transition temperature of the phospholipid mixtures used.

RESULTS

Various types of proteolipid samples were prepared, and it was important to specify, for each of them, the lipid to protein ratio, Ri, tested. Assuming that, in a 2D network of annexin V at the bilayer surface, one molecule of annexin V occupies on average an area of 31 nm^2 (*12*) and taking a molecular area of 0.63 nm^2 for the lipids in the fluid phase (*29*), one annexin V is associated on average with about 50 lipids at saturation. In the present work, annexin V was added to preformed lipid bilayers and could interact with the external leaflet only. On account of the very high affinity of annexin V for acidic lipids (Kd ≈ 10^{-10} M) (*30*), this means that saturation was achieved for overall Ri value of ≤100.

1. Influence of Annexin V on the Conformational and Rotational Mobility of Lipids

a. Fluorescence Polarization Experiments. In LUVs (egg-PC/brain-PS 9:1 mol/mol) and in the absence of annexin V, increasing the temperature from 4 to 37 °C resulted in continuous depolarization of the probe DPH, from 0.28 down to 0.1, indicating the existence of lipids in a fluid phase and an absence of gel-to-liquid crystalline phase transition over the temperature range tested. This is an expected result when working with lipids of natural origin which are composed of mixed acyl chains. Addition of calcium ions (1 mM) and annexin V to the LUV suspension up to lipid to protein ratios (Ri) of 1300, 650, 325, 162, 108, and 65 yielded fluorescence depolarization curves which, within the experimental error, were similar to each other and to that recorded in the absence of protein (data not shown). Because the polarization of the probe depends on its fluorescence lifetime, τ, this parameter was measured. Whatever the protein concentration may be, τ remained unchanged and kept the mean value of 7.7 ± 0.2 ns (*31*), indicating no perturbation of the probe by the protein. From these experiments, it can be concluded that even near saturation, annexin V had no influence on the bilayer "fluidity" as experienced by the probe DPH.

b. NMR Spectroscopy. As shown in Figure 1, the ^{31}P NMR spectrum of IFVs in the absence of annexin V exhibited the well-known powder pattern with a $\Delta\sigma = 44 \pm 2$ ppm corresponding to the breadth of the powder spectrum of large unilamellar lipid vesicles. The sharp peak centered at 0 Hz of ^{31}P and ^{2}H NMR spectra originates from a small fraction of SUVs undergoing isotropic motions. In the

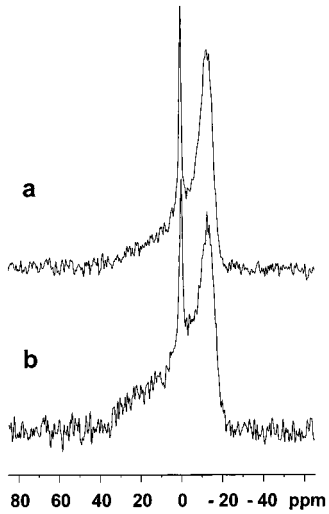

FIGURE 1: ^{31}P NMR spectrum of IFVs composed of DM(d_{27})PC/brain-PS (9:1 mol/mol) in the absence (a) and the presence (b) of the annexin V (Ri = 43). The deuterium-depleted buffer: 10 mM HEPES, 100 mM NaCl, and 1 mM $CaCl_2$, pH 7.5, was used. Experiments were performed at 37 °C.

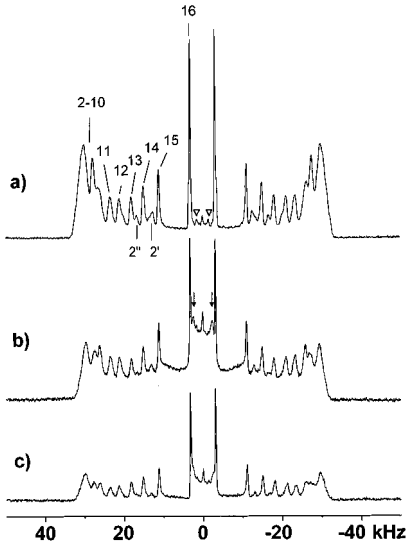

FIGURE 2: ^{2}H NMR spectra of oriented bilayers composed of P(d_{31})-OPC/brain-PS (9:1 mol/mol), with the bilayer normal parallel to the magnetic field; (a) sample without annexin V; numbering of peaks corresponds to the deuteron positions in the chain; the triangles mark the doublet due to unoriented lipids, (b and c) samples with annexin V (Ri = 144) with an echo delay of 40 and 300 μs, respectively. Two components appear in the spectrum: one is identical to the spectrum of pure lipids, the other (indicated by arrows) is a broad, nonaxial spectrum which disappears in the partially relaxed spectrum (Figure 2c). Experiments were performed at 30 °C.

presence of an excess of annexin V (Ri = 43) (Figure 1b) the general shape of the ^{31}P NMR spectrum was not modified, indicating that the bilayer structure was preserved. Careful inspection of the spectrum showed a slight increase in the intensity of the shoulder at 23 ppm, which may originate from a slight modification of the vesicle shape. It has already been observed that the shape of IFVs may not be strictly spherical, thus affecting the powder pattern of ^{31}P and ^{2}H NMR spectra (*32*).

^{2}H NMR experiments performed on DM(d_{27})PC/brain-PS (9:1 mol/mol) IFVs with a broad range of Ri (down to Ri = 43) did not reveal any modification of the spectra. However, since IFV samples give powder spectra in solid state NMR, we moved to the preparation of oriented bilayers which significantly improve the sensitivity and resolution (*24*). Various relative amounts of calcium and protein and also hydration conditions were tested which finally led to a fairly high degree of orientation: 90% of oriented bilayers in the absence and 70% in the presence of annexin V, estimated from the ^{31}P NMR spectrum of a sample with the bilayer normal oriented parallel to the magnetic field (0 degree orientation). These spectra revealed one single peak, corresponding to a CSA of 47 ± 2 ppm in the absence of annexin and of 41 ± 2 ppm in the presence of annexin. Figure 2 represents ^{2}H NMR spectra of P(d_{31})OPC oriented at 0°. In the absence of annexin V, the spectrum revealed well-resolved quadrupolar splittings characteristic of a homogenous phase and a unique orientation of the lipids (Figure 2a). The resolution allowed us to assign deuterated positions on the spectrum, assuming that quadrupolar splittings increased from the methyls to the C_3 position (*33*). The P(d_{31})OPC-labeled sample with annexin V (34% of membrane surface covered by annexin, Ri = 144) showed spectra with two components (Figure 2b). One component was identical to the spectrum in the absence of annexin. A second broad component was observed below the previous one and was responsible in particular for the doublet (4.8 kHz) indicated by arrows. In the partially relaxed spectrum (Figure 2c), this second component disappeared completely: it was therefore due to lipids having a very short T_{2e} (about 700 μs) and not to poorly oriented lipids.

These experiments revealed that, upon binding of annexin V, two populations of POPC existed which were in slow exchange on the deuterium NMR time scale, i.e., on the microsecond time scale. Part of the POPC molecules were not affected either in their order parameters (identical quadrupolar splittings) or in their dynamics (see below identical relaxation times). The other POPC molecules were in a completely different environment, and in particular have lost the line shapes typical of molecules in fast axial diffusion.

The effect of annexin V on POPS in labeled bilayers was also studied (Figure 3). In the absence of annexin V, the sample displayed a standard axially symmetric oriented spectrum, with quadrupolar splittings slightly larger than those of P(d_{31})OPC/brain-PS in the same conditions. The broad quadrupolar splitting up to 120 kHz was due to the $Ca(PS)_2$ phase, known to be formed in the presence of PS and calcium (*34*). This phase increased with calcium concentration, so that the axially symmetric spectrum component (corresponding to PS in the standard fluid phase) disappeared for a lipid to calcium ratio of 4.8. This static phase was not observed in the P(d_{31})OPS-labeled sample with annexin V (Ri = 144) because of the binding calcium ions to the protein (Figure 3b). In contrast with the results obtained on P(d_{31})OPC-labeled samples, annexin V affected all P(d_{31})OPS molecules. This resulted in a dramatic loss of resolution and sn-1 deuterium quadrupolar splittings close to those of POPC in the same conditions.

Peak broadening in the presence of annexin V may originate from heterogeneous distribution of quadrupolar splittings or from a modification of relaxation times. The longitudinal (T_1) and transverse (T_{2e}) relaxation times were

Table 1: ^{2}H Relaxation Times T_1 and T_{2e} of Acyl Deuterated Phospholipids of Oriented Bilayers (PC/PS 9:1 mol/mol) in Absence and in Presence of Annexin V[a]

sample, carbon position	T_1, ms ($\Delta\nu_Q$, kHz) lipids	T_1, ms ($\Delta\nu_Q$, kHz) lipids + annexin V	T_{2e}, μs ($\Delta\nu_Q$, kHz) lipids	T_{2e}, μs ($\Delta\nu_Q$, kHz) lipids + annexin V
P(d_{31})OPC, C_3-C_{10}	36 ± 9% (60)	32 ± 9% (60)	587 ± 10% (60)	531 ± 10% (60)
P(d_{31})OPC, C_{13}	71 ± 9% (36)	67 ± 9% (36)	1164 ± 10% (36)	986 ± 10% (36)
P(d_{31})OPC, C_{16}	304 ± 9% (6.3)	303 ± 9% (6.3)	2246 ± 10% (6.3)	2197 ± 10% (6.3)
P(d_{31})OPS, C_3-C_{10}			620 ± 10% (67)	426 ± 15% (60)
P(d_{31})OPS, C_{13}			607 ± 10% (38)	301 ± 15% (37)
P(d_{31})OPS, C_{16}			2322 ± 7% (8.4)	1460 ± 7% (7.1)

[a] Relaxation times were determined by a single exponential fitting of intensities of specific quadrupolar splittings; hence, the contribution of nearest resonances was neglected. Measurements were performed at 30 °C with the bilayer normal parallel to the magnetic field. Errors are given with 95% level of confidence.

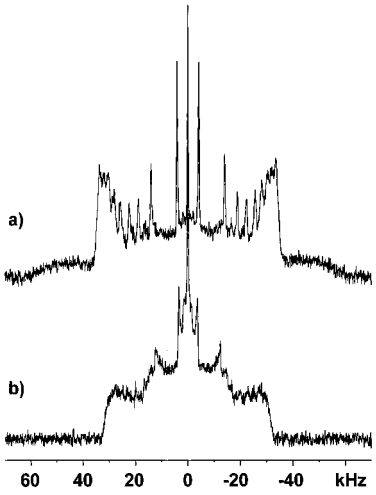

FIGURE 3: ^{2}H NMR spectra of oriented bilayers composed of POPC/P(d_{31})OPS (9:1 mol/mol), with the bilayer normal parallel to the magnetic field; (a) sample without annexin V and (b) sample with annexin V (Ri = 144). Experiments were performed at 30 °C.

therefore measured. Measurements were carried out for quadrupolar splittings corresponding to three positions along the acyl chain, i.e., the plateau region (C_3−C_8), the C_{13} position, and the terminal methyl group. The data presented in Table 1 shows that, in all positions, T_1 and T_{2e} of P(d_{31})-OPC were not significantly affected by the interaction of the protein with the lipids. In contrast, T_{2e} of P(d_{31})OPS were decreased by about 40% on average. From these experiments, one can conclude that lipid motions in the subnanosecond time scale were not affected by the lipid−protein interactions (T_1 unaffected), while slow motions of POPS molecules in the millisecond to microsecond time scale were modified (T_{2e} decreased).

2. Influence of Annexin V on the Lateral Mobility of Lipids

a. Characterization of Planar Supported Lipid Bilayers. Planar supported lipid bilayers were formed in contact with cleaned glass coverslips by adsorption and fusion of small unilamellar lipid vesicles (*19*−*22*). The lipid composition was 90 mol % egg-PC, 10 mol % brain-PS, and 1 mol % C_{12}-NBD-PC. Adsorption and fusion of SUVs was carried out in phosphate buffer (10 mM Na_2HPO_4 and 100 mM NaCl, pH 7.0). A relatively high ion concentration (110 mM) was necessary to prevent electrostatic repulsions between the negatively charged lipid vesicles and the negatively charged glass surface. Because phosphate anions can form complexes with the calcium ions which are required for the binding of annexin V to the acidic phospholipids, the phosphate buffer was removed and replaced by the HEPES buffer (10 mM HEPES and 100 mM NaCl, pH 7.2).

To verify that only one bilayer was formed in contact with the glass coverslips (*35*), fluorescence quenching experiments were performed using cobalt ions as quenchers. These cations do not penetrate the lipid bilayer in the concentration range used (0−100 mM) (*36*). The NBD group is located at the water−lipid interface, in the region of the lipid polar headgroups (*37*). In addition, and owing to the way the bilayers were prepared, the probe molecules can be assumed to be evenly distributed between the two leaflets of the lipid bilayer. Upon addition of $CoCl_2$ in the water subphase underneath the bilayer, the fluorescence signal progressively decreased, and for cobalt ion concentration above 40 mM, a plateau was reached corresponding to 45 ± 5% fluorescence quenching (data not shown). This clearly indicates both an absence of permeability of the bilayer to cobalt ions and the existence of a single bilayer of which only the distal leaflet (exposed to the subphase) was available to the quenchers.

In the absence of annexin V, C_{12}-NBD-PC exhibited a diffusion coefficient D of 0.8 ± 0.05 10^{-8} cm^2/s and a mobile fraction M of 95 ± 5% characteristic of a well organized bilayer noncontaminated by residual lipid vesicles (*19*, *20*). The probes present in the distal leaflet were quenched by addition of cobalt ions in the subphase. Similar D (0.75 ± 0.05 10^{-8} cm^2/s) and *M* (95 ± 5%) values were measured for the probes present in the proximal leaflet, demonstrating that lipids diffused laterally at the same rate in both leaflets. Finally, in the presence of 1 mM $CaCl_2$ in the water phase, *D* (0.7 ± 0.05 10^{-8} cm^2/s) and (95 ± 5%) remained practically unchanged, indicating no influence of calcium on the lateral motion of lipids.

b. FRAP Experiments at Fixed Annexin V Concentration and Variable Calcium Concentration. Because the binding of annexin V to lipids depends on both the protein and calcium concentrations (*38*, *39*), the influence of the protein on the lateral motion of lipids was checked first under the conditions of fixed protein concentration and varying calcium concentration. FRAP experiments were carried out with the probe C_{12}-NBD-PC on the same supported planar bilayer, in the presence of 80 nM annexin V and after successive additions of calcium in the water phase. For all the experiments described, *D* was measured after 30 min incubation of the protein with the bilayer. This was the time required for protein−lipid interactions to reach equilibrium and for the bilayer to stabilize as was inferred from the

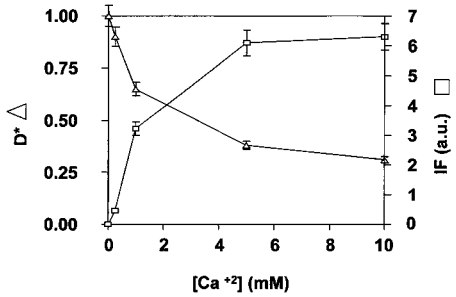

FIGURE 4: Influence of calcium concentration on the lateral motion ($D^* = D/D_0$) of C_{12}-NBD-PC (1 mol %) inserted in planar supported bilayers composed of egg-PC/brain-PS (9:1 mol/mol) mixtures at fixed annexin V concentration of 80 nM (△). Also shown is the binding of the FITC-labeled annexin V to the lipid bilayer in relation to the calcium concentration in the water subphase (□).

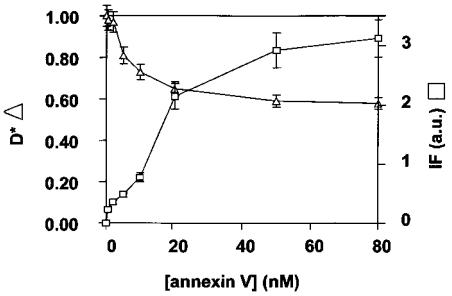

FIGURE 5: Influence of annexin V concentration on the lateral motion ($D^* = D/D_0$) of C_{12}-NBD-PC (1 mol %) inserted in planar supported bilayers composed of egg-PC/brain-PS (9:1 mol/mol) mixtures at fixed calcium concentration of 1 mM (△). Also shown is the binding of the FITC-labeled annexin V to the lipid bilayer in relation to its concentration in the water subphase (□).

stability of the fluorescence signal. Note that on account of the lipid bilayer surface (864 mm^2) and the aqueous phase volume (7 mL), 80 nM annexin V corresponded to a global Ri value of 8. In what follows and to account for possible fluctuations in D from one bilayer to the another, we will consider the relative diffusion coefficient D^* calculated as the ratio $D^* = D/D_0$ of the diffusion coefficients measured, respectively, in the presence (D) and in the absence (D_0) of annexin V and calcium for a given bilayer. In the absence of calcium, annexin V had no influence on D (not shown). Upon addition of calcium (Figure 4), D^* rapidly decreased to a value of 0.3 for 10 mM Ca^{2+}. In all cases, M remained unchanged at 100% (not shown). Because calcium was shown not to alter the lateral mobility of lipids, these decreases in D^* more than likely originate from interactions of annexin V with the lipids. As a control experiment, binding of the protein to the lipids was monitored using FITC-labeled annexin V and lipid bilayers depleted in NBD-labeled phospholipids (Figure 4). In the presence of 80 nM FITC-annexin V and in the absence of calcium, no fluorescence was detected by the FRAP apparatus at the level of the lipid bilayer. In contrast, addition of Ca^{2+} to the subphase brought about the appearance of a fluorescence signal (If) from the lipid bilayer which increased progressively upon further additions of Ca^{2+}. Good concordance was observed between the changes in If and D^*, strongly suggesting that decreased lipid lateral motions were the result of increased protein−lipid interactions.

To check whether insertion of the protein into the lipids occurred, control experiments were carried out with the FRAP apparatus by measuring the fluorescence signal I_0 originating from a small surface of C_{12}-NBD-PC-labeled planar supported bilayers and by varying the concentration of annexin V in the subphase. Any insertion of the protein within the lipid molecules would result in a decrease of the lipid probe surface density and therefore a decrease in I_0. In fact and within experimental error (less than 5%), addition of nonlabeled annexin V up to the 80 nM concentration did not affect I_0, indicating no change in the probe surface density (data not shown). Absence of annexin V insertion into the lipids can also be inferred from recent monolayer experiments carried out on PC/PG and PC/PS mixtures and showing that the protein had only little influence on the film surface pressure (*40*).

c. FRAP Experiments at Fixed Calcium Concentration and Variable Annexin V Concentration. In these experiments, the calcium concentration was 1 mM and the probes were C_{12}-NBD-PC or C_{12}-NBD-PS. For C_{12}-NBD-PC (Figure 5), D^* rapidly decreased down to 0.64 for a protein concentration of 20 nM (Ri = 32) and then stabilized around a value of 0.6 for a protein concentration of 80 nM (Ri = 8). In all cases, M remained unchanged at around 100%. Binding experiments using the FITC-labeled protein were also carried out (Figure 5). As above, a concomitant increase in If with a decrease in D^* was observed, supporting the concept that increased annexin V−lipid interactions were responsible for the observed decreases in lipid lateral mobility.

One could argue that the decrease in D induced by annexin V could originate from specific interactions between the protein and the NBD group of the probe at the water-lipid interface. For that reason, FRAP experiments were carried out with an anthracene-labeled phosphatidylcholine synthesized in our laboratory (*41*). In this molecule, which bears the 9-(2-anthryl)nonanoic acid at the sn-2 position, the anthacene chromophore is deeply buried in the hydrophobic core of the bilayer, near its center (*42*). For given annexin V and calcium concentrations, both probes yielded similar D^* values.

Finally, and for each annexin V and calcium concentration tested (Figures 4 and 5), FRAP experiments were performed after addition of EDTA (5 mM) to the subphase. D^* took values of 0.9−0.95, close to 1, indicating that the lipids recovered their initial diffusion rate.

Because PS is involved in the binding of annexin V to the lipid bilayer, it was interesting to check the influence of the protein on the lateral mobility of this lipid. FRAP experiments were carried out with the probe C_{12}-NBD-PS and at the fixed 1 mM calcium concentration as above. Addition of annexin V in the subphase at the concentrations of 5 nM (Ri = 144 as in NMR experiments) and 80 nM (Ri = 8), brought about large decreases in the lateral motion of C_{12}-NBD-PS with D^* values of 0.38 and 0.005, respectively.

The following conclusions can be drawn from these complementary approaches. The binding of annexin to lipids does not affect their molecular packing. Upon binding of annexin, the lateral diffusion of PS molecules is drastically reduced. The previous observations that the effects of annexin V (Figures 4 and 5) are due to a reversible calcium-dependent binding of the protein to lipids and not to nonspecific adsorption on the bilayer are confirmed and extended to planar supported bilayers.

DISCUSSION

The most accurate structural information on annexin V associated with phospholipids was obtained using electron microscopy at 20 and 8 Å resolution (*11*, *12*, *43*). From the data, a 3D reconstruction of annexin V associated with phospholipids showed that the protein was peripherally bound to them without drastic conformational modification as compared to the structure of the protein in its 3D crystal (*12*). Just slight change was observed and was attributed to a new conformation of annexin V in which a calcium binding site in domain III is revealed while at the same time tryptophan 187 is exposed outwardly and interacts as an anchor with the phospholipid bilayer. The same conclusion was reached from circular dichroism studies performed with annexin V interacting with SUVs (*44*). However, using the argument that 3D crystals of annexin XII, which is structurally closely related to annexin V, consist of hexameric repeat units (*45*), a different model has been proposed in which annexin V would also adopt a hexameric structure capable of penetrating a lipid bilayer. This could explain the voltage gated channel activity of annexin V in model membranes. However, extensive insertion of hexameric annexin V units in lipid bilayers would result in a large decrease of the lipid surface density. Instead, our control experiments with planar supported lipid bilayers showed that this parameter remained unchanged, even for a protein concentration in the subphase as high as 80 nM. Owing to the hydrophilic character of annexin V, extensive insertion of hexameric protein units would also induce significant changes in the lipid molecular packing and headgroup orientation. In the ^{31}P NMR spectra of both IFVs and oriented samples, no drastic modification was observed upon addition of the protein. The CSA reduction of oriented samples (about 10%) indicates that the average orientation of the polar headgroups was slightly affected. Moreover, no isotropic peak was observed in oriented spectra, as would be the case if micelle-like structures were formed in the vicinity of annexin. In the ^{2}H NMR spectra performed on large unilamellar vesicles, annexin V did not affect the acyl chain quadrupolar splitting of the deuterium-labeled DMPC probes even with a lipid to protein ratio of 43, a parameter which is very sensitive to any changes in the lipid molecular packing and in turn in the conformational mobility of the acyl chains. Consistently, the polarization of the probe DPH in lipid vesicles was not affected by the presence of the protein over a wide range of Ri. All these observations support a very simple model in which annexin V is adsorbed at the surface of the lipid bilayers, without significant insertion.

The use of oriented lipid bilayers in deuterium NMR is known to provide a higher sensitivity and resolution. It allowed us in this case to get new insights into the annexin V perturbation on lipid dynamics. It was shown that two distinct populations of POPC appeared upon binding of the protein: one which was not affected either in the amplitude of the sn-1-acyl chain motions (quadrupolar splitting unchanged) or in the time scale (relaxation times unaffected) and a second whose spectral parameters were deeply modified in particular due to the loss of fast axial diffusion. The existence of two distinct populations of POPC in slow exchange on the NMR time scale (microseconds) is not incompatible with only one diffusional component as determined by FRAP experiments, if the exchange rate is higher than FRAP time scale (seconds). In the same conditions, POPS molecules also show a strong interaction with the protein leading to a net reduction of T_{2e}. In deuterium NMR experiments, the spin-lattice relaxation times T_1 are dominated by motions occurring near or above the Larmor frequency ($\tau \approx 10^{-10}$ s), while the transverse relaxation times T_{2e} are also sensitive to slow motions due to the additional dependence of low-frequency spectral density (*46*). The different effects observed on T_1 and T_{2e} reveal that the interaction of the protein with the lipids induces slow motions in the system. The various motions of lipids in vesicles able to modulate T_{2e} have been described (*47*). Lateral diffusion along a curved surface and thermally excited collective undulations are excluded in the case of planar bilayers. A possible source of T_{2e} modification is therefore motions such as the reorientation of the lipids in proximity of the protein or chemical exchange between "free" and "bound" lipids. Our NMR results are consistent with a model of annexin V clusters adsorbed at the bilayer surface and segregating the PS molecules together with part of the PC molecules, while other lipidic domains remain essentially unaffected by the interaction.

The FRAP experiments clearly demonstrate that binding of annexin V to planar supported bilayers strongly affects the lateral motion of both PC and PS molecules, but not to the same extent. Experiments carried out with pyrene-labeled molecules also suggested that annexin V can affect the lateral motion of lipids without inducing lateral phase separation (*48*). Similarly, annexin IV was shown to alter the lateral motion of POPC, POPG, and their mixtures in planar supported bilayers. But in this case, the two probes used, NBD-PC and NBD-PG, exhibited similar behaviors and it was concluded that annexin IV induced fluid−fluid phase separation in these membranes rather than forming long-lived complexes with POPG molecules (*13*). Note that other extrinsic proteins like the bovine prothrombin fragment 1 (*49*) or antibodies specifically directed against phospholipid derivatives have also been shown to reduce the lateral diffusion rate of phospholipids (*50*−*52*). In the present work, the strict correspondence which exists between the decrease in D and the binding of FITC-annexin V to the planar supported bilayers clearly indicates that the decreases in D are a direct consequence of the interaction of the protein with the lipids. In addition, the considerable difference observed at saturation (80 nM annexin V in the subphase) between the PC ($D^* = 0.6$) and PS ($D^* = 0.05$) molecules strongly suggests that these protein−lipids interactions are specific. It is tempting to postulate that upon adsorption at the bilayer surface, annexin V enters a proteolipidic complex in the form of an extended 2D network in contact with the lipid bilayers (*12*, *53*, *54*) stabilized by specific interactions with phosphatidylserine molecules (*55*) and with reduced translational motions of the various components. In this complex, the lipids experience an environment which differs from that of the protein-free lipid domains, which could explain the NMR results. Computer simulations of this complex are under progress in our laboratory for a better understanding of the annexin−phopholipids interaction.

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