The Calcium-Dependent Binding of Annexin V to Phospholipid Vesicles Influences the Bilayer Inner Fluidity Gradient†

Francesco M. Megli,*,‡ Mariangela Selvaggi,‡ Susanne Liemann,§,|| Ernesto Quagliariello,‡ and Robert Huber§

Centro di Studio sui Mitocondri e Metabolismo Energetico, c/o Dipartimento di Biochimica e Biologia Molecolare, Università di Bari, V. Orabona, 4, 70126 Bari, Italy, and Max-Planck-Institut für Biochemie, Abteilung für Strukturforschung, Am Klopferspitz 18a, 82152 Planegg Martinsried, Germany

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ABSTRACT: The fluidity of the hydrophobic interior of phospholipid vesicles after calcium-dependent binding of human annexin V (AVH) was studied using EPR spectroscopy. Vesicles (SUVs) composed of PC or PE and an acidic phospholipid (alternatively PS, PA, or CL) were probed at different bilayer depths by either phosphatidylcholine, or the accompanying acidic phospholipid, bearing a spin label probe at position C-5, C-12, or C-16 of the *sn*-2 acyl chain. Alternatively, the vesicle surface was probed with a polar head spin labeled PE (PESL). The EPR spectra of annexin-bound bilayer domain(s) were obtained by computer spectral subtraction. The order parameter values (*S*) from the resulting difference spectra revealed that the bilayer hydrophobic interior has a greatly altered fluidity gradient, with an increased rigidity up to the C-12 position. Thereafter, the rigidification progressively vanished. The effect is not linked to the phospholipid class, since all the acidic phospholipid spectra, as well as phosphatidylcholine, shared the same sensitivity to the bound protein. The observed membrane rigidification appears to parallel the "crystallizing" tendency of vesicle-bound annexin V, but may not be involved in the calcium channeling activity of this protein.

Membrane fluidity is a well-established feature of natural biomembranes and is observed in artificial phospholipid model membranes as well (*1*−*4*). Although this feature can be predicted on a mere physicochemical basis, due to conformer interchange of the hydrocarbon chain (*5*), it has also been attributed a physiological role (*6*−*11*). From a functional point of view, the variation of membrane fluidity is considered key to understanding the action mechanism of some intrinsic enzymes (*12*−*14*). It is also a prerequisite for the definition of membrane effectors (*15*). The search for effectors of membrane fluidity is still active, as is the search for a better definition of the physiological role of this property of membranes in the living cell.

The annexins, a recently discovered family of proteins comprising more than thirteen members, bind to membranes containing acidic phospholipids in response to calcium concentrations as low as 0.1−0.01 μM, depending on the phospholipid. Their physiological role in the living cell is still unclear, even if many activities have been attributed to them, including calcium channeling and anticoagulant and antiinflammatory properties (*16*, *17*). Since the onset of all activities is triggered by calcium-dependent binding of annexins to membranes, the various aspects of this interaction are under continuous scrutiny.

From the structural point of view, human annexin V (AVH)[1] has been extensively studied, both in itself, and in association with membranes. Its tertiary structure has been defined (*18*) and, on this basis, calcium channeling properties have been hypothesized (*19*) and successively proven in liposomes (*20*). When bound to planar membranes, AVH forms trimers (*21*) and tends to polymerize, forming a 2-D crystal lattice (*22*). This tendency is so strong as to force LUVs to take on a faceted shape (*23*). Although a clear correlation between this behavior of AV and its functioning does not exist as yet, it is unlikely that effects exerted on the phospholipid bilayer are separate from the many hypothesized activities of the annexins.

The present study is aimed at establishing the properties of the phospholipid bilayer interior of various model membranes after Ca-dependent AVH binding. The response of the phospholipids involved in the interaction was studied by use of phospholipids spin labeled at positions C-5, C-12, and C-16 in the *sn*-2 fatty acid chain (*24*, *25*). Computer-aided spectral titration (*26*−*31*) allowed selective scrutiny of the interacting membrane domains. It will be shown that membrane-bound AVH shifts the bilayer fluidity gradient in its vicinity to a more immobilized profile, independently of the component phospholipid species, in a manner well correlated with the crystallizing tendency of AVH. However,

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* Author to whom correspondence should be addressed.

‡ Department of Biochemistry and Molecular Biology, University of Bari.

§ Max-Planck-Institut, Martinsried.

|| Current address: Institut für Molekularbiologie und Biophysik, ETH Hönggerberg HPM, 8093 Zürich Schweiz.

[1] Abbreviations: AVH, human annexin V; PE, phosphatidylethanolamine; PESL, polar head spin labeled PE; nDSA, *n*-doxylstearic acid; nDSPC, *sn*-*2*-(*n*-doxylstearoyl)phosphatidylcholine; nDSPS, *sn*-*2*-(*n*-doxylstearoyl)phosphatidylserine; nDSPA, *sn*-*2*-(*n*-doxylstearoyl)-phosphatidic acid; nDSCL, 1-[*sn*-*2*-(*n*-doxylstearoyl)phosphatidyl]-3-phosphatidylglycerol, cardiolipin spin label; SUV, small unilamellar vesicle; LUV, large unilamellar vesicle.

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correlating this finding with the Ca-channeling activity of the protein, though conceivable, is hazardous at this stage.

MATERIALS AND METHODS

Materials. Recombinant annexin V, in pure form, was prepared as described in ref *32*. Before use, an aliquot was taken from the stock and dialyzed three times against 1000 volumes of Hepes 20 mM, pH 7.4.

Egg yolk phosphatidylcholine (PC), *sn*-2-lysophosphatidylcholine, phosphatidic acid (PA), and phosphatidylethanolamine (PE), bovine brain phosphatidylserine (PS), and bovine heart cardiolipin (CL) were from the Sigma Chemical Company (St. Louis).

Doxylstearic acid, spin labeled at positions (*n*) 5, 12, and 16 (nDSA), was from Aldrich. Phospholipase D (phosphatidylcholine phosphatidohydrolase, EC 3.1.4.4) was purchased from Boehringer-Mannheim and *Trimeresurus flavoviridis* venom (phospholipase A_2, EC 3.1.1.4) from Sigma.

Preactivated silicic acid Unisil was from the Clarkson Chemical Company (Williamsport, PA), and silica gel H chromatoplates were from Merck. Microgranular preswollen carboxymethylcellulose CM-52 was from Whatman.

Phospholipid Spin Labeling. Spin labeled phosphatidylcholines, *sn*-2-(*n*-doxylstearoyl)phosphatidylcholine (nDSPC, $n = 5, 12, 16$), were obtained by acylation of *sn*-2-lysophosphatidylcholine with doxylstearoylimidazide as described in ref *33*. Transphosphatidylation of the appropriate spin labeled phosphatidylcholine by phospholipase D, in the presence of L-serine as described (*34*), yielded the corresponding *sn*-2-(doxylstearoyl)phosphatidylserines (nDSPS). In the same reaction, a convenient amount of the corresponding *sn*-2-(*n*-doxylstearic)phosphatidic acid was also formed. The two homologously spin labeled phospholipids were purified in one step by CM-column chromatography in methanol, as also described in ref *34*.

Monolysocardiolipin, the starting compound for cardiolipin spin labeling, was prepared by stereospecific hydrolysis of cardiolipin by phospholipase A_2 with *T. flavoviridis* venom and purified by preparative thin-layer chromatography as described in ref *35*. The product was acylated with the proper fatty acid spin label as mentioned above (*33*), yielding spin labeled cardiolipin, (nDSCL), which was purified as above.

Polar head spin labeled phosphatidylethanolamine (PESL) was prepared by reacting PE with 2,2,5,5-tetramethylpyrroline-*N*-oxide-3-carbonyl chloride (*36*, *37*). Alternatively, the corresponding carboxylic acid of the spin label was converted to an activated form by the method outlined in (*33*), and used in place of the carbonyl chloride.

Vesicle Preparation. Small unilamellar vesicles (SUVs) were prepared by sonication of phospholipid mixtures composed of PC/PS, PC/CL (1:1 by mole) or PC/PA (2:1 by mole). Either of the phospholipids in each mixture, contained a percentage of its spin labeled homologue: 5 mol % for the 5- and 12-DS species, 2.5 mol % for the 16-DS label to avoid spin−spin exchange. Surface spin labeled SUVs were prepared in the same way, with PESL in place of 5 mol % PC. After suspending the dried lipids in 50 mM KCl, 10 mM Hepes, 1 mM $MgCl_2$, pH 7.4, sonication was performed using a Branson metal-probe sonifier at 40 W output, in an ice bath under a N_2 stream, (six 2.5-min strokes with 1-min cooling intervals). From this preparation, the SUVs were selected in the following peak by Sepharose 4B gel chromatography (*38*) with a buffer composed of 100 mM KCl, 20 mM Hepes, 2 mM $MgCl_2$, pH 7.4.

Phosphorus and Protein Assay. Phospholipid phosphorus was assayed after digesting the lipids in concentrated $HClO_4$ at 180 °C, by the Nakamura method (*40*). Protein content was determined by the Bradford method (*41*).

Sample Preparation. Some 100 nmol phospholipid vesicle was mixed with annexin (1.06 mg/nmol protein:phospholipid) in a microvial, and the solution was made 1 mM Ca^{2+}, in a final volume of 50 mL. Since annexin is known to bind only to the outer membrane surface, the experimental protein:phospholipid ratio was kept as high as possible in order to completely coat the vesicles. Based on AVH molecular dimensions (*18*) and vesicular radius (*38*, *39*, *42*, *43*), the protein amount is calculated to be enough to cover an area twice as large as the one covered by 1 nmol SUV (0.53 μg/nmol). The sample was then transferred into a glass capillary (30−35 mL volume) for EPR spectrometry. Blank samples were prepared in the same way without annexin, or, alternatively, by adding 10 mM EGTA (final concentration) to the AVH-containing samples.

EPR Spectrometry. EPR spectra were recorded on a 9-GHz Varian E-9 Century Line spectrometer, and digitized by means of an HP9835b desktop computer connected to the spectrometer via a Varian data acquisition system. Alternatively, an EPR data system from STELAR (Mede, Italy) was used. Instrumental settings were 3396 G field set with 200 G scan range; 2×10^3, gain; 0.064−0.25 s, time constant. The signal was modulated at a frequency of 100 kHz and 2 G amplitude; radiating field power and frequency were 20 mW and 9.5 GHz, respectively; all spectra were recorded at room temperature, unless otherwise stated.

The magnetic parameters of PESL were measured from the spin labeled phospholipid immobilized onto silica gel. After treating an ether solution of the lipid with silica gel and evaporating the solvent, EPR spectra of PESL bound to the dry silica were recorded with decreasing temperature. No further change in the features of the outcoming strongly immobilized spectrum were observed below −50 °C, and reference spectra were recorded at −60 °C.

Spectral Analysis. The composite nature of the EPR spectra of spin labeled vesicles was assessed by means of spectral titration (*26*−*31*), using an interactive software written by Stan Sykora (Stelar), following the method outlined in ref *31*. To eliminate the contribution of noninteracting membrane domains, the spectrum of vesicles in the presence of Ca^{2+} alone, uninfluenced by annexin, was subtracted from that obtained in the presence of AVH and Ca^{2+} (raw spectrum) by carefully skimming the inversion of EPR spectral bands to avoid oversubtraction. The final difference spectra were matched to standard spectra by superimposition, and optimal fitting was confirmed after regression analysis (reduced χ^2 values ranged typically between 10^{-4} and 10^{-6}). To this aim, standard motionally restricted spectra were obtained from spin labeled SUVs scanned at various temperatures (*44*), and in a minority of cases they were taken from a library. All EPR spectra of spin labeled vesicles in the presence of AVH and Ca^{2+} (at the indicated concentration) were obtained by this method, and from each spectrum the corresponding value of the order

parameter *S* was evaluated (*24*, *25*). The increase in the *S* value of difference spectra in the presence of AVH over that of the spectra from SUVs without AVH, ΔS, was taken as the measure of the bound annexin related fluidity change at a given acyl chain position. The ratio between the computed second integral of the resulting spectrum and that of the raw one yielded an estimate of the percentage of the annexin-influenced phospholipid spin label (*31*). As a control, addition of the estimated percentage of a titrated spectrum to the complementary percentage of the corresponding annexin-free one reproduced the raw spectrum, as judged by its outer hyperfine splitting ($2A_{max}$), with an error in spin quantitation ranging between 5 and 10%. The upper limit of uncertainty in *S* and ΔS was estimated to be about 0.05; in all figures, this is represented by figure symbols drawn to scale with a 0.05-width in *S* units, instead of error bars.

Simulation of EPR spectra of strongly immobilized PESL was performed using a software from Varian, and the closest match with the experimental spectrum was obtained with the following values: $A_{||} = 36$ G, $A_{\perp} = 6.5$ G; $g_{||} = 2.0029$, $g_{\perp} = 2.0081$. These values were used for calculating the order parameter of vesicles labeled with this phospholipid spin label.

RESULTS

The effect of bound annexin on the EPR spectra of vesicles spin labeled in the bilayer interior with 5DSPS, 12DSPS, or 16DSPS (PC/PS/nDSPS mole ratio: either 50:45:5 for $n = 5$ and 12, or 52.5:45:2.5 for $n = 16$) is reported in Figure 1 and in Table 1. In the figure, direct comparison of spectra *a* and *b* ($n = 5$), *d* and *e* ($n = 12$), and *g* and *h* ($n = 16$) is inadequate to reveal the effect of bound AVH (*b*, *e*, *h*) with respect to those without protein (*a*, *d*, *g*). Nonetheless, in Table 2 a reproducible and highly significant, though small, increase of the maximum hyperfine splitting ($2A_{max}$) value of spectra *b*, *e*, and *h* is clearly evident, especially when the probe is placed at positions C-5 (*b*) and C-12 (*e*), with $2A_{max}$ increasing from 53.4 to 55.4 G, and from 36.4 to 37.8 G, respectively. The effect on the mobility of the 16DS probe is better shown by the increase in the τ_c value from 1.3 to 1.4 ns, even if the significance level is far too low for such a small difference. The feature strongly suggests the presence of a more immobilized component in spectra *b*, *e* and, possibly, *h*. By applying spectral titration to the putative composite spectra (*b*, *e*, *h*), using the first member (*a*, *d*, *g*) of each triplet as the one to be subtracted, nonvanishing difference spectra were systematically obtained, shown in Figure 1, *c*, *f*, *i*, confirming the presence of a motionally restricted component in the raw spectra, as anticipated by their $2A_{max}$ values. Inclusion of 10 mM EGTA in the samples when AVH and 1 mM Ca^{2+} were present, zeroed the difference spectra and the $2A_{max}$ increase as well, while making no difference when only Ca^{2+} was present, demonstrating the motionally restricted spectrum to be related to AVH Ca-dependent binding and not to Ca ions. This behavior allowed attribution of the nonzero final "immobilized" spectra to the spin labeled membrane region involved in annexin Ca-dependent binding. The difference spectra in Figure 1, *c*, *f*, *i*, were matched to variously motion-restricted standard spectra, obtained (in most cases) by scanning the same phospholipid spin label SUV preparation in the presence of Ca^{2+} at lower temperatures. Best fits were sought by superimposing the spectra by trial and error (*3*, *26*, *27*, *31*), and confirmed by a reduced-χ^2 test. From the difference spectra the order parameter *S* values were

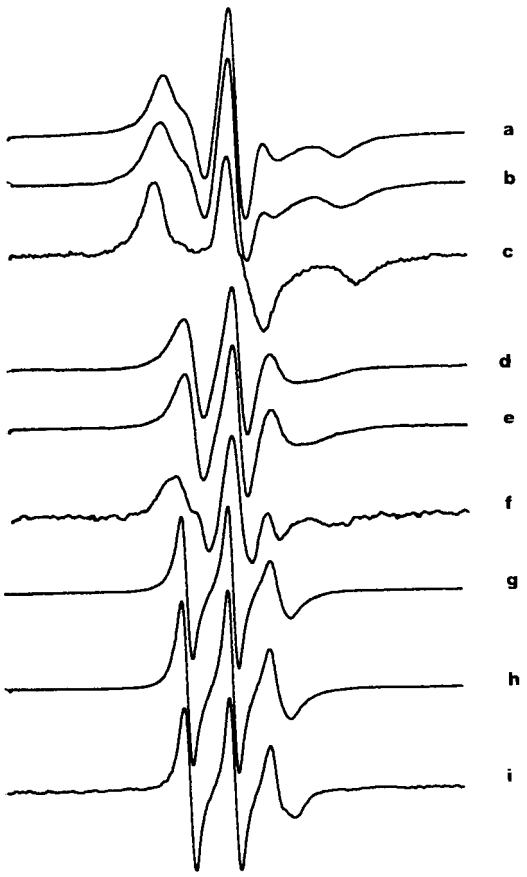

FIGURE 1: EPR spectra of PC/PS (1:1 by moles) SUVs spin labeled with nDSPS. Doxyl ring position on the fatty acyl chain: a − c: C-5; d − f: C-12; g − i: C-16. In each triplet, the top spectrum (*a*, *d*, *g*) is recorded in the absence of AVH (see also Table 2); the middle spectrum (*b*, *e*, *h*) is the raw composite spectrum (see Table 2) in the presence of annexin; the bottom spectrum (*c*, *f*, *i*) is for the rigid component, obtained by subtraction of a given percentage of the top spectrum from the mid one (see Spectral Analysis). Total scan width was 140 G; scaling factors of spectra *c*, *f*, *i* were comprised between 5 and 8.

Table 1: Order Parameter (*S*) Values in Various SUV Types[a] at Various Acyl Phospholipid Chain Positions and Its Variation (ΔS) after Ca-Dependent Binding of AVH

	n[b]									
	5			12			16			
	−	+	ΔS	−	+	ΔS	−	+	ΔS	Δ%[c]
PC/**PS**[a]	0.62	0.91	0.29	0.31	0.58	0.27	0.14	0.21	0.07	22
PC/PS	0.62	0.87	0.25	0.34	0.60	0.26	0.14	0.20	0.06	19
PC/**PA**	0.66	0.84	0.18	0.31	0.49	0.18	0.14	0.18	0.04	21
PC/PA	0.62	0.82	0.20	0.30	0.50	0.20	0.14	0.21	0.07	20
PC/**CL**	0.64	0.91	0.27	0.33	0.59	0.26	0.13	0.17	0.04	22
PC/CL	0.61	0.86	0.25	0.35	0.60	0.25	0.14	0.21	0.07	20

[a] Vesicles were composed as described in Materials and Methods; the species in bold was the phospholipid carrying the spin label at the [b]carbon atom number indicated. Figures are the order parameter *S* values as calculated from (−) spectra recorded from samples without annexin, and from (+) difference spectra obtained by subtraction of a given percentage of the former (−) spectra from the composite spectra (*S* not reported). [c] Percentage of immobilized phospholipid spin label calculated by double integration of the rigid component spectrum (+) at positions C-5 and C-12.

Table 2: Influence of AVH Binding on the EPR Spectral Parameters of nDSPS Labeled Small Unilamellar Vesicles[a]

n^b	$2A_{max}{}^c$ (G)		P^d	$\tau_c{}^e$ (ns)		P	m^f
	−AVH	+AVH		−AVH	+AVH		
5	53.42 ± 0.93	55.40 ± 0.96	<0.005				(9)
12	36.44 ± 0.92	37.83 ± 0.82	<0.05				(5)
16	32.86 ± 0.89	33.02 ± 0.86		1.29 ± 0.11	1.39 ± 0.12	<0.1	(6)

[a] Vesicles composed of nDSPS/PS/PC as described in Material and Methods were incubated with (+AVH) or without (−AVH) annexin, in the presence of 1 mM $CaCl_2$. Figures are the mean of experimental values, ± SD. [b] Spin label position. [c] Maximum spectral hyperfine splitting (first maximum to last minimum). [d] Significance level. [e] Rotational correlation time. [f] Number of experiments.

calculated and reported in Table 1. In the case of nDSPS−SUVs, the order parameter reflecting the mobility of the acyl chain segments of the phospholipid molecules influenced by annexin binding is seen to increase from 0.62 to 0.91, and from 0.31 to 0.58, (for $n = 5$ and 12, respectively) with a net increment between 0.3 and 0.25 at both positions. Position C-16 appears to be less affected, since the *S* value for this spin label increases much less, from 0.14 to 0.21. More properly, this is also confirmed by a small increase of the rotational correlation time, from $\tau_c = 1.29$ ns (*g*) to 1.88 ns (*i*). The percentage of the motionally restricted phospholipid spin label building up the difference spectra is estimated to be around 20% of the total vesicular phospholipid (Table 1), based on the second integral values of spectra *c*/*b* and *f*/*e*. The experimental annexin:phospholipid ratio, chosen on the basis of vesicle geometry and protein dimensions, was positively checked for maximum spin label response. In fact, in nDSPC/CL SUVs, the dependence of the percentage of the motionally restricted phospholipid spin label upon the incubated AVH/SUV ratio was found to be linear up to 0.26 μg/nmol (resulting in 16−17% immobilized phospholipid spin label). Thereafter the percentage of motionally restricted spin label showed a saturation trend with increasing AVH/SUV, and reached a limiting value of 23.2% ± 2.1% (semisaturation AVH/SUV ratio 0.21 ± 0.042 μg/nmol). The AVH/SUV ratio adopted in our experiments, namely 1.06 μg/nmol (as calculated after geometrical considerations), lies inside the saturation range, being thus validated.

Very similar results, from an analogous set of spectra (not shown) were obtained from similarly composed SUVs, in which nDSPS was replaced by nDSPC in the same molar ratio. In fact, from Table 1, the order parameter of this spin label is seen to increase by 0.25−0.26 at positions C-5 and C-12, with a negligible variation at position C-16. Table 1 also contains the order parameters of SUVs composed of PC/PA/nDSPA/nDSPC, either 67:28:5:0 or 62:33:0:5 mole ratio, and PC/CL/nDSCL/nDSPC vesicles, either 50:45:5:0 or 45:50:0:5 by mole, as calculated from sets of spectra similar to those in Figure 1, analyzed in the same way. These spectra are not shown, owing to their strong resemblance to the reported ones (representative of all SUV spectra), while the outcoming data are plotted in the figures to be described next.

The resulting fluidity profile of free and of annexin-bound PS−SUVs is plotted in Figure 2. In these vesicles, one can see that the bilayer appears to be more rigid in the section close to the phospholipid polar head region, where the protein is known to bind. Panel B of Figure 2 shows the influence of bound annexin on the bilayer fluidity, as sensed by the acyl chain of nonbinding PC, which now bears the spin label instead of PS. Interestingly, even if PC proved not to be involved in annexin binding, the same variation in the fluidity profile is observed. In fact, an increase of 0.25−0.26 in the *S* value is estimated at positions C-5 and C-12, while a more modest increase of about 0.06 is revealed at position C-16. In each panel, the trend of DS is also shown, indicating a similar rigidification of the acyl chain up to the C-12 position.

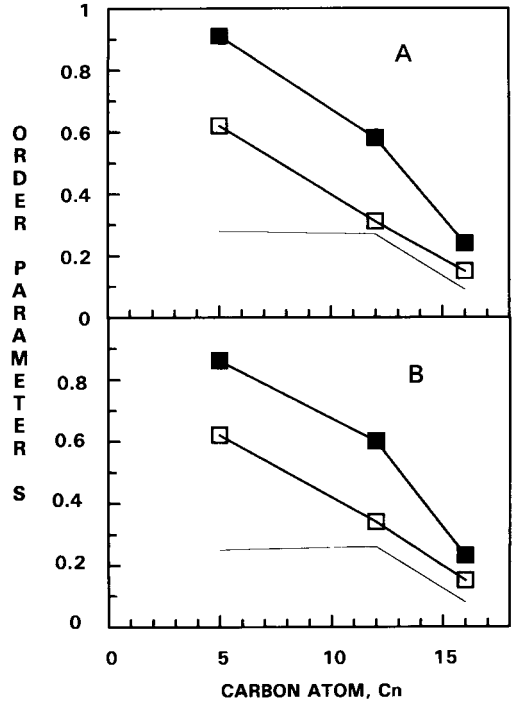

FIGURE 2: Fluidity profile of PC/PS/nDSPS/nDSPC SUVs, with molar composition A, 50:45:5:0; B, 45:50:0:5 (the 16 doxylstearoyl phospholipid was 2.5%). Order parameter values (*S*) at the given carbon atom number (C_n) of the (■) rigid component (from difference spectra) in the presence of AVH, or of the (□) unaffected spectrum in the absence of AVH. (—), trend of Δ*S*, the increase of the order parameter, upon AVH binding, with C_n.

The results of experiments with PA-containing SUVs (Table 1) are reported in Figure 3, panels A and B. A different molar ratio was adopted since SUV formation was difficult with a higher PA percentage, as revealed by the absence of the following peak after Sepharose 4B chromatography. The bilayer's fluidity profile is influenced by annexin binding also in these vesicles, and its trend reveals a rigidification, similar to that observed in PS−SUVs. The bilayer section in the vicinity of the polar heads appears to be slightly less immobilized, with a slightly lower increase in *S* (about 0.20) than for PS−SUVs, that is, from 0.66 to 0.84 at position C-5, and from 0.31 to 0.49 at position C-12 (see Table 1). Position C-16 remains almost unaffected, showing an increase of only 0.04 in the *S* value. As in the case of PS-containing SUVs, from Figure 3B, PC acyl chains

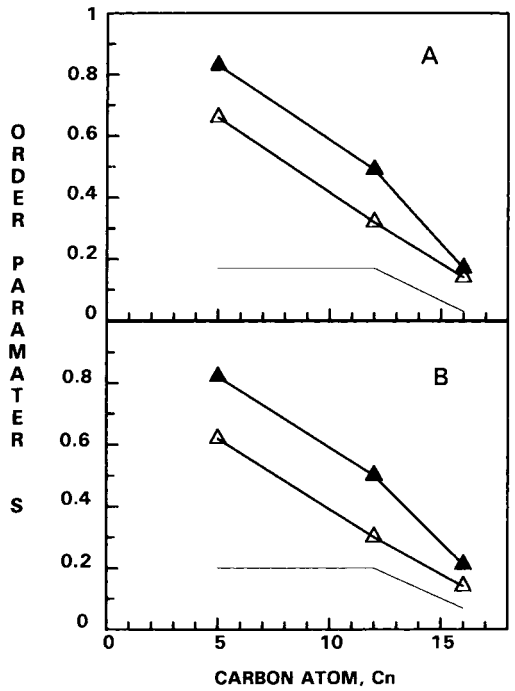

FIGURE 3: Fluidity profile of PC/PA/nDSPA/nDSPC SUVs, with molar composition A, 67:28:5:0; B, 62:33:0:5 (the 16 doxylstearoyl phospholipid was 2.5%). Order parameter values (*S*) at the given carbon atom number (C_n) of the (▲) rigid component (from difference spectra) in the presence of AVH, or of the (△) unaffected spectrum in the absence of AVH. (—), trend of ΔS, the increase of the order parameter, upon AVH binding, with C_n.

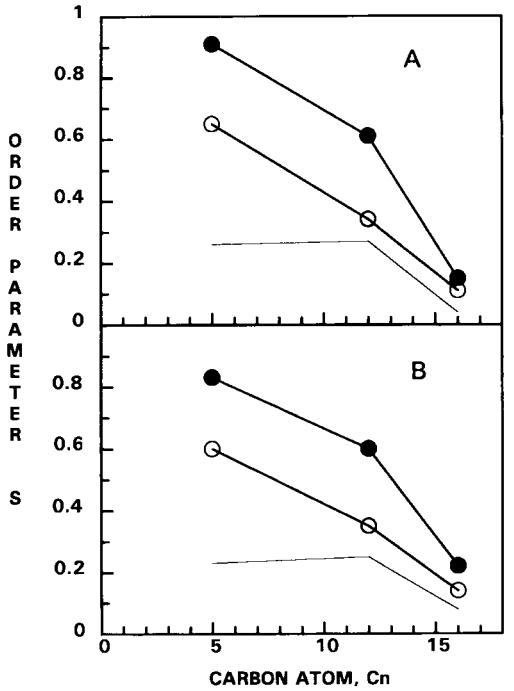

FIGURE 4: Fluidity profile of PC/CL/nDSCL/nDSPC SUVs, with molar composition A, 50:45:5:0; B, 45:50:0:5 (the 16 doxylstearoyl phospholipid was 2.5%). Order parameter values (*S*) at the given carbon atom number (C_n) of the (●) rigid component (from difference spectra) in the presence of AVH, or of the (○) unaffected spectrum in the absence of AVH. (—), trend of ΔS, the increase of the order parameter, upon AVH binding, with C_n.

are seen to experience the same rigidification as those of acidic PS, though not directly involved in annexin binding, with *S* increasing by 0.20 (C-5, C-12) and 0.07 (C-16, Table 1). As in the previous cases, double integration of the difference spectra yields an estimated amount of the phospholipids affected by annexin binding of about 20% (Table 1).

Cardiolipin-containing SUVs also behaved similarly, as shown in Figure 4. From this figure, the already familiar shift to a more rigid profile appears. Again, rigidification is prominent in the region close to the phospholipids' polar head (up to about the 12th carbon atom, as also shown from the trend of ΔS reported therein), and is sensed by both the acidic phospholipid directly involved in the interaction (panel A) and noninteracting phosphatidylcholine (panel B), to a similar extent. In fact, in both panels, the order parameter *S* is seen to increase by a similar amount of 0.25−0.27, both at the C-5 and C-12 positions, with a minor jump of 0.04−0.07 at position C-16 (Table 1). Also in this case, the percentage of phospholipid molecules affected by the protein the presence is estimated around 20%.

The effect exerted by the bound annexin on the vesicle surface was monitored by using PE-SL, a phospholipid spin label suited for probing the membrane surface (*36*, *37*, *45*). Variously composed small unilamellar vesicles were made of PC/X/PE-SL 45:50:5 by mole, with X being either PS or CL, and of PC/PA/PE-SL 62:33:5 mole ratio. The spin label order parameter *S* from the vesicles in the presence of 1 mM Ca^{2+} and without protein, was close to 0.20, as shown in Table 3. Controls showed that addition of AVH was ineffective when 10 mM EGTA was already present. In the presence of 1 mM Ca^{2+} and annexin (1.06 μg/nmol P) the *S* value, calculated from difference spectra, following the same criteria and methods used with the nDS spin labels, is seen to rise to about 0.50. In the same table, it is indicated that the titration endpoint is reached by subtracting about 70% of the unaffected spectrum, which yields an estimated 30% of phospholipid molecules sensing the protein's presence.

Table 3: Interaction of AVH with Surface Spin Labeled Phospholipid Vesicles[a]

vesicle composition		order parameter, *S* −AVH[c]	order parameter, *S* +AVH[d]	ΔS	Corr.[e] (%)
PC/PS/PESL	45:50:5[b]	0.22	0.51	+0.29	65
PC/CL/PESL	45:50:5	0.21	0.53	+0.32	68
PC/PA/PESL	62:33:5	0.24	0.51	+0.27	70

[a]SUVs, with the indicated [b]molar ratio composition, were surface labeled by means of polar head spin labeled PE (PESL, see Materials and Methods). *S* values calculated from spectra of vesicles [c]without annexin, and from [d]difference spectra, obtained by subtraction (see Spectral Analysis) of the indicated [e]percentages of the spectra without annexin from raw spectra (*S* not reported) in the presence of AVH.

DISCUSSION

In the absence of calcium, annexins exhibit no phospholipid binding affinity, their membrane binding capacity relying uniquely on Ca ions, at least in the physiological experimental conditions commonly adopted. From this aspect, these proteins may not be included in the classification of peripheral proteins recently made by Swamy and Marsh (*46*). The annexin:phospholipid binding stoichiometry is controlled by the number of Ca-binding sites, three to four per protein molecule (*47*−*49*), so that the percentage of directly bound phospholipids in a vesicle should be as small

as 3−4%, while the number of phospholipid molecules indirectly involved should coincide with that covered by one protein molecule, that is 26−28 per annexin (*49*, *50*). Additional interactions of annexins with the membrane cannot be excluded (*51*), rendering difficult, if not impossible, the definition of a binding parameter for these proteins, as stated (*52*). Owing to multisite cooperativeness, despite bound/free rapid Ca-ions and phospholipid exchange (*53*, *54*), the annexin−Ca−membrane complex is quite stable (*55*). Following these characteristics, it seems unlikely that the annexin−Ca−phospholipid interaction could fit the lipid−protein interaction model reviewed by Marsh (*30*). Consequently, the present study is aimed at the overall effect on phospholipid mobility in annexin-bound membrane domains (*51*), and at its possible relationship with the protein binding specificity for a particular acidic phospholipid class.

Spectral titration successfully shows the effect of bound annexin on the phospholipid bilayer, even though the raw spectra barely betrayed the presence of the bound protein. No effect of calcium alone was detected, and Mg ions (mimicking the effect of Ca^{2+} on membrane surface (*56*)) in the SUV buffer ensure cation balance across the vesicle bilayer.

Our results clearly show that annexin binding to the membrane affects the bilayer interior, resulting in a marked rigidification of the hydrophobic part of the bilayer close to the phospholipid polar head, and that the membrane fluidity gradient is largely modified with respect to that of unbound vesicles.

Remarkably, all kinds of SUVs labeled with doxyl-stearoylphospholipids in the bilayer interior gave similar sets of EPR spectra. The resulting membrane fluidity profiles in the presence of AVH at the level of the binding acidic phospholipid (Figures 2A−4A) are quite similar in the differently composed SUVs. The observed large decrease in membrane fluidity up to position 12 in the acyl chain is noteworthy (shown by the ΔS plot) and appears to be independent of the acidic phospholipid class, thus excluding any specificity of the rigidifying effect of the bound annexin and confirming that negatively charged polar heads are the only requirement for annexin binding, as previously suggested (*54*). More notably, in panel B of each figure, spin labeled phosphatidylcholine, though not directly involved in annexin binding, is seen to experience a rigidification almost identical to its acidic neighbor. This suggests that the membrane site occupied by the protein is influenced as a whole, with the annexin effect aspecifically extending along and across the membrane plane until about the 12th carbon atom and almost vanishing while approaching the ω position.

The invariant amount of about 20% immobilized phospholipid was unexpected, and considered too low in comparison with the number of phospholipid molecules in the outer leaflet (60−70% by mol) (*42*). The expected outward facing phospholipid spin labels do not appear to be completely involved, despite the fact that the annexin/vesicle ratio was appropriate for maximum effect. An asymmetric, mainly inward, transmembrane distribution of acidic phospholipids, as occurring in many membrane types (*57*−*61*), could explain this figure, since it might result in a lower number of binding sites than expected on the outer surface. Of course, the same applies also to nDSPC, the sensitivity to AVH of which is dependent on the presence of its acidic phospholipid companion. Alternatively, one might speculate that despite the proper protein:phospholipid ratio, annexin is unable to occupy all available binding sites, in agreement with the observation that the high curvature of SUVs impedes complete coating of the outer surface by the protein (*23*). In any case, our results do indicate that membrane rigidification occurs at the membrane site involved in annexin binding, whatever its width.

Due to the average spherical shape of liposomes, the spin labels in our SUV system should be viewed as a randomly oriented sample, unable to reveal variations of the molecular order. Thus, ascribing the observed order parameter increase to some ordering effect of the annexin into the bilayer would be misleading. On the other hand, it is known that interactions involving the outer surface can extend their effect into the bilayer, as happens with cations and cationic polypeptides or proteins, whose rigidifying effect on the acyl chains is linked to charge neutralization and consequent condensing of phospholipid polar heads (*56*, *62*). As a more specific example, avidin binding to spin labeled biotin−PE at the level of the polar head is reflected on the whole acyl chain (*46*). We therefore favor the idea that the observed membrane rigidification is due to surface AVH binding, extending its effect into the bilayer, to a considerable extent within intermediate depth ($n = 12$) and fading in the long range ($n = 16$). This interpretation is in close agreement with the observed tendency of AVH to form hexagonal 2-D crystalline arrays of trimers on phospholipid surfaces (*22*), since the formation of rigid patches on the membrane surface should similarly extend its effect also into the bilayer interior. In fact, strong hindrance from bound AVH at the vesicle surface level is clearly reported by phosphatidylethanolamine spin labeled at the polar group (Table 3). Binding of AV (*63*) to these vesicles has been previously assessed (*64*, *65*, *43*). The appearance of a rigid component spectrum after spectral titration, with a definite increase in the order parameter of the label, reveals a rigidification of the membrane surface similar to that experienced by the fatty acyl chain at position C-5, independently of the composition of the phospholipid vesicles. Other authors' observations also favor membrane rigidification independently of the phospholipid polar head and vesicle composition, such as the high exothermic ΔH_{assoc} of annexin V and VI with SUVs similar to ours (*66*), and the 35-fold decreased phospholipid lateral diffusion constant found by FRAP measurements after annexin binding (*67*). Significantly, extreme impediment in phospholipid freedom is also suggested by the hydrophobic interaction of annexin with the *sn*-2-acyl chain (*68*).

Membrane rigidification after Ca-dependent binding of AV is confirmed by these results and enriched with a more detailed description of the bilayer fluidity gradient. Whether and how modification of this membrane parameter will influence the calcium channel activity of annexin V, though beyond the scope of this study, remains an appealing issue, deserving further and deeper consideration.

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