Annexin V Binding Perturbs the Cardiolipin Fluidity Gradient in Isolated Mitochondria. Can It Affect Mitochondrial Function?†

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ABSTRACT: The phosholipid bilayer fluidity of isolated mitochondria and phospholipid vesicles after calcium-dependent binding of annexin V was studied using EPR spectroscopy. The membranes were probed at different depths by alternatively using cardiolipin, phosphatidylcholine, or phosphatidylethanolamine spin labeled at position C-5 or C-12 or C-16 of the β acyl chain. Computer-aided spectral titration facilitated observing and quantitating the EPR spectrum from phospholipid spin labels affected by annexin binding, and spectral mobility was calibrated by comparison with standard spectra scanned at various temperatures. In most cases it was found that binding of the protein to the membranes makes the inner bilayer more rigid up to acyl position C-12 than afterward, in agreement with the previously observed effect in SUVs [Megli, F. M., Selvaggi, M., Liemann, S., Quagliariello, E., and Huber, R. (1998) *Biochemistry 37*, 10540−10546]. Moreover, in isolated mitochondrial membranes, cardiolipin apparently is more readily affected than the other main phospholipids, while in vesicles made from mitochondrial phospholipids, the different species are affected in essentially the same way. This behavior is consistent with the existence of distinct cardiolipin pools in mitochondria, and with the already advanced hypothesis that these domains are the binding site for annexin V to the isolated organelles [Megli, F. M., Selvaggi, M., De Lisi, A., and Quagliariello, E. (1995) *Biochim. Biophys. Acta 1236*, 273−278]. Keeping in mind the funcional importance of cardiolipin in the mitochondrial membrane, the question is raised as to whether the observed influence of annexin V binding to this phospholipid and its consequent local fluidity alteration might affect the mitochondrial functionality, at least in vitro.

In the search for the physiological role of annexins in the living cell, the interaction of these cytosolic proteins with subcellular membranes has been scarcely considered. Among cell organelles, mitochondria contain a specific acidic phospholipid and involve an intense traffic of Ca-ions at the membrane, at least under certain conditions; both are requisite for annexin binding to membranes. In fact, cardiolipin, virtually the only acidic phospholipid in the organelles, is abundant in the inner membrane and also present in the outer membrane (*1*, *2*). Calcium plays an important role in the mitochondrial energy metabolism, and its concentration is known to reach transient high values in the vicinity of these membranes (*3*−*6*). These considerations triggered our interest in studying annexin V (AV)[1] affinity for cardiolipin (*7*, *8*), and annexin binding to isolated mitochondria, possibly to cardiolipin-rich microenvironments, was also advanced in one of our papers (*9*). In the past decade, the functional role of cardiolipin in the activation of energy metabolism enzymes has received increasing consensus (*10*), and the mechanism of this regulation is under intense study (*11*−*14*). Furthermore, the physical state and composition of the membrane is receiving growing consideration under the physiological aspect (*15*−*18*), and it is widely accepted that modifications of those properties can affect the optimal functioning of the embedded enzymes (*19*). Following these considerations, we argue that modifications to membrane organization/fluidity at the level of cardiolipin after annexin binding to mitochondria is of interest from the functional point of view. Noteworthy, we have recently (*20*) shown AV binding-related rigidification of vesicle phospholipid bilayers containing cardiolipin or other acidic phospholipids, by monitoring the phospholipid acyl chain mobility with phospholipid spin labels.

In this article, we present a study of the fluidity of the phospholipid bilayer in isolated mitochondria as a function of annexin V Ca-dependent binding, at the level of the major mitochondrial phospholipids: cardiolipin, phosphatidylcholine, and phosphatidylethanolamine. The bilayer was probed at increasing depths by use of phospholipid spin labels carrying a nitroxide ring on the β fatty acyl residue, at

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[1] Abbreviations: AV, annexin V; PC, phosphatidylcholine; PE, phosphatidylethanolamine; CL, cardiolipin; *n*DSA, *n*-doxylstearic acid; *n*DSPC, *sn-2*-(*n*-doxylstearoyl) phosphatidylcholine; *n*DSPE, *sn-2*-(*n*-doxylstearoyl) phosphatidylethanolamine; *n*DSCL, 1-[*sn-2*-(*n*-doxylstearoyl)phosphatidyl]-3-phosphatidylglycerol, cardiolipin spin label; REV, reversed-phase evaporation vesicles; MtPL, mitochondrial phospholipids; SUV, small unilamellar vesicles; RLM, rat liver mitochondria; EIT, equivalent immobilization temperature; $2A_{max}$, maximun hyperfine coupling constant; $R_{1/2}$, incubation ratio for half-maximum effect.

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positions C-5, C-12, or C-16, and the fluidity of the membrane was inferred from the shape of the outcoming EPR spectrum, after computer-aided spectral analysis. The different immobilization trend of the different phospholipid spin labels in mitochondria, in contrast with the similarity observed in the topologically homogeneous vesicles, suggests a site-specific localization of the probes in these membranes, conceivably at homologous phospholipid microdomains, enabling us to discriminate between differently immobilized phospholipid pools. Our results show that annexin V binding rigidifies the phospholipid bilayer, especially as far as position C-12, in both phospholipid vesicles and mitochondrial membranes. More specifically, mitochondrial cardiolipin is immobilized more promptly and more strongly than PC and PE, the latter being the least affected, in a way indicating that annexin binds directly to mitochondrial cardiolipin, and suggesting that the effect of this binding is extended more to cardiolipin domains than to the others.

If the fluidity of cardiolipin microdomains in mitochondria has an effect on cardiolipin-dependent enzymes, such as respiratory complexes or some membrane carrier functioning, the possibility that annexin binding influences some mitochondrial function is considered.

EXPERIMENTAL PROCEDURES

Materials. Recombinant annexin V, in pure form, was prepared as described (*21*). Before use, an aliquot was taken from the stock and dialyzed three times against 1000 vol of Hepes 20 mM, pH 7.4. Bovine lung annexin V was isolated and purified as described (*7*, *22*), and equilibrated in the same buffer.

Egg yolk phosphatidylcholine (PC), *sn*-2-lysophosphatidylcholine, phosphatidylethanolamine (PE), and bovine heart cardiolipin (CL) were from Sigma Chemical Co. (St. Louis).

Lipids were extracted from rat liver mitochondria by the method of Fleischer and Fleischer (*23*). Mitochondrial phospholipids (MtPL) were freed from neutral lipids by silicagel 60 column chromatography by elution with $CHCl_3$ followed by CH_3OH, yielding approximately 220 nmol of phospholipid P (0.195 mg)/mg of mitochondrial protein on average, in close agreement with the value of 0.175 mg/mg reported in ref 1.

Doxylstearic acid, spin labeled at positions (*n*) C-5, C-12, and C-16 (*n*DSA), was from Aldrich. Phospholipase D (phosphatidylcholine phosphatidohydrolase, EC 3.1.4.4) was prepared from Savoy cabbage leaves (*24*), and Trimeresurus flavoviridis venom (phospholipase A_2, EC 3.1.1.4) was purchased from Sigma.

Silicagel 60 for column chromatography and silicagel H chromatoplates were from Merck. Prior to use, silicagel 60 was activated at 110 °C for 1 h.

Phospholipid Spin Labeling. Spin-labeled phosphatidylcholines, *sn*-2-(n-doxylstearoyl)-phosphatidylcholine (*n*DSPC, $n = 5, 12, 16$), were obtained by acylation of *sn*-2-lysophosphatidylcholine with doxylstearoylimidazide as described in ref *25*. Transphosphatidylation of the appropriate spin-labeled phosphatidylcholine by phospholipase D in the presence of ethanolamine (*26*, *27*) yielded the corresponding *sn*-2-(doxylstearoyl)-phosphatidylethanolamines (*n*DSPE). Pure phospholipid spin labels were eluted from silicagel 60 columns with $CHCl_3/CH_3OH$ mixtures as described (*28*). TLC, with $CHCl_3/CH_3OH/H_2O$ 65:25:4 as the developing solvent, was the routine purity check. Monolysocardiolipin, the starting compound for cardiolipin spin labeling, was prepared by stereospecific hydrolisis of cardiolipin by phospholipase A2 with Trimeresurus Flavoviridis venom (*29*), and purified by silicagel 60 column chromatography using $CHCl_3/CH_3OH/NH_4OH_{concentrated}$ 65:25:1.5 by vol as the eluting solvent. The product was acylated with the proper fatty acid spin label as mentioned previously (*25*) yielding spin-labeled cardiolipin (*n*DSCL), which was purified in the same way. After each step, purity was checked by TLC using $CHCl_3/CH_3OH/NH_4OH_{concentrated}$ 65:25:5 by vol as the developing solvent.

Vesicle Preparation. MtPLs were doped alternatively with 5% (by mole) 5- or 12- or 2.5% 16-DS phospholipid spin labels. Reversed-phase evaporation vesicles (REVs) were prepared from these mixtures according to ref *30*, using 250 mM Sucrose, 125 mM KCl, 25 mM Hepes, and 2.5 mM $MgCl_2$, pH 7.4, as the acqueous buffer. The resulting REV suspension was diluted with 3 vol of the same buffer without sucrose and the vesicles were collected by centrifugation at 9000*g* for 3 min, washed twice, and resuspended in the sucrose-free buffer.

Small unilamellar vesicles (SUVs) were prepared by sonication of phospholipid mixtures composed of PC/*n*DSCL, PC/*n*DSPC, or PC/*n*DSPE in 2:1 molar ratio. The dried lipids, suspended in 125 mM KCl, 25 mM Hepes, and 2.5 mM $MgCl_2$, pH 7.4, were sonicated with a Branson metal-probe sonifier at 40 W output, in an ice bath under an N_2 stream (six 2.5 min strokes with 1 min cooling intervals). Traces of tungsten and a few coarse aggregates were removed by centrifugation at 9000*g* for 5 min, and the preparation was concentrated to 250−300 μL in an Amicon ultrafilter.

Preparation and Spin Labeling of Rat Liver Mitochondria. RLMs were prepared (*31*) in 0.25 M Sucrose, 5 mM EDTA, and 10 mM Tris, pH 7.4, and purified from peroxysomes and lysosomes by centrifugation on a Percoll density gradient (*32*). Purified mitochondria, suspended in the isolation buffer, were quickly frozen to −80 °C, stored at this temperature, and used within a week. After thawing, an aliquot containing 20 mg of mitochondrial protein (corresponding to about 4.4 μmol of phospholipid P) was pelleted, washed once with the SUV buffer and treated with 2 μmol of SUVs (250−300 μL) containing the desired phospholipid spin label, at 37 °C for 20 min, with gentle shaking. The procedure was essentially the one described for phospholipid exchange experiments (*33*−*35*). After incubation, the mixture was centrifuged onto a cushion of 14% w/v sucrose in order to separate the mitochondria from donor SUVs (*36*) and pelleted spin-labeled mitochondria were washed twice and resuspended in 125 mM KCl, 25 mM Hepes, 2.5 mM $MgCl_2$, pH 7.4. Other sets of spin-labeling experiments were performed under the same experimental conditions, with amounts of SUVs varying from 0 to 0.3 nmol of P/mg of mitochondrial protein.

Phosphorus and Protein Assay. Phospholipid phosphorus was assayed after digesting the lipids in concentrated $HClO_4$ at 180 °C (*37*), and protein content was determined by use of Coomassie brilliant blue (*38*). Alternatively, the biuret assay or the method of Waddel-Hill (*39*) was also used.

Sample Preparation. Annexin was added to either spin-labeled REVs or mitochondria in amounts increasing from

Table 1: Characteristics of Spin-Labeled Cardiolipin in Mitochondrial Membranes and Phospholipid Vesicles after Binding of Annexin V

	mitochondria					rev				
DSCL	P_{max}[a] (%)	$R_{1/2}$[b] (μg/nmol P)	n[c]	χ^2[d]	t[e] (°C)	P_{max} (%)	$R_{1/2}$ (μg/nmol P)	n	χ^2	t (°C)
5	25.6 ± 0.9	0.11 ± 0.03		0.124	2	32.1 ± 1.2	0.20 ± 0.04		0.181	2
12	32.6 ± 1.2	0.15 ± 0.02		0.148	2	36.6 ± 0.9	0.17 ± 0.02		0.174	2
16	28.9 ± 1.3	0.12 ± 0.04		0.156	12	27.9 ± 1.5	0.92 ± 0.04	4.9	0.163	16

[a] Maximum percentage of spin labeled cardiolipin immobilized by annexin binding. [b] Annexin:membrane ratio yielding semimaximal percentage of immobilized cardiolipin. [c] Cooperativity index. [d] χ^2 test value of best fitting curve (GRAFIT 3.0, Erithacus) yielding parameters $a-c$. [d] EIT, temperature of matching reference EPR spectrum (Figures 2−4).

Table 2: Characteristics of Spin-Labeled Phosphatidylcholine in Mitochondrial Membranes and Phospholipid Vesicles after Binding of Annexin V

	mitochondria					rev				
DSPC	P_{max}[a] (%)	$R_{1/2}$[b] (μg/nmol P)	n[c]	χ^2[d]	t[d] (°C)	P_{max} (%)	$R_{1/2}$ (μg/nmol P)	n	χ^2	t (°C)
5	32.5 ± 1.1	0.36 ± 0.05		0.138	8	36.9 ± 0.8	0.22 ± 0.04		0.153	8
12	34.3 ± 0.9	0.43 ± 0.06		0.188	8	31.7 ± 1.2	0.24 ± 0.04		0.176	8
16	28.9 ± 0.9	1.18 ± 0.09		0.176	16					

[a] Maximum percentage of spin labeled phosphatidylcholine immobilized by annexin binding. [b] Annexin:membrane ratio yielding semimaximal percentage of immobilized phosphatidylcholine. [c] Cooperativity index. [d] χ^2 test value of best fitting curve (GRAFIT 3.0, Erithacus) yielding parameters $a-c$. [d] EIT, temperature of matching reference EPR spectrum (not shown).

0.1 to 2 μg of AV/nmol of P (25−450 μg of Av/mg of mitochondrial protein). This ratio ranges around the values estimated sufficient to completely coat LPVs and SUVs (*20*). The suspension was made 1 mM Ca^{2+} and centrifuged at 9000*g* for 3 min. The pellet was resuspended in 20 μL of the supernatant and transferred to a calibrated glass capillary for EPR measurement. Bulk spectra were obtained from blank samples to which only 1 mM Ca^{2+} was added, without annexin. Each blank, scanned at various temperatures, also provided a series of standard spectra of the spin-labeled membrane under scrutiny with varying degrees of rigidification, to be used for calibrating the immobilization presented by the AV-bound phospholipid spin labels.

EPR Spectrometry. EPR spectra were recorded at room temperature by use of a 9-GHz Varian E-9 Century Line spectrometer and digitized by means of an EPR-Data System from STELAR (Mede, Italy) interfaced to the spectrometer. Instrumental settings were 3271 G field set with 200 G scan range; $1-2 \times 10^3$, gain; 0.016−0.064 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. Blank samples were also scanned at various temperatures using the Varian Variable Temperature Controller. The temperature was lowered from 24 °C to −6 °C in steps of 2 °C, as directly checked in the quartz sample holder inside the cavity by means of a calibrated thermocouple and the EPR spectrum was recorded at each step.

Spectral Analysis. EPR spectra of annexin V-affected spin-labeled phospholipids in the membranes were freed from bulk spectra by means of spectral titration as described (*40*−*45*), by use of interactive software written by Stan Sykora (Stelar) following the method outlined in ref *44*. Accordingly, the contribution from noninteracting membrane domains to the total EPR spectrum, obtained in the presence of AVH and Ca^{2+}, was determined by subtracting the varying tentative weight of the blank spectrum of vesicles in the presence of Ca^{2+} alone, and by superimposing the difference to the variable temperature standard spectra on the computer screen until the best match was obtained. The latter's second integral ratio to that of the total spectrum yielded the percentage of phospholipid immobilized by the interaction with AV. At the same time, the temperature of the matching standard spectrum was taken as a measure of the immobilization degree of the difference spectrum and named equivalent immobilization temperature (EIT), that is, the temperature bringing about a rigidification of the AV-free membrane equivalent to that provoked by annexin binding. As a back control, the difference spectra were added to the blanks with the calculated weight and matched to the starting composite spectrum, yielding an error in the second integral ranging around 5%. In several instances, difference and standard spectra were also compared by regression analysis, yielding reduced χ^2 values $\leq 10^{-4}$.

The calculated percentage of immobilized phospholipid, with an uncertainty in immobilization temperature of 2 °C, was plotted as a function of the incubated annexinV/mitochondrial (or REV) P ratio. Curve fitting, accomplished by use of GRAFIT 3.0 (Erithacus Software), yielded the parameters given in Tables 1−3.

RESULTS

Spin labeling of mitochondria was achieved by incubation with SUVs enriched with spin-labeled phospholipids, under the experimental conditions in which spontaneous/catalyzed phospholipid exchange occurs (*33*−*36*). After labeling, sedimented mitochondrial protein was assayed, while the second integral of the EPR spectrum yielded phospholid spin label content on the basis of known standards. On the basis of the determined mitochondrial phospholipid:protein ratio of 220 nmol of P/mg (*1*), the uptake of spin-labeled phospholipids into mitochondria was expressed as a percentage of endogenous total phospholipids and plotted as a function of the incubated donor vesicles per milligram of mitochondrial protein, as reported in Figure 1. As can be

Table 3: Characteristics of Spin-Labeled Phosphatidylethanolamine in Mitochondrial Membranes and Phospholipid Vesicles after Binding of Annexin V

	mitochondria					rev				
DSPE	P_{max}[a] (%)	$R_{1/2}$[b] (% μg/nmol P)	n[c]	χ^2[d]	t[e] (°C)	P_{max} (%)	$R_{1/2}$ (μg/nmol P)	n	χ^2	t (°C)
5	13.5 ± 0.7	0.32 ± 0.03	2.1	0.192	8	27.1 ± 1.3	0.18 ± 0.02		0.115	8
12	12.5 ± 1.1	0.40 ± 0.05	2.4	0.137	8	24.5 ± 1.4	0.20 ± 0.04		0.174	8
16	12.3 ± 1.0	0.72 ± 0.03	5.7	0.181	16					

[a] Maximum percentage of spin labeled phosphatidylethanolamine immobilized by annexin binding. [b] Annexin:membrane ratio yielding semimaximal percentage of immobilized phosphatidylethanolamine. [c] Cooperativity index. [d] χ^2 test value of best fitting curve (GRAFIT 3.0, Erithacus) yielding parameters $a-c$. [e] EIT, temperature of matching reference EPR spectrum (not shown).

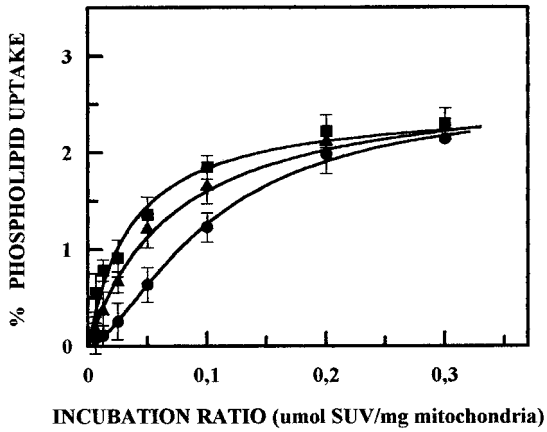

FIGURE 1: Percentage of (●) 16DSCL, (■) 16DSPC, and (▲) 16DSPE found in mitochondria after incubation with different vesicle/mitochondria ratios of the correspondingly spin-labeled SUVs. Vertical bars represent the standard deviation calculated from duplicate experiments at least.

seen, each phospholipid spin label is incorporated into acceptor mitochondria following a saturation profile, up to a similar maximum amount, that is 2.76% of the acceptor mitochondrial phospholipids for 16DSCL, 2.51% for 16DSPC, and 2.76% for 16DSPE. However, CL appears to be adsorbed with more difficulty than PE and PC, since maximum spin labeling by this phospholipid is reached following a sigmoid profile with an apparent coperativity coefficient of 1.44. On the contrary, from the percent uptake profile of PC and PE, it can be seen that these two phospholipids are more readily adsorbed, the former even slightly more than the latter with an incubation ratio for half-maximum labeling of 0.036 and 0.072 nmol of SUV/mg of mitochondrial protein, respectively.

The EPR spectrum of 5DSCL-spin-labeled mitochondria, in the presence of 1 mM Ca^{2+}, is reported in Figure 2A, superimposed on the spectrum of the same vesicles incubated with 1.8 μg of annexin V/mg of mitochondrial protein, and 1 mM Ca^{2+} (asterisk). The superimposition clearly shows an increased $2A_{max}$ value of 54.6 G of the latter over 52.2 G of AV-free spectrum, revealing a decrease in the acyl chain mobility at position C-5 in the annexin-bound membrane. Spectral titration of this spectrum with the AV-free one, yields the spectrum reported in Figure 2B with still more rigid features ($2A_{max}$ = 59.8), very closely matching the spectrum of AV-free mitochondria at the temperature of 2 °C, superimposed onto it in part B of the figure. The ratio of the second integral of this spectrum to that of the AV-free one establishes that it pertains to 25% of the total 5DSCL in the AV-free mitochondria.

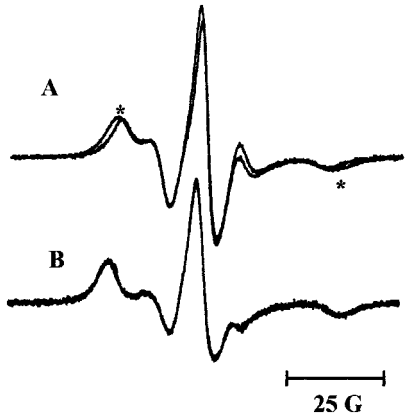

FIGURE 2: EPR spectrum of panel A (*), 5DSCL-labeled mitochondria in the presence of AV/1 mM Ca^{2+} superimposed on the spectrum of the same membranes in the presence of 1 mM Ca^{2+}; (B) difference spectrum of spectra in panel A superimposed on the spectrum of 5DSCL-labeled mitochondria/1 mM Ca^{2+} scanned at 2 °C.

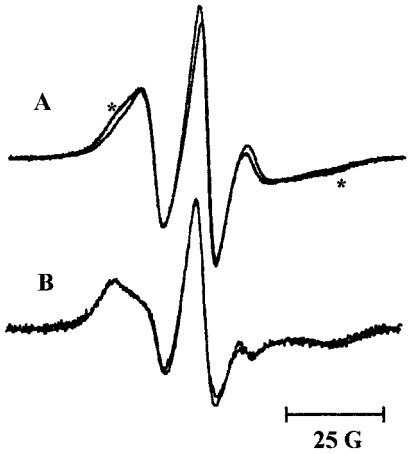

FIGURE 3: EPR spectrum of panel A (*), 12DSCL-labeled mitochondria in the presence of AV/1 mM Ca^{2+} superimposed on the spectrum of the same membranes in the presence of 1 mM Ca^{2+}; (B) difference spectrum of spectra in panel A superimposed on the spectrum of 12DSCL-labeled mitochondria/1 mM Ca^{2+} scanned at 2 °C.

A similar situation occurs at position C-12 of spin-labeled cardiolipin in mitochondrial membranes, as reported by the spectra in Figure 3. After spectral titration, a more immobilized spectrum (part B of the figure) appears in place of the telltale shoulders indicated by the asterisks in Figure 2A in the spectrum of AV-bound mitochondria, which are not observed in the superimposed spectrum of 12DSCL in AV-free mitochondria. Also in this case, the difference most closely matches the spectrum of 12DSCL-labeled mitochondria at 2 °C as shown by superimposition of this spectrum

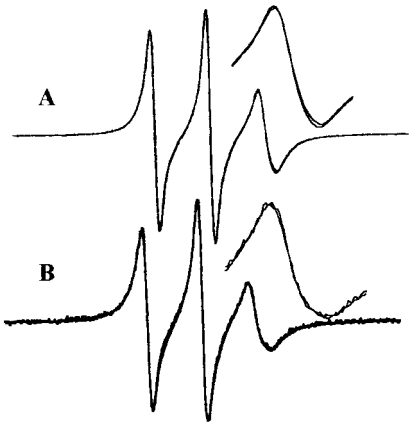

FIGURE 4: EPR spectrum of panel A, 16DSCL-labeled mitochondria in the presence of AV/1 mM Ca^{2+} superimposed on the spectrum of the same membranes in the presence of 1 mM Ca^{2+}; (B) difference spectrum of spectra in A superimposed on the spectrum of 16DSCL-labeled mitochondria/1 mM Ca^{2+} scanned at 12 °C.

in part B of the figure, and its second integral allows determination of 30% motionally restricted 12DSCL in the AV-bound membranes.

Spectral analysis of EPR spectra from 16DSCL-mitochondria in the absence and in the presence of AV (Figure 4) reveals a more fluid terminal segment of the acyl chain in AV-bound mitochondrial membranes, with an equivalent immobilization temperature of 12 °C, and immobilized cardiolipin percentage of 27%, close to those of the other probes.

Similar sets of experimental and difference EPR spectra were also obtained for *n*DSCL-labeled REVs and for mitochondrial membranes and REVs labeled with *n*DSPC and *n*DSPE. Given the overall similarity, their presentation is omitted.

The spectra shown in Figures 2−4 are relative to experiments performed with the highest experimental AV/membrane incubation ratio. However, many experiments were performed with a variable annexin amount, to study the dependence of the bound annexin-dependent rigidification of the spin-labeled phospholipid acyl chain upon the protein binding, both in mitochondrial and vesicular bilayers. Each experimental spectrum, obtained with a different AV/membrane incubation ratio, was titrated with the blank (containing 1 mM Ca^{2+}) spectrum. After finding the difference best matching the appropriate temperature standard spectrum, the calculated percent ratio of the difference's second integral to that of the blank, measuring the AV-immobilized phospholipid probe percentage, was plotted as a function of the incubation ratio. The range of this ratio was chosen on the basis of the previously (*8*) observed increasing binding of AVSL to mitochondria in the range 2.5−20 μg of mitochondria/μg of AV, corresponding to a range of 1.8−0.04 μg of AV/nmol of P. In Figure 5A, as the incubated annexin amount increases from 0 to 1.8 μg per nmol phospholipid phosphorus, the calculated percentage of immobilized cardiolipin in mitochondria is seen to increase following a saturation profile, up to a leveling value ranging from 26 to 33% at positions C-5, C-12 (EIT = 2 °C) and C-16 (EIT = 12 °C, see also Table 1). The incubation ratio for the half-maximum percentage of immobilized lipid is calculated to range from 0.11 to 0.15 μg/nmol of P, as also reported in Table 1. Figure 5B reports the trend of the

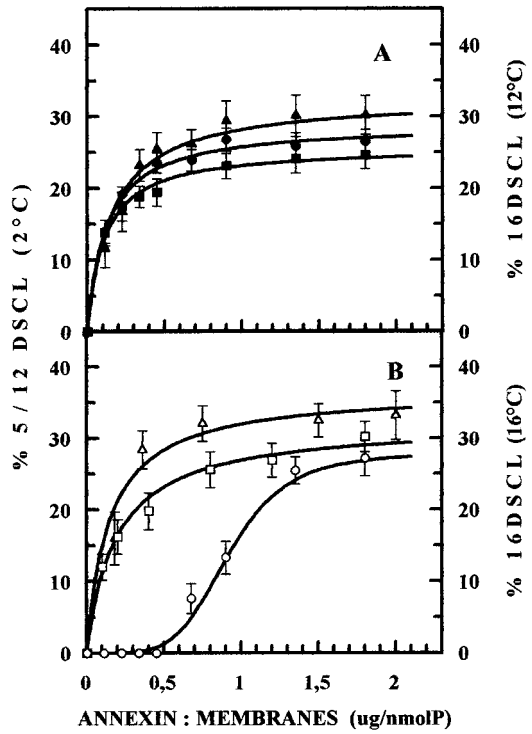

FIGURE 5: Percentage of immobilized *n*-DSCL as a function of the amount of incubated annexin V in the presence of 1 mM Ca^{2+}. Protein amount is expressed as the ratio of incubated annexin to membrane phospholipid phosphorus. (■) 5-, (▲) 12-, (●) 16-DSCL mitochondria; empty symbols, REVs. Vertical bars represent the standard deviation calculated from triplicate experiments at least.

percentage of motionally restricted *n*DSCL with increasing amounts of bound AV in REV bilayers, after incubation with increasing amounts of annexin in the same range as with mitochondria. In this case, the profile of the motionally affected lipid percentage is also saturation limited, with maximum amount of rigidified phospholipid ranging from 28 to 37% (Table 1), as reported by the spin label in acyl chain positions C-5 and C-12. These two positions respond readily to the increasing binding of AV as shown by the low semimaximal incubation ratio ranging from 0.17 to 0.2 μg of AV/nmol of P. On the contrary, position C-16 in these membranes responds more sluggishly, following a sigmoidal trend, with an apparent cooperativity coefficient as high as 5 (see Table 1). The EIT at position C-5 and C-12 (2 °C) is similar to that in mitochondria and higher at position C-16 (16 °C, see also Table 1), indicating that annexin binding is scarcely reflected by the terminal acyl segment.

The results of similar experiments from isolated mitochondria and REVs labeled with *n*DSPC are reported in Figure 6 and summarized in Table 2. Increased binding of AV to mitochondria also rigidifies increasing amounts of PC acyl chains at positions C-5 and C-12 (Figure 6A), and a maximum of about one-third of the PC molecules are affected (32 and 34%, respectively, Table 2). The incubation ratio for semimaximal motion-restricted percentage is about 0.4 μg of annexin/mg of mitochondrial protein, and the rigidification strength is equivalent to that brought about by a temperature of 8 °C at both positions. Remarkably, position C-16 appears to be reached with more difficulty by the protein's effect, as indicated by the high $R_{1/2}$ value (1.18 μg/nmol of P) and high EIT of 16 °C. Results from AV-

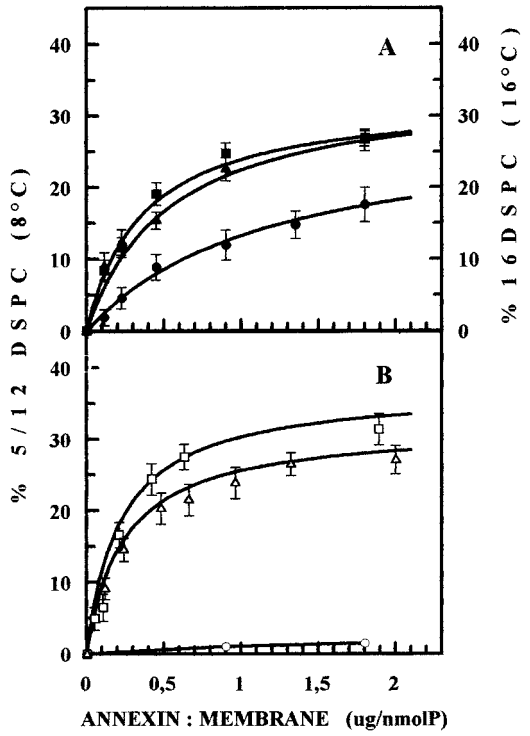

FIGURE 6: Percentage of immobilized *n*-PCDS as a function of the amount of incubated annexin V in the presence of 1 mM Ca^{2+}. Protein amount is expressed as the ratio of incubated annexin to membrane phospholipid phosphorus. (■) 5-, (▲) 12-, (●) 16-DSPC mitochondria; empty symbols, REVs. Vertical bars represent the standard deviation calculated from triplicate experiments at least.

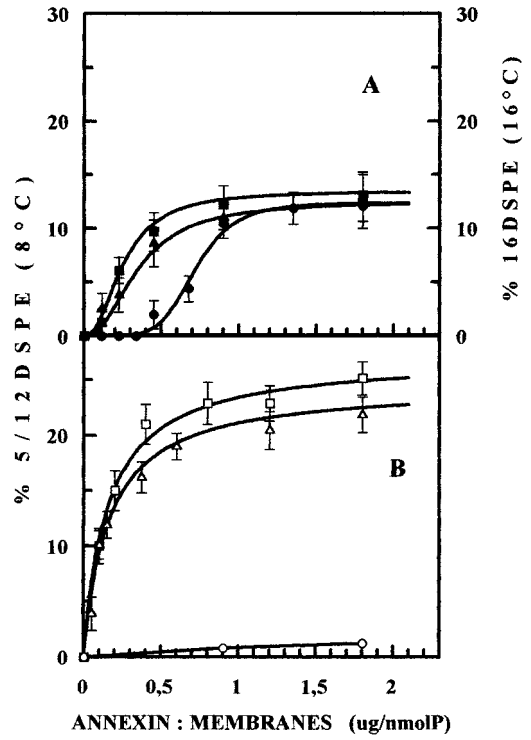

FIGURE 7: Percentage of immobilized *n*-DSPE as a function of the amount of incubated annexin V in the presence of 1 mM Ca^{2+}. Protein amount is expressed as the ratio of incubated annexin to membrane phospholipid phosphorus. (■) 5-, (▲) 12-, (●) 16-DSPE mitochondria; empty symbols, REVs. Vertical bars represent the standard deviation calculated from triplicate experiments at least.

bound REVs reveal a similar percentage of PC experiencing motional restriction at position C-5 and C-12 (37 and 32%, respectively, Table 2), but a higher responsivity to the bound protein as shown by a half-maximum incubation ratio ranging around 0.23 μg/nmol of P. The strength of rigidification is also the same as in mitochondrial membranes, with an EIT of 8 °C at both positions, while position C-16 appears to be completely out of reach.

Binding of AV to mitochondrial membranes is sensed to a markedly lesser extent by PE β-acyl chains, as shown by plots in Figure 7A, the details of which are given in Table 3. As can be seen, the maximum immobilized *n*DSPE percentage is lower than for the previous spin labels, ranging between 12% (16DSPE) and 14% (5DSPE), as reported in Table 3. The profile of the response to AV binding is increasingly sigmoidal, in the order 5DSPE < 12DSPE < 16DSPE, with cooperativity indexes of 2.1, 2.4, and 5.7 μg of AV/nmol of P. The strength of the immobilization experienced by PE acyl chains is expressed by an EIT of 8 °C at position C-5 and C-12 and of 16 °C at position C-16. On the contrary, in the REV bilayer, the trend of *n*DSPE percentage affected by increasing Ca-bound annexin, resembles those from previous probes and is leveled to about a maximum of 20% spin-labeled phospholipids, not so different from the *n*DSPC response in mitochondria. Annexin binding is more readily experienced in REVs, as shown by the hyperbolic profile with semimaximal incubation ratios of 0.18 and 0.2 μg/nmol of P at positions C-5 and C-12, respectively. Also in this case, motional restriction of acyl chains at both C positions is measured by an EIT of 8 °C, not reaching C-16 either.

DISCUSSION

Mitochondria were spin labeled under the experimental condititons used for studying spontaneous phospholipid exchange (*33*−*36*). The EPR signal was strongly quenched in donor SUVs (*36*) thanks to the high spin label molar ratio (33%), while the signal from spin-labeled mitochondria was fully unquenched, as confirmed by the low probe incorporation (<3%). Under our experimental conditions, the uptake profile of the probes shown in Figure 1 indicates a more hindered diffusion into mitochondrial membranes of CL in comparison to PE and PC, suggesting that the phospholipid is adsorbed in some membrane compartment less accessible than the main mitochondrial phospholipids'. In this aspect, PE also seems to exchange with less exposed mitochondrial compartments than the ones of PC, and the overall idea is that the different phospholipids are adsorbed at different mitochondrial microdomains. In fact, this behavior is in agreement with the general assumption that a given phospholipid species is targeted to its homologous phospholipid pool.

Reversed-phase evaporation vesicles were chosen to mimic mitochondria since they are reported to be single-shelled and to have a curvature radius high enough to present low surface curvature on the scale of annexin molecular dimensions, as mitochondrial membranes would. Even though the previously used (*20*) large phospholipid vesicles (*46*) would be better from the geometry point of view, their membrane is reportedly 2-3-shelled. In addition, to better simulate mitochondrial membranes, REVs were prepared with phospholipids extracted from the organelles. However, such vesicles should not simulate mitochondria phospholipid topology. In

Table 4: Effect of AV Binding on the Order Parameter S of Spin-Labeled Phospholipids in Mitochondrial Membranes and in Lipid Vesicles

n^a	5		12		16			
mitochondria								
DSCL	0.62	0.86	0.36	0.63	0.11	*1.21*[b]	0.16	*1.51*
DSPC	0.61	0.77	0.27	0.43	0.12	*1.23*	0.15	*1.39*
DSPE	0.64	0.76	0.29	0.42	0.12	*1.21*	0.15	*1.35*
AV	−[c]	+[d]	−	+	−		+	
REVs								
DSCL	0.61	0.85	0.34	0.64	0.11	*0.95*	0.14	*1.19*
DSPC	0.57	0.75	0.25	0.39	0.12	*0.78*	0.13	*0.78*
DSPE	0.56	0.76	0.24	0.38	0.11	*0.75*	0.12	*0.76*

[a] Acylic position of the spin label. [b] Correlation time in nanoseconds. [c] Values calculated from blank spectra. [d] Values calculated from difference spectra.

fact, the artificially prepared membranes are expected to lose their natural membrane phospholipid distribution and to take up the mean phospholipid mitochondrial composition.

In the EPR spectra of spin-labeled mitochondria and REVs, in the presence of annexin V and 1 mM Ca^{2+}, the features of a more immobilized component appear in sharper contrast against the blank than in the previously studied SUV spectra (*20*), maybe because of the tighter bilayer of the latter (*47*, *48*). Consequently, spectral titration with the blank spectrum of the membranes in the presence of Ca^{2+} was adopted to highlight the immobilized component. Nonzero difference spectra were always obtained in the presence of annexin and 1 mM Ca^{2+}, unless 10 mM EGTA was added to the samples as a control. Matching the difference spectra to standard variable temperature spectra (*49*) of the same membrane under scrutiny allowed calibration of the spin-labeled acyl chain rigidification by means of temperature instead of the order parameter S (*40*, *41*).We observe that the 25−0 °C EIT-scale of spectra can be more finely tuned to the experimental spectra than the 0−1 S-scale. However, values of the order parameters (and of correlation time τ_c, when evaluable) from our experiments are reported in Table 4, for comparison.

Besides the well-known annexin binding to acidic phospholipid-containing vesicles, binding of annexin to mitochondria as a function of the incubated protein amount has also been previously assessed (*8*). In vesicular lipid bilayers, this binding perturbs the hydrophobic region close to the polar head, and its effect fades toward the methyl end of the chain, provoking a change in the fluidity gradient of the membrane (*20*). The effect is extended to all phospholipid molecules, and has been related to neutralization of the polar head charge upon annexin Ca-dependent binding with a consequent tighter packing of the phospholipid molecules directly involved along with their neighbors (*20*). Extension of this effect to all phospholipid molecules is ascribed to the homogeneous mixing of acidic (binding) with nonbinding phospholipids in the model membrane architecture. On the contrary, natural membranes conserve a high topology of phospholipid microdomains (*16*, *50*, *51*). This is probably linked to the localization of the different enzymes in the membrane, and in the case of mitochondria, cardiolipin-rich microdomains can be thought to exist in the surroundings of cardiolipin dependent enzymes, such as cytochrome P450scc, FOF1 ATPase, cytochrome *c*, cytochrome *c* oxidase, ADP/ATP carrier (*51*−*57*). If the binding phospholipid is segregated into different mitochondrial microdomains, contrarily to that which occurs in artificial phospholipid bilayers, a homogeneous response from different phospholipids to annexin V binding could not be expected. Comparison of panel A of Figures 2−4 shows binding of annexin to mitochondria to be experienced in fairly different ways by the different phospholipids. Tables 1−3 show that the maximum immobilized PC and CL percentages are similar (around 30%), but CL responds better than PC, with an $R_{1/2}$ value of 0.13 μg of AV/nmol of P against 0.4 μg of AV/nmol of P respectively, on average for the different acyl C positions. PE displays the toughest response, as indicated by the sigmoid profile with high *n* values (Table 3), and by the lower motionally restricted maximum percentage of 13%, on average. The different response may be due to segregation of PE molecules from annexin-binding CL molecules (as expected for example from local asymmetric transbilayer phospholipid distribution), while overspread PC molecules more probably also reside close enough to CL to reflect more its rigidification than PE would, though not to the same extent as CL itself. Taken together, these data are consistent with a nonhomogeneous distribution of mitochondrial phospholipids, at least in the probed AV-bound domains, as already suggested by the trend of spin-labeled phospholipid uptake (Figure 1). Implicit in our data is the observation that the fluidity gradient of the different phospholipid domains is also influenced to a different extent. In fact, from the EIT values in Tables 1−3 it can be seen that CL better reflects the protein binding with a higher rigidification along the whole acyl chain than PC and PE, strongly indicating that cardiolipin could actually be directly involved in the annexin-mitochondria interaction.

Comparison between panels B of Figures 2−4 (Tables 1−3), reveals a more leveled response of the various spin-labeled phospholipids in the structurally homogeneous REVs. Apart from C-16 position and more importantly, the sensitivity of the three phospholipids at position C-5 and C-12 appears to be more similar than in mitochondria. This is shown both by values of $R_{1/2}$ evenly ranging around 0.20 μg of AV/nmol of P and by the similar saturation profile, as would be expected from a membrane structure homogeneous in composition and topology. Since the range of the incubation ratio of the REVs is the same as that of isolated mitochondria, a similar availability of phospholipids for AV binding can deduced, thus validating these membranes as a proper model of the mitochondrial bilayer in composition and size, apart from topology differences.

These results suggest that AV binding to mitochondrial membranes is able to discriminate different phospholipid microdomains, and indicate that those rich in CL are more influenced by this binding. In contrast with phospholipid vesicles, in which homogeneity of the different probes' rigidification reflects structural homogeneity, the different degree of immobilization of the same probes in mitochondria could reflect the different distance of the different phospholipid pools from the AV-binding site.

Even if structural details of some phospholipid−protein complexes appear in the literature (*59*, *60*), imaging the architecture of those complexes in the mitochondrial membrane is a difficult goal, far from the aim of the present paper. Moreover, in both REVs and mitochondrial membranes, the

effect of bound AV is limited to no more than two-thirds of the probed membrane phospholipid population (Tables 1−3). In fact, we do not expect the protein to reach the inner membrane leaflet or patches of the inner mitochondrial membrane. Therefore, our conclusions are limited to those membrane domains in which fluidity variations are apparent. The presented observations indicate that among the altered membrane regions, the most affected are cardiolipin-rich microdomains which are further confirmed as the putative-binding site of AV to mitochondrial membranes, as already proposed (*8*). The AV-altered domains topology in the mitochondria is left undetermined by our experiments. Nonetheless, one must consider that most CL is localized in the inner mitochondrial membrane (*1*), and that its presence has also been well demonstrated in the outer membrane (*2*), particularly at the level of junction sites (*51*, *61*). In addition, besides the well-known cardiolipin-dependent mitochondrial enzymes present in the inner membrane (*52*−*58*), the outer membrane also embeds some of them (*62*). Our in vitro experiments were performed with mitochondria subjected to a long experimental protocol (purification, freezing and thawing, spin labeling), after which the integrity of the outer membrane is not guaranteed so that we cannot determine to what extent the inner membrane of our spin-labeled mitochondria was exposed. However, since experiments with mitoplasts (*8*) have shown binding of annexin V to the inner mitochondrial membrane to occur as well, this consideration is no drawback to advancing the conclusion that, in vitro, annexin binding to mitochondria perturbs mostly the cardiolipin pool. Therefore, from a speculative point of view, we can reasonably expect that some perturbation could reach those enzymes inserted in cardiolipin domains, raising special interest when affecting those whose function critically depends on the presence of cardiolipin (*63*−*65*). On this basis, the effects of annexin binding to isolated mitochondria on the energy metabolism are currently under scrutiny, to open a new perspective on the physiological role of annexin V in the living cell.

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