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EPR study of annexin V–cardiolipin Ca-mediated interaction in phospholipid vesicles and isolated mitochondria

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Abstract

The properties of the binding of annexin V to variously composed phospholipid vesicles have been studied by applying a recently developed EPR method, using an annexin V spin label. By this approach, this protein is seen to bind to acidic phospholipid-containing vesicles, as reported, thus confirming the reliability of the method. In addition, binding of this annexin to cardiolipin-containing vesicles has been studied in more depth, and the protein has been shown to have a distinct affinity for this phospholipid. As a cardiolipin-rich natural membrane system, mitochondrial membranes and mitoplasts from rat liver were considered, and a strong binding of AV to these membranes was observed. Having compared this binding with that to phospholipid vesicles, cardiolipin-rich microdomains in the mitochondrial membranes are proposed as the putative mitochondrial binding sites for annexin V.

Keywords: Annexin V; EPR; Spin labeling; Phospholipid; Cardiolipin; Mitochondrion

1. Introduction

The calcium-dependent binding of annexins [1] to membrane acidic phospholipids appears to be the key step to the onset of the various well documented biological effects exerted by these proteins, such as the inhibition of phospholipase A_2, anticoagulatory activity, exocytosis, calcium channelling and others [1]. Nonetheless, systematic studies of the interaction of each annexin with different phospholipid classes are scarcely represented in the literature [2,3]. This paper is a first step in an attempt to define the phospholipid affinity pattern of each annexin, which, in turn, will help to establish whether or not the different proteins are membrane specific. According to the phospholipid composition of a given membrane (or a given membrane microdomain), this definition might provide a clue to the possibility of a sort of targeting of each annexin in a cell to a particular subcellular membrane (or membrane microdomain). This possibility may help in understanding the observed variability of the annexin ensemble present in all tissues studied to date [1].

Following this reasoning, this paper considers in more depth the previously observed binding of annexin V (from bovine lung) spin label (AVSL) to isolated rat liver mitochondria [4], in comparison with the binding of this spin label to variously composed liposomes (SUVs). Beyond the acidic phospholipids previously studied [2,3,5], cardiolipin, an important mitochondrial phospholipid [6] and a well known modulator of functionally important mitochondrial enzymes [7], has been specifically investigated. Our results indicate that AV binds to acidic phospholipids, as already established by different methods [2,5], and that, accordingly, optimal Ca-regulation of this binding occurs between 10 and 0.1 μM Ca^{2+}. In addition, a high binding affinity of AV for cardiolipin-rich liposomes has been observed; moreover, such liposomes prove to be the only ones to undergo aggregation following AV binding. Application of the EPR method to the study of the interaction of AV with mitochondrial membranes, revealed a fair Ca-dependent binding ability of these membranes towards the protein. Finally, the present results indicate that cardiolipin

Abbreviations: EPR, electron paramagnetic resonance; AVSL, annexin V spin label; SUV, small unilamellar vesicle; LPV, large phospholipid vesicles; RLM, rat liver mitochondria; FCCP, carbonyl cyanide *p*-(trifluoromethoxy)phenylhydrazone; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PA, phosphatidic acid; PI, phosphatidylinositol; PS, phosphatidylserine; DPG, diphosphatidylglycerol (cardiolipin).

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is probably the main lipid component of the mitochondrial annexin binding site.

2. Materials and methods

2.1. Protein preparation

Annexin V (p34) was isolated and purified from beef lung as described elsewhere [5,8]. The spin labeling procedure, using 3-(2-iodoacetamido)-2,2,5,5-tetramethylpyrrolidinyl-*N*-oxyl (iodoacetamidoproxyl, Aldrich) has also been outlined previously [4,8]. Protein content was assayed by the Bradford method [9].

2.2. Phospholipid vesicles

Liposomes (SUVs) were prepared by ultrasonic irradiation of phospholipid suspensions in 125 mM KCl/2.5 mM $MgCl_2$/25 mM Hepes (pH 7.4) (Buffer B), at 40 W output (three or four 5 min strokes, intercalated with 1 min cooling intervals) in an ice bath under N_2. In some instances, these preparations were further subjected to Sepharose 4B chromatography [10], and control experiments were run separately with either the first or the second eluting peak (larger and smaller phospholipid vesicles, respectively). Phospholipid phosphorus was assayed according to Nakamura [11].

2.3. Mitochondrial membranes

Mitochondria were prepared according to Hogeboom [12] from freshly excised rat livers, in 0.25 M sucrose/10 mM Tris-HCl (pH 7.4). In order to suppress reductive side-effects by the organelles on the spin labeled protein, the former were resuspended in Buffer B and subjected to 30 min incubation at 37° C in the presence of 2 μM FCCP and air, as described [4]. This preparation contained 20–30 μM Ca^{2+}, as previously determined by atomic absorption [4]; this amount was taken into account in calculating the final concentrations of Ca-EGTA buffers yielding the desired experimental free p*Ca* [13].

Delipidated mitochondria were obtained by treating this preparation with acetone and ammonia, as described by Fleischer and Fleischer [14,15].

Mitoplasts were also prepared from the starting mitochondrial fraction, by digitonin treatment according to Greenawalt [16].

Purification of isolated mitochondria from accompanying peroxisomes and lysosomes was accomplished by centrifugation on a Percoll density gradient [17]. The purity of the preparation was checked by assaying the marker enzymes of peroxisomes (catalase) and lysosomes (acid phosphatase) as described [18,19]. Mitochondrial protein was determined by the Biuret method.

2.4. EPR spectrometry and AVSL binding assay

The binding of AVSL to membranes was measured by EPR spectroscopy, as described in a previous paper [4]. Briefly, 40 μl samples in 100 mM KCl/2 mM $MgCl_2$/20 mM Hepes (pH 7.4) (Buffer A), containing up to 300 nmol SUV (as phospholipid phosphorus), 10–15 μg AVSL and variable calcium and EGTA concentrations, depending on the desired free Ca^{2+} concentration [13], were incubated for 10 min at room temperature and transferred into glass capillaries. Binding to variously treated mitochondria was measured on similar samples in which the SUVs were replaced by 50–500 μg mitochondrial protein. EPR spectra were recorded by means of a Varian E-Line ESR spectrometer, coupled to an HP 9835B desk computer through the Varian Data Acquisition System. Instrumental settings were: 3396-G field set, with 200-G scan range; 100 kHz and 2 G modulation frequency and amplitude, respectively; 20 mW and 9.5 GHz radiating field power and frequency, respectively; $1/1.6 \cdot 10^4$ gain; 0.5/2 s time constant; and 30/120 min scan time.

Quantitation of the bound AVSL percentage was achieved as previously described [4]. In summary, the method requires the following measurements: as a first step, the EPR spectra of the fully bound/unbound AVSL (in the presence of either 1 mM Ca^{2+} or 10 mM EGTA, respectively) are recorded, and the two sets of bandwidths are estimated for the two specimens. As a second step, the EPR spectrum of the sample(s) in given conditions is recorded, and the rotational correlation time [20], τ_c, evaluated [21]. Finally, the bound AVSL percentage, q, is calculated [4] by solving the equation:

$$\tau_c = c\{I_U I_B/[I_B - q(I_B - I_U)] + K_U K_B/[K_B - q(K_B - K_U)] - 2J_U J_B/[J_B - q(J_B - J_U)]\}$$

where capital I, J, K stand for the above-cited set of low-, mid- and high-field bandwidths, with suffixes U and B indicating the unbound and bound AVSL, respectively, and c is a constant dependent on the particular spin label used. The theoretical background and experimental procedure of this method are given in Ref. [4].

2.5. Vesicle aggregation

SUV aggregation promoted by AV was observed by phase-contrast microscopy [2]. Each 25 μl sample contained 50 nmol phospholipid vesicles, 10 μg AV and 0.1 mM $CaCl_2$ in Buffer A. In the case of turbidity development, the sample was centrifuged, and after phase-contrast observation, one drop of 100 mM EGTA was added to the pellet directly on the glass slide using a microsyringe. Measurement of aggregation was performed spectrophotometrically [2] by means of a Beckman DU-8 spectrophotometer. 0.5 ml cuvettes contained 0.35 ml final vol-

ume samples in Buffer A, comprising 100 nmol vesicle, and 15 μg annexin. The absorbance of the samples was monitored at 350 nm, a value outside the annexin absorption band and the closest to 300 nm, the wavelength commonly used in spectrophotometrical studies of SUVs [10,22]. After recording the absorbance for 1–2 min, calcium was added to a final concentration of 0.1 mM. The absorbance increase was monitored in time until a constant value was reached, and then EGTA, at a final 10 mM concentration, was added.

3. Results

Fig. 1 shows the percentage binding of AVSL to variously composed SUVs upon variable calcium concentration, as determined by the method previously outlined [4]. From Fig. 1A, the maximum binding of the protein to SUVs made up of PC and PS (1:1 mol ratio) at p*Ca* 3 (1 mM free Ca^{2+}) can be seen to decrease, with increasing p*Ca*, with modulation of the binding by the cation in the p*Ca* range between 6 and 8 (free Ca^{2+} concentration 1–0.01 μM). Binding of AVSL to PI- and PE-containing SUVs (in a 1:1 molar ratio mixture with PC), whilst apparently high at p*Ca* 3, is seen to drop to negligible values at higher p*Ca*, with no Ca-modulation features. The results in Fig. 1B show the behaviour of 50% PA- or DPG-containing SUVs to be similar to that of those containing PS, with a Ca-regulated binding range between p*Ca* values of 5 and 7 (10 and 0.1 mM free Ca^{2+} concentration). In addition, from the same figure, it can be seen that lowering the acidic phospholipid content in the vesicles from 50% to 10% is reflected in a lower AVSL

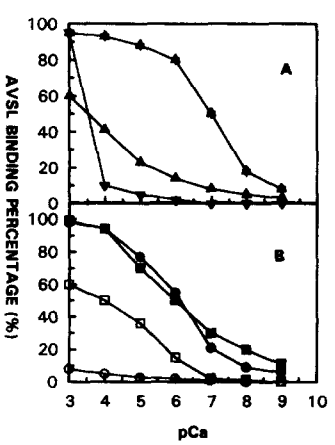

Fig. 1. Percentage binding of AVSL to variously composed SUVs as a function of Ca concentration (p*Ca* = $-\log[Ca^{2+}]$). Phospholipid vesicles were made up of 1:1 mole ratio mixtures of (A): ⋆, PS/PC; ▼, PI/PC; ▲, PE/PC; (B) PA/PC in a molar ratio of either, ■, 1:1, or, □, 1:9; DPG/PC either, ●, 1:1; or, ○, 1:9 molar ratio. Vesicle amount in each experiment was 27.5 nmol SUV phospholipid phosphorus per μg AVSL. 10 to 15 μg annexin was incubated. More experimental details are given under Materials and methods.

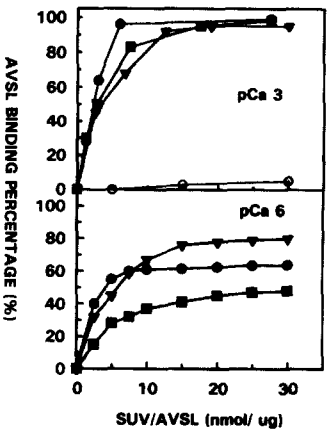

Fig. 2. Percentage binding of AVSL to variously composed SUVs with variable membrane amounts, at p*Ca* 3 and p*Ca* 6, as indicated. PC and either, ▼, PS, or, ■, PA; or, ●, DPG, 1:1 molar ratio; ○, PC/DPG, 1:9 molar ratio. The variable membrane amount is expressed as nmol SUVs per μg incubated annexin (SUV/AVSL); other experimental conditions were as in Fig. 1.

binding ability of these membranes. This effect is particularly evident for cardiolipin-containing SUVs.

Fig. 2 reports the dependence on vesicular membrane availability (expressed as the nmol SUV to μg AVSL ratio), of the percentage of AVSL binding to vesicles made of 50% PC and either PS, PA, or DPG (1:1 mol ratio). This figure, at p*Ca* 3, shows that DPG-containing SUVs, bind 100% AVSL at a ratio of approx. 5 nmol/μg SUV/AVSL, whilst PA- and PS-containing vesicles bind the same annexin percentage at approx. 15 nmol/μg. In the same figure, at p*Ca* 6, the SUV amount yielding the maximal percent binding (Fig. 1, p*Ca* 6) is unchanged for DPG and is somewhat higher (20 nmol/μg) for PA and PS. Referring to p*Ca* 3 and 100% binding, it can be calculated that approximately 170 nmol DPG-SUVs binds 1 nmol AVSL, whilst more than 500 nmol PA- or PS-SUVs is required to bind the same annexin amount. From the data at p*Ca* 6, equivalent requirements of 280, 850 and 1700 nmol SUV per nmol annexin can be estimated for DPG, PS and PA, respectively, to reach maximal binding.

The aggregation of SUVs promoted by AV, was first observed visually in the EPR capillaries and then detected by phase-contrast microscopy on 50% DPG-containing SUVs or LPVs. In the presence of 0.1 mM Ca^{2+}, aggregates of either vesicle type were clearly visible; the formations readily disappeared when EGTA was added. More precisely, vesicle aggregation was studied by spectrophotometrically measuring the consequent change in turbidity of the SUVs as the variation of optical density at 350 nm (Fig. 3), similar to as described elsewhere [2]. As can be seen, upon addition of final 0.1 mM Ca^{2+}, only cardiolipin-containing SUVs (a) undergo aggregation by AV, while vesicles containing the other binding acidic phospholipids (b and c) remain unaffected [2]. Noteworthy from this figure is that this distinctive feature of DPG-SUVs is

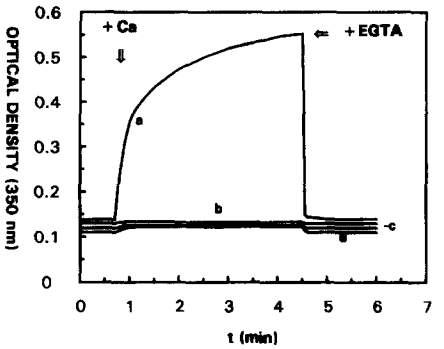

Fig. 3. Rate of Ca-induced aggregation of variously composed SUVs in the presence of AV. PC and either (a) DPG, (b) PA, or (c) PS, 1:1 molar ratio. (d) DPG/PC, 1:9 molar ratio.

seen to vanish when the phospholipid content therein is lowered to 10% (d). Another important result in this figure is the apparent full reversibility of the Ca-induced effect upon addition of final 10 mM EGTA.

Binding of AVSL to mitochondrial membranes was studied by using a mitochondrial fraction purified from peroxisomes and lysosomes by centrifugation on a Percoll density gradient. The respiratory activity of the preparation was also blocked, as described. The calcium dependence of AVSL binding to purified rat liver mitochondrial membranes, under various conditions, is reported in Fig. 4. The membranes bind 100% AVSL at p*Ca* 3 (1 mM free Ca^{2+}) and a ratio of mitochondrial protein/AVSL of 24:1 ($\mu g/\mu g$). The binding percentage gradually decreases with increasing p*Ca*, with optimal Ca-regulated binding range comprised between p*Ca* 5 and p*Ca* 7 (10 to 0.1 μM free Ca^{2+}). Binding is still visible when the mitochondria/AVSL ratio is lowered to 3:1, even if a limiting effect of the incubated membrane amount is evident. On the contrary, from the same figure, it is seen that the delipidated mitochondrial preparation [6,23], incubated at a 24:1 ratio to the protein, does not respond at all to the Ca-dependent binding of AVSL.

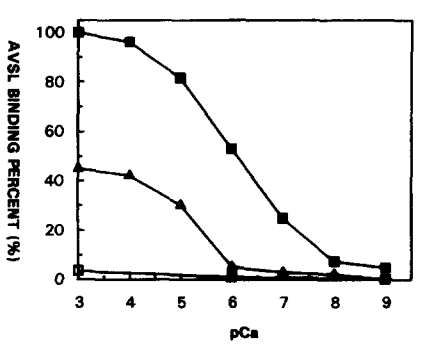

Fig. 4. Percentage binding of AVSL to purified mitochondria with variable Ca^{2+} concentration (p*Ca*). Ratio of mitochondrial protein to annexin ($\mu g/\mu g$): ■, 24:1; ▲, 3:1; lipid-depleted mitochondria, □, 24:1.

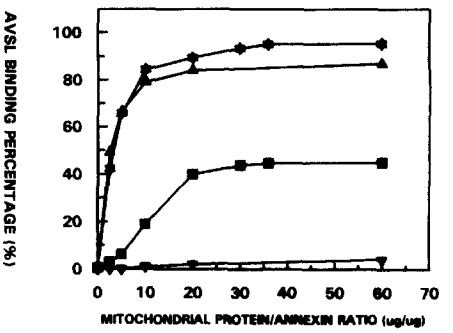

Fig. 5. Percentage binding of AVSL to variously treated mitochondrial membranes with variable membrane amounts. The incubated membrane amount is expressed as the ratio ($\mu g/\mu g$) of mitochondrial protein to annexin (mitochondrial protein/annexin ratio). Untreated mitochondria at either p*Ca* 3 (⋆) or p*Ca* 6 (■). Mitoplasts, p*Ca* 3 (▲). Lipid-depleted mitochondria, p*Ca* 3 (▼).

The AVSL binding ability of the variously treated mitochondrial membranes was compared by studying the dependence of AVSL binding percentage upon the incubated amount of each membrane preparation. The results are reported in Fig. 5. From this figure it is seen that the maximum annexin binding, at p*Ca* 3, to both mitochondria and mitoplasts occurs at a minimum ratio (μg mitochondrial protein/μg AVSL) of 10–20:1. At p*Ca* 6, binding is still operative, with a maximum binding ratio shifted to somewhat higher values (20–30:1 $\mu g/\mu g$), while delipidated mitochondrial membranes, as shown, are no longer able to bind AVSL at any ratio.

4. Discussion

In comparison with other methods for studying the Ca-mediated binding of AV to acidic phospholipids [3,5], the EPR methodology previously presented [4] proves to be more advantageous in directly monitoring the binding, without further handling of the incubated samples [23,24]. From this aspect, the EPR method is similar to more recently developed methods [25–27]. Its reliability is confirmed by the binding profile of AVSL to acidic phospholipids with variable p*Ca* (Fig. 1). This fits very well the already reported AV binding properties [2,5]; as an example, 50%-PI LPVs are reported to bind AV only at 1 mM Ca^{2+}. As the only exception we found PE, which, unlike the others, is zwitterionic at the experimental pH.

In the same figure, more detail appear about AV binding to DPG. In fact, contrarily to what observed for PA, a high molar ratio of DPG seems to be critical for the annexin binding in the whole p*Ca* range tested. This observation strongly indicates a peculiar affinity of AV for cardiolipin-rich membranes or, at least, membrane microdomains. In addition, even if PA, PS and DPG appear

to be the most effective in annexin binding at physiological p*Ca*, estimation of the binding molar ratio from the results reported in Fig. 2, allow the conclusion that cardiolipin-containing SUVs have an approximately three-fold binding affinity (ca. 170 nmol SUV per nmol AVSL) with respect to PA and PS (ca. 510 nmol SUV per nmol AVSL), at p*Ca* 3, and even more at physiological p*Ca*. The figure of 510 nmol SUV per nmol AVSL for the binding molar ratio for PS-SUVs, estimated from Fig. 2, is very close to the one estimated by other authors [26] for 20% PS-containing vesicles at p*Ca* 3 (ca. 400 nmol vesicles per nmol AV), by using a fluorescence-labeled AV. The high binding affinity of DPG for AV has already been observed [29]. An explanation might be the presence of an additional distinct phosphate group in its polar head, in comparison with the additional carboxyl group of PS, and the second negative charge (partial, at neutral pH) on the single phosphate group of PA.

Whilst most annexins promote vesicle aggregation, AV is reportedly non-aggregating towards the commonly studied acidic phospholipids [2,25,28]. Accordingly, our results (Fig. 3) confirm no aggregation of PA- and PS-SUVs, while strongly evidencing the occurrence of DPG-SUVs aggregation after calcium addition, in the presence of AV. The EGTA-dependent abolition of the absorbance increase rules out vesicle fusion, whilst control runs excluded any side-effect by calcium alone. Moreover, failure of AV in aggregating 10% DPG-containing SUVs is in keeping with the above results, further arguing in favour of a peculiar relationship of cardiolipin-rich membranes (or membrane microdomains) with AV.

Binding of AV to cardiolipin-containing bilayers has already been reported [29–31]. This annexin showed the highest affinity for planar bilayers in the mM Ca^{2+} concentration range [29], and for LPVs between 1 and 10 μM Ca^{2+} [3]. Our results, obtained with SUVs, a third and again different membrane system, resemble more those from LPVs, most probably due to their similar vesicular shape. With these membranes, modulation of AV binding occurs via both the DPG content and the Ca^{2+} concentration in a range (0.1–10 μM) considered functionally important for AV binding in the living cell [29].

These considerations prompted the study of the interaction of AV with mitochondria, the most cardiolipin-rich membrane in the cell, using the EPR method which, as stated above, is particularly well-suited to studies involving natural membranes [23,24,38]. As an additional reason, few studies have appeared dealing with natural membrane binding of annexins [32–37]. From the present study, mitochondrial membranes show a strong ability of AVSL binding at p*Ca* 3 and exhibit a well-characterized Ca-modulated binding profile upon increasing p*Ca*, with optimal modulation in the physiological p*Ca* range (Fig. 4, p*Ca* 5–7). Whilst lowering the RLM to AVSL ratio from 24:1 to 3:1 (μg/μg) only shows an expectable limiting effect of membrane availability, delipidation of mitochondria (a well established method, useful to decide the role of the phospholipid component in membrane biochemistry, [6,14,15]) results in no binding at all, even at the maximum binding-ratio for untreated mitochondria, i.e., 24:1. This behaviour confirms the phospholipid component of the mitochondrial membrane as being responsible for the binding of AV. In more detail, by considering the phospholipid composition of rat liver mitochondria [6], it can be seen that, amongst the significantly represented phospholipids, only DPG is capable of AV binding. Thus, the reported absence of PA and PS suggests that failure in AVSL binding to lipid-depleted mitochondrial membranes (Fig. 4) should be ascribed to removal of cardiolipin. Furthermore, comparison of the profile and extent of AVSL binding to mitochondrial membranes with those to the SUVs (Fig. 1), particularly in the physiological calcium concentration range, favours attribution of the mitochondrial AVSL binding capacity to DPG-rich microdomains.

Mitoplasts exhibit essentially the same AVSL binding affinity as the whole mitochondrial membranes, since both preparations appear to reach quasi-maximal binding (90%) at the same membrane to AVSL ratio of 10 μg per μg spin labeled annexin V (Fig. 5). This result was to be expected on the basis of the cardiolipin content of the inner mitochondrial membrane, confirming that these membranes owe their AV binding ability to the presence of the phospholipid. The decrease in the binding of mitochondria (and mitoplasts, not shown) to about 50% at p*Ca* 6 (compatible with the value expected from Fig. 4), compared with the negligible binding of AV to PE and PI at the same p*Ca* value (see Fig. 1A), confirms the correct attribution of the observed binding to DPG. In addition, the observed binding to mitoplasts suggests that rupture of the outer membrane, possibly provoked by prolonged handling during the purification and respiration steps, does not detract from our results. However, due to asymmetrical transmembrane DPG distribution [6] and different phospholipid/protein mass ratio in the mitochondrial membranes [16], these data are not suited to give quantitative assessments of the phenomenon observed.

In conclusion, the data presented indicate a peculiar affinity of AV for cardiolipin, and suggest that this protein might be targeted to mitochondria via this phospholipid in the living cell. Targeting of annexins to membranes (or membrane subdomains), via the phospholipid composition, has already been proposed [30], as has the possibility of a regulatory role of bound annexins on membrane proteins [30,31]. Our results show that the Ca^{2+} concentration in the living cell is appropriate for AV binding to DPG-rich curved bilayers, in contrast with what has been reported for 20% DPG-containing planar bilayers [29]. In this context, the similarity between the cardiolipin requirements for the optimal functioning of many important mitochondrial enzymes [39–43] and those for AV binding to mitochondria, raises interesting ideas worthy of further investigation.

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