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The effect of annexin IV and VI on the fluidity of phosphatidylserine/phosphatidylcholine bilayers studied with the use of 5-deoxylstearate spin label

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An effect of annexin IV and VI on the fluidity of phosphatidylserine/phosphatidylcholine (PS/PC) membranes was studied by spin labeling technique with the use of 5-doxylstearic acid. It was found that calcium ions at micromolar concentrations induced a marked decrease in the order parameter of PS/PC membranes. This effect was completely abolished by both annexins. The influence of annexins on the mobility of fatty acid chains in the studied region in PE/PC membranes was insignificant.

Annexins IV and VI; Calcium ion; Membrane fluidity; Spin label

1. INTRODUCTION

Many cellular events proceeding at the membrane level, e.g. exocytosis, membrane transport and fusion, are triggered and modulated by changes of the concentration of free calcium ions [1–4]. Action of calcium is based on binding to the specific proteins of several classes [5,6]. Recently, a group of homologous Ca^{2+}-sensitive, phospholipid-binding proteins named annexins has been discovered in many different cells and tissues [7,8]. In the presence of Ca^{2+} ions these proteins interact synergistically with negatively charged phospholipids forming ternary complexes [7–10]. Within the cells, the sites of action of annexins are obviously membrane anionic phospholipids which serve as target/docking molecules for them. As already has been documented, annexins play a role in the initiation of fusion of membrane vesicles as well as in exocytosis [11–13].

It is well known that the interaction of calcium ions with anionic phospholipid domains induces structural changes in the membranes [14–19]. This effect is related to the ability of Ca^{2+} to evoke phase transition and separation in membranes containing negatively charged phospholipids leading to a transient destabilization of the bilayer that facilitates fusion of the membranes [17–19]. So far these effects have been observed at millimolar concentrations of calcium ions. Annexins, on the other hand, interact with phospholipids and induce fusion of the membranes at low (10–50 μM) concentration of free Ca^{2+} [13,20,21].

Apart from that little is known about the influence of these proteins binding on the physical properties, e.g. fluidity of membrane bilayer.

In this study we examine the effect of two annexins (IV and VI) on the order parameter of artificial membranes composed of phosphatidylserine/phosphatidylcholine and phosphatidylethanolamine/phosphatidylcholine in the presence of micromolar concentrations of Ca^{2+} ions with the use of 5-doxylstearic acid spin probe.

2. MATERIALS AND METHODS

The phospholipid vesicles were formed according to the procedure of Mimms et al. [22]. Briefly, 20 mg of phospholipids phosphatidylserine (egg PS; Sigma Chem. Co., St Louis, MO)/phosphatidylcholine (brain PC, Avanti Polar Lipids, Birmingham, AL) or phosphatidylethanolamine (dipalmitoyl-PE; Serva, Heidelberg, Germany/phosphatidylcholine (60:40) were dissolved after evaporation of organic solvent under vacuum in 1 ml of reconstitution buffer: 300 mM sucrose, 150 mM NaCl, 1 mM EGTA, 10 mM Tris-HCl, pH 7.5, supplemented with 30 mg of *n*-octyl-β-D-glucopyranoside (Sigma). Reconstitution of vesicles was achieved after removal of the detergent by gel filtration on Sephadex G-50 (Medium, 1.5 × 35 cm) column equilibrated with the reconstitution buffer. The vesicles were 10-fold diluted with the above buffer without sucrose and sedimented at 30,000 × *g* for 30 min. The diameter of the vesicles was examined by electron microscopy. For this purpose, a drop of vesicle preparation was layered onto 300 mesh copper grid coated with collodion/carbon

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Abbreviations: PC, phosphatidylcholine; PE, phosphatidyl ethanolamine; PS, phosphatidylserine; EPR, electron paramagnetic resonance.

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films. One minute later the liquid was removed with filter paper and the preparation was negatively stained with 1% uranyl acetate for 30 s and examined in an electron microscope. The diameter of the prepared lipid vesicles was in the range of 150–300 nm. The concentration of PS/PC and PE/PC were 9.7 and 6.4 mg/ml, respectively.

Annexins IV and VI were isolated according to the procedure described earlier [23]. Phospholipid phosphorus was determined according to Rouser et al. [24] and protein according to Bradford [25] method.

Spin labeling with 5-doxylstearic acid (Syva, Palo Alto, CA), and EPR spectroscopy was performed as described previously [26]. Briefly, spin label was evaporated from ethanolic solution. Vesicles in appropriate buffer were incubated on a thin film of spin label for 60 min at room temperature. The concentration of 5-doxylstearate was 4×10^{-5} M and 5-doxylstearic acid methyl ester 4.9×10^{-5} M. The calculation of the order parameter was performed according to Sefton and Gaffney [27]. The R_1 parameter related to the rotational correlation time was calculated according to Swartz [28]. The concentration of free calcium ions was predicted from Ca^{2+}/EGTA buffer system [29] and finally measured with a Ca^{2+} selective electrode (Orion Res. Inc.)

3. RESULTS

The dependence of order parameter of PS/PC (60:40) membranes spin labeled with 5-doxylstearate on temperature in the presence or absence of annexins and Ca^{2+} ions is shown in Fig. 1. It appears that the treatment of PS/PC vesicles with free calcium ions at the concentration of 100 μM significantly (10–21%) decreased an order parameter of the membrane reflecting marked fluidization of the membrane lipids (compare the order parameter values for PS/PC/Ca^{2+} with PS/PC control sample). The significance of these differences was rather high, since standard deviation values calculated for chosen data points (not shown) were in the range of 0.5–2.0%. That large changes of this parameter are rather seldom in the measurements of membrane fluidity. This rather unexpected result concerning Ca^{2+}-evoked phenomenon was substantiated by further experiments in which the effect of various concentrations Ca^{2+} on order parameter of reconstituted from detergent solution PS/PC vesicles, as well as 'hand-shaken' PS/PC suspensions was examined (Fig. 2). In both cases, calcium ions in a range of 5–1000 μM caused similar fluidization of membrane lipids, although the absolute values of order parameters were different (Fig. 2A). Furthermore, even if 5-doxylstearic acid was used for membrane labeling, the increase, although not so large, of spin label mobility could also be observed (Fig. 2B).

When PS/PC membranes fluidized by calcium ions were exposed to annexin VI (2.9×10^{-6} M), the values of the order parameter of the membranes dramatically increased (compare PS/PC/Ca^{2+}/annexin VI versus PS/PC/Ca^{2+}) and were even higher (1.6–16.5%) than the control values, i.e. membranes containing neither Ca^{2+} nor annexin VI (Fig. 1A). These differences were more significant at higher (over 30°C) temperatures (Fig. 1A).

Similarly to annexin VI, annexin IV at the concentration of 6.25×10^{-6} M acting on PS/PC/Ca^{2+} complex also completely abolished the fluidization effect of calcium ions (Fig. 1B). The values of the order parameter were also higher (0.3–14%) than control membranes.

In Fig. 3 of the order parameter on the concentration of both annexins (0–200 μg/ml) at a constant concentration of Ca^{2+} (100 μM) is shown. The gradual addition

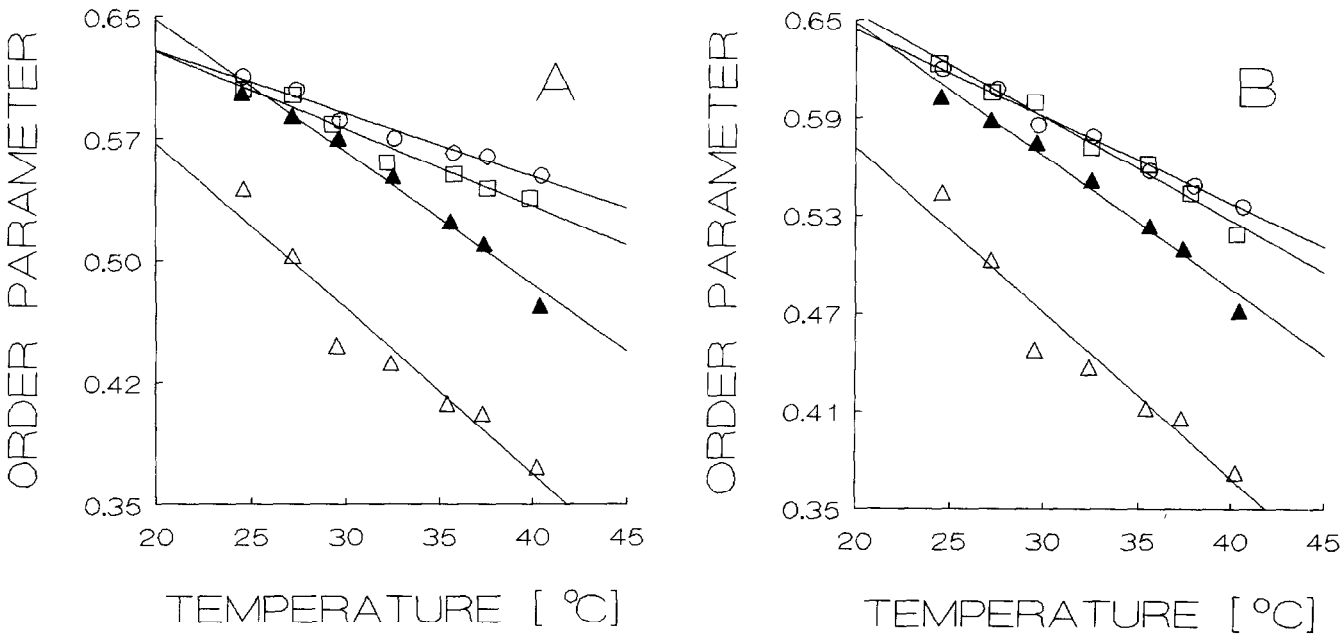

Fig. 1. Effect of annexin VI (p68) (A) and IV (p32) (B) on the order parameter of PS/PC membranes. (▲) PS/PC membranes; (△) PS/PC membranes in the presence of 100 μM free Ca^{2+}; (○) PS/PC membranes in the presence of 100 μM Ca^{2+} and 200 μg/ml annexin; □, PS/PC membranes in the presence of the same concentrations of annexins.

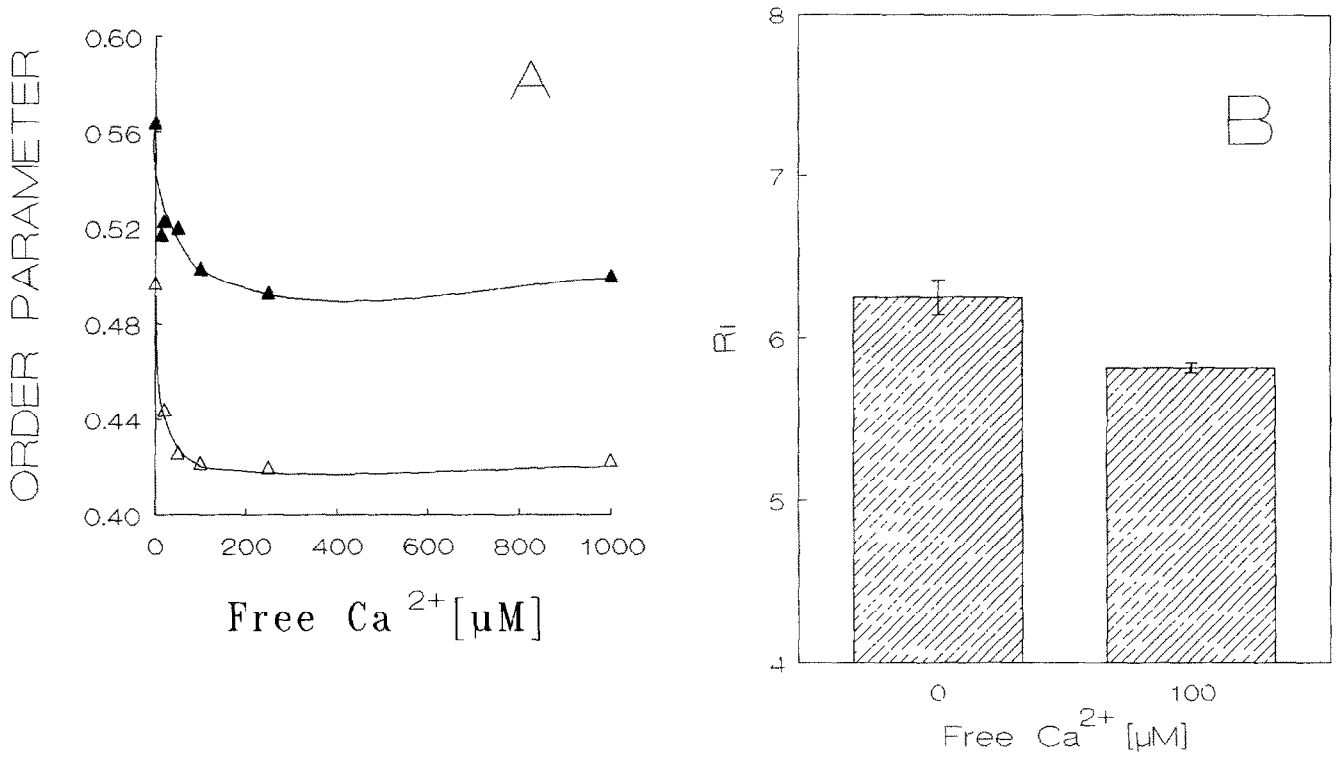

Fig. 2. The effect of various concentrations of free Ca^{2+} on the fluidity of PS/PC membranes. (A) 5-Doxylstearate; (△) 'hand-shaken' vesicles; (▲) reconstituted from detergent solution vesicles (order parameter). (B) 5-Doxylstearic acid methyl ester, 'hand shaken' vesicles, R_i parameter.

of both annexins to PS/PC/Ca^{2+} system changed progressively an order parameter of the membranes. More pronounced 'saturation' is observed in the case of annexin IV, probably due to the higher molar concentration of the protein.

It is well known that PS containing membranes are affected by calcium ions. To compare the effect of annexins as well as of Ca^{2+} ions on the fluidity of other membranes which do not contain PS we used PE/PC (60:40) vesicles prepared in the same way. The results of the measurements performed in the variable temperatures are shown in Fig. 4. As could be expected, neither significant effect of calcium ions at 100 μM nor that of both proteins on the order parameter was observed. Therefore it may be concluded that the primary effect of annexins on membranes, i.e. abolishing Ca^{2+} induced 'fluidization' is rather PS-specific.

4. DISCUSSION

The results of the experiments indicate that the primary effect of annexin IV and VI on PS/PC membranes implies abolishing the fluidization of the membrane evoked by micromolar concentrations of calcium ions. This effect may be a result of: (i) chelation of Ca^{2+} ions by annexins and therefore competition with PS for Ca^{2+}; or (ii) formation of Ca 'bridges' connecting PS and annexins. The first possibility seems to be rather unlikely since at the molar concentration used (maximally ~ 6 μM) annexins are not able to bind more than twice as much Ca^{2+}. In such a cases as applied here concentrations of this ion effect of annexins should not be ob-

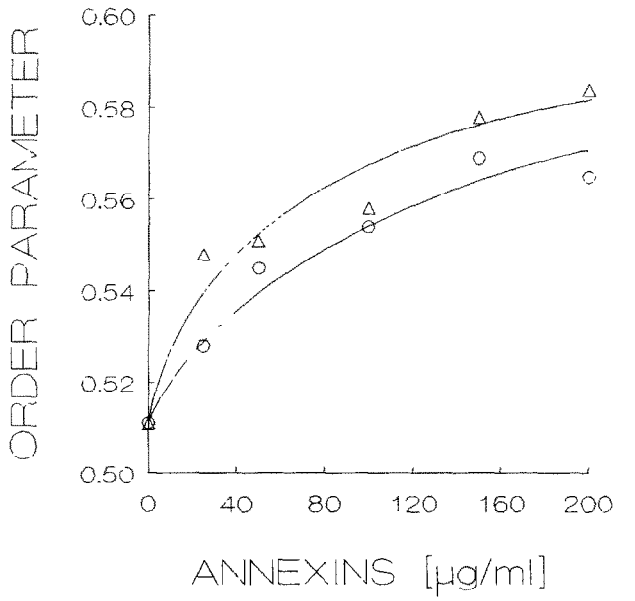

Fig. 3. The effect of various concentrations of of annexin VI (△) and IV (○) on the order parameter of PS/PC membranes labeled with 5-doxylsterate.

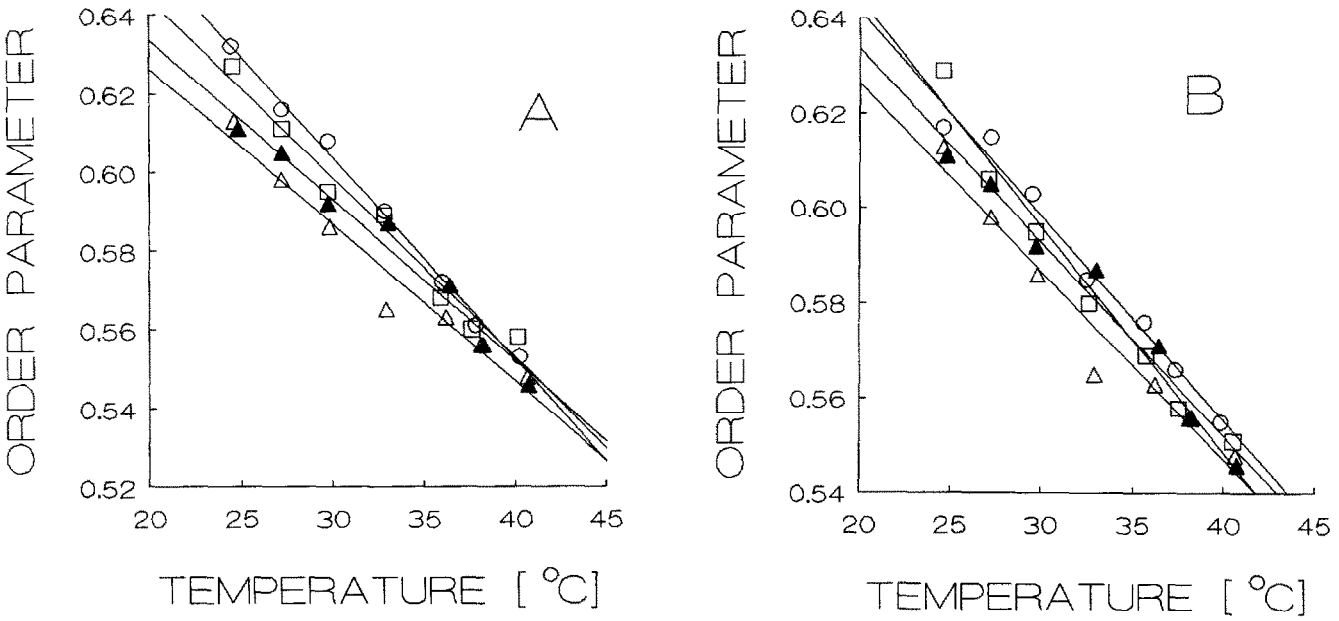

Fig. 4. The effect of annexins (A) VI and (B) IV on the order parameter of PE/PC (60:40) membranes. (▲) PE/PC vesicles; (△) PE/PC vesicles in the presence of Ca^{2+} (100 μM); (○) PE/PC in the presence of 100 μM Ca^{2+} and 200 μg/ml annexin; (□) PE/PC in the presence of the same concentrations of annexins.

served. The second possibility should be discussed in connection with the effect of Ca^{2+} on PS/PC membranes. Most of the data on the influence of this ion on the physical state of PS indicate the formation of $Ca(PS)_2$ complex and the gel phase. The $Ca(PS)_2$ phase appears to be different from thermotropic gel phase. It has been demonstrated to exhibit low water content and highly ordered acyl chains that results in unusually high melting temperatures [17,30–32]. It should be noted that most of the data were obtained at the millimolar concentration of calcium ions. However, the data on PS in the mixtures with PC suggests that at room temperature calcium ions induce aggregation of PS and separation into rigid PS phase that exclude PC and a PC-rich fluid phase [16,33]. In the case of PS/PC membranes spin labeled with fatty acid probe the situation could be similar: the formation of small gel phase domains might (i) induce exclusion of spin probe into more liquid regions, and/or (ii) induce defects in membrane phospholipid packing resulting in the increased mobility of fatty acid chains in the region of carbon 5, the site of spin labelling. The described effect could not have been a result of the probe (5-doxylstearate)–Ca^{2+} interaction since a similar although lower effect was observed in the case of 5-doxylstearic acid methyl ester which localizes closer to the core region of the membrane. It should be noted that our experiments have been performed in the presence of micromolar Ca^{2+} concentrations. The fluidization effect of PS/PC membranes after addition of millimolar Ca^{2+} could be elicited from the data of Silvius [33].

The addition of annexin and formation of the calcium 'bridge' between the protein and PS might eliminate the cross-linking capacity of PS by Ca^{2+}. This could explain the major effect of annexins on PS/PC membranes. The participation of Ca^{2+} ions in binding has been suggested also by Meers et al. [34] although the major mechanism seems to be ionic interactions [10,34,35] of positively charged annexin groups, including highly conserved arginine in the consensus sequence, with anionic sites in phospholipids [36,37]. Binding of calcium ion by the protein probably leads also to the exposure of regions of the molecule responsible for binding of the protein to the membrane. Such binding might be involved in preventing phase separation in the membrane.

Further studies are necessary for the detailed explanation of the mechanisms of the effects of these proteins on the structure and function of biological membranes.

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