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ARTICLE

Annexin A4 binding to anionic phospholipid vesicles modulated by pH and calcium

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Abstract Annexin A4 belongs to a class of Ca2+-binding proteins for which different functions in the cell have proposed, e.g. involvement in exocytosis and in the coagulation process. All these functions are related to the ability of the annexins to bind to acidic phospholipids. In this study the interaction of annexin A4 with large unilamellar vesicles (LUV) prepared from phosphatidylserine (PS) or from phosphatidic acid (PA) is investigated at neutral and acidic pH. Annexin A4 strongly binds to either lipid at acidic pH, whereas at neutral pH only weak binding to PA and no binding to PS occurs. Addition of 40 μM Ca2+ leads to a strong binding to the lipids also at neutral pH. This is caused by the different electric charge of the protein below and above its isoelectric point. Binding of annexin A4 induces dehydration of the vesicle surface. The strength of the effects is much greater at pH 4 than at pH 7.4. At pH 7.4 annexin A4 reduces the Ca2+-threshold concentration necessary to induce fusion of PA LUV. The Ca2+ induced fusion of PS LUV is not affected by annexin A4 at pH 7.4. At pH 4 annexin A4 induces fusion of either vesicles without Ca2+. Despite the low binding extents at neutral pH annexin A4 induces a Ca2+ independent leakage of PS- or PA-LUV. The leakage extent is increased at acidic pH. From the data two suggestions are made: (1) At pH 4 annexin A4 (at least partially) penetrates into the bilayer in contrast to the preferred location at the vesicle surface at neutral pH. The conformation of annexin A4 seems to be different at the two conditions. (2) At neutral pH, Annexin A4 seems to be able to bind two PA vesicles simultaneously; however, only one PS vesicle at the same time. This behavior might be related to a recently described double Ca2+ binding site, which appears to be uniquely suited for PS.

Keywords Annexins · Binding · Calcium · Fusion · Dielectric constant · Liposome

Abbreviations

DPE	*N*-(6-dimethylaminonaphthalene-2-sulfonyl)-phosphatidylethanolamine
FRET	Fluorescence resonance energy transfer
HEPES	*N*-(2-hydroxyethyl)piperazine-*N*′-(2-ethansulfonic acid)
LUV	Large unilamellar vesicles
MLV	Multilamellar vesicles
NBD-PE	1-4-nitrobenz-2-oxa-1,3-diazol-phosphatidylethanolamine
PA	Phosphatidic acid
PC	Phosphatidylcholine
PE	Phosphatidylethanolamine
PS	Phosphatidylserine
Pyr-PC	3-Palmitoyl-2-(1-pyrenedecanoyl)-L-α-phosphatidylcholine
Rh-PE	Lissamine rhodamine B sulfonyl-phosphatidylethanolamine
Trp	Tryptophan
TMR	Tetramethylrhodamine
ANTS	1-aminonaphthalene-3,6,8-trisulfonic acid
DPX	*p*-xylenebis(pyridiium bromide)

Dedicated to Prof. K. Arnold on the occasion of his 65th birthday.

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Introduction

The annexins are a family of highly conserved Ca2+-dependent membrane-binding proteins, which are present in a variety of species and cell types (for review see Burgoyne and Geisow 1989; Creutz et al. 1992; Moss et al. 1992; Raynal and Pollard 1994; Gerke and Moss 2002; Rescher and Gerke 2004; Gerke et al. 2005). They share structural homology in a conserved core consisting of four domains of about 70 amino acids each, containing the annexin consensus sequence. On the other hand, the N-terminal tails of the individual annexins strongly differ in length and amino acid sequence (Geisow et al. 1986). The crystal structure of annexin A4 and its Ca2+ binding sites has been described (Sutton and Sprang 2000). Annexins are clearly distinguished from the well-known 'EF-hand' proteins like calmodulin and troponin C, in the sequence and geometry of their calcium-binding sites as well as in their affinity for calcium (Burgoyne and Geisow 1989). Their conservation and abundance suggest important functions. For this group of proteins an involvement in exocytosis (Piljic and Schultz 2006), inhibition of protein kinase C and phospholipase A_2, involvement in the coagulation process (Moss et al. 1992; Raynal and Pollard 1994) and participation in the calcification of cartilage (Genge et al. 1991; Kirsch and Wuthier 1994) are discussed. All these functions stem from the ability of the annexins to bind to negatively charged phospholipid membranes.

Several authors reported that in the absence of membranes annexins have a much lower affinity for Ca2+ than the 'EF-hand' family (Burgoyne and Geisow 1989). When annexins are bound to the phospholipid bilayer, their apparent Ca2+ binding affinity is greatly enhanced (Blackwood and Ernst 1990). With respect to their influence on the Ca2+-induced membrane aggregation and fusion the annexins have been divided into two different classes: (1) annexins which enhance the action of Ca2+ (annexins I, II, IV, VII) and (2) annexins which inhibit it (annexins V, VI) (Meers et al. 1991a; Moss et al. 1991). Especially of interest in the first group is the effect of reducing the Ca2+ concentration necessary to induce fusion of phospholipid vesicles. Because the structural homologies between the different annexins are high, the question arises which regions or structural requirements are responsible for such a different behavior. Hoekstra et al. (1993) compared annexin V and a chimeric annexin which contained the N-terminal region of annexin I and the consensus region of annexin V. The authors found differences in the action of these two annexin forms on the leakage and aggregation of phosphatidylserine (PS) vesicles. A different binding strength of annexin V and I in the presence of Ca2+ to phospholipid vesicles has been measured (Ernst et al. 1994). Annexin VII (formerly called synexin), as an example for enhancing interactions with phospholipids, has been studied in detail (Hong et al. 1981, 1982; Meers et al. 1988, 1991a, b). Nevertheless, the detailed mechanism of the combined action of Ca2+ and annexins on phospholipid vesicles is still not fully explained (Hill et al. 2003). It is the main topic of this study to further clarify the nature of binding of annexin A4 to PS and PA vesicles in the absence and presence of Ca2+ under different conditions (pH, concentration of protein).

Materials and methods

Chemicals

Phosphatidylserine and phosphatidic acid (PA) were purchased from Avanti Polar Lipids (Alabaster, AL). The purity of the phospholipids was checked by thin layer chromatography. Both phospholipids gave only one spot each. 3-Palmitoyl-2-(1-pyrenedecanoyl)-L-α-phosphatidylcholine (Pyr-PC) was obtained from Molecular Probes (Eugene, OR), dansyl phosphatidylethanolamine (DPE), nitrobenzoxadiazole phosphatidylethanolamine (NBD-PE), and lissamine rhodamine B sulfonyl phosphatidylethanolamine (Rh-PE) were from Avanti Polar Lipids (Alabaster, AL).

Annexin A4

Annexin A4 was isolated from fresh or frozen bovine liver by the modified procedure of Bandorowicz et al. (1992). The tissue of the bovine liver was cut into small pieces, which were homogenized in a washing buffer solution A_1 (20 mM Tris–HCl, pH 7.5, 100 mM NaCl, 0.2 mM phenylmethylsulfonylfluoride, 5 TIU/l aprotinin, 1 mM $CaCl_2$, 1 mM NaN_3 and 1% (v/v) Triton X-100) using an Ultra-Turrax T25 homogenizer (Janke & Kunkel, IKA®-Labortechnik). For disruption of cells a Potter S-homogenizer (B. Braun Melsungen AG) at 0°C and 900/min was used. Triton X-100 was necessary to bring the membrane-bound annexin fraction in solution. The homogenate was centrifuged at 15,000×*g* and 4°C for 15 min. This step was followed by resuspending the sediment and centrifugation of the resuspension at 15,000×*g* (4°C, 10 min) three times repeated (washing steps). After the last washing step the suspension was centrifuged at 50,000×*g* (4°C, 20 min). The obtained sediment was gently stirred overnight in the washing buffer solution A_2 (1 mM $CaCl_2$ was replaced by 5 mM ethylene glycol-bis(*β*-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid). The samples were centrifuged 1 h at 80,000×*g* (Beckman OPTIMA™ L-60 preparative ultracentrifuge with rotor type 80Ti) at 4°C. After these procedures proteins were separated by precipitation with pH or ammonium sulfate and ion exchange column chromatography (NaCl gradient: 0–0.5 M, flow rate 1 mL/min) with the Pharmacia FPLC equipment.

Identification of annexin A4

The purity of the resulted protein fraction after the ion exchange chromatography was checked by sodium dodecyl sulfate-polyacrylamide gel electrophoresis according to Schagger and Von Jagow (1987) in the presence of 2% (v/v) 2-mercaptoethanol, stained with coomassie blue. With monoclonal antibody probes from ICN Biomedicals (Costa Mesa, CA) against human and bovine annexin A4 the identity was checked by western blot.

Buffers

Experiments were performed in buffer solutions of 10 mM citrat (pH 4.0), 10 mM 2-(*N*-morpholino)ethansulfonic acid (MES; pH 5.5), or 10 mM *N*-(2-hydroxyethyl)piperazine-*N*′-(2-ethansulfonic acid) (HEPES; pH 7.4), respectively. All solutions contained 0.1 M NaCl.

Vesicle preparation

Multilamellar phospholipid vesicles (MLV) were prepared using the method of Bangham et al. (1968). The lipid was initially dried from chloroform, subsequently dispersed in buffer solution (10 mM HEPES and 100 mM NaCl, adjusted at pH 7.4) and shaken at a temperature above the gel-to-liquid-crystalline transition temperature for 10 min. Large unilamellar vesicles (LUV) were prepared by five freeze-thawing cycles of MLV and following extrusion (five times) through 0.1 μm Nucleopore filter membranes using an extruder (Lipex Biomembranes, Vancouver, Canada) at 30°C. Final phospholipid concentrations were determined on the basis of total phosphate determination according to Chen et al. (1956).

Fluorescence measurements

All fluorescence assays were carried out on a Perkin-Elmer LS-50B spectrofluorimeter (Beaconsfield, England) or a Jobin Yvon Spex FluoroMax-2 spectrofluorimeter (Edison, NJ). We used quartz cuvettes equipped with a magnetic stirrer. Vesicles were suspended in a 1.0 mL buffer solution, reaching a final concentration of 80 μM lipid. The recording started after the system had reached equilibrium (about 3–5 min). Appropriate aliquots of aqueous protein stock solutions were added to the vesicle suspension and continuously stirred. The experiments were performed at 37°C. All experiments were carried out at least twice or thrice. The reproducibility of different runs was within a 5–10% error. For the shown figures, representative experiments were chosen.

Protein binding to vesicles

Pyr-PC was mixed with phospholipids before evaporation at a concentration of 10% (mol/mol). Protein adsorption was evaluated by measuring the fluorescence resonance energy transfer (FRET) from protein tryptophan (Trp) residues to the pyrene fluorophore of Pyr-PC (Arnold et al. 1992). Emission spectra were recorded from 300 to 560 nm. The extent of the FRET was calculated as the pyrene fluorescence intensity integrated over the wavelength range 370–550 nm, corrected as follows:

$$\mathrm{FRET} = \frac{I_{\mathrm{Pyr}} - I^{0}_{\mathrm{Pyr}}}{I^{0}_{\mathrm{Pyr}}} \quad (1)$$

where I_{Pyr} is the pyrene fluorescence intensity and I^{0}_{Pyr} is the initial pyrene fluorescence in the absence of any Trp. Trp fluorescence was excited at 290 nm in order to minimize initial pyrene fluorescence.

Measurement of the surface dielectric constant

Dansyl phosphatidylethanolamine was mixed with the phospholipids in chloroform at a molar ratio of phospholipid/DPE of 200–300 before evaporation. The excitation wavelength was 340 nm, the emission was measured in the range of 400–600 nm. The wavelength at the maximum intensity of the emission spectrum depends on the dielectric properties of the environment of the probe. The wavelength increases with increasing dielectric constant. Dielectric constants can practically be calculated from an empiric law obtained by use of a set of organic solvents (Kimura and Ikegami 1985; Ohki and Arnold 1990a).

Phospholipid mixing assay (NBD-PE/Rh-PE)

The intermixing of phospholipids was followed by a FRET method, using NBD-PE and Rh-PE (Struck et al. 1981). Labeled vesicles were composed of PA or PS, respectively, and 1% (mol/mol) each of both NBD-PE and Rh-PE. One part of the fluorophore-labeled vesicles and four parts of unlabelled vesicles were suspended in the appropriate buffer solution (with/without annexin). The fluorescence measurements were carried out by exciting the fluorescence at 470 nm and recording the emission in the wavelength range from 500 to 620 nm. We modified the calculation method as follows: The extent of the phospholipid mixing M was evaluated from the ratio of the fluorescence intensities of NBD-PE at 520 nm and Rh-PE at 588 nm

$$r = \frac{I_{520}}{I_{588}}$$
$$M = \frac{r - r^0}{r^{100} - r^0} \quad (2)$$

where r is the fluorescence amplitudes ratio after addition of the fusogen, $r0$ is the ratio before addition, and $r100$ is the ratio after solubilization of the vesicles in 0.1% (v/v) Triton X-100, respectively. Using the ratio, we did not need to correct intensities for light scattering or quenching of the NBD fluorescence by Triton X-100.

Measurement of vesicle leakage

Release of vesicle contents was monitored using procedures based on the quenching of ANTS fluorescence by DPX. For the preparation of vesicles containing ANTS and DPX, the phospholipids were evaporated and subsequently suspended in a buffer solution containing 12.5 mM ANTS, 45 mM DPX, and 20 mM NaCl (Ellens et al. 1985). LUV were produced as described above. Free dyes were removed by passage through a Sephadex G-75 column. The leakage assay was carried out on a Perkin-Elmer LS-50B spectrofluorimeter (Beaconsfield, England). The excitation wavelength was set to 360 nm, the time course of the emission was measured above 530 nm using a corning filter and the total emission attachment for LS-50B. We used quartz cuvettes equipped with a magnetic stirrer. The experiments were performed at 37°C. The phospholipid suspension was diluted with the appropriate buffer solution reaching a final concentration of 10 or 80 μM phospholipid, respectively. The recording started after the system had reached equilibrium (about 3–5 min). Appropriate aliquots of aqueous protein stock solutions were added to the vesicle suspension and continuously stirred. The change in fluorescence emission intensity was usually measured over a time period of 10 min. The final leakage extent (the emission intensity reached a stable value, typically after 1 min) was calculated as

$$L = \frac{I - I_0}{I_{100} - I_0} \times 100\% \quad (3)$$

with I fluorescence intensity, I_0 fluorescence intensity obtained from intact vesicles containing both ANTS and DPX, and I_{100} fluorescence intensity measured after lysis of the vesicles by 0.1% (v/v) Triton X-100.

As a second method for estimation of pore size of PS LUV induced by annexin A4 tetramethylrhodamine labelled dextran with molecular weights of 3 and 10 kDa (Molecular Probes, USA) were used. A buffer solution containing 20 mg TMR-dextran/ml was used for preparation of PS LUV. Free dye was separated using gel chromatography with Sephadex G-100. Excitation was 530 nm, emission intensities were measured at 585 nm.

Trp fluorescence of annexin A4

The Trp fluorescence was examined at different pH, in absence and presence of Ca2+ and PA or PS LUV. Excitation was set to 280 nm, emission was measured from 310 to 400 nm.

Results

Binding of annexin A4 to PS and PA LUV

Annexin A4 contains only one Trp residue in the peptide chain. Trp185 is situated close to the Ca2+ binding face of the molecule (Raynal and Pollard 1994). To proof the binding of annexin A4 to membranes it is promising to study the possible FRET from the Trp to pyrene probes residing in the phospholipid bilayer. We used the chain-labeled Pyr-PC, the fluorescence moiety of which is preferentially located in the hydrophobic region of the membrane (Vanderkooi et al. 1974). The FRET from Trp185 to Pyr-PC provides a measure of the binding of annexin A4 to the bilayer because the corresponding Förster distance is 27–31 Å (Dobretsov et al. 1989).

At pH 7.4 only a small FRET occurred after addition of annexin A4 to PA LUV in the absence of Ca2+ (Fig. 1a). However, the subsequent addition of 40 μM $CaCl_2$ increased the FRET about twice. Blackwood and Ernst (1990) have reported a Ca2+ concentration of 0.95 μM for half-maximum binding of annexin A4 to PA. Therefore, 40 μM $CaCl_2$ were sufficient to induce the binding. Lowering the pH to the isoelectric point of annexin A4 (pH 5.5) led to a significantly stronger FRET, which likewise was increased by addition of 40 μM $CaCl_2$. At pH 4.0 the FRET was far stronger than at pH 7.4 and pH 5.5 in the absence of Ca2+. Addition of 40 μM $CaCl_2$ had no increasing effect at pH 4.0.

The binding of annexin A4 to PS LUV was comparable to the binding of annexin A4 to PA LUV at pH 7.4. At pH 4.0 the FRET was much stronger than at pH 7.4, however, not as strong as in the case of PA, and was slightly Ca2+-dependent (Fig. 1b).

In a second set of binding experiments, annexin A4 Trp emission spectra were measured at different pH, additionally lipid vesicles (PA or PS LUV) were added. At low pH a maximum blue shift of the Trp emission peak of about 4 nm was measured for both LUVs in the absence of Ca2+ (data not shown). The corresponding protein/total PL ratios were in the range of 1/500.

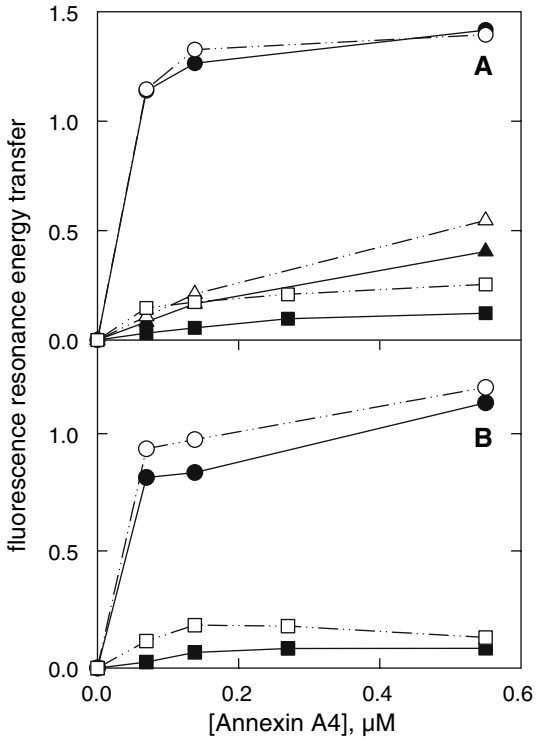

Fig. 1 Binding of annexin A4 to PA LUV (*panel A*) and PS LUV (*panel B*) at pH 4.0 *circle*, 5.5 *triangle*, and 7.4 *square*. *Closed symbols* refer to annexin A4, *open symbols* refer to annexin A4 with subsequent addition of 40 μM Ca2+

Membrane surface polarity

In order to determine changes in the surface dielectric constant of the phospholipid vesicles caused by annexin A4, we used the corresponding shift of the emission wavelength of DPE (Ohki and Arnold 1990a). Figure 2 shows the surface dielectric constants of PA LUV (panel A) and PS LUV (panel B) at pH 7.4 as a function of the Ca2+ concentration in the presence of different annexin A4 concentrations. The surface dielectric constants of either vesicles were almost constant up to a critical Ca2+ concentration (about 1 mM) in the absence of annexin A4. If the Ca2+ concentration exceeded the critical value the surface dielectric constant was dramatically lowered due to the formation of dehydrated areas at the contact zone of closely aggregated vesicles (Ohki and Arnold 1990b).

In the presence of annexin A4 the critical Ca2+ concentration was reduced. The higher the annexin concentration the stronger was the reduction. However, the two lipid species differed in their interaction with the protein. The same annexin concentration produced a much more pronounced reduction of the critical Ca2+ concentration with PA than with PS. Annexin A4 lowered the surface dielectric constant of PA LUV even in the absence of Ca2+.

At pH 4.0 the surface dielectric constant of PA LUV was only slightly lowered, compared to the same annexin A4 and Ca2+ concentrations at pH 7.4 (not shown). Obviously almost maximum depolarization was reached at pH 7.4. The surface dielectric constant of PS LUV was largely changed at pH 4.0 compared to pH 7.4 (Fig. 3). The strength of the depolarization was similar to that obtained with PA.

Phospholipid intermixing of PS and PA LUV, respectively

In order to evaluate the influence of annexins on the Ca2+-induced phospholipid intermixing we measured the intermixing extents at various pH in the presence of different annexin concentrations. Phospholipid intermixing was followed using the NBD-PE/Rh-PE assay (Struck et al. 1981). The influence of annexin A4 on the phospholipid intermixing at pH 7.4 is represented in Fig. 4. In order to induce phospholipid intermixing the Ca2+ concentration had to exceed a critical value. This critical value was the same as that for the Ca2+-induced decrease of the surface polarity of the vesicles (cf. above). The presence of increasing annexin A4 concentrations more and more lowered the critical Ca2+ concentration for the phospholipid intermixing between PA LUV (Fig. 4b), but not between PS LUV (Fig. 4a). Moreover, the presence of annexin A4 slightly diminished the extent of the Ca2+-induced intermixing of PS LUV.

At pH 4.0 annexin A4 induced phospholipid intermixing in the absence of Ca2+ (Fig. 5). The extent of the annexin A4-induced intermixing increased with increasing annexin A4 concentration. Addition of subcritical Ca2+ concentration only slightly enhanced the intermixing extent obtained by annexin A4 alone. Addition of supercritical Ca2+ concentrations brought about an additional increase of the intermixing extent. Interestingly, we did not find any lowering of the threshold concentration of this additional effect as one could expect from the results obtained at pH 7.4.

Leakage of PS- and PA-LUV

As shown in Fig. 6, at pH 7.4 annexin A4 at concentrations higher than 0.1 μM induces in the absence of Ca2+ a remarkable leakage of PA and PS LUV. The interaction of annexin A4 with PA or PS LUV is concentration dependent. Concentrations of annexin A4 higher than 0.1 μM induce a leakage extent of about 20% for PA LUV, about 30% for PS LUV. Further increase of annexin A4 concentrations did not result in higher leakage extents. Measurements at pH 5.5 at annexin A4 concentrations of about

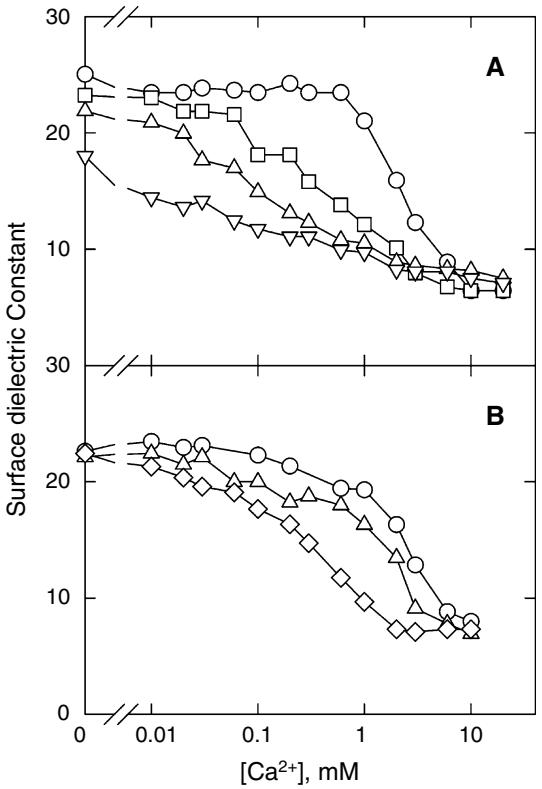

Fig. 2 Surface dielectric constants of PA LUV (*panel A*) and PS LUV (*panel B*) at pH 7.4 in dependence on Ca2+ at different annexin A4 concentrations (*circle* 0 μM annexin A4, *square* 0.034 μM annexin A4, *up triangle* 0.07 μM annexin A4, *down triangle* 0.14 μM annexin A4, *diamond* 0.27 μM annexin A4)

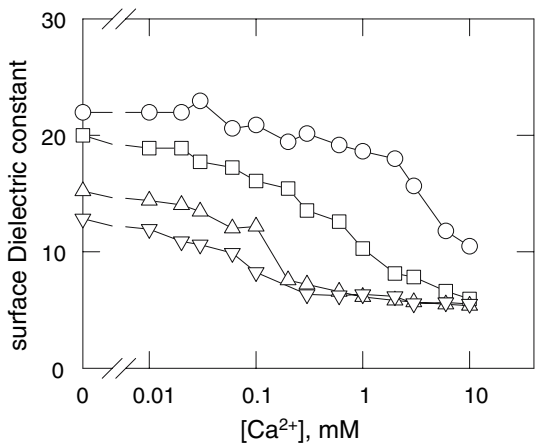

Fig. 3 Surface dielectric constant of PS LUV at pH 4.0 in dependence on Ca2+ at different annexin A4 concentrations. (*circle* 0 μM annexin A4, *square* 0.034 μM annexin A4, *up triangle* 0.07 μM annexin A4, *down triangle* 0.14 μM annexin A4)

0.05 μM indicate leakage extents of more than 50% for PA LUV, about 65% for PS LUV. Also at low pH there exist a saturation concentration for the induction of leakage by annexin A4.

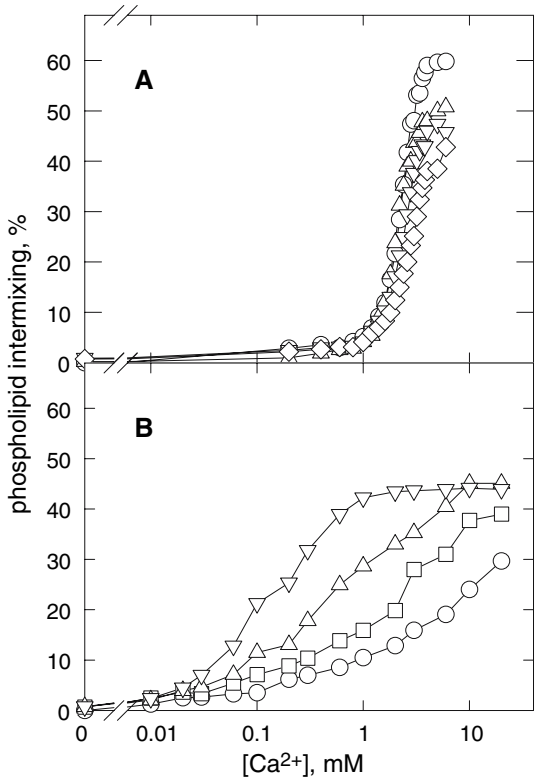

Fig. 4 Phospholipid intermixing between PS LUV (panel A) or PA LUV (panel B), respectively, in dependence on Ca2+ at different annexin A4 concentrations at pH 7.4 (*circle* 0 μM annexin A4, *square* 0.034 μM annexin A4, *up triangle* 0.07 μM annexin A4, *down triangle* 0.14 μM annexin A4, *diamond* 0.27 μM annexin A4)

To get more information about the pore size of the liposomes induced by the action of annexin A4, encapsulated TMR-Dextran 3k was used. The data are shown in Fig. 7 as time curves of fluorescence intensities of TMR-dextran 3k and 10k. Using concentrations of 0.14 μM annexin A4 about 22% leakage extent (TMR-dextran 3k) was measured. TMR-dextran 10k (encapsulated in PS LUV) was released into the bulk phase after addition of annexin A4 at comparable concentrations to a much lesser extent (0.14 μM annexin A4 induces about 9% leakage). It should be noted that the maximum leakage extents of PS LUV induced by annexin A4 are reached within nearly 2 h. In contrast, the time for a maximal ANTS/DPX leakage extent were in the range of about 5 min.

The combined action of annexin A4 and Ca2+ on the stability of PS LUV is shown in Fig. 8. For these measurements the ANTS/DPX assay was used. At pH 7.4 in the absence of Ca2+ the addition of 0.14 μM annexin A4 to PS vesicles result in an increase of the leakage extent to about 35%. Subsequent addition of Ca2+ increases moderately the leakage extent of the vesicles. Reaching Ca2+ concentrations of about 1 mM, the leakage extent increases

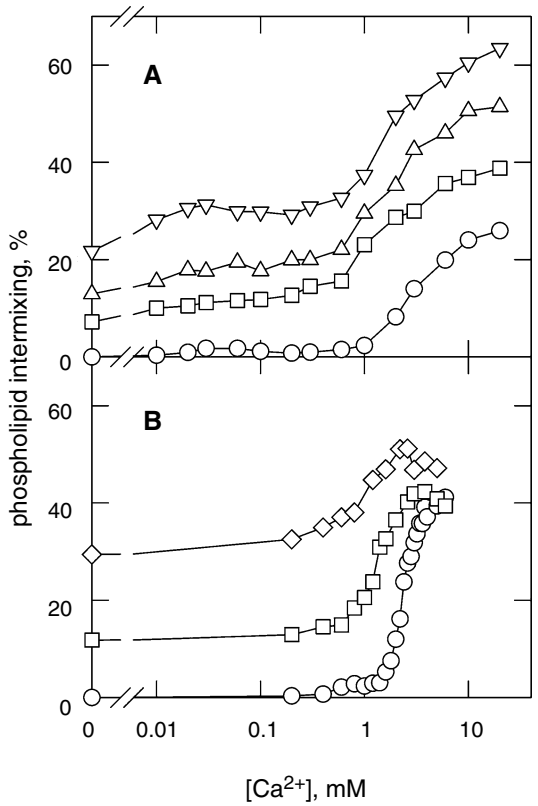

Fig. 5 Phospholipid intermixing between PA LUV (*panel A*) or PS LUV (*panel B*), respectively, in dependence on Ca2+ at different annexin A4 concentrations at pH 4.0 (*circle* 0 μM annexin A4, *square* 0.034 μM annexin A4, *up triangle* 0.07 μM annexin A4, *down triangle* 0.14 μM annexin A4, *diamond* 0.27 μM annexin A4)

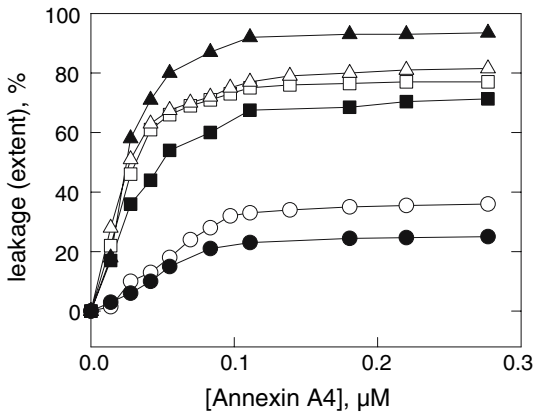

Fig. 6 Leakage extents as measured by the ANTS/DPX assay for PS (*open symbols*) and PA LUV (*closed symbols*) at different pH values in dependence on the annexin A4 concentration in absence of Ca2+ (*circle* pH 7.4, *square* pH 5.5, *up triangle* pH 4.0)

dramatically. There is no strict difference to samples without Ca2+. The threshold concentration of Ca2+ for the induction of leakage is not shifted. In the absence of Ca2+, at pH 4.0 the leakage extent of PS LUV is much higher for

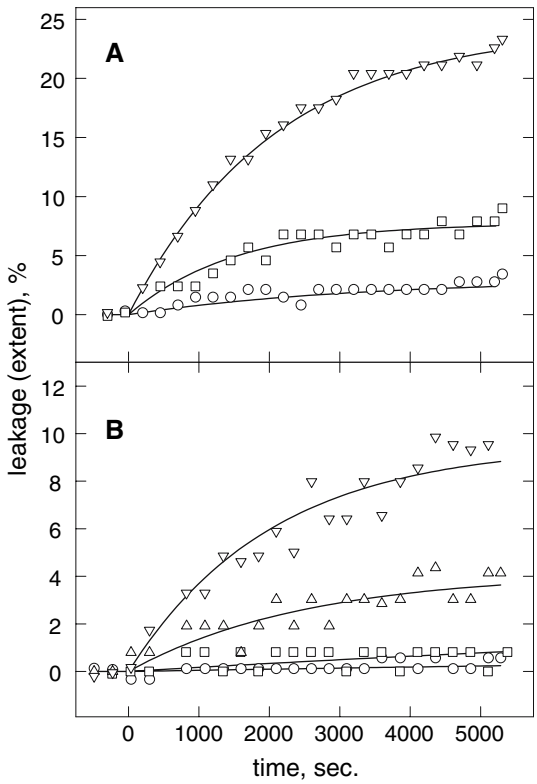

Fig. 7 Time trace of the leakage extent as measured by the TMR-dextran 3k assay (*panel A*) and TMR-dextran10k (*panel B*) encapsulated in PS LUV in dependence on annexin A4 at concentrations of 0 μM annexin A4 (*circle*), 0.034 μM annexin A4 (*square*), 0.07 μM annexin A4(*up triangle*),0.14 μM annexin A4(*down triangle*) in the absence of Ca2+ at pH 7.4

given annexin A4 concentrations compared to pH 7.4. The leakage extents are 60% for 0.35 μM annexin A4, reaching more than 80% for 0.14 μM annexin A4. The additional presence of Ca2+ increases the leakage extents also at lower threshold concentrations, but the effect is not so obvious because of the initial high leakage extents induced by annexin A4 itself.

Discussion

All proposed functions of the annexins directly depend on their ability to bind to phospholipid bilayers (Benz and Hofmann 1997). Since biological membranes are negatively charged we studied the interaction of annexin A4 with vesicles made from negatively charged phospholipids. PS is the most common negatively charged phospholipid at those membrane faces, which are relevant for fusion. PA is not very common in membranes; however, PA is produced by phospholipase D after stimulation of the cell and may therefore play a role in processes like degranulation or exocytosis which are expected to proceed under involvement of annexins (Blackwood et al. 1997).

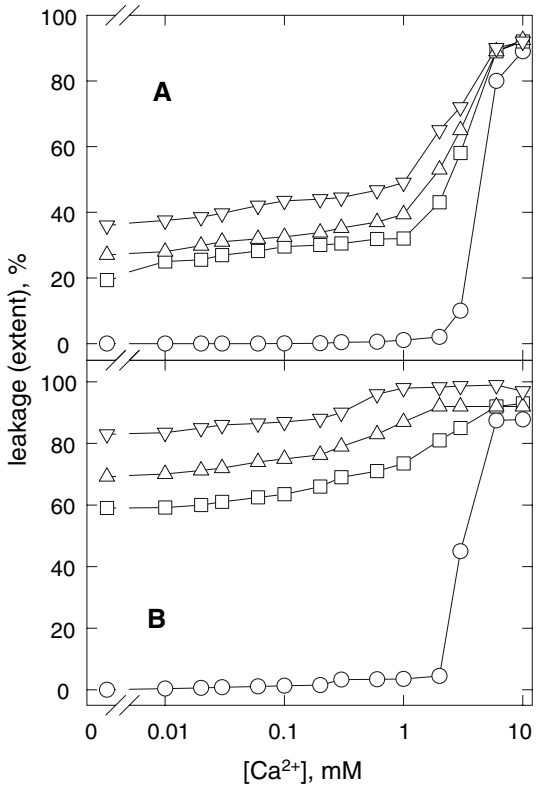

Fig. 8 Leakage extents (ANTS/DPX assay) of PS LUV at pH 7.4 (*panel A*) and pH 4.0 (*panel B*) for different annexin A4 concentrations (*circle* 0 μM annexin A4, *square* 0.034 μM annexin A4, *up triangle* 0.07 μM annexin A4, *down triangle* 0.14 μM annexin A4) in dependence on Ca2+ concentrations

The annexin A4 binding to PS LUV as well as to PA LUV was studied in the absence and presence of Ca2+. Only weak binding of annexin A4 to either phospholipid occurs in the absence of Ca2+ at pH 7.4. Under these conditions annexin A4 has a negative net charge like the phospholipids. Thus, annexin A4 feels a repulsive force when it approaches towards the bilayer. Addition of Ca2+ induces and mediates a strong binding between annexin A4 and the phospholipids. This is due to electrostatic attraction forces. However, the binding is very effective because of the high affinity of the annexin-phospholipid system for the Ca2+ ions. This affinity originates from the special design of the conserved type II Ca2+ binding sites of the annexin which together with the phosphoryl moiety of phospholipids form a coordinative Ca2+ complex (Huber et al. 1990; Weng et al. 1993).

When pH falls below the isoelectric point of annexin A4 {5.4–5.8 (Geisow et al. 1987)} electrostatic attraction between annexin A4 and PA or PS occurs. Consequently, there is no need for Ca2+ to mediate the binding at such acidic pH. Interestingly, at pH 4.0 the FRET from annexin A4 Trp185 to bilayer-residing pyrene is six to eightfold that at pH 7.4 and 40 μM Ca2+. Since >80% of the added annexin A4 should be already membrane-bound at pH 7.4 with Ca2+ (Blackwood and Ernst 1990) we have to rule out a binding of a six to eightfold higher amount of annexin A4 at pH 4.0, compared to pH 7.4. Instead of a higher binding we assume that this finding monitors the existence of two different binding modes of annexin A4, characterized by different environments and/or locations (with respect to the bilayer) of Trp185. The environment and location of Trp185 at pH 7.4 is known: Although Trp185 is a part of an interhelical loop at the Ca2+-binding face of the protein, its fluorescent moiety is buried in the hydrophobic interior of the protein (Meers 1990). In the membrane-bound annexin A4 Trp185 is located roughly in the depth of the glycerol backbone of the membrane (Meers 1990). Similar findings have been made for the corresponding Trp187 of annexin V (Meers 1990). Obviously, at pH 4.0 the distance between Trp and the pyrene moiety of Pyr-PC {situated near the center of the bilayer} is reduced leading to the observed increase of FRET. Additionally, the partial quenching of the Trp fluorescence occurring in the native protein (Meers 1990) may be reduced. From the largely increased FRET we therefore deduce a different conformation of annexin A4 at pH 4.0 compared to pH 7.4.

We conclude from our findings a conformational change and a partially penetration of the annexin A4 molecule into the phospholipid membrane at pH 4.0. Such a conclusion has also been made for annexin V at the same pH (Kohler et al. 1997). A penetration of annexin A4 is facilitated at low pH because of the increased hydrophobicity of the protein due to protonation of the acidic residues.

One of the basic findings of studies on ion-induced aggregation and fusion of PS vesicles is that the membrane surface hydrophobicity is an important factor contributing to membrane fusion. The induction of phospholipid intermixing of PS or PA SUV by cations is accompanied by a decrease of the hydration of the vesicles (Ohki and Zschornig 1993). The correlation between hydrophobization and occurrence of fusion has also been demonstrated for the combined action of poly-(ethylene glycol) and cations on PS SUV (Ohki and Arnold 1990a). The binding of annexin A4 to PS or PA LUV likewise caused an increase of the vesicle surface hydrophobicity measured here as the decrease of the surface dielectric constant. This decrease can be interpreted as a decrease of the hydration. This is not a unique effect of annexin A4, also other proteins like annexin V or lysozyme and peptides like melittin induce a decrease of the vesicle surface hydration (Arnold et al. 1992; Kohler et al. 1997; Lafleur et al. 1991). However, one must note that dehydration is a necessary but not sufficient condition for membrane fusion to occur. Annexin V inhibits the fusion at neutral pH despite potent dehydration (Kohler et al. 1997).

We found different strengths of the annexin A4-induced hydrophobization of PA LUV compared to PS LUV. Annexin A4 decreased the hydrophobicity of PA but not PS in the absence of Ca2+ at pH 7.4. Combined action of annexin A4 and Ca2+ produced a stronger hydrophobization than the individual agents. Especially, the threshold concentration of Ca2+-induced hydrophobization is lowered in the presence of annexin A4. All described effects also depend on the applied pH: Decrease of pH increases the effects.

The vesicle fusion measured as phospholipid intermixing does not in every case correlate with the found hydrophobization of the vesicles. While annexin A4 decreased the hydrophobicity of PA in the absence of Ca2+ at pH 7.4, the fusion starts only after the addition of some 10 μM Ca2+. However, at pH 4.0 both qualities correlate well. Annexin A4 produces potent phospholipid intermixing of either vesicles with or without Ca2+ at pH 4.0.

The physiologically most interesting finding is that annexin A4 reduces the threshold concentration of the Ca2+-induced fusion of PA LUV in a concentration-dependent way. The reduction amounts to two orders of magnitude in the presence of 0.14 μM annexin A4. A similar reduction is well known for the Ca2+-induced chromaffin granule fusion in the presence of annexin A4 or annexin VII (Zaks and Creutz 1991a). In contrast to PA, PS is not susceptible to a threshold-reducing action of annexin A4 (Blackwood and Ernst 1990, this paper). A similar finding has been made for annexin I (Blackwood et al. 1997): annexin I was not able to reduce the Ca2+ requirement for the fusion of PC/PE/PS/phosphatidylinositol LUV. If the vesicles were incubated with phospholipase D prior to the addition of annexin I, the Ca2+ requirement was reduced by almost three orders of magnitude since phospholipase D had converted PC to PA.

While there are clear differences between PS and PA concerning vesicle hydrophobization and fusion at pH 7.4, the annexin A4-phospholipid binding characteristics do not allow to distinguish the two phospholipid membrane systems. One might assume that the accessibility of the phosphoryl moiety and/or the headgroup size influence the Ca2+/annexin-induced membrane fusion rather than the annexin-phospholipid binding. Especially, the serine moiety of PS might allow aggregation of the vesicles (leading to the observed less pronounced dehydration of PS) but hinder a close molecular contact which is necessary for fusion. Nevertheless, these differences disappear at pH below the isoelectric point of annexin A4. The suggested low-pH conformation of annexin A4 may be produced independent of the phospholipid headgroup structure. Solely the negative phospholipid charge seems to be of importance for the electrostatically driven binding of the currently positively charged annexin to the bilayer.

It has been formerly suggested that annexin A4 may be able to interact with two membranes simultaneously by tightly binding the first membrane only with its domains I + IV and reorientating the domains II + III away from that membrane, caused by an interaction of the concave faces of the two halves of the molecule with one another (Zaks and Creutz 1991b). Such a reorientation may occur with PA but is obviously hindered in the presence of PS. It has been demonstrated that besides the common phosphoryl-Ca2+-annexin interaction the carboxylate group of the serine moiety of PS can bind to an additional Ca2+ coordinated at the same interhelical loop forming a double Ca2+ binding site which appears to be uniquely suited for PS (Swairjo et al. 1995). This may lead to a tighter binding of all four annexin domains.

References

Arnold K, Hoekstra D, Ohki S (1992) Association of lysozyme to phospholipid surfaces and vesicle fusion. Biochim Biophys Acta 1124:88–94

Bandorowicz J, Pikula S, Sobota A (1992) Annexins IV (p32) and VI (p68) interact with erythrocyte membrane in a calcium-dependent manner. Biochim Biophys Acta 1105:201–206

Bangham AD, Hill MW, Miller NGA (1968) Preparation and use of liposomes as models of biological membranes. Methods Membr Biol 1:1–68

Benz J, Hofmann A (1997) Annexins: from structure to function. Biol Chem 378:177–183

Blackwood RA, Ernst JD (1990) Characterization of Ca2(+)-dependent phospholipid binding, vesicle aggregation and membrane fusion by annexins. Biochem J 266:195–200

Blackwood RA, Smolen JE, Transue A, Hessler RJ, Harsh DM, Brower RC, French S (1997) Phospholipase D activity facilitates Ca2+-induced aggregation and fusion of complex liposomes. Am J Physiol 272:C1279–C1285

Burgoyne RD, Geisow MJ (1989) The annexin family of calcium-binding proteins. Cell Calcium 10:1–10

Chen PS, Toribara TY, Warner H (1956) Microdetermination of phosphorus. Anal Chem 28:1756–1758

Creutz CE, Moss S, Edwardson JM, Hide I, Gomperts B (1992) Differential recognition of secretory vesicles by annexins. European Molecular Biology Organization Course ''Advanced Techniques for Studying Secretion''. Biochem Biophys Res Commun 184:347–352

Dobretsov GE, Kurek NK, Machov VN, Syrejshchikova TI, Yakimenko MN (1989) Determination of fluorescent probes localization in membranes by nonradiative energy transfer. J Biochem Biophys Methods 19:259–274

Ellens H, Bentz J, Szoka FC (1985) H+- and Ca2+-induced fusion and destabilization of liposomes. Biochemistry 24:3099–3106

Ernst JD, Mall A, Chew G (1994) Annexins possess functionally distinguishable Ca2+ and phospholipid binding domains. Biochem Biophys Res Commun 200:867–876

Gerke V, Moss SE (2002) Annexins: from structure to function. Physiol Rev 82:331–371

Gerke V, Creutz CE, Moss SE (2005) Annexins: linking Ca2+ signalling to membrane dynamics. Nat Rev Mol Cell Biol 6:449–461

Geisow MJ, Fritsche U, Hexham JM, Dash B, Johnson T (1986) A consensus amino-acid sequence repeat in Torpedo and mamma-

lian Ca2+-dependent membrane-binding proteins. Nature 320:636–638
Geisow MJ, Walker JH, Boustead C, Taylor W (1987) Localization and structure of novel calcium-regulated phospholipid-binding proteins. Biochem Soc Trans 15:800–802
Genge BR, Wu LN, Adkisson HD, Wuthier RE (1991) Matrix vesicle annexins exhibit proteolipid-like properties. Selective partitioning into lipophilic solvents under acidic conditions. J Biol Chem 266:10678–10685
Hill WG, Kaetzel MA, Kishore BK, Dedman JR, Zeidel ML (2003) Annexin A4 reduces water and proton permeability of model membranes but does not alter aquaporin 2-mediated water transport in isolated endosomes. J Gen Physiol 121:413–425
Hoekstra D, Buist Arkema R, Klappe K, Reutelingsperger CP (1993) Interaction of annexins with membranes: the N-terminus as a governing parameter as revealed with a chimeric annexin. Biochemistry 32:14194–14202
Hong K, Duzgunes N, Papahadjopoulos D (1981) Role of synexin in membrane fusion. Enhancement of calcium-dependent fusion of phospholipid vesicles. J Biol Chem 256:3641–3644
Hong K, Duzgunes N, Ekerdt R, Papahadjopoulos D (1982) Synexin facilitates fusion of specific phospholipid membranes at divalent cation concentrations found intracellularly. Proc Natl Acad Sci USA 79:4642–4644
Huber R, Schneider M, Mayr I, Romisch J, Paques EP (1990) The calcium binding sites in human annexin V by crystal structure analysis at 2.0 A resolution. Implications for membrane binding and calcium channel activity. FEBS Lett 275:15–21
Kimura Y, Ikegami A (1985) Local dielectric properties around polar region of lipid bilayer membranes. J Membr Biol 85:225–231
Kirsch T, Wuthier RE (1994) Stimulation of calcification of growth plate cartilage matrix vesicles by binding to type II and X collagens. J Biol Chem 269:11462–11469
Kohler G, Hering U, Zschornig O, Arnold K (1997) Annexin V interaction with phosphatidylserine-containing vesicles at low and neutral pH. Biochemistry 36:8189–8194
Lafleur M, Samson I, Pezolet M (1991) Investigation of the interaction between melittin and dipalmitoylphosphatidylglycerol bilayers by vibrational spectroscopy. Chem Phys Lipids 59:233–244
Meers P (1990) Location of tryptophans in membrane-bound annexins. Biochemistry 29:3325–3330
Meers P, Hong K, Papahadjopoulos D (1988) Free fatty acid enhancement of cation-induced fusion of liposomes: synergism with synexin and other promoters of vesicle aggregation. Biochemistry 27:6784–6794
Meers P, Hong K, Papahadjopoulos D (1991a) Annexin–phospholipid interactions in membrane fusion. In: Ohki S (ed) Cell, model membrane interactions. Plenum Press, New York, pp 115–134
Meers P, Daleke D, Hong K, Papahadjopoulos D (1991b) Interactions of annexins with membrane phospholipids. Biochemistry 30:2903–2908
Moss SE, Edwards HC, Crumpton MJ (1991) Diversity in the annexin family. In: Heizmann CW (ed) Novel calcium-binding proteins. Springer, New York, pp 535–66
Moss SE, Haigler HT, Schlaepfer DD, Horseman ND, Russo Marie R, Gerke V, Weber K, Burgoyne RD, Creutz CE, Comera C, Junker M, Kambouris NG, Klein JR, Nelson MR, Rock P, Snyder SL, Wang W, Pollard HB, Rojas E, Merezhinskaya N, Kuijpers GA, Srivastava M, Zhang Keck ZY, Shirvan A, Burns AL, Huber R, Berendes R, Burger A, Luecke H, Karshikov A, Dedman JR, Kaetzel MA, Hauptmann R, Reutelingsperger CP, Maki M, Shidara Y, Sato H (1992) The annexins. Portland Press, London, Chapel Hill
Ohki S, Arnold K (1990a) Surface dielectric constant, surface hydrophobicity and membrane fusion. J Membr Biol 114:195–203
Ohki S, Arnold K (1990b) Phospholipid vesicle fusion induced by cations and poly-(ethylene glycol). In: Glaser R, Gingell D (eds) Biophysics of the cell surface. Springer, New York, pp 193–219
Ohki S, Zschornig O (1993) Ion-induced fusion of phosphatidic acid vesicles and correlation between surface hydrophobicity and membrane fusion. Chem Phys Lipids 65:193–204
Piljic A, Schultz C (2006) Annexin A4 self-association modulates general membrane protein mobility in living cells. Mol Biol Cell 17:3318–3328
Raynal P, Pollard HB (1994) Annexins: the problem of assessing the biological role for a gene family of multifunctional calcium- and phospholipid-binding proteins. Biochim Biophys Acta 1197:63–93
Rescher U, Gerke V (2004) Annexins—unique membrane binding proteins with diverse functions. J Cell Sci 117:2631–2639
Schagger H, Von Jagow G (1987) Tricine-sodium dodecyl sulfate-polyacrylamide gel electrophoresis for the separation of proteins in the range from 1 to 100 kDa. Anal Biochem 166:368–379
Struck DK, Hoekstra D, Pagano RE (1981) Use of resonance energy transfer to monitor membrane fusion. Biochemistry 20:4093–4099
Sutton RB, Sprang SR (2000) Three dimensional structure of annexin IV. In: Seaton BA (ed) Annexins: molecular structure to cellular function. Springer, New York, pp 31–42
Swairjo MA, Concha NO, Kaetzel MA, Dedman JR, Seaton BA (1995) Ca(2+)-bridging mechanism and phospholipid head group recognition in the membrane-binding protein annexin V. Nat Struct Biol 2:968–974
Vanderkooi J, Fischkoff S, Chance B, Cooper RA (1974) Fluorescent probe analysis of the lipid architecture of natural and experimental cholesterol-rich membranes. Biochemistry 13:1589–1595
Weng X, Luecke H, Song IS, Kang DS, Kim SH, Huber R (1993) Crystal structure of human annexin I at 2.5 A resolution. Protein Sci 2:448–458
Zaks WJ, Creutz CE (1991b) Ca(2+)-dependent annexin self-association on membrane surfaces. Biochemistry 30:9607–9615
Zaks WJ, Creutz CE (1991a) Membrane fusion in model systems for exocytosis: characterization of chromaffin granule fusion mediated by synexin and calelectrin. In: Ohki S (ed) Cell and model membrane interactions. Plenum Press, New York, pp 325–340