Annexin V Interaction with Phosphatidylserine-Containing Vesicles at Low and Neutral pH†

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ABSTRACT: Annexin V belongs to a class of Ca^{2+}-binding proteins for which different functions in the cell are discussed, e.g., involvement in exocytosis, inhibition of protein kinase C, and calcium channel activity in cartilage matrix vesicles. All these functions are related to the ability of the annexins to bind to acidic phospholipids. In this study, the interaction of annexin V with large unilamellar vesicles (LUV) prepared from phosphatidylserine (PS) at low pH was compared to that at neutral pH. Annexin V strongly binds to PS LUV at low pH, whereas at neutral pH 20−100 μM Ca^{2+} are required to induce binding. This is caused by the different electric charge of the protein. The binding ability of the PS LUV for annexin V is higher at low pH. Binding of annexin V induces dehydration of the vesicle surface and a decrease of the lateral diffusion within the bilayer. While this dehydration is due to vesicle contact at pH 4, at pH 7.4 it is due to surface covering by annexin V. Annexin V promotes the phospholipid intermixing between LUVs at pH 5 and below but inhibits it at pH 7.4. A substitution of up to 80% of the PS by the uncharged phosphatidylcholine does not impair the extent of phospholipid intermixing at pH 4. The high binding capacity of PS LUV, the disappearance of the inhibiting action, and a calculated increase of the annexin V hydrophobicity make it likely that annexin V is able to penetrate into the membrane at low pH. At neutral pH, annexin V molecules act as steric barriers, preventing close apposition of two vesicles. At pH 5, annexin V lowers the threshold concentration of the Ca^{2+}-induced phospholipid intermixing. Such a promotion is well-known for annexin VII (synexin). The effect may be related to the isoelectric points of the two annexins which have been reported as 4.8 (annexin V) and 7.0 (annexin VII), respectively.

Annexin V belongs to a class of Ca^{2+}-binding proteins for which different functions in the cell are discussed, e.g., involvement in exocytosis, inhibition of protein kinase C, and calcium channel activity in cartilage matrix vesicles (for review, see ref 1). The crystal structure of annexin V and its Ca^{2+} binding sites have been described (*2*). With respect to the interaction of annexins with membranes in the presence of Ca^{2+}, two different classes within the family of annexins are distinguished: (1) annexins enhancing membrane interactions (I, II, IV, VII), and (2) annexins inhibiting membrane interaction (V, VI) (*3*, *4*). In the first group, the effect of the reduction of the Ca^{2+} concentration necessary to induce fusion of phospholipid (PL)[1] vesicles is of interest. Since the structural homology between the different annexins is high, the question arises which regions or structural requirements of the annexins are responsible for such a different behavior. Annexin V and a chimeric annexin, which consisted of the N-terminus of annexin I and the consensus region of annexin V, have been reported to differ in their influence on the leakage and aggregation of phosphatidylserine (PS) vesicles (*5*). A different binding strength of annexins V and I in the presence of Ca^{2+} to PL vesicles has been measured (*6*). Especially the interaction of annexin VII (also called synexin) with phospholipids has been studied in detail (*3*, *7*−*9*). One obvious difference between annexins V and VII is the isoelectric point, which is 7.0 for annexin VII (*10*), but 4.8 for annexin V (*4*). In this study, we address the question whether annexin V would have comparable properties close to its isoelectric point as annexin VII has.

For the description of the Ca^{2+}-induced interactions of PL membranes the concept of an increased membrane surface hydrophobicity in the process of adhesion of the vesicles has been suggested (*11*). In this paper, we will adopt these measurements on the interaction beween annexin V and PS-containing bilayers.

MATERIALS AND METHODS

Chemicals. Phosphatidylserine (PS) and dimyristoylphosphatidylcholine (DMPC) were purchased from Sigma (Deisenhofen, Germany). The purity was checked by thin layer chromatography. Recombinant human annexin V, purified from *Escherichia coli*, was obtained from Serva (Heidelberg, Germany). The *sn*-2 fluorophore-labeled PL pyrenedecanoyl phosphatidylcholine (Pyr-PC) was obtained from Molecular Probes (Eugene, OR). Dansyl phosphatidylethanolamine (DPE) was from Sigma (Deisenhofen, Germany); nitroben-

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[1] Abbreviations: DMPC, 1,2-dimyristoyl-*sn*-glycero-3-phosphatidylcholine; DPE, *N*-(6-dimethylaminonaphthalene-2-sulfonyl)-1,2-dihexadecanoyl-*sn*-glycero-3-phosphatidylethanolamine; HEPES, 2-[4-(2-hydroxyethyl)-1-piperazinyl]ethanesulfonic acid; LUV, large unilamellar vesicles; MLV, multilamellar vesicles; NBD-PE, *N*-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)-1,2-dihexadecanoyl-*sn*-glycero-3-phosphatidylethanolamine; PC, phosphatidylcholine; PL, phospholipid; PS, phosphatidylserine; Pyr-PC, 1-palmitoyl-2-(1-pyrenedecanoyl)-*sn*-glycero-3-phosphatidylcholine; RET, resonance energy transfer; Rh-PE, *N*-(lissamine rhodamine B sulfonyl)-1,2-dihexadecanoyl-*sn*-glycero-3-phosphatidylethanolamine; SUV, small unilamellar vesicles.

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zoxadiazole phosphatidylethanolamine (NBD-PE) and lissamine rhodamine B sulfonyl phosphatidylethanolamine (Rh-PE) were obtained from Avanti Polar Lipids (Alabaster, AL).

Vesicle Preparation. Multilamellar PL vesicles (MLV) were prepared using the method of Bangham et al. (*12*). The lipid was initially dried from chloroform, subsequently dispersed in buffer (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.

Fluorescence Measurements. All fluorescence assays were carried out on a Perkin-Elmer LS-50 B spectrofluorimeter (Beaconsfield, England). Vesicles were suspended in a 2.5 mL buffer solution, reaching a final concentration of 80 μM lipid. The recording started after the system had reached equilibrium (about 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. The agreement between different runs was well within a 10% error margin. For the 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 (RET) from protein tryptophan residues to the pyrene fluorophore of Pyr-PC (*13*). Emission spectra were recorded from 300 to 560 nm. The extent of the resonance energy transfer was calculated as the pyrene fluorescence intensity integrated over the wavelength range 370−560 nm, corrected as follows:

$$\mathrm{RET} = \frac{I_{\mathrm{Pyr}} - I^0_{\mathrm{Pyr}}}{I^0_{\mathrm{Pyr}}} \tag{1}$$

where I_{Pyr} is the pyrene fluorescence intensity and I^0_{Pyr} is the initial pyrene fluorescence in the absence of any tryptophan. Tryptophan fluorescence was not excited at its absorption maximum, but at 290 nm in order to minimize initial pyrene fluorescence.

Measurement of the Surface Dielectric Constant. DPE 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 400−600 nm. The wavelength at the maximum intensity of the emission spectrum is related to the dielectric properties of the probe environment, the wavelength increases with increasing dielectric constant (*14*). Dielectric constants can practically be calculated from an empiric law obtained by use of a set of organic solvents (*11*, *15*).

Lateral Phospholipid Diffusion. For consideration of the lateral PL diffusion within the membrane, Pyr-PC was mixed with phospholipids before evaporation at a concentration of 10% (mol/mol). Pyrene excimers (excited dimers) arise from collision of an excited monomer with a ground state monomer. Therefore, the excimer by monomer intensity ratio is proportional to both pyrene concentration and diffusion (*16*). We calculated the ratio of the emission intensities at 470 nm and 394 nm, which are the characteristic maxima of pyrene excimers and monomers, respectively (*16*). The ratio is related to the excimer formation rate $k_a c$ by

$$\frac{\Phi^{\mathrm{E}}}{\Phi^{\mathrm{M}}} = \kappa\frac{I_{470}}{I_{394}} = \frac{k_{\mathrm{f}}^{\mathrm{E}}}{k_{\mathrm{f}}^{\mathrm{M}}}\tau_0^{\mathrm{E}} k_{\mathrm{a}} c \tag{2}$$

where Φ are the quantum yields of excimer (E) and monomer (M) fluorescences, respectively, κ is a spectral conversion factor, I are the respective intensities, k_{f} are the fluorescence rate constants, τ_0 is the natural lifetime of the excited state, k_{a} is the second-order rate constant of the excimer formation, and c is the probe concentration within the membrane. In order to use intensities instead of quantum yields, κ was introduced. We determined its value as 1.64. The diffusion coefficient can be calculated from k_{a} by use of a "random walk" model for the 2-D PL diffusion. For details refer to ref 17. Briefly, this model considers the PL diffusion as random jumps of the molecules between neighbored lattice sites. The diffusion coefficient is determined from jump length and jump frequency.

Phospholipid Mixing Assay. The mixing of phospholipids was followed by the fluorescence energy transfer method, using NBD-PE and Rh-PE (*18*). The vesicles were composed of PS and 1% (mol/mol) each of both NBD-PE and Rh-PE. One part of the fluorophore-labeled vesicles and four parts of unlabeled vesicles were suspended in the appropriate buffer solution (with/without annexin). The fluorescence measurements were carried out by exciting at 470 nm and recording the fluorescence in the wavelength range from 500 to 620 nm. We modified the calculation method as follows: the extent of phospholipid mixing M was evaluated from the ratio of the fluorescence intensities of NBD-PE at 520 nm and Rh-PE at 588 nm

$$r = \frac{I_{520}}{I_{588}}$$

$$M = \frac{r - r^0}{r^{100} - r^0} \tag{3}$$

where r is the fluorescence amplitudes' ratio after addition of the fusogen, r^0 is the ratio before addition, and r^{100} 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.

RESULTS

Binding of Annexin V to PS LUV. The annexin V molecule contains only one tryptophan residue at the 187 position of the peptide chain (*1*). This tryptophan is located at one loop of the third repeat and thus situated close to the Ca^{2+} binding face of the molecule. Therefore, it is promising to study the possible fluorescence resonance energy transfer from this tryptophan to pyrene probes residing in the phospholipid bilayer. We used the chain-labeled Pyr-PC, the fluorescent moiety of which is preferentially located in the hydrophobic region of the membrane (*19*). The reso-

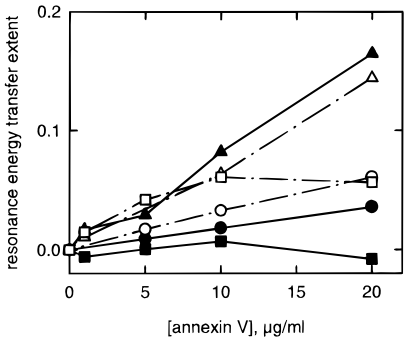

FIGURE 1: Binding of annexin V to PS LUV as monitored by the energy transfer from annexin V Trp187 to Pyr-PC incorporated at a 1:9 ratio in PS LUV. The applied pH was 7.4 (squares), 5.0 (circles), or 4.0 (triangles). Closed symbols refer to addition of only annexin V, open symbols of annexin V and, subsequently, 40 μM $CaCl_2$. For the calculation of the RET extents, see Materials and Methods.

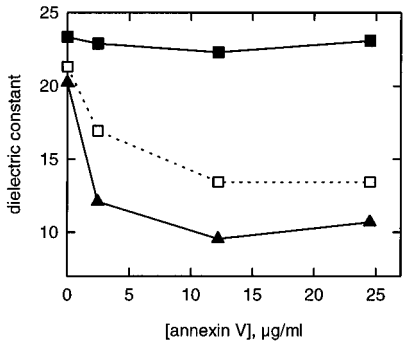

FIGURE 2: Surface dielectric constant of PS LUV after addition of annexin V at pH 7.4 (closed squares), at pH 7.4 in the presence of 200 μM Ca^{2+} (open squares), and at pH 4.0 (triangles). Values were calculated from the emission blue shifts of DPE.

nance energy transfer from Trp187 to Pyr-PC should be able to monitor the binding of annexin V to the bilayer, because the corresponding Förster distance is 27−31 Å (*20*).

At pH 4.0, we found a large energy transfer which was nearly proportional to the annexin V concentration up to 20 μg/mL (Figure 1). At pH 5.0, much less energy transfer occurred whereas, at pH 7.4, transfer totally disappeared. However, subsequent addition of 40 μM $CaCl_2$ resulted in a significant energy transfer even at pH 7.4 with an annexin V concentration dependence in a saturation-like manner rather than linear. At pH 5.0, addition of $CaCl_2$ induced only a slight enhancement of the transfer extent, whereas, at pH 4.0, the extent was not enhanced. It should be noted that the calculation of the energy transfer extent was corrected for the pH dependence of the initial tryptophan fluorescence.

Membrane Surface Hydrophobicity. In order to determine changes in the surface dielectric constant of the vesicles caused by annexin V, we used the shift of the emission wavelength of DPE (*11*). Figure 2 shows the annexin V-induced DPE shifts in PS LUV. At pH 4.0 in the presence of annexin V, a strong blue shift of the emission maximum was observed. This indicates a strong dehydration of the surface of the PS vesicles by annexin V. Already as little concentrations as 2.5 μg/mL annexin V resulted in potent surface dehydration. At pH 7.4, no shift of the DPE fluorescence was observed, which is in agreement with the fact that no annexin V binding occured. DMPC LUV did not show any DPE shift after addition of 0−24.5 μg/mL annexin V neither at pH 7.4 nor at pH 4.0 (data not shown).

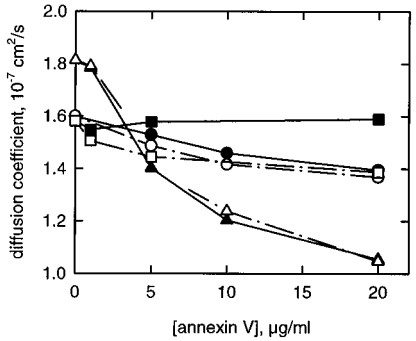

FIGURE 3: Lateral diffusion coefficient of Pyr-PC incorporated at a 1:9 ratio in PS LUV. The applied pH was 7.4 (squares), 5.0 (circles), or 4.0 (triangles). Closed symbols refer to addition of only annexin V, open symbols of annexin V and, subsequently, 40 μM $CaCl_2$.

Addition of 100 μM Ca^{2+} to PS LUV in the presence of annexin V induced a large blue shift of the emission peak. This blue shift was enlarged with increasing annexin V concentration. The surface dielectric constant decreases dramatically if water-free contacts of two vesicle surfaces are formed in the aggregation state (*11*). Although annexin V did not induce vesicle aggregation (*5*), a strong DPE shift occurred in the presence of both annexin V and Ca^{2+}. This can be understood as a strong binding of annexin V which obviously creates zones of nearly water-free PS headgroups, too. Increasing the Ca^{2+} concentration to 4 mM did not lead to a further blue shift (data not shown).

Membrane Lateral Diffusion. The lateral diffusion of the phospholipids within the membrane was quantified using the excimer formation of Pyr-PC (*16*). A strong binding of phospholipids to annexin V would cause a decrease of the overall diffusion rate resulting in a diminished excimer formation. On the other hand, a segregation of the differently charged phospholipid species, i.e., PS and Pyr-PC, would lead to a clustering of the probe molecules with an enhanced excimer formation.

We found a strong decrease of the excimer by monomer intensity ratio at pH 4.0 after addition of annexin V to the vesicle suspension (Figure 3), indicating a largely reduced lateral PL diffusion rate. Subsequent addition of $CaCl_2$ did not alter the intensity ratio. At pH 5.0, attenuated effects were obtained. At pH 7.4, annexin V did not alter the diffusion rate itself, only addition of 40 μM $CaCl_2$ produced an alteration comparable to that at pH 5.0. A similarly reduced diffusion rate was reported for annexin IV (*21*).

Phospholipid Intermixing of PS LUV at Different pH. In order to evaluate the influence of annexin V on the Ca^{2+}-induced PL intermixing, we measured the mixing extents at various pH in the presence of different concentrations of annexin V. PL intermixing was followed using the NBD-Rhodamine assay (*18*).

Ca^{2+} alone induces PL intermixing if added at critical concentrations. The fusion kinetics and, therefore, the critical Ca^{2+} concentration depends on the temperature (*22*), the membrane curvature (*23*), the PL species used (*24*), and the ionic strength of the buffer (*25*). The threshold Ca^{2+} concentration for PS LUV (0.1 μm diameter) was described to be about 1 mM (*11*). At decreased pH, the critical Ca^{2+} concentration increased (cf. Figures 4−6, closed symbols). Similar observations for the pH dependence of the Ca^{2+}-induced PL intermixing were made for PA SUV (*26*).

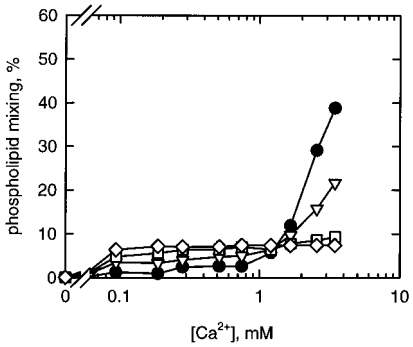

FIGURE 4: Ca^{2+}-induced PL mixing (NBD-PE/Rh-PE assay) of PS LUV at pH 7.4 in the presence of different annexin V concentrations: 0 μg/mL (closed circles), 2.5 μg/mL (triangles), 12.2 μg/mL (squares), and 24.5 μg/mL (diamonds).

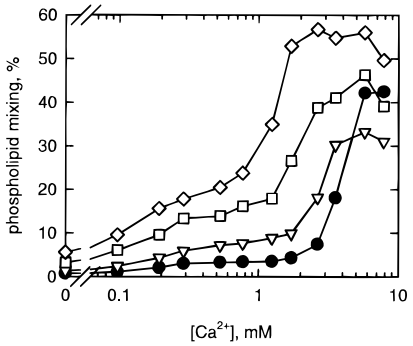

FIGURE 5: Ca^{2+}-induced PL mixing (NBD-PE/Rh-PE assay) of PS LUV at pH 5.0 in the presence of different annexin V concentrations: 0 μg/mL (closed circles), 2.5 μg/mL (triangles), 12.2 μg/mL (squares), and 24.5 μg/mL (diamonds).

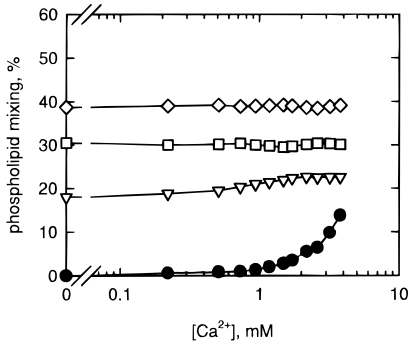

FIGURE 6: Ca^{2+}-induced PL mixing (NBD-PE/Rh-PE assay) of PS LUV at pH 4.0 in the presence of different annexin V concentrations: 0 μg/mL (closed circles), 2.5 μg/mL (triangles), 12.2 μg/mL (squares), and 24.5 μg/mL (diamonds).

Although H^+ themselves are able to induce PL intermixing [pH 3 (11)], they compete with Ca^{2+} for the membrane binding sites.

At pH 7.4, annexin V did not induce any PL intermixing in the absence of Ca^{2+} (Figure 4). After addition of 100 μM Ca^{2+}, annexin V was bound. The binding caused the NBD intensity to slightly increase and the rhodamine intensity to decrease, respectively. However, this effect was not due to PL intermixing. Experiments performed with only labeled vesicles showed similar alterations of the probe fluorescence (up to 7 % for 24.5 μg/mL annexin V, not shown) and revealed that this effect was an artifact of the assay. Such artifacts induced by 5 mM Ca^{2+} have been described (*27*). If the Ca^{2+} concentration exceeded the critical concentration, the PL intermixing was inhibited by annexin V. The extent of PL intermixing decreased with

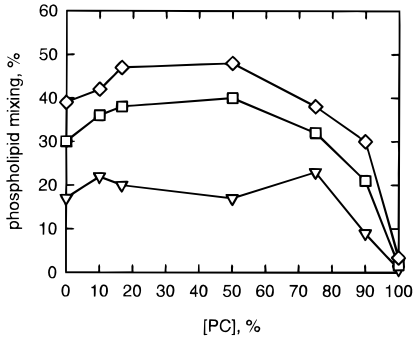

FIGURE 7: Annexin V-induced PL mixing (NBD-PE/Rh-PE assay) of PS/PC LUV at pH 4.0 in dependence on the PC contents. The applied annexin V concentration was 2.5 μg/mL (triangles), 12.2 μg/mL (squares), or 24.5 μg/mL (diamonds).

increasing annexin V concentration. This inhibiting action of annexin V is well-known (*3*, *4*).

At pH 5.0, PL intermixing occurred even in the absence of Ca^{2+} (Figure 5). Addition of 100−500 μM Ca^{2+} significantly enhanced the extent of mixing. This effect did not originate from an artifact. Control experiments yielded apparent mixing extents of 0% in the absence of Ca^{2+} and 2−5% in the presence of 500 μM Ca^{2+}, respectively. At higher Ca^{2+} concentrations, annexin V and Ca^{2+} exhibited a synergism with respect to PL intermixing. Annexin V reduced the critical Ca^{2+} concentration. This action was similar to that of annexin VII (*3*, *7*−*9*).

At pH 4.0, annexin V alone induced potent PL intermixing (Figure 6). There was almost no influence of Ca^{2+} on the mixing extent, even at the lowest annexin V concentration of 2.5 μg/mL. Obviously, the behavior of annexin V is completely different at acidic and at neutral pH. The turning point may be about pH 5. The electric charge is most likely that annexin V property which is responsible for this finding. The isoelectric point of annexin V is reported to be at 4.8 (*4*). To check whether the transition is reversible, we incubated annexin V for 10 min in pH 4 buffer solution. The pretreated annexin V was then added to PS LUV in pH 7.4 buffer. No difference between treated and untreated annexin V was observed (data not shown).

Phospholipid Intermixing of PS/PC LUV. Biological membranes usually consist of both charged and uncharged lipid species. Therefore, we studied the dependence of the mixing extent on the concentration of uncharged PL within the membrane. As a model, we used PS/PC LUV. Neither at pH 7.4 nor at pH 4.0 did DMPC LUV show PL mixing of more than 2% after addition of 0−24.5 μg/mL annexin V or 0−6.5 mM Ca^{2+} or both combined (data not shown). At pH 7.4, Ca^{2+} is necessary to induce the annexin V−PL interaction. Since the PC−Ca^{2+} interaction is weak, the PL intermixing induced by 4 mM Ca^{2+} completely disappeared if the LUV membrane contained 50% PC or more (not shown). Annexin V generally inhibited Ca^{2+}-induced PL intermixing at pH 7.4.

At pH 4.0, annexin V interacted with PS LUV in the absence of Ca^{2+}. The probable reason is that annexin V is positively charged itself. The dependence of the PL intermixing on the PC contents is shown in Figure 7. Annexin V did not induce PL intermixing of pure PC LUV. However, already small amounts of PS (10%) resulted in a significant PL intermixing. In the broad range 20−100% PS, the extent of mixing did not strongly depend on the PL charge. This

finding is very remarkable with respect to its possible biological relevance.

DISCUSSION

In the present work, the pH dependence of the annexin V interaction with negatively charged PL vesicles was studied. The binding of annexin V results in a change of the physicochemical properties of the membrane. At pH 7.4, a concentration of 20−100 μM Ca^{2+} is required in order to induce binding, dehydration of the PS headgroups, and reduction of the lateral PL diffusion within the membrane. This value agrees well with a threshold concentration of 16 μM Ca^{2+} for the binding between annexin V and PS (*28*). At pH 4.0, annexin V binds to PS LUV even in the absence of Ca^{2+}. Since annexin V does not bind to PC LUV, we conclude that the binding is driven by electrostatic forces at either pH. The charge of the annexin V molecule is positive at pH 4.0, but negative at pH 7.4.

Besides the electrostatic differences, the binding capacity of the PS LUV is different at low and neutral pH. At pH 7.4, the membrane is saturated at an annexin V concentration of about 10 μg/mL. In contrast, at pH 4.0, no such saturation behavior was found up to 20 μg/mL annexin V.

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 (*11*). A decrease of the surface dielectric constant was also found for the combined action of poly(ethylene glycol) and cations on vesicle fusion. The validity of this concept for the annexin V−Ca^{2+} system is supported by the observation, that the induction of PL mixing of PS LUV by annexin V at low pH is accompanied by a decrease in the hydration of the PL surface. This is not a unique effect of annexin V, also other proteins like lysozyme and melittin induce a decrease of the PL surface hydration (*29*, *30*). On the other hand, despite potent dehydration, the PL mixing remains weak at neutral pH. Therefore, we can assume that the adsorption of sufficient amounts of annexin V dehydrates the vesicle surface due to a covering by the protein rather than to an aggregation of two vesicles. The number of phospholipids influenced by one annexin V molecule has been calculated as to be 59 (*31*). Using this value, we obtain a vesicle coverage of some 33% for 10 μg/mL annexin V. This amount of annexin V is sufficient to produce a dehydration comparable to Ca^{2+}-induced aggregation, since in the latter case also only part of the vesicle surface is affected.

The binding of annexin V to the vesicles leads to a strong decrease of the lateral PL diffusion within the bilayer. At pH 4.0, the lateral diffusion coefficient decreases about 4 times as large as at pH 5.0. At the same time, the resonance energy transfer from Trp187 to Pyr-PC is, at pH 4.0, only 2.5 times as large as at pH 5.0. From this finding and from the observed PL intermixing at pH 4.0 (in contrast to inhibition at pH 7.4), we conclude a different binding geometry. Ca^{2+}-bound annexin V is located at the vesicle surface (*32*). NMR studies revealed a significant annexin V-induced effect on the PS head groups but not on the hydrocarbon-chain segmental motions (*33*). The Ca^{2+}-bound annexin molecules act as steric obstacles, preventing a close approach of the vesicles. In contrast, at pH 4.0, annexin V does not prevent vesicle approach. Obviously a considerable

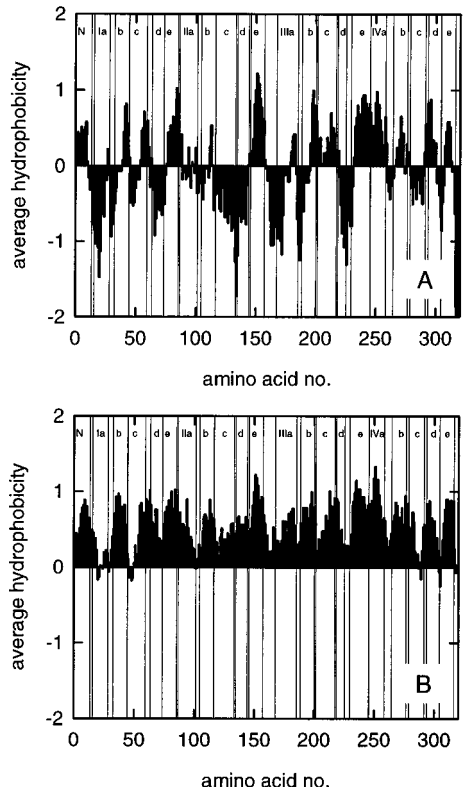

FIGURE 8: Hydrophobicity of annexin V at pH 7.4 (panel A) and pH 4.0 (panel B). The hydrophobicity values were calculated using the scale of Abraham and Leo (*34*). A gliding window with a length of 11 amino acids was used for avaraging. The corresponding elements of the secondary structure are indicated as follows: N refers to the N-terminal tail, I−IV refer to the four annexin repeats, and a−e refer to the five α-helices which each repeat consists of, respectively.

penetration of annexin V into the bilayer occurs, reducing the lateral PL diffusion more effectively and allowing the vesicle surfaces to come in contact.

Why should the positively charged annexin V be able to penetrate into the membrane but the negatively charged should not? Lowering the pH does not only change the electric charge of the molecule. Protonation of the acidic residues increases their hydrophobicity. Abraham and Leo introduced a hydrophobicity scale reflecting this change of polarity (*34*). Using that scale, we computed the hydrophobicities of the amino acid residues of annexin V at pH 7.4 and 4.0, respectively. As seen in Figure 8 the hydrophobicity of annexin V is considerably enlarged at pH 4. This may facilitate the insertion of annexin V into the hydrophobic core of a membrane.

While at pH 7.4 the Ca^{2+}-induced PL mixing of PS LUV is inhibited by increasing concentrations of annexin V, at pH 5.0 the Ca^{2+}-induced PL mixing extent is increased in the presence of annexin V. Close to the isoelectric point, Ca^{2+}, annexin V, and PS act synergistically: potent PL mixing reveals a strong interaction of annexin V and PS even at pH 5.0. At this pH, the net charge of annexin V is still negative, so that Ca^{2+} is required to mediate the interaction of annexin V with vesicles. Reaching the vesicle surface annexin V now may become positive since near the vesicle surface the H^+ concentration is increased [about 1.3 pH units lower than in bulk, calculated from a surface potential of −80 mV for PS (*35*)].

Annexin V has the lowest isoelectric point within the annexin family (*4*). Among other annexins, annexin VII has

been described to promote Ca^{2+}-induced fusion (*3*, *7*−*9*). Its isoelectric point is 7.0 (*10*). Annexin VII has been reported to enter and span target membranes in the presence of Ca^{2+} (*36*). In spite of the role of the N-terminal end (*5*, *37*), which is very variable between the different annexins (*1*), it appears that near the isoelectric points all annexins are able to penetrate into membranes and to support Ca^{2+}−PL interactions. From this point of view, the inhibiting action of annexin V would be caused by its low isoelectric point.

Our studies of the interaction of annexin V with phospholipids at pH 4.0 suggest that annexin V is able to penetrate into the PL bilayer. It is unlikely that pH 4.0 can be found *in vivo*. However, this behavior is maintained at pH 5.0 in the presence of Ca^{2+}. For matrix vesicle annexin (anchorin CII), which shares close homology with annexin V, a partitioning into the lipophilic milieu under acidic conditions has been described (*38*). From this finding, a hydrophobic structural change was concluded. This structural change should allow specific members of the annexin family to transverse the membrane in order to form Ca^{2+} channels, to assume proteolipid-like properties, or to become externalized.

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