Interaction of Annexins with Membranes: The N-Terminus as a Governing Parameter As Revealed with a Chimeric Annexin[†]

Dick Hoekstra,[*,‡] Rennie Buist-Arkema,[‡] Karin Klappe,[‡] and Chris P. M. Reutelingsperger[§]

Laboratory of Physiological Chemistry, University of Groningen, Bloemsingel 10, 9712 KZ Groningen, The Netherlands, and Department of Biochemistry, University of Limburg, 6200 MD Maastricht, The Netherlands

Received June 10, 1993; Revised Manuscript Received September 14, 1993[®]

ABSTRACT: The modulating effect of the variable N-terminus of annexins on the properties of these Ca^{2+}-binding proteins was investigated. To this end, the interaction of annexin V and a mutant annexin, I_NV_C, consisting of the N-terminus of annexin I (amino acids 1-45) and the core of annexin V (19-320), with large unilamellar phosphatidylserine (PS) vesicles was examined. In contrast to annexin V, the mutant annexin mediated Ca^{2+}-dependent aggregation of the lipid vesicles at neutral pH. However, annexin V induces Ca^{2+}-dependent aggregation at mild acidic pH. Moreover, both proteins can engage in hydrophobic interactions with PS vesicles, which results in release of the vesicle contents. These membrane-perturbing properties are expressed by both annexins in the absence of Ca^{2+} and occur at neutral and mild acidic pH. Interestingly, addition of Ca^{2+} inhibits annexin V-induced release, but sustains the release induced by the mutant annexin I_NV_C. The Ca^{2+}-dependent effects on the release of vesicle contents are reversed upon EDTA addition. Conformational changes revealed by binding of the hydrophobic probe, 4,4′-bis(1-anilino-8-naphthalenesulfonate), underly the observed Ca^{2+}-modulated effects on leakage. However, low-pH-mediated aggregation by annexin V does not seem to be related to macroscopic conformational changes. Annexin I_NV_C also affects Ca^{2+}-induced fusion of PS vesicles, displaying synergistic properties in conjunction with Ca^{2+} at neutral pH. By contrast, annexin V does not display similar properties at mild acidic pH, in spite of its ability to aggregate vesicles under such conditions. Since the cores of annexins V and I_NV_C are identical, the present results emphasize the role of the N-terminus in governing annexin-membrane interaction properties. It is furthermore of interest that, in addition, the properties of annexins might be regulated by pH, which would extend their physiological range of operation.

The annexins form a family of Ca^{2+}-dependent membrane-binding proteins, which are found in many tissues and which are thought to play important functional roles in numerous intra- and extracellular processes (Geisow et al., 1987; Drust & Creutz, 1988; Klee, 1988). Their putative functions include the ability to inhibit blood coagulation and phospholipase activity (Haigler et al., 1987; Reutelingsperger et al., 1985; Tait et al., 1988). Annexins also seem to be involved in cytoskeletal interactions (Ikebuchi & Waisman, 1990), while their potency in membrane binding has led to proposals concerning potential involvement in the aggregation and fusion of membranes (Drust & Creutz, 1988; Hong et al., 1981; Nir et al., 1987; Meers et al., 1988; Blackwood & Ernst, 1990). However, their exact physiological role remains largely obscure.

The structural features of annexins are gradually emerging. Thus far, some 12 different annexins have been identified. They share the structural homology of a conserved core consisting of four (or eight in the case of annexin VI) 60-80 amino acid repeat sequences and a 17 amino acid consensus sequence appearing in each repeat (Geisow et al., 1986). Highly characteristic for each annexin is its N-terminal domain (Geisow, 1986). Compositional variability of the latter domain is thought to be of crucial significance in dictating and governing (cell-)specific functions of annexins (Drust & Creutz, 1988). Their sensitivity toward Ca^{2+} also seems to be located in the variable N-terminus, as modulating effects directed toward this sequence also modulated Ca^{2+}-binding sensitivity (Drust & Creutz, 1988; Varticovski et al., 1988; Ando et al., 1989; Glenney, 1986; Huang et al., 1987; Schlaepfer & Haigler, 1987). Nevertheless, the N-terminus is not needed for binding to phospholipids, as this Ca^{2+}-dependent function appears to be located in the consensus sequence repeats (Glenney, 1986; Schlaepfer & Haigler, 1987). However, the ability to aggregate membranes after binding may well be related to a specific feature(s) of the N-terminus.

With artificial membrane vesicles, it has been shown that annexins can be subdivided into two functional catagories: one group stimulating and the other inhibiting or preventing the aggregation of the vesicles (Blackwood & Ernst, 1990; Tsao, 1990). Annexin I belongs to the former group, while annexin V is a member of the latter. Since the N-terminal domains of annexins I and V are directed away from the surface upon binding to a membrane, these domains could thus display the functional flexibility that might be involved in determining subsequent membrane aggregation (Meers, 1990; Hubert et al., 1990a). Whether the ability to trigger membrane aggregation after initial protein binding involves monomeric protein-lipid interactions, protein polymerization, and/or protein-protein interactions remains to be determined (Meers et al., 1988; Creutz et al., 1979; Mosser et al., 1991; Andree et al., 1990, 1992).

To further define the potential role of the N-terminus in this process, a chimeric annexin was constructed (Maurer-Fogy et al., 1988; Andree et al., 1993), consisting of the "active" N-terminus of annexin I (amino acids 1-45) and the

---

[†] C.P.M.R. was supported by a grant from the Royal Dutch Academy of Sciences (KNAW).

[*] Corresponding author (FAX 31-50-632728).

[‡] University of Groningen.

[§] University of Limburg.

[®] Abstract published in *Advance ACS Abstracts,* December 1, 1993.

0006-2960/93/0432-14194$04.00/0

C-terminus of the *aggregation-inactive* annexin V (amino acids 19-320). The hybrid annexin thus formed is defined as I_NV_C. Previously (Andree et al., 1993), it was shown that I_NV_C could bring about a Ca^{2+}-dependent attachment of small unilamellar lipid vesicles to planar stacked bilayers. Moreover, since zwitterionic phosphatidylcholine vesicles could also be cross-linked to the planar bilayers in this manner, but only after prior binding of the annexin to the planar membrane, it was proposed that I_NV_C may contain two binding sites. One site would be Ca^{2+}-dependent, while the other is supposedly hydrophobic in nature (Andree et al., 1993). The overall binding properties (e.g., binding kinetics and Ca^{2+} dependence) of annexin V and I_NV_C were similar, consistent with the notion that the initial, Ca^{2+}-dependent binding properties are primarily governed by the core of the molecule (Glenney, 1986; Schlaepfer & Haigler, 1987), which is identical for both proteins.

In the present work, we have used large unilamellar PS vesicles to examine the role of the annexin N-terminus in annexin–phospholipid interactions per se and its relevance in modulating intermembrane interactions. To this end, the properties of I_NV_C were compared with those of annexin V with regard to the abilities of both proteins to bind to vesicles, to induce leakage of vesicle contents, and to cause vesicle aggregation and fusion. *Large* unilamellar vesicles were employed to eliminate curvature-related artifacts frequently arising with *small* unilamellar vesicles. The involvement of protein conformational changes that could be required for each of these events to occur was also examined.

MATERIALS AND METHODS

Materials. Phosphatidylserine (PS,[1] bovine brain), *N*-(7-nitro-2,1,3-benzoxadiazol-4-yl)dioleoylphosphatidylethanolamine (N-NBD-PE), and *N*-(lissamine rhodamine B sulfonyl)-phosphatidylethanolamine (N-Rh-PE) were obtained from Avanti Polar Lipids (Birmingham, AL). 8-Aminonaphthalene-1,3,6-trisulfonic acid, disodium salt (ANTS), *p*-xylenebis-(pyridinium bromide) (DPX), and 4,4′-bis(1-anilino-8-naphthalenesulfonate), (bis-ANS) were from Molecular Probes, Inc.

Annexin V was prepared by cDNA recombinant techniques with plasmid pRH291 and purified as described previously (Maurer-Fogy et al., 1988). The mutant annexin I_NV_C was constructed as detailed elsewhere (Maurer et al., 1988; Andree et al., 1993).

Preparation of Lipid Vesicles. Large unilamellar PS vesicles were prepared following the reverse-phase evaporation procedure, as previously described (Hoekstra & Düzgünes, 1986). The vesicles were sized by extrusion through a polycarbonate filter to an average diameter of 0.1 μm. In some binding experiments, multilamellar PS vesicles were used. They were prepared by vortexing (5 min) after the addition of a buffer solution to the dried lipid film. The vesicle suspension was then sized to 0.4 μm as above, followed by centrifugation for 15 min at top speed in an Eppendorf table centrifuge. The pelleted fraction was used to determine annexin binding.

Annexin-Mediated Vesicle Aggregation. Annexin-mediated aggregation of PS vesicles was monitored by turbidity changes detected at 450 nm measured in an LKB Biochrom Ultrospec Plus spectrophotometer, equipped with a magnetic stirrer and a thermostated cuvette holder (Ter Beest & Hoekstra, 1993). Vesicles (100 nmol of lipid) were suspended in a buffer consisting of 140 mM NaCl/10 mM sodium acetate/5 mM HEPES (NAH buffer) at the indicated pH values (see figure legends). The reaction was initiated by adding annexins and Ca^{2+}. The divalent cation was present at a final concentration of 1 mM. The final incubation volume was 2 mL, and the incubation temperature was 37 °C.

Annexin-Mediated Lipid Mixing. Lipid mixing was monitored with an assay based on resonance energy transfer, employing the fluorescently tagged lipids N-NBD-PE and N-Rh-PE (Struck et al., 1981; Hoekstra, 1990a). The probes were incorporated into the vesicles at a density of 0.8 mol % each. Vesicles thus labeled were mixed with an equimolar amount of nonlabeled vesicles at a final lipid concentration of 50 μM in NAH buffer at the desired pH. Lipid mixing was initiated by adding annexin V or the mutant annexin I_NV_C, followed by Ca^{2+}, at a final concentration of 1 mM. The relief of energy transfer was monitored continuously by measuring NBD fluorescence (excitation and emission wavelengths were 465 and 530 nm, respectively). The fluorescence scale was calibrated by setting the initial fluorescence of the labeled vesicles to the zero level and the fluorescence obtained after the addition of Triton X-100 (1%, v/v), after correction for sample dilution and quenching of NBD fluorescence by the detergent, to 100%. It should be noted that, as described in detail elsewhere (Hoekstra, 1990a; Hoekstra & Düzgünes, 1993), the fluorescence level will rach 50% (on the scale of 100% as obtained by the detergent procedure) when all available surface area of a 1:1 mixture of unlabeled and labeled vesicles merges.

Asymmetrically labeled (with respect to NBD) vesicles were prepared by treating symmetrically labeled vesicles (1–2 mM) with sodium dithionite (20 mM for 20 min, at 37 °C). This treatment, which irreversibly destroys the NBD fluorescence by a reduction reaction (McIntyre & Sleight, 1991), eliminates the fluorescence signal derived from N-NBD-PE present in the outer leaflet. This can be inferred from the observation that the intensity decreases by approximately 50–60% (not shown). Subsequent addition of Triton X-100 also allows access of thionite to the inner leaflet, which is reflected by an immediate quenching of the remaining fluorescence. "Asymmetrically" labeled vesicles can be obtained by separating the treated vesicles from sodium dithionite by elution on a Sephadex G75 column. Lipid dilution experiments were carried out with this vesicle fraction, as described above.

Leakage Measurements and Contents Mixing. Release and mixing of contents were carried out using procedures based on the ANTS/DPX assay (Ellens et al., 1985). For leakage measurements, the fluorescent ANTS probe (25 mM in 100 mM NaCl, 10 mM sodium acetate, and 5 mM HEPES, pH 7.0) was mixed with its quencher, DPX (90 mM in 60 mM NaCl, 10 mM sodium acetate, and 5 mM HEPES, pH 7.0), at a ratio of 1:1 (v/v). The mixture was subsequently encapsulated in PS vesicles prepared as described above. Nonencapsulated material was removed by gel filtration on a Sephadex G100 column. Upon release of contents, the probes were diluted in exogenous medium, giving rise to instantaneous relief of the quenching of ANTS fluorescence. Calibration was done by setting the zero level with vesicles containing both ANTS and DPX and the 100% level (complete release)

---

[1] Abbreviations: PS, phosphatidylserine; N-NBD-PE, *N*-(7-nitro-2,1,3-benzoxadiazol-4-yl)dioleoylphosphatidylethanolamine; N-Rh-PE, *N*-(lissamine rhodamine B sulfonyl) phosphatidylethanolamine; ANTS, 8-aminonaphthalene-1,3,6-trisulfonic acid, disodium salt; DPX, *p*-xylenebis(pyridinium bromide); NAH buffer, 140 mM NaCl/10 mM sodium acetate/5 mM HEPES; bis-ANS, 4,4′-bis(1-anilino-8-naphthalenesulfonate); MLV, multiamellar vesicles; PC, phosphatidylcholine; LUV, large unilamellar vesicles.

after treatment of the vesicles with Triton X-100 (1%, v/v).

Contents mixing was done by preparing ANTS- and DPX-containing vesicles, respectively. These vesicles were freed from nonencapsulated compounds as described above. Upon fusion, the ANTS fluorescence becomes quenched when mixed with DPX. In the experiments, ANTS- and DPX-containing vesicles were mixed at a ratio of 1:1 and a final lipid concentration of 50 μM. The fluorescence scale was calibrated by setting the fluorescence of the ANTS-containing vesicles at 100% (0% fusion) and that of vesicles containing both ANTS and DPX (see above) at 0%. ANTS fluorescence was measured continuously at excitation and emission wavelengths of 384 and 530 nm, respectively. All measurements were done at 37 °C.

Miscellaneous Procedures. Labeling of Annexins with Bis-ANS (Rosen & Weber, 1969; Lambers et al., 1984). A stock solution of bis-ANS (0.6 mM) in NAH buffer was freshly prepared for each experiment. For protein labeling, an aliquot of the stock solution was mixed in a cuvette at a final concentration of 60 μM. Fluorescence emission spectra were recorded at an excitation wavelength of 365 nm (Lambers et al., 1984). Other experimental conditions are described in the figure legends.

Binding of Annexins to Lipid Vesicles. PS MLV, prepared as described above, were used for this purpose (Seppen et al., 1992). The lipid vesicles were suspended in NAH buffer at a final concentration of 50 μM. Annexins were then added, followed by Ca^{2+}. After an incubation period of 10 min at 37 °C, the mixtures were centrifuged in an Eppendorf table centrifuge at top speed. To quantify protein binding, protein was determined in both the pellet and supernatant fractions.

RESULTS

Effect of pH on Annexin-Induced Aggregation. As shown in Figure 1A, annexin V does not display a significant ability to induce the aggregation of vesicles at neutral pH, consistent with previously published results (Blackwood & Ernst, 1990; Oshry et al., 1991). However, depending on the protein concentration *and* the presence of Ca^{2+}, annexin V does cause the aggregation of large unilamellar PS vesicles at *mild acidic pH*. Binding experiments using multilamellar PS vesicles and separating bound and nonbound annexin by centrifugation revealed that the pH did not affect the extent of Ca^{2+}-mediated protein binding (not shown). Under similar conditions (Figure 1B), the hybrid annexin, I_NV_C, also induces vesicle aggregation. However, in contrast to annexin V, aggregation induced by I_NV_C is much more pronounced at neutral pH. As for annexin V-induced aggregation, annexin I_NV_C-induced aggregation requires the presence of Ca^{2+}. Note that the efficiency of aggregation, in terms of a requirement for a minimal protein concentration, is substantially higher for the mutant annexin.

Nature of the Annexin–Membrane Interaction. Effect of Ca^{2+}. To further characterize the nature of the interaction of annexin with the PS bilayer, we determined whether protein binding, as reflected by protein-induced aggregation of the vesicles, also resulted in membrane perturbation. To this end, an aqueous contents marker, ANTS/DPX, was entrapped in the vesicles. The release of the complex, which results in an immediate relief of fluorescence quenching of ANTS by DPX, was monitored continuously after the addition of either annexin V or the hybrid annexin, I_NV_C. As shown in Figure 2A, at both pH 5.0 and 7.0 the release of contents occurred upon the addition of annexin V. The rate and extent of release increased with increasing protein concentration. Furthermore, the leakage increased further when the pH was lowered. Hence, concomitant with an enhancement of annexin-induced aggregation, leakage becomes more extensive at mild acidic pH. However, in contrast to aggregation, the release of contents occurred in the absence of (exogenously added) Ca^{2+}. In this context, it should be noted that leakage was not affected by prior addition of EDTA (2 mM). In fact, when Ca^{2+} is added prior to or after initiating annexin V-induced leakage, the release of contents is either prevented or interrupted, respectively (Figure 3A). The inhibitory effect of Ca^{2+} is relieved, i.e., leakage resumes, when the divalent cation is complexed by the chelator EDTA. Furthermore, the effect of Ca^{2+} is seen, irrespective of the order of cation and protein addition.

Analogously, the mutant annexin I_NV_C also induces the leakage of vesicle contents in a protein concentration- and pH-dependent fashion. Thus, leakage increases at acidic pH and with increasing protein concentration (Figure 2B). As observed for aggregation, I_NV_C displays a much higher potency in inducing leakage than annexin V. Interestingly, in contrast to the inhibitory effect of Ca^{2+} seen for annexin V, the leakage potency of I_NV_C is maintained when Ca^{2+} is added to the system (Figure 3B). The divalent cation does not cause a substantial increase in the *rate* of leakage per se (compare curve a *vs* c in Figure 3B), but rather sustains the rate of release, which eventually results in the total release of vesicle contents. In the presence of Ca^{2+} (Figure 3B), the pH dependence of leakage induced by I_NV_C, as shown in Figure 2B, becomes less apparent since at both pH 5.0 and 7.0 massive leakage is seen when the divalent cation is present.

Annexin Mediates Ca^{2+}-Induced Lipid Mixing and Membrane Fusion. The ability of both annexin V and I_NV_C to cause aggregation and substantial membrane perturbation, as reflected by the release of vesicle contents, prompted us to examine whether these processes are accompanied by lipid mixing and, eventually, membrane fusion. Initial efforts to monitor fusion by means of aqueous contents mixing assays revealed a virtual lack of contents mixing. This could imply that fusion either did not occur or that, given the leakage induced by both annexins, the rate of release of contents is faster than the rate of contents mixing. As a measure of fusion, we therefore had to rely on the mixing of membranes monitored by a lipid mixing assay based on resonance energy transfer.

In the absence of Ca^{2+}, annexin V did not induce lipid mixing (not shown), consistent with the inability of annexins per se to induce lipid/membrane mixing. In the presence of 1 mM Ca^{2+}, annexin V-mediated lipid mixing was seen at pH 5.0, but not at pH 7.0. As shown in Figure 4A, at pH 5.0 the rate of lipid mixing was dependent on the concentration of annexin V. At relatively high concentrations the rate actually decreased, suggesting steric interference when the occupancy of protein at the vesicle surface increases. The maximal extent of lipid mixing was quite moderate, reaching approximately 5% of the maximal fluorescence level.

In the presence of Ca^{2+} and at pH values below 6.0, I_NV_C did not induce any significant lipid mixing. Between pH 6.0 and 8.0, lipid mixing was induced but no distinct optimum was apparent in the rate of I_NV_C-mediated, Ca^{2+}-induced lipid mixing in this pH range, which amounted to around 6%/min (not shown). As shown in Figure 4B, the rate did depend on the protein concentration. The trend of this dependence was similar to that observed for annexin V, in that the rate also decreases after having reached an optimal rate when the protein concentration is further increased. Note that the efficiency of I_NV_C to bring about lipid mixing in combination with Ca^{2+} is again more prominent than that

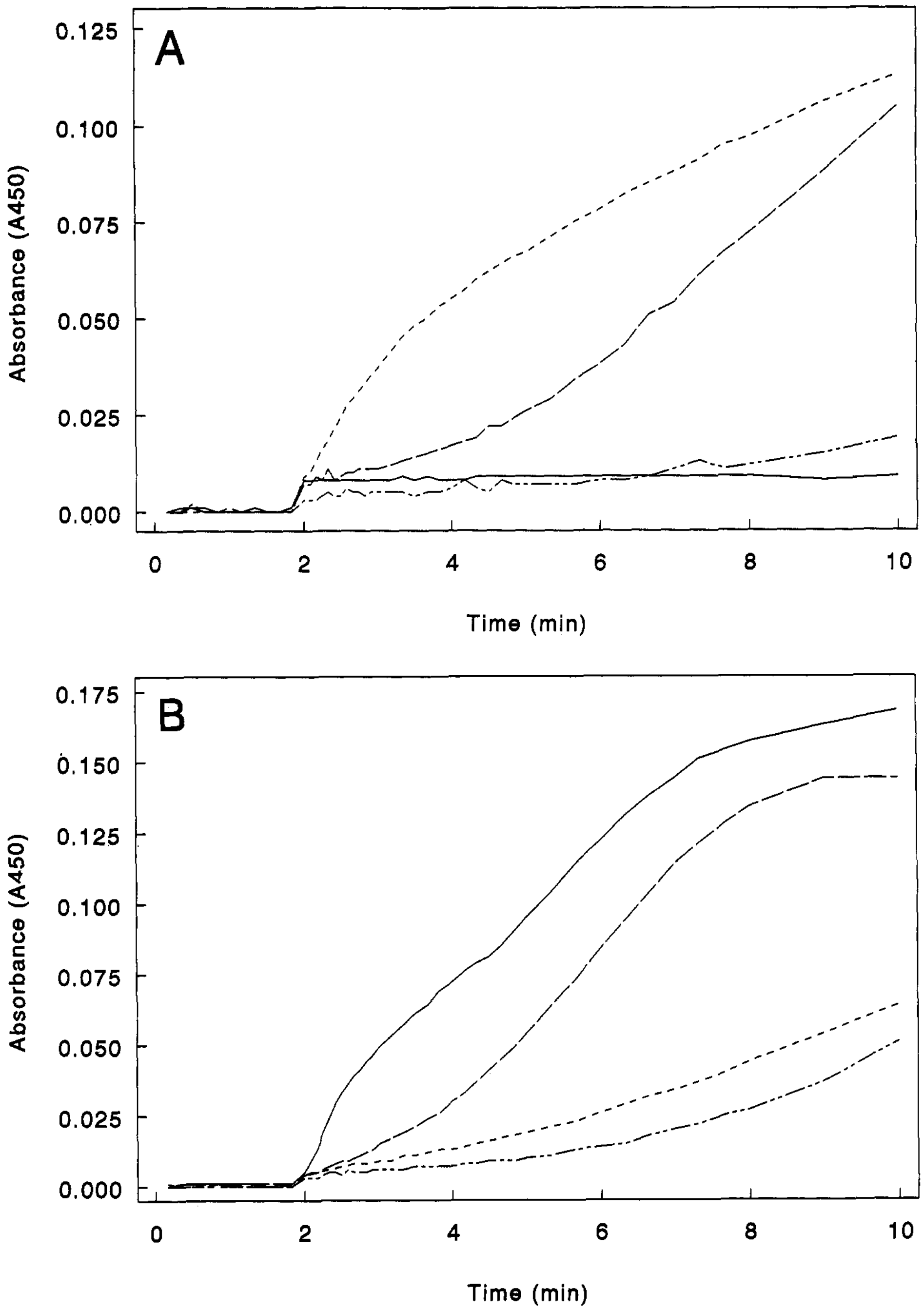

FIGURE 1: Annexin-induced aggregation of lipid vesicles. Effect of Ca^{2+} and pH. In A, annexin V was added to lipid vesicles (100 nmol of PS LUV) at $t = 0$; after 2 min, 1 mM Ca^{2+} (final concentration) was injected. Changes in turbidity, as a measure of aggregation, were monitored by following the absorbance at 450 nm. Conditions were as follows: pH 5.0 and 23 (– –), 18 (- - -), and 14 μg (– - - –) of annexin V; pH 7.0 and 23 μg of annexin V (—). In B, the results of a similar experiment are shown using I_NV_C. The protein was added at $t = 0$, and Ca^{2+} was included at $t = 2$ min. Conditions were as follows: pH 7.0 and 4.6 (—), 1.2 (- - -), and 0.6 μg (– - - –) of annexin I_NV_C; pH 5.0 and 4.6 μg (– –) of protein.

observed for the combined action of Ca^{2+} and annexin V. The final extent of lipid dilution reached in this case centered around 30% (not shown).

Finally, since neither 1 mM Ca^{2+} nor annexins alone cause any lipid mixing, it is evident that the proteins act as synergistic compounds in the process of Ca^{2+}-induced lipid mixing. Whether or not the mixing of lipids actually reflects the mixing of membranes was examined next. To this end, vesicles were prepared containing both N-Rh-PE and N-NBD-PE. The fluorescently labeled vesicles were subsequently treated with sodium dithionite to produce asymmetrically labeled vesicles (see Materials and Methods). As a result of this treatment, the NBD fluorescence diminished by about 50–60%, suggesting that the pool of N-NBD-PE, present in the outer leaflet of the vesicles, becomes (irreversibly) quenched. With these vesicles, the lipid mixing experiments as described above were repeated. Because of effective destruction of the NBD-labeled lipid in the outer leaflet, it is assumed that changes in resonance energy transfer potentially occurring as a result of lipid exchange between outer leaflets of labeled and nonlabeled vesicles will no longer take place. N-NBD-PE, still present in the *inner* leaflet, may in principle translocate to the outer leaflet where it will be quenched by N-Rh-PE, but upon subsequent exchange it may similarly give rise to an increase in NBD fluorescence. However, the overall kinetics of this process most likely would be different (i.e., slower) from those of fusion. It seems reasonable to assume, therefore, that if true fusion were to occur, the rate of lipid mixing determined with

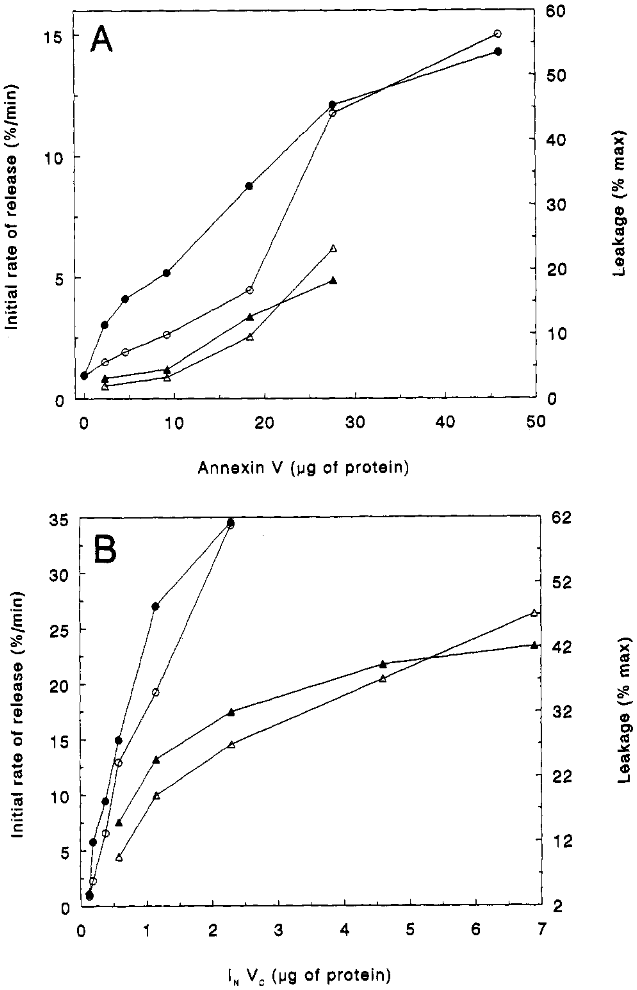

FIGURE 2: Interaction of annexins with PS membranes, as reflected by leakage of the vesicle contents. Effect of annexin concentration. Large unilamellar PS vesicles (100 nmol) containing the fluorescent complex ANTS/DPX as the aqueous contents marker were incubated with annexin V (A) or annexin I_NV_C (B), at either pH 5.0 (●, ○) or pH 7.0 (▲, △). The rate of leakage (○, △) was calculated from the tangents drawn to the leakage curves (see Figure 3), monitored as described in Materials and Methods, at time zero. The extent of leakage (●, ▲, right axis) was determined after the fluorescence development had leveled off, when no further change was observed. Note that the leakage experiments were carried out in the *absence* of Ca^{2+}.

asymmetrically labeled vesicles should be the same as that observed for symmetrically labeled vesicles. As shown in Table 1, addition of annexin I_NV_C and Ca^{2+} resulted in a rate of lipid mixing that was essentially indistinguishable from the type of vesicles used (symmetrically *vs* asymmetrically labeled). By contrast, significant lipid mixing with asymmetrically labeled vesicles in the presence of annexin V and Ca^{2+} was no longer detectable (Table 1), suggesting that lipid mixing observed for the symmetrically labeled vesicles was primarily due to lipid exchange. This extent of lipid exchange is about 5%, as noted above.

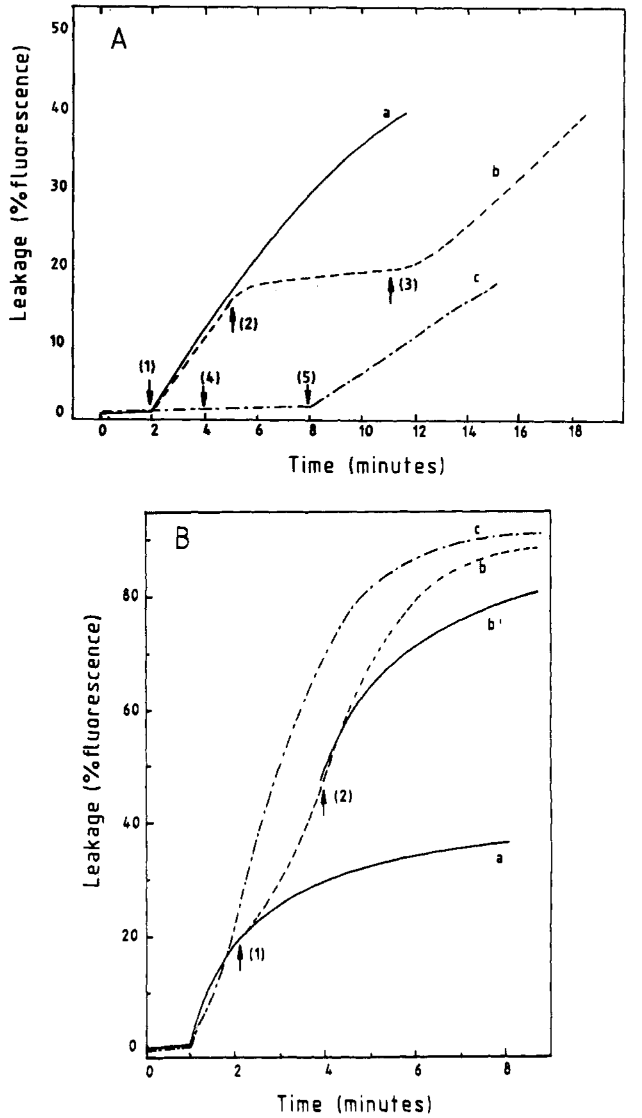

FIGURE 3: Effect of Ca^{2+} on the release of vesicle contents induced by annexin V (A) and annexin I_NV_C (B). Contents release was monitored by following the relief of ANTS fluorescence quenching by DPX, as described in Materials and Methods. Part A, curve a, shows the leakage of PS vesicle (100 nmol) contents induced by annexin V (18.4 µg, arrow 1) at pH 5.0, in the absence of Ca^{2+}. The experiment was repeated (curve b), and during release, Ca^{2+} (1 mM) was added (arrow 2, $t = 5$ min), followed by 2 mM EDTA (arrow 3). Curve c was obtained after sequential addition of the following compounds: 1 mM Ca^{2+} (arrow 1), 18.4 µg of annexin V (arrow 4), and 2 mM EDTA (arrow 5). Part B, curve a, shows annexin I_NV_C (4.6 µg of protein) induced release of contents from PS vesicles (100 nmol) in NAH buffer, pH 7. The protein was added at $t = 1$ min. When, during leakage, 1 mM Ca^{2+} is added (arrow 1, at $t = 2$ min), the release of contents proceeds (curve b); addition of EDTA (arrow 2) causes a moderate leveling of the rate (curve b′). Curve c is obtained when the hybrid annexin is preincubated with 1 mM Ca^{2+} prior to its addition to 100 nmol of PS vesicles at $t = 1$ min.

Role of Annexin Conformation. The three-dimensional structure of annexins can change when the free protein binds to phospholipid membranes [see, for example, Meers (1990)]. Conformational changes may govern and reflect the expression of particular protein properties, such as the ability to induce membrane perturbation and to cause aggregation and fusion. In this regard, annexin V and the mutant annexin I_NV_C display different properties, including distinctions in bringing about fusion and also with respect to the effect of Ca^{2+} on the protein-induced release of vesicle contents. To examine whether these functional distinctions between both proteins were related to structural parameters, we have investigated such potential changes using bis-ANS. This fluorescent probe is virtually nonfluorescent in aqueous solutions, but becomes strongly fluorescent when it is bound to hydrophobic sites in proteins (Rosen & Weber, 1969). Concomitantly, the fluorescence emission maximum shifts from 525 nm, in an aqueous environment, toward shorter wavelengths of around 500 nm when the probe senses a hydrophobic environment. In this way, a qualitative assessment was made of potential confor-

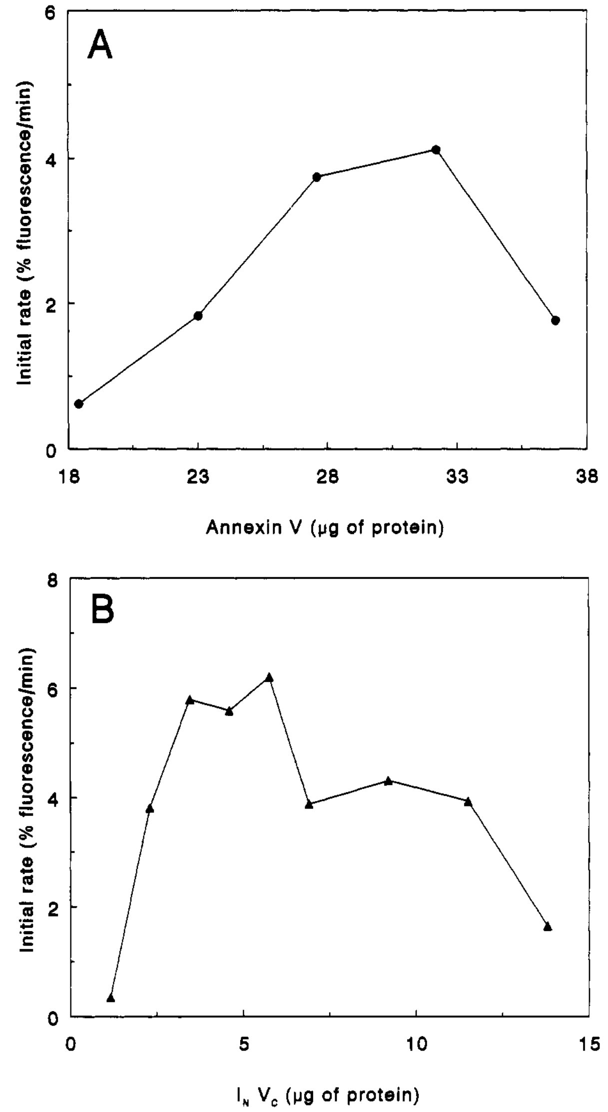

FIGURE 4: Annexin-mediated mixing of vesicle membrane lipids. Effect of the annexin concentration. Large unilamellar PS vesicles, labeled with equal amounts of N-Rh-PE and N-NBD-PE (0.8 mol % each), were mixed with nonlabeled PS vesicles as described in Materials and Methods. Either annexin V (A) or annexin I_NV_C (B) was then added at the indicated amount, followed by the addition of 1 mM Ca^{2+} (final concentration). The initial rate of lipid dilution was calculated and plotted as a function of the amount of protein added. The experiments in A were done at pH 5.0, those in B at pH 7.0 (see text). The incubation temperature was 37 °C, and the final incubation volume was 2 mL.

mational changes, induced in the annexins, in order to further define the structure–function relationship.

When annexin V or I_NV_C was injected into an aqueous solution containing bis-ANS, the fluorescence intensity increased considerably (about 10-fold), accompanied by a blue shift of the emission maximum (Figure 5A, spectrum b). When equal amounts of protein and a fixed concentration of bis-ANS (60 μM) were used, the maximal fluorescence values obtained for either annexin were identical, as were the emission maxima. This would suggest that the core of both proteins (which is identical) harbors the hydrophobic binding site(s) for the fluorophore. An additional effect on the spectral fluorescence properties was seen when the incubation medium also contained the lipid vesicles (Figure 5A, spectrum c). When PS vesicles were added to the bis-ANS-labeled proteins, an additional enhancement in the fluorescence intensity was seen. Also, a further downward shift of the emission maximum of about 12 nm became apparent. This suggests that additional binding sites become exposed on the proteins and/or that a further shift in conformation, hydrophobic in nature, occurs since the blue shift in the emission maximum would not be consistent with the availability of phospholipid binding sites for bis-ANS. Binding to the latter would have given rise to a red shift (Lambers et al., 1984). Subsequent addition of Ca^{2+} does not significantly affect the fluorescence spectra (Figure 5A, spectrum d). However, addition of Ca^{2+} to the vesicle-containing incubation medium prior to the addition of annexin V almost completely eliminates labeling of the protein with bis-ANS, given the strongly reduced fluorescence signal (Figure 5B spectrum d *vs* Figure 5A spectrum d). This effect, in turn, is completely reversed when Ca^{2+} is subsequently chelated with EDTA. Interestingly, when the same experiment was repeated for I_NV_C, only a minor effect of the prior addition of Ca^{2+} (i.e., relative to the addition of vesicles) was seen. Whereas in the case of annexin V, EDTA addition increases the fluorescence approximately 5-fold (Figure 5B, spectrum d *vs* e), an increase in bis-ANS fluorescence of only about 1.3-fold was obtained when EDTA was added to Ca^{2+}-pretreated annexin I_NV_C, which had subsequently been added to the vesicles. Note in this context that Ca^{2+} inhibits annexin V-induced leakage, whereas leakage induced by annexin I_NV_C is relatively unaffected by the divalent cation (Figure 3).

Table 1: Ca^{2+}-Induced Lipid Mixing, Mediated by Annexin V or I_NV_C, of Symmetrically *vs* Asymmetrically Labeled PS Vesicles[a]

	initial rate of lipid mixing (%/min)	
lipid vesicles	annexin I_NV_C	annexin V
symmetrically labeled	11.4 ± 2.0	1.68 ± 0.05
asymmetrically labeled	12.0 ± 0.6	0.0

[a] Equal amounts of fluorescently labeled and unlabeled PS vesicles (final concentration 50 μM) were mixed in NAH buffer (pH 7.0 for annexin I_NV_C; pH 5.0 for annexin V). Asymmetrically labeled vesicles were prepared by sodium dithionite treatment of the symmetrically labeled ones, as described in Materials and Methods. Lipid mixing was induced by adding 4.6 μg of annexin I_NV_C or 18.4 μg of annexin V, followed by the addition of 1 mM Ca^{2+} (final concentration). The initial rates were calculated from the tangents drawn to the fluorescence curves at time zero. Values are the means of three separate experiments.

Finally, when equal amounts of annexin I_NV_C or annexin V (23 μg) were incubated with 60 μM bis-ANS, the fluorescence intensities and emission maxima obtained at pH 7.0 or 5.0, either in the presence or absence of vesicles, were very similar (not shown). Hence, the distinction of pH-dependent aggregation of PS vesicles (Figure 1), with the mutant annexin I_NV_C inducing Ca^{2+}-dependent aggregation at neutral pH whereas annexin V induces this process at pH 5.0, does not appear to be related to macroscopic pH-dependent changes in the structure of either protein. Hence, if structural changes do occur, they do not appear to give rise to the exposure of previously buried hydrophobic binding sites/pockets for the fluorophore.

DISCUSSION

In the present work, we have demonstrated that the nature of the N-terminus of annexin molecules can have profound functional consequences with regard to the ability of these proteins to modulate intra- and intermembrane interactions. To reveal this functional significance, the interaction of annexin V and a hybrid annexin, I_NV_C, consisting of the core of annexin V and the N-terminus of annexin I, with PS vesicles was examined. The modulating properties of the N-terminus were revealed, among others, by the distinct differences in the

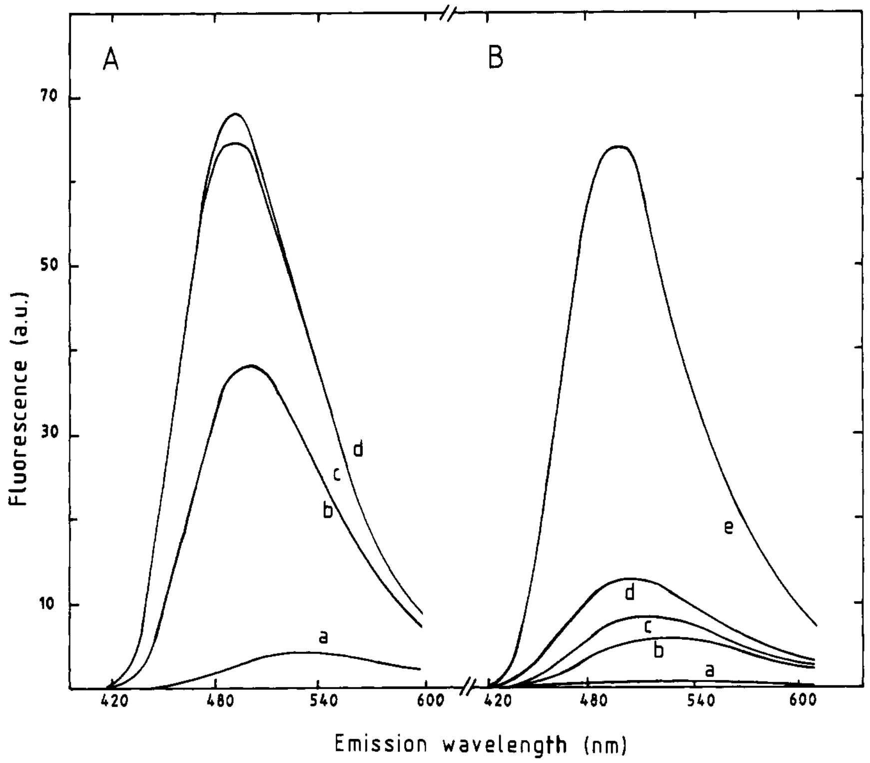

FIGURE 5: Alterations of annexin V structural properties, as monitored by changes in bis-ANS emission spectra. Emission spectra on the left (A) were recorded at an excitation wavelength of 365 nm at the following conditions: (a) 60 μM bis-ANS was mixed in NAH buffer, pH 7.0. Subsequently, 46 μg of annexin V was added (b), followed by (100 nmol) large unilamellar PS vesicles (c). Finally, 1 mM Ca^{2+} was added (d). Essentially the same spectrum as d is obtained when EDTA (2 mM) is added following the addition of the divalent cation (not shown). Emission spectra at the right (B) were recorded similarly and were sequentially monitored at the following conditions: (a) 100 nmol of PS vesicles were suspended in the buffer solution, followed by the addition (b) of 60 μM bis-ANS. Then 1 mM Ca^{2+} was added, giving rise to spectrum c. Subsequent addition of 46 μg of annexin V results in an emission spectrum indicated as in d, whereas spectrum e was obtained after the inclusion of EDTA (2 mM).

efficiency by which annexin V *vs* I_NV_C triggered protein-mediated effects when interacting with the lipid vesicles. Much lower protein concentrations were required for annexin I_NV_C than for annexin V to trigger a distinct effect, and given their similarities in binding properties (Andree et al., 1993; see below), it is evident that the amount of *bound* protein is not a crucial parameter. It thus follows that the N-terminus must be a determining factor in terms of the nature and efficiency of a certain protein-mediated effect.

Elsewhere (Ernst et al., 1991) it has been suggested that the first repeat sequence of annexins may play an important functional role in vesicle aggregation. This was inferred from studies in which a chimera consisting of the first repeat of annexin I and the core of annexin V was used. However, the significance of the N-terminus as a particularly relevant molecular factor is further supported by a number of observations in the present work. Thus, a distinct role of the N-terminus can be inferred from the annexin-induced leakage experiments (Figure 2). In these experiments, similar pH-dependent trends were observed for leakage induced by both annexins, provided that Ca^{2+} was not included in the incubation mixture (Figure 3). Yet when bound as a result of Ca^{2+} addition, the leak-inducing capacity of both annexins becomes entirely different, with annexin I_NV_C-induced leakage becoming more potent (see below) while annexin V-induced leakage is inhibited (Figure 3). In this context, it is then interesting to note that previous work has shown that annexins V and I_NV_C have similar phospholipid-binding properties (Andree et al., 1993). This is consistent with the notion that the core of annexins is particularly important for Ca^{2+} binding and subsequent interaction of the annexin with phospholipids (Glenney, 1986; Huang et al., 1987; Schlaepfer & Haigler, 1987).

Furthermore, as opposed to annexin V, the hybrid annexin appears to be capable of mediating Ca^{2+}-induced fusion (Table 1). Opposing effects, likely related to the difference in N-termini, are equally apparent for aggregation at neutral pH, where I_NV_C induces Ca^{2+}-dependent aggregation whereas annexin V-mediated aggregation is negligible (Figure 1). The inability of annexin V to mediate aggregation is consistent with the findings of others (Blackwood & Ernst, 1990; Oshry et al., 1991). However, of particular interest is the observation that annexin V-mediated aggregation *does* occur at mild acidic pH which, moreover, appears Ca^{2+}-dependent. Hence, apart from shifting from a non-aggregation- to an aggregation-inducing protein at mild acidic pH, annexin V maintains features with regard to the Ca^{2+}-dependent aggregation properties characteristic for annexins in general. The difference in pH dependence of the ability of both annexins to induce aggregation, or the distinction in pH-dependent aggregation-mediating properties of annexin V per se, could not be correlated to gross, pH-dependent structural changes, i.e., changes that would be reflected by the exposure of additional hydrophobic sites. The bis-ANS fluorescence signals were very similar for both proteins and were only marginally affected by pH (in the range pH 5.0–7.0).

Annexin II is the only annexin of which a similar, low-pH-enhancement of vesiclar aggregation has been reported, but this annexin also induces vesicle aggregation at neutral pH (Drust & Creutz, 1988). From a physiological point of view, pH-modulated activation of the properties of distinct annexins is of obvious interest, providing an additional regulatory mechanism in the expression of functional properties of some of these proteins.

The present work also reveals that different membrane interaction modes may exist for annexins. One mode involves stable adsorption, mediated by Ca^{2+}, which is required for aggregation to occur (Figure 1). The other involves a dynamic, Ca^{2+}-independent mode of interaction, which is presumably hydrophobic in nature. This dynamic interaction and its hydrophobic character are reflected by changes in membrane permeability and in spectral changes, typical for hydrophobic interactions, of bis-ANS upon interaction of annexin with PS vesicles *in the absence of Ca^{2+}* (Figure 5). Evidently, the hydrophobic mode of interaction is modifiable by Ca^{2+}, presumably as a result of stable membrane attachment of the annexins, but distinctly different for either one (Figure 3). This feature as such would suggest that an effect on the C-terminus, where Ca^{2+}-binding sites are located, can be propagated to the N-terminus, in analogy to previous reports on the reverse (Drust & Creutz, 1988; Ando et al., 1989; Schlaepfer & Haigler, 1987; see the introduction). Since the net effect of this binding on leakage is remarkably different for annexin V *vs* annexin I_NV_C (see Figure 3), and while it simultaneously indicates that annexin-induced aggregation and leakage are independent, unrelated processes, it is apparent that the N-terminus must be of crucial significance to this phenomenon. This significance could imply a direct involvement per se or a modulating effect on the structure of the C-terminus. Given the similarity in binding properties noted previously (Andree et al., 1993), the latter possibility is less likely.

The experiments further emphasize the conformational change that apparently may occur upon annexin binding to a bilayer [see also Meers (1990)]. In the case of annexin I_NV_C, the binding stabilizes the hydrophobic interaction mode, as it sustains the release of contents. It could be argued, however, that a leaky fusion event may contribute to the observed release. However, leakage is also strongly pronounced at mild acidic pH (Figure 2B), i.e., conditions at which fusion does not occur. Furthermore, upon the addition of EDTA, leakage does not abruptly halt, but continues at a rate similar to that in the absence of Ca^{2+}, thus suggesting the cation independence of the phenomenon. Finally, the rate of lipid mixing, as a measure of fusion, appears to be significantly slower than that of leakage (see also below). These arguments would thus further support a structural cause for the modulation of leakage rather than trivial events, for example, as a consequence of fusion. This conclusion is strengthened, particularly when one takes into account previous observations (Andree et al., 1993) showing that annexin I_NV_C can mediate the binding of PC vesicles to stacked planar bilayers only *after* prior Ca^{2+}-mediated binding of the hybrid annexin to the planar membrane. A similar secondary change in conformation after binding, exposing hydrophobic interaction sites, has been reported for annexin I (Tsao, 1990). In the presence of Ca^{2+}, the expression of a hydrophobic binding quality is severely reduced, but not eliminated, for annexin V (Figure 5B). Nevertheless, as a result, leakage is impeded. Release of contents resumes, however, when EDTA is added, and apparently, as reflected by the subsequent increase in fluorescence intensity, a minimal degree of hydrophobic capacity is "restored".

Our work does not allow conclusions as to the extent to which the annexins might penetrate into the bilayer in order to cause the release of contents. However, given that these interactions are too weak to cause aggregation (leakage occurs in the absence of aggregation, cf. Figure 1A *vs* Figure 2A), transmembrane penetration is unlikely, as has been suggested to occur for synexin, on the basis of single-channel current measurements (Burns et al., 1989). Channel activity has also been suggested for annexin V (Rojas et al., 1990), but its specificity becomes uncertain in light of the protein's ability to perturb the stability of membranes, as described in the present work.

Although annexin V induces aggregation of the lipid vesicles at mild acidic pH and membrane perturbations, these events are not accompanied by membrane fusion, although a limited degree of lipid mixing (approximately 5%) occurs at acidic conditions. In this context, it should thus be emphasized that at mild acidic pH, annexin V does not acquire nonspecific fusion-inducing properties, as has been reported for a variety of proteins at such conditions (Hoekstra, 1990b; Arnold et al., 1992). By contrast, the lipid mixing observed for the mutant annexin I_NV_C, which only occurs at pH >6, appears to reflect genuine fusion, as revealed by lipid mixing occurring with asymmetrically labeled vesicles (Table 1). Note that fusion is not induced by the protein, but requires the presence of Ca^{2+}. Since the concentration of Ca^{2+} used is not sufficient for triggering fusion itself, it would appear that the combined effect is synergistic, with the protein aggregating the vesicles. The closely associated vesicles will then readily allow formation of a fusion-inducing trans Ca^{2+}/PS complex, which can be formed at a lower Ca^{2+} threshold concentration in the presence of the protein.

Such synergistic effects have been observed for a variety of systems involving Ca^{2+} and a membrane-aggregating catalyst, including other annexin species (Drust & Creutz, 1988; Nir et al., 1987; Meers et al., 1988; Blackwood & Ernst, 1990; Hoekstra & Düzgünes, 1986; Hong et al., 1982). It is therefore not surprising to observe an inhibition of fusion, as reflected by lipid mixing, when the protein concentration increases beyond a certain concentration. Apparently, above this concentration bound protein molecules act as steric inhibitors, preventing the apposed bilayers from getting into "fusion-susceptible" contact. The inhibitory effect is seen for both annexins (Figure 4), indicating that the lipid transfer observed for annexin V also requires close intermembrane interaction. Aggregation proceeds normally and does not display a critical dependence of the concentration. This further supports a steric interference which, given that a simple monomeric binding mechanism has been described for annexin V (Tait et al., 1989), could be accomplished by the density-dependent crowding of monomers and/or the organization of monomers into a two-dimensional lattice formation on the vesicle surface (Meers et al., 1988; Andree et al., 1992; Zaks & Creutz, 1991; Newman et al., 1989). Whatever the precise mechanism, in both cases a sufficient narrowing of the intervesicular gap, allowing Ca^{2+} bridging, no longer occurs, thus eliminating (lipid transfer and) membrane fusion. Also, the fusion-mediating properties of annexins, i.e., at the level of inducing close intermembrane aggregation, are thus likely dominated by the nature of the N-terminus, allowing intermembrane interaction sufficiently close for a trans Ca^{2+}/PS complex to be formed. Evidently, at neutral pH the N-terminus of annexin I_NV_C provides such a possibility. By contrast, in spite of allowing aggregation, the annexin V terminus does not, although a limited degree of lipid mixing may occur.

The observed annexin/Ca^{2+}-mediated fusion event differs considerably from that of Ca^{2+}-induced fusion per se. In the latter case it has been shown that leakage of contents results from collapse of the vesicles, occurring as a consequence of fusion. We were unable to measure any contents mixing in the present system, suggesting, as noted above, that leakage occurs faster than the fusion event. Indeed, when comparing the kinetics of lipid mixing (Figure 2B) and those of contents release (Figure 4B), the rate of lipid mixing is 2–4-fold slower than that of leakage.

In summary, it appears that a variety of effects can be triggered by annexins when interacting with lipid membranes, which include aggregation, leakage, and fusion. These events are not necessarily related to each other, i.e., aggregation does not necessarily lead to fusion, nor does binding of annexins cause membrane perturbation (leakage). It appears, however, that many of these effects can be modulated by the N-terminus of the annexin, as indicated in the present work. Possibly, these modulating effects may in part result from tail-propagating effects into the phospholipid-interacting module of the core [see, for example, Hubert et al. (1990a,b)]. Of particular interest is also tht annexins can engage in hydrophobic interactions and that annexin V can display pH- and Ca^{2+}-dependent membrane aggregation properties.

ACKNOWLEDGMENTS

Martin ter Beest is gratefully acknowledged for helpful suggestions. The secretarial assistance of Aly Schirring and Rinske Kuperus is much appreciated.

REFERENCES

Ando, Y., Imamura, S., Hong, Y.-M., Owada, M. K., Kanunaga, T., & Kannag, R. (1989) *J. Biol. Chem. 264*, 6948–6955.

Andree, H. A. M., Reutelingsperger, C. P. M., Hauptmann, R., Hemker, H. C., Hermens, W. T., & Willems, G. M. (1990) *J. Biol. Chem. 265*, 4923–4928.

Andree, H. A. M., Stuart, M. C. A., Hermens, W. T., Reutelingsperger, C. P. M., Frederik, P. M., & Willems, G. M. (1992) *J. Biol. Chem. 267*, 17907–17912.

Andree, H. A. M., Willems, G. M., Hauptmann, R., Maurer-Fogy, I., Stuart, M. C. A., Hermens, W. T., Frederik, P. M., & Reutelingsperger, C. P. M. (1993) *Biochemistry 32*, 4634–4640.

Arnold, K., Hoekstra, D., & Ohki, S. (1992) *Biochim. Biophys. Acta 1124*, 88–94.

Blackwood, R. A., & Ernst, J. D. (1990) *Biochem. J. 266*, 195–200.

Burns, A. L., Magendzo, K., Shirvan, A., Meera, S., Rojas, E., Alijani, M. R., & Pollard, H. B. (1989) *Proc. Natl. Acad. Sci. U.S.A. 86*, 3798–3802.

Creutz, C. E., Pazoles, C. J., & Polard, H. B. (1979) *J. Biol. Chem. 254*, 553–558.

Drust, D. S., & Creutz, C. E. (1988) *Nature 331*, 88–91.

Ellens, H., Bentz, J., & Szoka, F. C. (1985) *Biochemistry 24*, 3099–3106.

Ernst, J. D., Hoye, E., Blackwood, R. A., & Mok, T. L. (1991) *J. Biol. Chem. 266*, 6670–6673.

Geisow, M. J. (1986) *FEBS Lett. 203*, 99–103.

Geisow, M. J., Fritsche, U., Hexham, J. M., Dash, B., & Johnson, T. (1986) *Nature 320*, 636–638.

Geisow, M. J., Walker, J. H., Boustead, C., & Taylor, W. (1987) *Biosci. Rep. 7*, 289–298.

Glenney, J. (1986) *J. Biol. Chem. 261*, 7247–7252.

Haigler, H. T., Schlaepfer, D. D., & Burgess, W. H. (1987) *J. Biol. Chem. 262*, 6921–6930.

Hoekstra, D. (1990a) *Hepatology 12*, 61a–66a.

Hoekstra, D. (1990b) *J. Bioenerg. Biomembr. 22*, 121–155.

Hoekstra, D., & Düzgünes, N. (1986) *Biochemistry 25*, 1321–1330.

Hoekstra, D., & Düzgünes, N. (1993) *Methods Enzymol. 220*, 15–32.

Hong, K., Düzgünes, N., & Papahadjopoulos, D. (1981) *J. Biol. Chem. 256*, 3641–3644.

Hong, K., Düzgünes, N., & Papahadjopoulos, D. (1982) *Biophys. J. 37*, 297–305.

Huang, K.-S., McGray, P., Mattaliano, R. J., Burne, C., Chow, E. P., Sinclair, L. K., & Pepinski, R. B. (1987) *J. Biol. Chem. 262*, 7639–7645.

Hubert, R., Schneider, M., Mayer, I., Römisch, J., & Paques, E.-P. (1990a) *FEBS Lett. 275*, 15–21.

Hubert, R., Römisch, J., & Paques, E.-P. (1990b) *EMBO J. 9*, 3867–3874.

Ikebuchi, N. W., & Waisman, D. M. (1990) *J. Biol. Chem. 265*, 3392–3400.

Klee, C. B. (1988) *Biochemistry 27*, 6645–6653.

Lambers, J. J. W., Terpstra, W., & Levine, Y. (1984) *Biochim. Biophys. Acta 789*, 188–196.

Maurer-Fogy, I., Reutelingsperger, C. P. M., Pieters, J., Bodo, G., Stratowa, C., & Hauptmann, R. (1988) *Eur. J. Biochem. 174*, 585–592.

McIntyre, J. C., & Sleight, R. G. (1991) *Biochemistry 30*, 11819–11827.

Meers, P. (1990) *Biochemistry 29*, 3325–3330.

Meers, P., Bentz, J., Alford, D., Nir, S., Papahadjopoulos, D., & Hong, K. (1988) *Biochemistry 27*, 4430–4439.

Mosser, G., Ravanat, C., Freyssinet, J.-M., & Brisson, A. J. (1991) *J. Mol. Biol. 217*, 241–245.

Newman, R., Tucker, A., Ferguson, C., Tsernoglou, D., Leonard, K., & Crumpton, M. J. (1989) *J. Mol. Biol. 206*, 213–219.

Nir, S., Stutzin, A., & Pollard, H. (1987) *Biochim. Biophys. Acta 903*, 309–318.

Oshry, L. Meers, P., Mealy, T., & Tauber, A. I. (1991) *Biochim. Biophys. Acta 1066*, 239–244.

Reutelingsperger, C. P. M., Hornstra, G., & Hemker, H. C. (1985) *Eur. J. Biochem. 151*, 625–629.

Rojas, E., Pollard, H. B., Haigler, H. T., Parra, C., & Burns, L. (1990) *J. Biol. Chem. 256*, 21207–21215.

Rosen, C.-G., & Weber, G. (1969) *Biochemistry 8*, 3915–3920.

Schlaepfer, D. D., & Haigler, H. T. (1987) *J. Biol. Chem. 262*, 6931–6938.

Seppen, J., Ramalho-Santos, J., de Carvalho, A. P., ter Beest, M., Kok, J. W., Pedroso de Lima, M. C., & Hoekstra, D. (1992) *Biochim. Biophys. Acta 1106*, 209–215.

Struck, D. K., Hoekstra, D., & Pagano, R. E. (1981) *Biochemistry 20*, 4093–4099.

Tait, J. F., Sakata, M., McMullen, B. A., Miao, C. H., Funakoshi, T., Hendrickson, L. E., & Fujikawa, K. (1988) *Biochemistry 27*, 6268–6276.

Tait, J., Gibson, D., & Fujikawa, K. (1989) *J. Biol. Chem. 264*, 7944–7949.

Ter Beest, M. B. A., & Hoekstra, D. (1993) *Eur. J. Biochem. 211*, 689–696.

Tsao, F. H. C. (1990) *Biochim. Biophys. Acta 1045*, 29–39.

Varticovski, L., Chahwala, S. B., Whitman, M., Cantley, L., Schindler, D., Chow, E. P., Sinclair, L., & Pepinsky, R. B. (1988) *Biochemistry 27*, 3682–3690.

Zaks, W., & Creutz, C. E. (1991) *Biochemistry 30*, 9607–9615.