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## Clustering of Lipid-bound Annexin V May Explain Its Anticoagulant Effect\*

(Received for publication, April 30, 1992)

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In 1985 we isolated a new vascular anticoagulant protein VAC $\alpha$ , now called annexin V, with a high binding affinity ( $K_d < 10^{-10}$  M) for phospholipids. Its anticoagulant effect was attributed to displacement of coagulation factors from the phospholipid membrane. The present study demonstrates that the inhibition of prothrombinase activity by annexin V strongly depends on the curvature of the membrane surface and on the calcium concentration. Half-maximal inhibition of prothrombinase on and binding of annexin V to small vesicles, composed of 20% phosphatidylserine and 80% phosphatidylcholine, requires 2–3 mM calcium. With large vesicles and planar bilayers considerably less calcium is required for inhibition of prothrombinase and for lipid binding. Half-maximal binding of annexin V to large vesicles and to planar bilayers occurs at 0.7 and 0.2 mM calcium, respectively. This seemingly confirms the displacement model. The displacement of coagulation factors, however, proved to be incomplete, with residual surface concentrations of factors Xa, Va, and prothrombin sufficient for effective production of thrombin. Cryoelectron microscopy revealed that annexin V binding to large vesicles caused planar facets, indicating the formation of large sheets of clustered annexin V. Apparently, the formation of these two-dimensional arrays is promoted by calcium and hampered by high surface curvature. It is speculated that the complete inhibition (>99%) of prothrombinase activity by annexin V is caused by the reduced lateral mobility of prothrombin and factor Xa in rigid sheets of annexin V covering the membrane.

Several steps in the complicated process of blood coagulation, for instance the activation of factor X by the factor IXa/factor VIIIa complex or the conversion of prothrombin (factor II) to thrombin (factor IIa) by the factor Xa/factor Va (prothrombinase) complex, are greatly stimulated by the adsorption of both the enzyme/cofactor complex and the substrate to phospholipid membranes. For instance, the thrombin-generating capacity of factor Xa is enhanced by four to six orders of magnitude after its assembly with factor Va on a lipid surface (see Ref. 1 for a review).

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Annexin V (formerly called vascular anticoagulant  $\alpha$  (2)) has a high calcium-dependent binding affinity for negatively charged phospholipids (3–5) and blood platelets (6). The protein was discovered by its anticoagulant effect (2). Since then it has been demonstrated to inhibit several phospholipid-dependent reactions, such as lipid degradation by phospholipase A<sub>2</sub> (7, 8), production of thrombin by the prothrombinase complex (2, 9–11), and activation of factor X by the tissue factor/factor VIIa complex (12–15). Annexin V does not inhibit factor Xa activity in the absence of phospholipids and does not bind to factor Xa (11). It also has no proteolytic activity (2). Therefore, it is generally assumed that annexin V inhibits blood coagulation by displacement of blood coagulation factors from the procoagulant phospholipid surface.

In the present study it is observed that, for low (3 mM) calcium concentrations, the inhibition of prothrombinase activity by annexin V is considerably less for small unilamellar vesicles (SUV)<sup>1</sup> than for large vesicles (LV), platelets, or planar bilayers. It is verified that indeed less annexin V binds to SUV than to planar bilayers. These findings seem to confirm the displacement concept, but a separate series of experiments demonstrates that displacement of coagulation factors from the phospholipid surface by annexin V is incomplete. The remaining surface concentrations of factor Xa, factor Va, and prothrombin are sufficient for effective production of thrombin. Annexin V thus seems to interfere with the conversion process itself. Using cryoelectron microscopy it was found that adsorption of annexin V on large phospholipid vesicles, with a low surface curvature, results in the formation of facets on the vesicles. Apparently, sheets of clustered annexin V molecules are formed on the surface, with sufficient rigidity to deform the bilayer. It is suggested that these clusters may provide a fencing mechanism, interfering with the lateral transport of prothrombin on the membrane toward the prothrombinase complex.

### MATERIALS AND METHODS

**Phospholipids**—1,2-Dioleoyl-*sn*-glycero-3-phosphatidylcholine (PC) was purchased from Sigma Chemical Co. 1,2-dioleoyl-*sn*-glycero-3-phosphatidylserine (PS) was bought from Avanti Polar Lipids. Unless stated otherwise a mixture of 20% PS/80% PC (PS/PC) was used. Small unilamellar vesicles (20–30 nm diameter, SUV) were prepared by sonication of a nitrogen-dried phospholipid mixture in buffer (16). Large vesicles (70–200 nm diameter, LV) were obtained by lipid extrusion through a 200-nm polycarbonate filter under moderate nitrogen pressure (17). Planar phospholipid bilayers were stacked on hydrophilic silicon slides (Wacker Chemie) by dipping the slides in a stirred-vesicles solution as described (18).

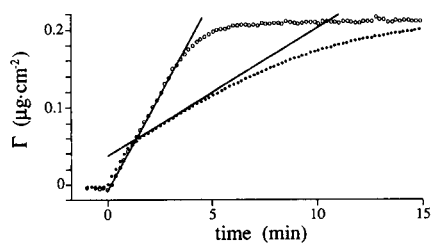
<sup>1</sup> The abbreviations used are: SUV, small unilamellar vesicles; LV, large vesicles; PC, 1,2-dioleoyl-*sn*-glycero-3-phosphatidylcholine; PS, 1,2-dioleoyl-*sn*-glycero-3-phosphatidylserine; PG, phosphatidylglycerol; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

**Buffers**—All experiments were performed at room temperature (20–22 °C) in a Tris-HCl buffer, pH 7.5, unless platelets were used. The Tris-HCl buffer, unless stated otherwise, contained 50 mM Tris, 100 mM NaCl, 3 mM Ca<sup>2+</sup>, and 0.5 g/liter bovine serum albumin (Sigma, A-7030 fatty acid free) to prevent protein depletion by adsorption to cuvette walls, stirrer etc. Experiments with platelets were performed in Hepes buffer, pH 7.4, containing 3 mM Ca<sup>2+</sup>, 137 mM NaCl, 2.7 mM KCl, 1.7 mM MgCl<sub>2</sub>, 10 mM Hepes, 25 mM glucose, and 0.5 g/liter bovine serum albumin.

**Proteins**—Human recombinant annexin V, prepared as described in Ref. 13, was a kind gift of Boehringer Ingelheim. Coagulation factors II, X, and V were purified from bovine plasma according to Lindhout (19). Factors X and V were activated with Russels Viper Venom-X (Sigma) (20) and thrombin (19), respectively. Concentrations of factor Xa preparations were determined by active-site titration with *p*-nitrophenyl-*p*'-guanidinobenzoate hydrochloride (ICN Nutritional Biochemicals). Low factor Xa concentrations were determined with a prothrombinase assay with excess factor Va (1 nM), 20% PS/80% PC vesicles (50 μM), and prothrombin (1 μM). Standard curves from 0.1 to 1 pM factor Xa yielded  $k_{cat}$  values of  $3850 \pm 350 \text{ min}^{-1}$  (mean  $\pm$  S.D.) at 37 °C. Prothrombin was incubated for at least 10 min with the indicated calcium concentration at 20 °C before the start of the experiment. Prothrombin concentrations were determined after activation with *Echis carinata* venom (Sigma). Prothrombinase activity was determined in serial samples quenched with 20 mM EDTA and 0.1 g/liter soybean trypsin inhibitor (Sigma). The cups were stored on ice until thrombin concentrations were determined with the chromogenic substrate S-2238 (Kabi), using a Cobas Bio spectrophotometer (Hoffmann-La Roche) at 37 °C (18). Factor Va concentrations were determined by assessment of prothrombinase activity with excess factor Xa (100 pM), vesicles (50 μM), and prothrombin (1 μM) at 37 °C.

**Measurement of Protein Adsorption on Planar Bilayers**—Binding of proteins to planar phospholipid bilayers was measured by ellipsometry (5, 21). Experiments were performed in a quartz cuvette filled with 5 ml of buffer and rendered hydrophobic by pretreatment with Sigmacote (Sigma SL-2). The solution was stirred with a Teflon-coated magnetic stirrer (length, 8 mm; diameter, 2 mm), rotating at 1800 rpm and separated 1–2 mm from the site of measurement on the slide.

**Measurement of the Adsorption of Annexin V on Vesicles**—The initial adsorption rate of annexin V on planar PS/PC bilayers is transport-limited and satisfies the relation:  $d\Gamma/dt = C_{bulk} \cdot \Delta$ , with  $\Gamma$  representing the surface concentration of protein ( $\mu\text{g} \cdot \text{cm}^{-2}$ ),  $C_{bulk}$  representing the bulk concentration of protein ( $\mu\text{g} \cdot \text{cm}^{-3}$ ), and  $\Delta$  representing the mass transfer constant ( $\text{cm} \cdot \text{s}^{-1}$ ), which depends on stirring conditions, the buffer viscosity, and the diffusion coefficient of the protein (5, 22). Experimentally this was verified for annexin V concentrations from 3 to 100 nM. These observations are used to estimate the annexin V binding to PS/PC vesicles through measurement of the reduction of the free annexin V concentration caused by binding to the lipid vesicles. This is illustrated in Fig. 1: without addition of lipid vesicles the adsorption rate is constant up to 75% of maximal surface coverage ( $\Gamma_{max}$ ). Addition of 1 μM PS/PC vesicles 1 min after the start of the adsorption results in a rapid decrease of the



**FIG. 1. Ellipsometric measurement of annexin V binding to vesicles.** At  $t = 0$  annexin V (15 nM) was added to the cuvette (○) and annexin V adsorption to a PS/PC bilayer was measured. After 1 min PS/PC SUV (1 μM) was added (●), and this resulted within 15 s after addition in a decrease of the adsorption rate from  $8.5 \times 10^{-4} \mu\text{g} \cdot \text{cm}^{-2} \cdot \text{s}^{-1}$  before vesicle addition to  $2.8 \times 10^{-4} \mu\text{g} \cdot \text{cm}^{-2} \cdot \text{s}^{-1}$ . Straight lines represent the best-fitting regression line to the annexin adsorption up to 1 min and the annexin adsorption 1 min after addition of the vesicles. The experiment was performed at room temperature (20–22 °C) in Tris-HCl buffer (pH 7.5, 50 mM Tris, 100 mM NaCl, 10 mM CaCl<sub>2</sub>, and 0.5 g/liter bovine serum albumin).

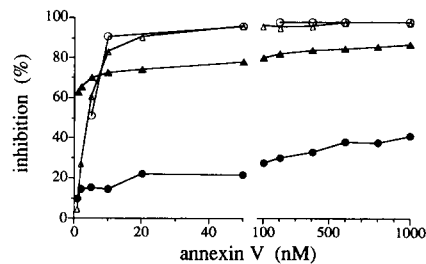
adsorption rate to about 30% of the initial adsorption rate. For an annexin V concentration of 15 nM and 1 μM PS/PC SUV the transient is completed within 10–15 s. Thereafter, a new, lower, steady state adsorption rate is established, corresponding to the new free annexin V concentration. Addition of excess lipid vesicles (10 μM PS/PC) to 15 nM annexin V at 3 mM Ca<sup>2+</sup> completely stops the adsorption. From these data it is concluded that the steady-state free annexin V concentration is rapidly established and that the quasi-steady-state adsorption rate observed after addition of vesicles is proportional to the free annexin V concentration. It is unaffected by lipid vesicles *per se* or by lipid-bound annexin V. In the binding experiments (presented in Fig. 3) the vesicles were added to the cuvette prior to the addition of annexin V, and the free annexin V concentration was determined using the calibration line mentioned before. Best accuracy is attained if binding to vesicles causes a reduction of the free annexin V concentration exceeding 25%. For experiments with 15 nM annexin V this required 1 μM SUV and 2 μM LV. Experiments with 100 nM annexin V were performed with 5 μM SUV.

**Measurement of Prothrombinase Activity on Blood Platelets**—Washed platelets were prepared as described (23). Stirred platelets ( $1.2 \times 10^6$  platelets per ml  $\approx$  0.1 μM phospholipid) were activated by incubation with 10 μM calcium ionophore A-23187 (Sigma) for 10 min. Since platelets contain factor V, a factor Xa-limited assay was used. Factor Va (1 nM) and factor Xa (10 pM) were allowed to bind for 5 min. Annexin V was added and after 2 min the reaction was started by addition of a final concentration of 1 μM prothrombin.

**Visualization of Vesicles**—Vesicles were visualized with the use of cryoelectron microscopy (24). No stains were used. A 700-mesh grid of 3.5-μm thickness was dipped into the vesicle suspension (PS/PC concentration, 5 mM). The adhering film was vitrified by rapid plunging into liquid ethane. Films were stored in liquid nitrogen and observed in a Gatan 626 cryoholder at  $-170$  °C with a Philips CM12 microscope. Electron micrographs were taken at 100 kV under low dose conditions, and 1–2-μm defocus was used to improve contrast.

## RESULTS

Fig. 2 presents the inhibition of prothrombinase activity on SUV (0.1 μM PS/PC), LV (0.1 μM PS/PC), planar bilayers (0.6 cm<sup>2</sup> in 5 ml of  $\sim$ 0.06 μM PS/PC) and platelets ( $1.2 \times 10^6 \text{ ml}^{-1}$  of  $\sim$ 0.1 μM phospholipid) at a calcium concentration of 3 mM as function of the annexin V concentration. Without addition of annexin the prothrombinase activities, expressed as moles of thrombin produced per mol of factor Va (Xa for platelets) per min were 1900, on SUV, 1400 on LV, 2200 on planar bilayers, and 1200 on platelets. Complete inhibition, with a half-maximal annexin V concentration of 4–5 nM, is observed for platelets and planar bilayers. Inhibition on SUV is incomplete with a maximal inhibition of only 40%, while



**FIG. 2. Inhibition of prothrombinase.** Inhibition of prothrombinase activity on planar bilayers (○), on activated platelets (Δ), and on small (●) and large vesicles (▲). Lipid concentrations were 0.06–0.1 μM. Prothrombinase activity was produced as follows. On planar bilayers 2 fmol/cm<sup>2</sup> prothrombinase was assembled by adsorption of 10 pM Va and 50 pM factor Xa during 7 min (18). The assembly was stopped by replacement of the buffer. Calcium ionophore-activated platelets were incubated during 5 min with 10 pM factor Xa and 1 nM factor Va. Vesicles were incubated during 5 min with 10 pM factor Va and 100 pM factor Xa. After assembly of prothrombinase annexin V was added to the final concentration indicated on the horizontal axis, and after 5–10 min prothrombin was added to start thrombin production. Calcium concentration was 3 mM, and the experiments were performed at room temperature.

for LV an intermediate inhibition of 90% is observed. Depletion of annexin V, due to lipid binding, plays only a minor role because 0.1  $\mu\text{M}$  vesicles or planar bilayers could maximally bind 1.2 nM annexin V (see below). The horizontal axis in Fig. 2 therefore represents, apart for this minor correction, the free annexin V concentration.

The disparity between prothrombinase inhibition on low curvature (planar bilayers and platelets) and high curvature (SUV) lipid is investigated further in Fig. 3 by comparison of prothrombinase inhibition by annexin V and lipid binding of annexin V as a function of the calcium concentration. The left panel shows that prothrombinase inhibition increases with increasing  $\text{Ca}^{2+}$  concentration and decreases with surface curvature. Ultimately, at 10 mM calcium, even for SUV complete inhibition of prothrombinase is observed. Measurement of prothrombinase activity was restricted to calcium concentrations above 1 mM as the prothrombinase complex tends to dissociate below this concentration. The right panel of Fig. 3 shows the parallel effect of calcium on lipid binding of annexin V. Binding at 10 mM  $\text{Ca}^{2+}$  was 12 mmol of annexin V/mol PS/PC for planar bilayers and SUV and 6 mmol of annexin V/mol PS/PC for LV. The half-maximal calcium concentration for inhibition as well as binding was 2–3 mM on SUV and 0.7 mM on LV. At 1 mM calcium prothrombinase inhibition on planar bilayers is still nearly complete (87%), and half-maximal binding on planar bilayers required only 0.2 mM calcium. Frequent lipid inclusions as observed for LV (see Fig. 7), caused a lower annexin V binding at 10 mM  $\text{Ca}^{2+}$  (5.5–6.3 mmol of annexin V/mol PS/PC). Therefore, titrations were performed with 2  $\mu\text{M}$  LV instead of 1  $\mu\text{M}$  SUV.

Fig. 4 demonstrates that annexin V is unable to completely displace factor Va from the phospholipid surface. In the control situation, with 2  $\text{fmol}\cdot\text{cm}^{-2}$  of factor Va adsorbed on the surface and 50 pM factor Xa in solution, a thrombin production of  $4.4 \pm 0.7 \text{ pmol}\cdot\text{cm}^{-2}$  was observed after addition of 1  $\mu\text{M}$  prothrombin. Complete coverage of the PS/PC bilayer with annexin V reduced the thrombin production to only 6% of the original conversion rate. As annexin V was removed from the buffer prior to the measurement of prothrombin conversion this demonstrates that surface-bound annexin V interferes with the conversion process. Thrombin generation after removal of annexin V from the surface and reconstitution of prothrombinase by addition of 50 pM factor Xa 7 min prior to prothrombin addition amounted to  $3.0 \text{ pmol}\cdot\text{cm}^{-2}$ . Thus 68% of the original factor Va adsorbed to the surface was retained after displacement by annexin V.

Fig. 5 shows that annexin V does not completely displace Factor Xa from the surface. About  $20 \text{ fmol}\cdot\text{cm}^{-2}$  of factor Xa was adsorbed on a planar bilayer during incubation with 50 pM factor Xa for 14 min. The cuvette was flushed to remove

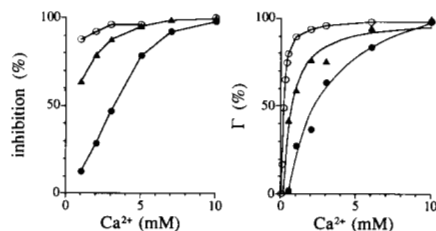


FIG. 3. Effect of calcium on inhibition of prothrombinase activity by annexin V and on binding of annexin V to PS/PC membranes. Left panel shows the extent of inhibition of prothrombinase on planar bilayers (○), SUV (●), and LV (▲) by 1  $\mu\text{M}$  annexin V as function of the calcium concentration. Experiments were performed under conditions as described in Fig. 2. Right panel shows the PS/PC binding of 100 nM annexin V to planar bilayers (○) and of 15 nM annexin V to 1  $\mu\text{M}$  SUV (●) and 2  $\mu\text{M}$  LV (▲).

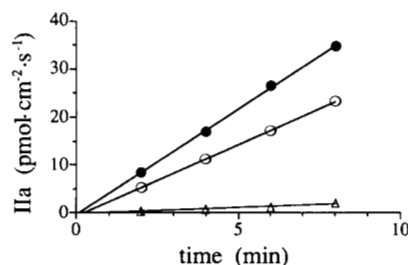


FIG. 4. Incomplete displacement of factor Va from the PS/PC membrane. Prothrombinase was assembled on the PS/PC bilayer as described in Fig. 2. Thrombin production after addition of 1  $\mu\text{M}$  prothrombin is shown in the control situation (●), after complete coverage of the lipid surface with annexin V (Δ), and when annexin V was removed after complete surface coverage (○). Complete surface coverage with annexin V was attained by adsorption of 100 nM annexin V during 5 min. This was followed by flushing with 50 ml of buffer containing 3 mM  $\text{Ca}^{2+}$  in order to remove annexin V from the buffer. Prior to addition of prothrombin the bilayer was incubated for 7 min with 50 pM factor Xa. Annexin V was removed from the PS/PC surface by flushing the cuvette with buffer containing only 50  $\mu\text{M}$   $\text{Ca}^{2+}$ , and thrombin production was measured after reconstitution of prothrombinase by incubation for 7 min with 3 mM calcium and 50 pM factor Xa prior to prothrombin addition.

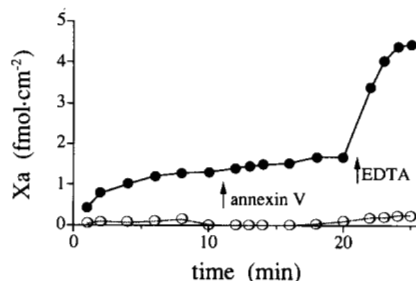


FIG. 5. Incomplete displacement of factor Xa from the PS/PC membrane. About  $20 \text{ fmol}\cdot\text{cm}^{-2}$  of factor Xa was adsorbed to a planar PS/PC bilayer. A mask, preventing exposure to air, was placed on the lipid membrane, and the slide was transferred to a clean cuvette. The release of factor Xa from the bilayer was measured by determination of the factor Xa concentration in serial samples from the buffer. After measurement of spontaneous release of factor Xa during 10 min 100 nM annexin V was added, and factor Xa release was followed for another 10 min. Finally, 3.5 mM EDTA was added in order to quantify the remaining membrane-bound factor Xa. Experiments on PS/PC (●) and pure PC (○) bilayers are shown.

free factor Xa and allowed to equilibrate for 7 min. During this period part of the factor Xa desorbs from the lipid surface. Regrettably, the accuracy of ellipsometry is insufficient to monitor these minute adsorbed quantities ( $\sim 1 \text{ ng}\cdot\text{cm}^{-2}$ ), but for a higher surface coverage of  $0.044 \mu\text{g}\cdot\text{cm}^{-2}$  ( $<10\%$  of  $\Gamma_{\text{max}}$ ) a value for the desorption rate constant,  $k_{\text{off}} = 0.15\text{--}0.3 \text{ min}^{-1}$  is observed. A mask was placed over the phospholipid membrane, preventing exposure to air, and the slide was transferred to a clean cuvette with buffer. This precaution is needed to prevent nonspecific release of factor Xa from the cuvette walls. As shown in Fig. 5, some factor Xa slowly desorbs spontaneously from the surface. Annexin V was added but hardly stimulated factor Xa release. Addition of EDTA, however, released  $2.7 \text{ fmol}\cdot\text{cm}^{-2}$  of factor Xa from the bilayer, and this quantity exceeds the surface concentration of factor Va used in the previous experiments. Thus the total release after transfer to the clean cuvette amounts to only  $4.7 \text{ fmol}\cdot\text{cm}^{-2}$ , i.e. 20–25% of the  $20 \text{ fmol}\cdot\text{cm}^{-2}$  initially adsorbed. This discrepancy is, however, only apparent due to neglect of factor Xa desorption during the 7-min equilibration, which amounts to 60–80% of the initially adsorbed quantity. A phospholipid

bilayer of 100% PC did not show factor Xa release, excluding nonspecific effects.

Binding of prothrombin to a phospholipid membrane fully covered with annexin V is demonstrated in Fig. 6. Annexin V was adsorbed to its maximal coverage of  $200 \text{ ng}\cdot\text{cm}^{-2}$  ( $5.7 \text{ pmol}\cdot\text{cm}^{-2}$ ), and prothrombin was added to a final concentration of  $1 \mu\text{M}$ . After 2 min, the extra adsorption of prothrombin was  $29 \pm 3.2 \text{ ng}\cdot\text{cm}^{-2}$  ( $0.4 \text{ pmol}\cdot\text{cm}^{-2}$ ). As discussed below, this surface concentration largely exceeds the concentration required for efficient thrombin production. For buffer concentrations in the micromolar range, similar effects as shown in Fig. 6 can be shown for factor Xa and factor Va. However, for picomolar factor Xa and Va concentrations, used in the assessment of prothrombinase activities, the adsorptions are correspondingly smaller and cannot be detected by ellipsometry.

Fig. 7 shows that annexin V forms sheets of clustered protein on surfaces with low curvature. The vesicles assumed bizarre, sharply edged shapes like rods, cubes, pyramids, etc. Some of the rods were straight up to 300 nm, with a deviation of less than 3 nm. Although the control vesicles contained occasional bulges or were bilamellar, they never displayed these straight sides. Vesicles in the presence of human serum albumin were not different from control vesicles.

## DISCUSSION

**Ellipsometric Determination of Annexin V Binding to Vesicles**—As explained in Fig. 1 the measurement of annexin V binding to vesicles consists of the determination of the free annexin V concentration in a suspension of vesicles. This

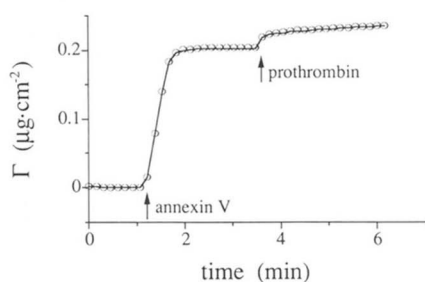


FIG. 6. Prothrombin adsorption to a PS/PC bilayer covered with annexin V. Adsorption of annexin V ( $100 \text{ nM}$ ) to a PS/PC bilayer was allowed to proceed until  $\Gamma_{\text{max}}$  was reached. About 1.5 min thereafter  $1 \mu\text{M}$  prothrombin was added, and the additional adsorption of at least  $30 \text{ ng}/\text{cm}^2$  was observed. Calcium concentration was  $3 \text{ mM}$ .

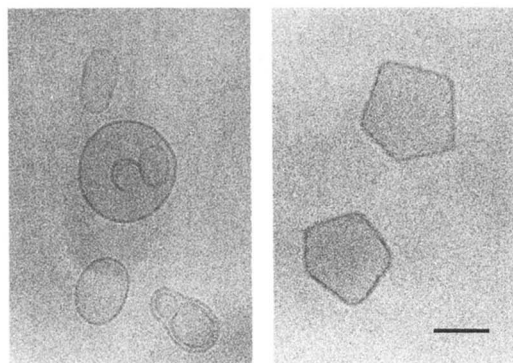


FIG. 7. Visualization of shape changes induced by annexin V binding to large vesicles. Samples of large vesicles, 70–200-nm diameter, and PS/PC concentration of  $5 \text{ mM}$ , were rapidly vitrified in liquid ethane, and electron micrographs were taken at  $-170 \text{ }^\circ\text{C}$ . The left panel shows vesicles without annexin V, and the right panel shows vesicles incubated for 10 min at  $20 \text{ }^\circ\text{C}$  with  $200 \mu\text{M}$  annexin V. Buffer contained  $3 \text{ mM}$  calcium. Bar indicates  $100 \text{ nm}$ .

concentration of free protein is determined by ellipsometry from the initial transport-limited adsorption rate to a PS/PC bilayer, which was calibrated by performing control experiments without added vesicles. This technique requires that the redistribution of annexin V between solution and vesicles is rapid compared to the duration of the initial phase of adsorption to the planar bilayer. This was demonstrated in Fig. 1 for  $1 \mu\text{M}$  PS/PC SUV and  $15 \text{ nM}$  annexin V.

**Incomplete Inhibition of Prothrombinase on Small Vesicles**—As was shown in Fig. 2, inhibition of prothrombinase by annexin V was incomplete on small phospholipid vesicles. This contrasts with results reported by other authors (2, 7, 10, 11), but in these studies higher calcium concentrations ( $5\text{--}10 \text{ mM}$ ) were used and vesicle size was not given. The poor inhibition of prothrombinase on SUV is correlated to a high calcium requirement for the binding of annexin V to these surfaces (Fig. 3). It seems as if the rigid clusters, shown in Fig. 7, are more difficult to form on highly curved surfaces.

**Calcium Requirement of Annexin V Binding**—The calcium concentration required for binding of annexin V is a function of the membrane content of anionic phospholipid such as PS (5). The PS in small vesicles may be preferentially located in the inner membrane leaflet, and this could explain the reduced binding of annexin V. However, for sonicated vesicles it has been estimated that PS concentration in the outer leaflet was only 30% reduced compared to a symmetrical distribution (25). This implies that in the SUV preparations used in this study the amount of PS in the outer leaflet would be about 13%. However, only a PS content below 5% could explain the increased calcium requirement (5). Furthermore, symmetrical distribution, or even preferential accumulation of phosphatidylglycerol (PG) in the outer leaflet, has been reported (26–29). Taking sonicated vesicles instead of planar bilayers, we found a similar shift in the calcium concentration required for half-maximal binding of annexin V to PG/PC vesicles (From  $0.15 \text{ mM}$  for 20% PG/80% PC planar bilayers to  $4.4 \text{ mM}$  for 20% PG/80% PC vesicles. Results not shown). It is concluded that asymmetrical distribution of lipids in the bilayer cannot explain the observed effects, and that surface curvature is probably more important.

Reduced binding of annexin V on SUV and LV could be caused by a lower binding affinity, a reduced number of binding sites, or both. For SUV we checked the effect of the free annexin V concentration on the binding to vesicles and observed that increasing the free annexin V concentration from 9 to  $70 \text{ nM}$  hardly affected the binding (data not shown). Thus the decreased binding reflects a decrease in the number of binding sites. This is consistent with the high binding affinity ( $K_d < 0.1 \text{ nM}$ ) reported for vesicles at  $1.2 \text{ mM Ca}^{2+}$  (4), a value two to three orders below the concentrations used in Fig. 3. The maximal surface coverage of  $2 \text{ mmol}$  of annexin V per mol of lipid, reported in the same study (4), is close to the value of  $2.2$  found in the present study for  $1 \text{ mM}$  calcium (c.f. Fig. 3).

**Displacement of Adsorbed Coagulation Factors by Annexin V**—Annexin V is able to displace  $>90\%$  of maximal surface coverage of the coagulation factors Xa, Va, and II. However, Figs. 4–6 show that the remaining amounts of adsorbed coagulation factors are sufficient for effective production of thrombin. For prothrombin this result is confirmed by the observation that prothrombin is able to compete for annexin V binding (4). The residual binding of more than  $400 \text{ fmol}\cdot\text{cm}^{-2}$ , is 10 times lower than the maximal binding of prothrombin of  $3500 \text{ fmol}\cdot\text{cm}^{-2}$  (30), but for the low prothrombinase activity on the surface it was estimated that a surface concentration of only  $2 \text{ fmol}\cdot\text{cm}^{-2}$  of prothrombin is required for

maximal production of thrombin (18). Similarly it was calculated that assembly of prothrombinase is already half-maximal for a surface concentration of  $0.44 \text{ fmol} \cdot \text{cm}^{-2}$  factor Xa, which is 5–6-fold below the quantity of factor Xa retained after displacement by annexin V (c.f. Fig. 5). It is concluded that the anticoagulant effect of annexin V cannot be explained by displacement of coagulation factors from the lipid surface.

**Formation of Ordered Clusters of Annexin V**—As demonstrated in Fig. 7 the cryoelectron micrographs revealed multifaceted phospholipid structures with sharp edges. These shape changes can only be explained by assuming that large clusters of annexin V induce surface deformation. Mosser *et al.* (31) have shown that annexin V binds on planar phospholipid monolayers in a two-dimensional array of repeated trimers. Their data could not confirm or reject the hypothesis that the clusters extend to include more than three protein molecules. The straight facets observed in the present study appeared to be limited by the size of the vesicles and sometimes measured more than  $100 \times 100 \text{ nm}$ . This involves up to 400 molecules of annexin V, as can be calculated from the mean area per annexin V molecule (5, 31). No changes in the shape of small sonicated vesicles in the presence of annexin V were observed, even at  $10 \text{ mM}$  calcium (data not shown). This may be due to the defocus of the objective lens ( $0.5\text{--}2 \mu\text{m}$ ) required for sufficient contrast. Using such defocus facilitates the recognition of straight facets but also results in a blurring of the image, which may obscure the high resolution information required to detect facets on small vesicles. However, if the vesicle diameter of  $20\text{--}30 \text{ nm}$  of SUV is compared to the average diameter of an annexin V molecule ( $5 \text{ nm}$ ), only  $50\text{--}100$  molecules of annexin V would fit onto one vesicle and the plane of adsorption of each annexin would make an angle of  $10\text{--}20^\circ$  with the planes of its neighbors. On large vesicles, the molecules adsorb in extended planes with an average angle between neighboring molecules of less than  $1^\circ$ . It is therefore likely that steric constraints counteract clustering of annexin V molecules on highly curved bilayers and this may cause the higher calcium requirement for annexin V binding observed in Fig. 3.

**Inhibition of Membrane-bound Transport of Prothrombin**—The data presented in Figs. 2–4 show that prothrombinase activity on PS/PC membranes can be completely abolished by annexin V, provided that annexin V binding to the membranes is maximal. Incomplete coverage with annexin V does not inhibit prothrombin conversion, and this excludes a direct interference of annexin V with either the assembly or the coagulation factors. This is illustrated in experiments similar to those shown in Fig. 4, where preadsorption of  $0.14\text{--}0.17 \mu\text{g}/\text{cm}^2$  annexin V ( $70\text{--}80\%$  of  $\Gamma_{\text{max}}$ ) to the PS/PC bilayer prior to prothrombinase assembly did not affect the prothrombin conversion rate (data not shown). These data, together with our observation that sufficient coagulation factors remain bound to the membrane for assembly of prothrombinase and for prothrombin conversion, suggest that the complete, closed-packed coverage of the surface in itself interferes with these processes (either the assembly and/or conversion). For other proteins, however, this is not supported by available data. Examples of poor inhibition in spite of complete surface coverage are:  $50\%$  inhibition of prothrombinase activity by  $5 \mu\text{M}$  prothrombin fragment 1 and fragment 1–2 (33) and by  $4 \mu\text{M}$  bone Gla protein (34) and less than  $50\%$  inhibition of tissue factor-factor VIIa activity by  $0.4 \mu\text{M}$  apolipoprotein A-I,  $0.4 \mu\text{M}$  apolipoprotein A-II,  $0.7 \mu\text{M}$  C-reactive protein, or  $0.5 \mu\text{M}$   $\beta_2$ -glycoprotein I (35). This poor inhibition by adsorption of independent protein molecules is seemingly in contrast with the recently proposed concept that the lipid membrane

accelerates coagulation reactions primarily by providing efficient lateral transport of the reactants (1, 18, 36, 37). It was calculated, however, that due to the high lateral mobility and the high collisional efficiency of two-dimensional diffusion even very low surface concentrations, in the order of  $\text{fmol} \cdot \text{cm}^{-2}$ , of the reactants are sufficient to attain the observed reaction rates. This lateral mobility of isolated inhibitor molecules also causes continuous rearrangement of adsorbed molecules on the lipid surface, and this reshuffling will produce empty spaces on which, e.g. prothrombin may adsorb (32).

In this framework it is attractive to speculate that the exceptionally complete inhibition of prothrombinase by annexin V is caused by the formation of rigid clusters of annexin V on the membrane. The empty spaces in the clusters may be large enough to allow binding of coagulation factors, but adsorbed reactants, e.g. prothrombin, are unable to diffuse through the surrounding cluster and no collisional complexes will be formed. The present study suggests that this fencing mechanism makes annexin V an effective anticoagulant.

**Physiological Importance**—Because plasma levels of annexin V are low ( $<0.2 \text{ nM}$ ) (39) a significant role of annexin V as a circulating anticoagulant is unlikely. On the other hand, annexin V concentrations in cultured endothelial cells are as high as  $0.5\%$  of the total amount of protein (38). Annexin V may, therefore, protect against thrombosis after endothelial cell damage. This study shows that, for a limited procoagulant lipid surface from platelets, only a small amount of annexin V is required for effective inhibition of prothrombinase activity.

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