Mode of action of annexin V (vascular anticoagulant alpha)

MODE OF ACTION OF ANNEXIN V (VASCULAR ANTICOAGULANT ALPHA), A PROTEIN SYNTHESIZED BY THE VESSEL WALL

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INTRODUCTION

Negatively charged phospholipids play a mandatory role in the coagulation process in that they serve as an essential integral part of the procoagulant enzyme complexes formed by vitamin K-dependent coagulation factors and tissue factor (1).

Binding of the coagulation factors to the phospholipid surface improves the kinetic parameters of the procoagulant reaction such that it proceeds readily under blood plasma conditions (2,3).

Annexin V (formerly vascular anticoagulant alpha) was first discovered in a particular protein fraction of an arterial tissue homogenate as a consequence of its pronounced anticoagulant activity (4). Its mechanism of anticoagulation appeared to be distinct from those of the at that time known anticoagulants like antithrombin III/heparin and activated protein C. In contrast to these annexin V showed no direct effect on the coagulation factors per se but interfered with the participation of negatively charged phospholipids in the procoagulant process (5).

Molecular cloning of its cDNA (6) revealed annexin V to be identical to placental anticoagulant protein (7), endonexin II (8), PP4 (9), lipocortin V (10) and calphobindin (11).

The deduced primary structure of annexin V showed an organisation in a 4-fold repeat of a 67/68 amino acid long conserved sequence, containing a consensus first described for a number of

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Ca^{2+}-dependent membrane binding proteins (12). This structural feature defined a new family of calcium/phospholipid binding proteins named the annexins (13).

Up to date eight different annexins have been discovered and their primary structures have been elucidated by means of protein sequencing or cDNA cloning techniques (for refs. of annexin I-VI see 10, annexin VII (14) and annexin VIII (15)).

Next to the accumulated chemical information a number of biological activities have been established for the annexins e.g. anicoagulant (4,6,15-18), anti-phospholipase activity (10,15,19,20), membrane-cytoskeleton linkage (21) and exocytosis (22,23). All these biological activities are based on the calcium/phospholipid binding properties and seem to be common features of the annexins. However, the individual annexins exhibit different specific activities and require different Ca^{2+}-levels for membrane binding suggesting a diversification of physiological functions.

Despite all the elaborated knowledge no clear picture of the true physiological functions of the annexins exists as yet.

MATERIALS AND METHODS

Protein

Annexin V was prepared by cDNA recombinant techniques with plasmid pRH291 and purified as described before (6). The protein preparation was more then 99% pure.

Lipids

Dioleoyl-phosphatidylcholine (DOPC), dioleoyl-phosphatidylserine (DOPS), dioleoyl-phosphatidylglycerol (DOPG), dioleoyl-phosphatidylinositol (DOPI), dioleoyl-phosphatidylethanolamine (PE), cardiolipin (CL) and sphingomyelin (SPH) were purchased from Sigma.

Annexin V binding to phospholipids

Adsorption of annexin V to phospholipid bilayers was studied using an automated ellipsometer as described in detail elsewhere (24).

Antibodies

Monospecific antibodies against annexin V were raised in rabbits employing standard techniques. The antibodies were affinity purified by using annexin V immobilized on Immobilon-P membranes (Milipore)

Swine anti-rabbit IgG conjugated with horse radish peroxidase or fluorescein were products of Dakopatts.

Cell culture

Endothelial cells (HUVEC) were isolated from human umbilical cord veins using established techniques. The isolated cells were cultured on fibronectin coated culture plastics in RPMI 1640, containing 20% human serum, 2 mM L-glutamine and streptomycine/penicilline. When the cells reached confluence they were passaged using 0.25% trypsin in PBS.

Western analysis of endothelial fractions

Confluent HUVEC were collected with a policeman in 10 mM Hepes/NaOH, 140 mM NaCl, 1 mM PMSF, 0.1 mM leupeptin and 30 uM EGTA, pH 7.4. The cell suspension was froozen and thawn three times and subsequently homogenized using a Potter Elvejhem. The lysed suspension was adjusted to the desired Ca^{2+} concentration and then centrifuged for 30 min at 100,000 x g. The supernatant was made 4% of sodium dodecyl sulfate (SDS) and 5 mM 2-mercaptoethanol (+SH). The pellet was washed and finally suspended in SDS/+SH-buffer.

Protein samples were submitted to SDS-gelelectrophoresis under reducing conditions according to Laemmli (25). The separated proteins were then transferred from the gel into nitrocellulose sheets (BioRad) using the technique as described by Towbin et al. (26). Annexin V was stained by indirect immunochemistry using anti-annexin V antibodies and peroxidase conjugated anti-IgG antibodies.

Immunofluorescence

HUVEC of the second passage were seeded (8.10^4 cells/ml) on gelatin coated glass coverslips and allowed to attach for 24 hours in RPMI 1640, 20% serum. The cells were washed with phosphate buffered saline (PBS, 1.5 mM KH_2PO_4, 6.5 mM Na_2HPO_4, 2.7 mM KCl, 137 mM NaCl, pH 7.4)

and subsequently treated the following procedure. Cells were fixed by a 25 minutes incubation with 3.5% formaldehyde in 10 mM Hepes, 150 mM NaCl, pH 7.4 with or without 2 mM $CaCl_2$. Subsequently the fixed cells were either used for immunofluorescence directly or first permeabilized by an incubation with 0.5% Triton X-100 in PBS.

Annexin V in the obtained cell preparations was visualized by indirect immunofluorescent staining using the affinity purified monospecific antibodies. Control experiments comprised either no anti-annexin V antibodies or antibodies, that were neutralized by purified annexin V.

Annexin V synthesis by HUVEC

A confluent layer of HUVEC was washed with Methionine-free MEM, containing 2 mM L-glutamine and 4% human serum. Endogenous methionine was depleted by a 6 hours incubation of HUVEC in the latter medium. Then ^{35}S-methionine was added and the incubation was prolonged for another 24 hours. In experiments where protein and RNA synthesis was blocked, cycloheximide (10 µg/ml) and actinomycine D (10 µg/ml) was added respectively, 3 hours prior to ^{35}S-methionine addition.

Following the incubation with labelled methionine the cells were washed and subsequently lysis of the cells was provoked by the addition of lysis buffer (10 mM Tris/HCl, 300 mM NaCl, 1% Triton X-100, 1.5 mM PMSF, 0.1 mM leupeptin, 20 ug/ ml soybean trypsin inhibitor, 2 mM EDTA, pH 7.4). For immunoprecipitation of annexin V the lysed suspension was incubated at 4^{o}C overnight with anti-annexin V conjugated to Sepharose beads. These beads were then washed thoroughly and subsequently suspended in an equal volume of 4% SDS, 5 mM 2-mercaptoethanol, 1.25 M Tris/HCl pH 6.8 and submitted to SDS-gel electrophoresis.

1. For qualitative analysis Western blotting was performed as described. The nitrocellulose sheet was firstly autoradiographed and secondly stained for annexin V with indirect immunochemistry using the peroxidase method.

2. For quantitative analysis the proteins in the gel were visualized with $CuCl_2$ and the annexin V band was excised and solved in Biofluor. Radioactivity was determined and the number of annexin V molecules was calculated, knowing that each molecule contains 8 residues of methionine.

RESULTS AND DISCUSSION

Phospholipid binding of annexin V

Annexin V does not associate with a phospholipid surface in the absence of Ca^{2+} (5,24). The presence of 3 mM Ca^{2+} induces a rapid adsorption of annexin V to a negatively charged phospholipid surface (fig. 1). The rate of adsorption is limited by the transport rate of annexin V to the surface and is directly proportional to annexin V-concentration. This is the case until 90% of the maximal adsorption is reached (24), indicating a high affinity binding. The association is completely reversible upon the addition of 3.5 mM EDTA. Repeated adsorption and desorption can be provoked by repeatedly alternating high and low Ca^{2+}-levels (fig. 1). Adorption kinetics

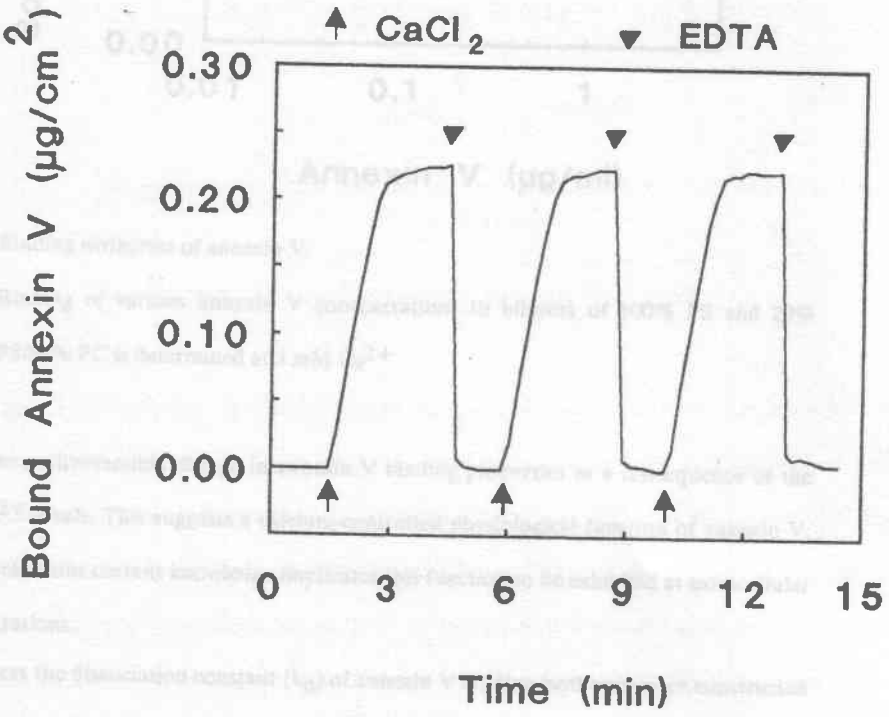


Fig. 1. Repeated adsorption and desorption of annexin V.

Adsorption of annexin V (ug/ml) to 25% PS/75% PC surface. Addition of Ca^{2+} (3, 4, 6 mM) and EDTA (3.5, 4.5,, 10 mM) is indicated by the arrow and arrowhead respectively

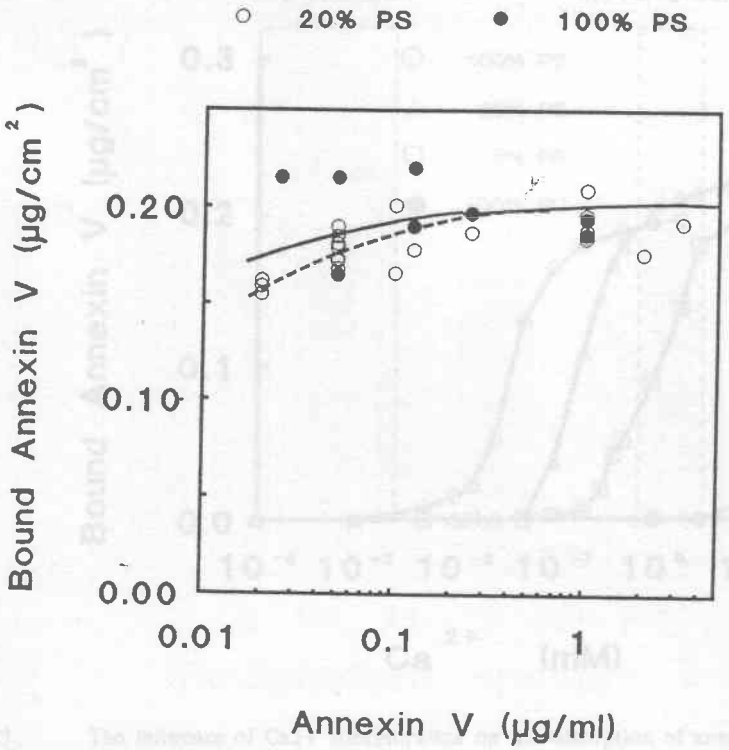


Fig. 2. Binding isotherms of annexin V.

Binding of various annexin V concentrations to bilayers of 100% PS and 20% PS/80% PC is determined at 3 mM Ca^{2+}

do not point to an irreversible change in annexin V binding properties as a consequence of the oscillating Ca^{2+}-levels. This suggests a calcium-controlled physiological function of annexin V. As discussed below our current knowledge implicates this function to be exhibited at extracellular Ca^{2+}-concentrations.

In order to assess the dissociation constant (k_d) of annexin V binding isotherms were constructed for adsorption to a 20% PS/80% PC and a 100% PS surface (fig. 2). Within the concentration range tested the surface coverage always exceeded 50%, indicating a $k_d < 10^{-10}$ M. Since adsorption times increased dramatically at low annexin V levels no k_d-determination better than the above given estimation was practicable.

There exists a Ca^{2+} requirement for annexin V binding to phospholipid surfaces. This requirement appears to depend on the nature of the phospholipid surface and is reversely related

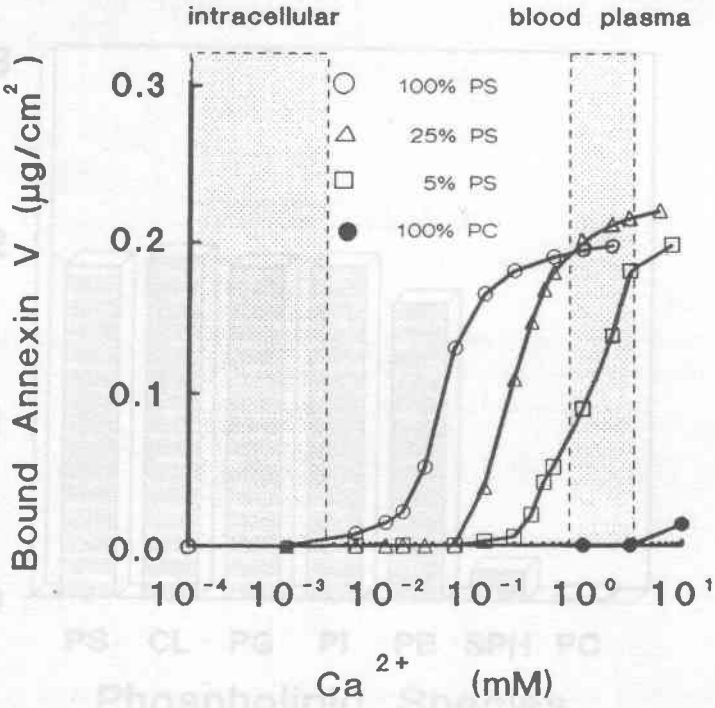


Fig. 3. The influence of Ca2+ concentration on the adsorption of annexin V to various surfaces.

All mixtures are supplemented with PC. [Annexin V]=1 µg/ml

to the PS contents (fig. 3). For binding to the optimal surface (100% PS) annexin V needs approximately 0.04 mM Ca^{2+} to cover the surface half maximally. This demand of Ca^{2+} increases to 0.22 and 1.5 mM when PS contents decreases to 20% and 5% respectively. Thus, annexin V binding to PS containing surfaces is unlikely to occur at intracellular Ca^{2+}-conditions but readily takes place at blood plasma Ca^{2+} levels if annexin V concentration is above 10^{-10} M. Although the binding of annexin V is stimulated by the presence of PS (fig. 4), the binding is not specific for this phospholipid. Cardiolipin (CL), phosphatidylglycerol (PG), phosphatidylinositol (PI), phosphatidylethanolamine (PE), sphingomyelin (SPH) and phosphatidylcholine (PC) all induce annexin V binding (fig. 4). However, Ca^{2+} requirement for half-maximal binding to these surfaces is distinct and is 0.04, 0.16, 0.47, 0.86, 7 and > 30 mM, respectively.

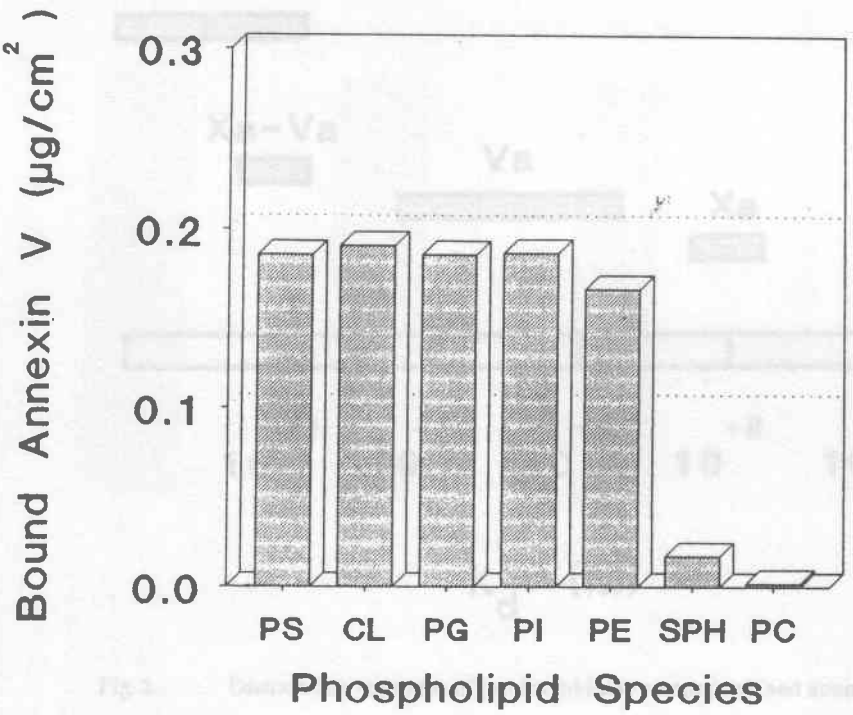
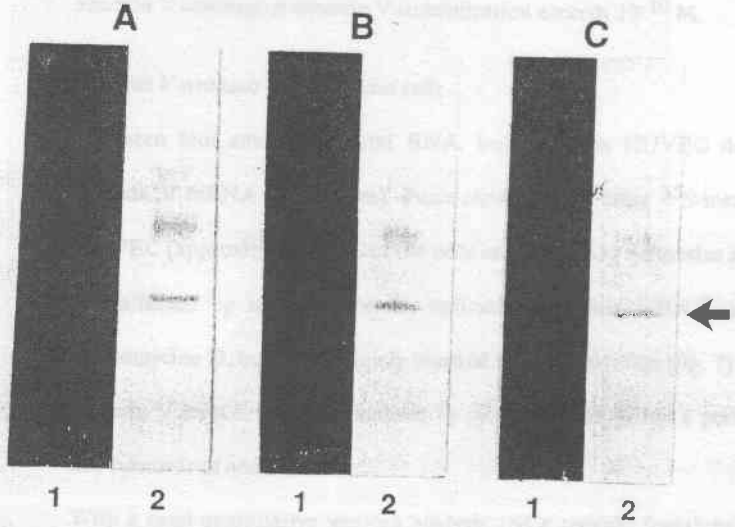


Fig. 4. Adsorption of annexin V to phospholipid bilayers of various composition. Annexin V adsorption to phosphatidylserine (PS), cardiolipin (CL), phosphatidylglycerol (PG), phosphatidylinositol (PI), phosphatidylethanolamine (PE), sphingomyeline (SPH), all mixed with 80% PC, and 100% phosphatidylcholine (PC). [Annexin V]=1 µg/ml, $[Ca^{2+}]$=3 mM

Anticoagulant mechanism of annexin V

The affinity of annexin V for a negatively charged phospholipid surface is a few orders of magnitude higher than those of factor Xa (27,28) and prothrombin (29). Even compared to factor Va and factor Xa-Va complex (28,30) annexin V binds better to a procoagulant phospholipid surface (fig. 5). In a competitive model it would implicate that annexin V displaces coagulation factors from the phospholipid surface. This, indeed, has been demonstrated for the proteins of the prothrombinase complex (5,31, Andree manuscript in prep.).

It is likely that annexin V also competes efficiently with the phospholipid binding of the



1 = Autoradiograph
2 = Western Blot
A = Control
B = Actinomycine D
C = Cycloheximide

Fig. 7. Immunoprecipitation of metabolically labelled endothelial annexin V. ^{35}S-methionine labbelled endothelial proteins are immunoprecipitated with anti-annexin V antibodies and analyzed by gel electrophoresis. [Actinomycine D]=10 µg/ml, [cycloheximide]=10 µg/ml

coagulation factors constituting the other phospholipid dependent procoagulant complexes (6,32,33).

Hence, annexin V is a novel anticoagulant interfering through a new mechanism on the phospholipid dependent procoagulant reactions and completes the set of anticoagulants acting on the four component procoagulant complex (fig. 6).

The phospholipids which promote annexin V binding most strongly are located in the intracellular leaflet of the plasma membrane (34) and are exposed to blood plasma components following activation of platelets or endothelial cells, or cell damage. The then prevailing Ca^{2+}

concentrations will induce sequestration of these phospholipids from the coagulation system by annexin V coverage if annexin V concentration exceeds 10^{-10} M.

Annexin V synthesis by endothelial cells

Northern blot analysis of total RNA, isolated from HUVEC demonstrates the presence of annexin V mRNA (not shown). Pulse-chase studies using ^{35}S-methionine show that confluent HUVEC (approximately 95% of the cells are in G0/G1) synthesize annexin V (fig. 7). Synthesis is not affected by actinomycine D, indicating a stable mRNA, at least in the presence of actinomycine D, but is completely blocked by cycloheximide (fig. 7). Western analysis shows that annexin V level is hardly influenced by cycloheximide within a period of 24 hours, suggesting a low turnover of annexin V.

With a semi-quantitative western analysis and a specific functional assay for annexin V it was determined that one endothelial cell contains $(3.1 \pm 0.5).10^6$ and $(4.5 \pm 0.9).10^6$ molecules of annexin V respectively (not shown).

Localisation of annexin V in the endothelial cell

The staining of endothelial cells with indirect immunofluoresence using anti-annexin V antibodies appears to depend on the conditions of fixation. Formaldehyde fixation with permeabilization leads to a diffuse cytoplasmic staining that changes into a granular one if Ca^{2+} is added to the fixative (fig. 8). During formaldehyde fixation endothelial cells respond vigorously within the first minutes of exposure. Membrane shuffling and vacuolization are observed and the annexin V positive granular structures (fig. 8A) are therefore difficult to identify. The experiments, however, confirm the extrapolation of the in vitro results, in respect to the Ca^{2+}/phospholipid binding properties, to in vivo situation.

Formaldehyde fixed cells, which are not permeabilized, show a patchy staining (fig. 8C). At the moment it is unknown, whether this originates from an extracellular localisation, as is observed for the avian counterpart of annexin V (35), or is an artifact of the formaldehyde fixation as an incident of localised damage.

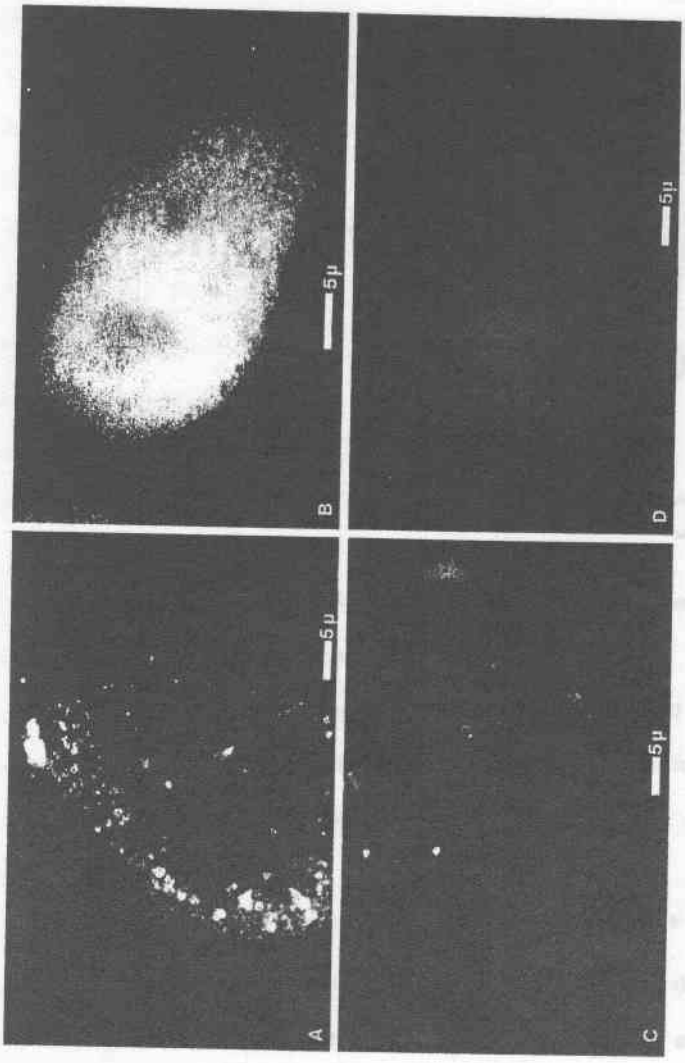


Fig. 8. Indirect immunofluorescence of endothelial cells using anti-annexin V antibodies. Endothelial cells are fixed with 3.5% formaldehyde and either permeabilized (A,B,D) with Triton X-100 or not (C). Fixation is performed either in the presence (A,C) or absence (B) of 2 mM Ca2+. Controls (D) comprised anti-annexin V antibodies which were neutralized with purified annexin V

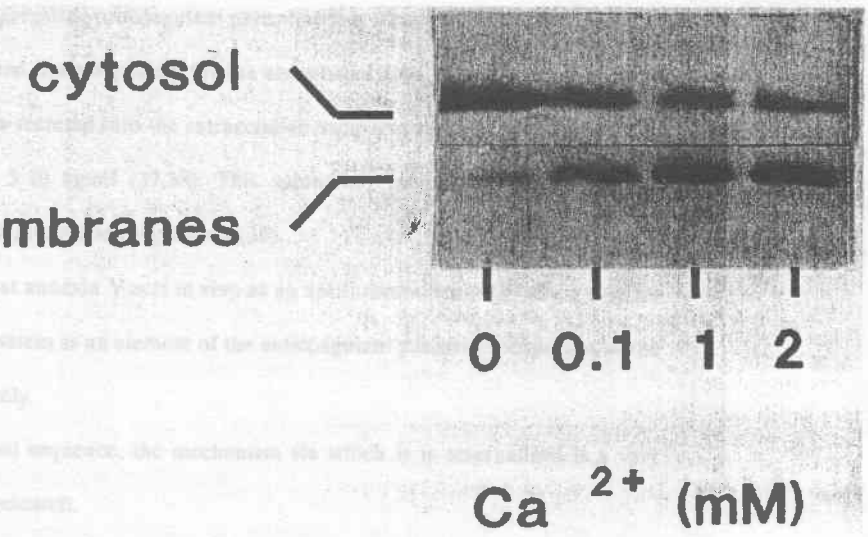


Fig. 9. Calcium dependent differential distribution of annexin V over the cellular compartments.

Endothelial cells are lysed and centrifuged in the presence of various Ca2+-concentrations. Cytosol and membrane fractions are analyzed by Western blotting using anti-annexin V antibodies

Fractionation of the endothelial cell at different Ca^{2+} concentrations results in a Ca^{2+}-dependent distribution of annexin V over the cytosol and the membrane fractions (fig. 9). Supra intracellular Ca^{2+}-levels are required for detectable association of annexin V with the membranes, which is in agreement with the in vitro observed phospholipid binding properties and and the immunofluorescence data. Furthermore this argues against the presence of a cofactor in confluent endothelial cells promoting phospholipid binding of annexin V at intracellular Ca^{2+} concentrations.

Conclusions

The presented results demonstrate that annexin V is abundantly present in the confluent endothelial cell (approximately 0.2-0.4% of total cellular protein) and occurs within the cell as a cytoplasmic phospholipid binding (anticoagulant) protein. Association with granular structures requires supra-intracellular Ca^{2+}-levels, which likely occurs upon cell damage. Annexin V then

acts as an anticoagulant by segregating procoagulant phospholipids from the coagulation process. Although not yet demonstrated unambiguously for the endothelial cells circumstantial evidence accumulates that annexin V is secreted into the extracellular compartment (36) and is present in blood plasma at a level of 5-10 ng/ml (37,38). This value may increase during myocardial infarction and disseminated intravascular coagulation (38).

Together with the findings that annexin V acts in vivo as an antithromboticum (39,40), a role for annexin V in the hemostatic system as an element of the anticoagulant paracrine properties of the endothelium becomes very likely.

Since annexin V lacks a signal sequence, the mechanism via which it is externalized is a very challenging subject of future research.

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