Ca^{2+} concentration during binding determines the manner in which annexin V binds to membranes

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Annexins are a family of calcium-binding proteins that have been implicated in a wide range of intracellular processes. We have previously reported that stimulation of platelets with thrombin can induce the association of intracellular annexin V with membranes in two distinct ways. First, in such a way that it can be eluted from the membrane with EGTA and secondly in a manner such that it is tightly bound to the membrane and requires the non-ionic detergent Triton X-100 for its solubilization. We report that exposure of platelets to the calcium ionophore A23187 mimics the relocation induced by stimulation with thrombin. In separate experiments we demonstrate that a calcium ion concentration [Ca^{2+}] of 0.8 μM is sufficient for maximum binding of the EGTA-resistant form to membranes. In contrast a higher [Ca^{2+}] was required to induce maximal binding of the annexin V which could be extracted with EGTA. We demonstrate that following temperature-induced phase separation in Triton X-114, the membrane-associated annexin V partitions predominantly into the aqueous phase. We also show that the isoelectric point of annexin V does not change following membrane association. These observations suggest that a covalent modification, of annexin V itself, is not responsible for its association with the membrane. Millimolar [Ca^{2+}] is required for maximal binding of purified annexin V to phospholipid vesicles. We show that binding to phospholipids can be reversed entirely by subsequent treatment with EGTA. This suggests that the EGTA-resistant form of annexin V is binding to a membrane component other than phosphatidylserine. Annexin V has previously been shown to bind to protein kinase C. Relocation of annexin V to membranes paralleled that of protein kinase C in thrombin-stimulated cells but not in cells treated with A23187, suggesting that these proteins are not functionally linked in platelet activation. Using bifunctional cross-linking reagents we have identified an 85 kDa complex containing annexin V. This may represent an association between annexin V and an annexin V-binding protein with a molecular mass of approximately 50 kDa.

INTRODUCTION

Annexins are a family of at least 12 proteins which contain repeats of a highly conserved 70-amino-acid sequence [1–5]. They have been identified in a diverse array of mammalian and plant tissues [6,7]. Individual annexins can also act as substrates for tyrosine kinases [8–11] and protein kinase C [12–14], suggesting that they may be involved in signal transduction. Although the exact physiological functions of annexins are not yet known, reconstitution experiments have implicated them in membrane trafficking [15–21], ion-channel regulation [22], inhibition of blood coagulation [23,24] and as inhibitors of phospholipase A_2 [25–27].

In heart, lung, liver and brain it has been observed that a proportion of the annexins remain associated with the membranes despite extraction with EGTA [28–31]. It has also been shown that activation of neutrophils with the chemoattractant formyl-methyl-leucyl-phenylalanine, an agonist which increases cytoplasmic Ca^{2+} concentration ($[Ca^{2+}]_i$) [32], induces relocation of annexin I to membranes [33,34].

Platelets are non-nucleated blood cells which on activation undergo changes in internal reorganization, secretion of granule contents and aggregation. Activation by an agonist, such as thrombin, stimulates signal transduction pathways which result in an increase in $[Ca^{2+}]_i$. However, the molecular events which couple the increase in $[Ca^{2+}]_i$ to the final platelet responses remain unexplained. It is probable that calcium-binding proteins such as annexins, which have previously been identified in platelets [35–39], play an important role following receptor-mediated activation of platelets. We have recently shown that, in platelets, annexin V binds to membranes following activation with the physiological agonist thrombin [39].

In this study we have used manipulations other than thrombin stimulation to generate two membrane-associated forms of annexin V. We have also demonstrated that the two membrane-associated forms of annexin V require different [Ca^{2+}] for their formation. We also present evidence that the membrane association is not a consequence of a covalent modification of annexin V itself, and that phospholipid binding cannot account for the EGTA-resistant annexin V.

MATERIALS AND METHODS

Materials

All chemicals were purchased from BDH or Sigma unless otherwise indicated. Affinity-purified horseradish peroxidase-conjugated sheep anti-(rabbit IgG) [HRP–anti-(rabbit IgG)] was purchased from Sigma. Monoclonal antibodies against protein kinase C-α and protein kinase C-β were purchased from Amersham as were the ECL reagents and Hyperfilm. Rabbit polyclonal antiserum specific for human annexin V and pure

Abbreviations used: $[Ca^{2+}]_i$, cytoplasmic Ca^{2+} concentration; DMS, dimethyl suberimidate; DSP, dithiobis(succinimidylpropionate); DTT, dithiothreitol; HRP–anti-(rabbit IgG), affinity-purified horseradish peroxidase-conjugated sheep anti-(rabbit IgG).

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human annexin V were gifts from Dr. F. Seiler and Dr. J. Römisch, Behring, Marburg, Germany.

Preparation and activation of platelets

Platelets from healthy volunteers were prepared as described previously [40] and suspended in Hepes-buffered Tyrode's solution (129 mM NaCl, 8.9 mM $NaHCO_3$, 2.8 mM KCl, 0.8 mM KH_2PO_4, 0.8 mM $MgCl_2$, 5.6 mM dextrose, 10 mM Hepes/NaOH, pH 7.4). The number of platelets and contaminating white blood cells were determined using a Coulter S Plus counter (courtesy of The Haematology Department, The General Infirmary, Leeds). The very low contamination by white blood cells was also determined by visual inspection.

Platelets (1×10^9/ml) were then incubated at 37 °C in the presence of 1 mM Ca^{2+} for 5 min before being incubated with either 0.1 unit/ml thrombin, 5 μM A23187 or buffer control for 10 min. The platelets were pelleted by centrifugation at 8000 *g* for 3 min in a microfuge and resuspended in 0.25 ml of buffer A [100 mM KCl, 5 mM EGTA, 0.5 mM dithiothreitol (DTT), 1 mM NaN_3, 10 mM Pipes, pH 7.4]. The samples were immediately frozen and stored at −20 °C.

When experiments were carried out to characterize the [Ca^{2+}] required for membrane association platelets were prepared and suspended in Hepes-buffered Tyrode's solution as above. They were then pelleted by centrifugation at 8000 *g* for 3 min in a microfuge and resuspended in 1 ml of the appropriate $CaCl_2$/EGTA buffer. All buffers contained 5 mM EGTA, 100 mM KCl, 0.5 mM DTT, 1 mM NaN_3, 10 mM Pipes, pH 7.4. The individual buffers varied in the amount of $CaCl_2$ added. $CaCl_2$was added to achieve free [Ca^{2+}] of 0.1 μM, 0.3 μM, 0.8 μM, 3.1 μM and 2 mM as predicted by the Metlig computer program [41,42]. The cells were then frozen at −20 °C.

Preparation and extraction of membranes

Platelets treated with thrombin or A23187 were ruptured (as confirmed by liberation of lactate dehydrogenase) by freeze–thawing and membranes sedimented by centrifugation at 100000 *g* for 15 min in a Beckman TL-centrifuge. For subcellular fractionation into cytosolic material, EGTA-elutable material and EGTA-resistant material, the supernatant (supernatant *a*) was removed and the pellet resuspended again in 0.25 ml of buffer A. The resuspended pellet was again centrifuged at 100000 *g* for 15 min. This procedure was repeated a further five times to produce six supernatants in total (supernatants *a–f*), representing the EGTA-elutable material. An aliquot from each of the supernatants was taken for measurement of annexin V content by quantitative immunoblotting. The washed pellet was then resuspended in 0.25 ml of buffer B [buffer A + 0.1 % (w/v) Triton X-100] and centrifuged at 100000 *g* for 15 min. The supernatant (supernatant *g*) was decanted and the pellet again resuspended in buffer B. The extraction was repeated to produce a second supernatant (supernatant *h*). Supernatants *g* and *h*, which contained the Triton X-100-soluble fraction, were then assayed. The final pellet was resuspended in 0.25 ml of buffer B and analysed for annexin V.

Platelets which were ruptured in the presence of different free [Ca^{2+}] were sedimented by centrifugation at 100000 *g* for 15 min. The cytosolic material was then removed and the pellet washed sequentially five times in the matching $CaCl_2$/EGTA buffer. The pellet was then resuspended in buffer B to produce a supernatant containing the total extractable annexin V. This was then assayed.

For separation of the EGTA-elutable from the EGTA-resistant material, platelets ruptured in the presence of Ca^{2+} were treated as described above for A23187-treated and thrombin-stimulated platelets.

Temperature-induced phase separation of Triton X-114

Hydrophobic proteins were separated from hydrophilic proteins by the method of Bordier [43]. Briefly, washed membranes, prepared as described above, were resuspended in 250 μl of 0.15 M NaCl/1 % (w/v) Triton X-114/10 mM Tris/HCl, pH 7.4, and left on ice for 5 min. The solubilized membranes were then incubated at 30 °C for 10 min and centrifuged at 3000 *g* for 3 min in a Sarstedt MH2 microfuge with a 12 × 1.5 ml swing-out rotor.

The lower (detergent-rich) phase was then separated from the upper (aqueous) phase. The two phases were then made up to equal volumes with 0.15 M NaCl/10 mM Tris/HCl, pH 7.4. The two phases were then assayed for annexin V as described below.

Determination of the isoelectric point of membrane-associated annexin V

Proteins were separated using a 3 % (w/v) polyacrylamide gel containing 5 M urea, 15 % (v/v) glycerol and 6 % (v/v) pH 4–6 or 3–10 ampholines on a vertical slab system as described by Giulian [44]. The gel was run at 20 W until equilibrium focusing was achieved. The pH gradient was determined using a flat-tip electrode and Western-blot analysis was carried out as described below.

Liposome binding assay

Large unilamellar phospholipid vesicles were formed in the presence of 240 mM sucrose by the method of Reeves and Dowben [45] using equimolar mixtures of phosphatidylcholine and phosphatidylserine.

Annexin V binding was performed as described by Boustead et al. [31]. Briefly, phospholipid vesicle preparations containing 0.5 μmol of each phospholipid were mixed with 0.5 μg of annexin V in either buffer A, buffer A containing 0.8 μM free Ca^{2+}, or buffer A containing 1 mM free Ca^{2+}in a final volume of 0.5 ml. The mixture was incubated at room temperature for 15 min and centrifuged at 40000 *g* for 10 min at 4 °C. The supernatant (supernatant *a*), representing the unbound material, was removed and the pellet resuspended in 0.5 ml of buffer A. The resuspended pellet was again centrifuged at 40000 *g* for 10 min and the supernatant (supernatant *b*) removed. This procedure was repeated a further four times to produce five supernatants (supernatants *b–f*) representing the EGTA-elutable material. The washed pellet was then suspended in 0.5 ml of buffer B and centrifuged at 40000 *g* for 10 min. The supernatant (supernatant *g*) was decanted and the pellet again resuspended in buffer B. The extraction was repeated to produce a second supernatant (supernatant *h*). Supernatants *g* and *h* again represent the Triton X-100-extractable fraction. The annexin V in each supernatant was then observed by qualitative immunoblotting.

Chemical cross-linking

EGTA-washed membranes were isolated from platelets (1×10^9 cells) lysed in the presence of 0.8 μM free Ca^{2+} as described above. The membranes were then incubated for 1 h at room temperature in a final volume of 100 μl in buffer C (0.2 M triethanolamine, 1 mM $CaCl_2$, pH 7.8) in either the presence or absence of the bifunctional reagent dimethyl suberimidate (DMS) added to a final concentration of 5 mM. The membranes were then sedimented by centrifugation at 100000 *g* and the

membrane-associated material extracted with 0.25 ml of buffer B. The Triton X-100-soluble material was taken up in sample buffer containing 10% (v/v) mercaptoethanol and 2% (w/v) SDS. Annexin V–protein complexes were identified by electrophoresis and Western blotting as described below.

Identification of annexin V by electrophoresis and Western-blot analyses

Proteins were separated by SDS/PAGE using a 10% (w/v) polyacrylamide gel and a discontinuous buffer system as described by Laemmli [46].

For Western-blot analysis proteins were transferred to nitrocellulose [47]. Complete transfer was confirmed by staining of the nitrocellulose with 0.1% (w/v) Ponceau S in 3% (w/v) trichloroacetic acid and destaining the background with 10% (v/v) acetic acid. Subsequently the blot was washed in Tris-buffered saline (TBS; 0.15 M NaCl, 10 mM Tris/HCl, pH 7.4) containing 0.1% (w/v) Triton X-100. The nitrocellulose membrane was then saturated with TBS containing 5% (w/v) non-fat dry skimmed milk (Safeway, PLC).

Nitrocellulose blots were probed overnight with a 1:1000 dilution of rabbit anti-(human annexin V) in TBS containing 0.1% Triton X-100. Immunoreactive bands were detected with HRP–anti-(rabbit IgG) (1:1000 dilution) as described below.

Quantitative analysis of annexin V

A quantitative assay for annexin V was developed. Standard annexin V and platelet-derived material were subjected to SDS/PAGE and annexin V was identified by Western blotting. Peroxidase activity was detected using the ECL detection system. Annexin V immunoreactivity was then quantified using an Apple One Scanner and the NIH-Image densitometry application program version 1.52. Annexin V band density was linear between 0 and 10 ng.

RESULTS AND DISCUSSION

We have previously shown that, when added to platelets, the physiological agonist thrombin can cause the association of annexin V with membranes in two ways. The association is such that a fraction of the annexin V can be eluted from the membrane by the calcium chelator EGTA, whereas a second fraction remains bound to the membranes despite extensive washing with EGTA. This EGTA-resistant form of annexin V was found to require the non-ionic detergent Triton X-100 for its solubilization [39] and may be exhibiting the same kind of EGTA-resistant binding previously identified in membranes isolated from lung, liver, heart and brain [28–31]. The mechanism by which this EGTA-resistant binding occurs has yet to be identified, although it is possible that Ca^{2+} ions play a role.

Thrombin activates many intracellular processes. We therefore used the calcium ionophore A23187, in the presence of 1 mM calcium, to investigate whether an increase in intracellular calcium alone can mimic the action of thrombin in inducing the association of annexin V with the membranes. The results are shown in Figure 1. Subcellular fractions from control (Figure 1, panel C), thrombin-stimulated (Figure 1, panel T) and A23187-treated (Figure 1, panel A) platelets were prepared and probed for annexin V. The EGTA-extractable material is shown in lanes a–f. The membrane-associated form of annexin V, which requires Triton X-100 for its extraction, is shown in lanes g and h. The Triton X-100-resistant pellet is shown in lane i. It is apparent that A23187, like thrombin, can induce the binding of annexin V to membranes in a manner which requires Triton X-100 for its

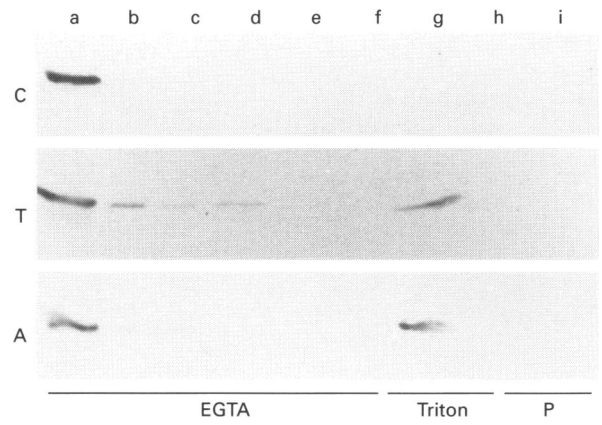

Figure 1 Subcellular distribution of annexin V in platelets by Western-blot analysis

Control platelets (C), thrombin-stimulated platelets (T) or A23187-treated platelets (A) were lysed and subfractionated as described in the Materials and methods section. The EGTA-elutable annexin V (lanes a–f), Triton X-100-extractable annexin V (lanes g and h) and Triton X-100-resistant annexin V (lane i) were detected by SDS/PAGE and Western blotting.

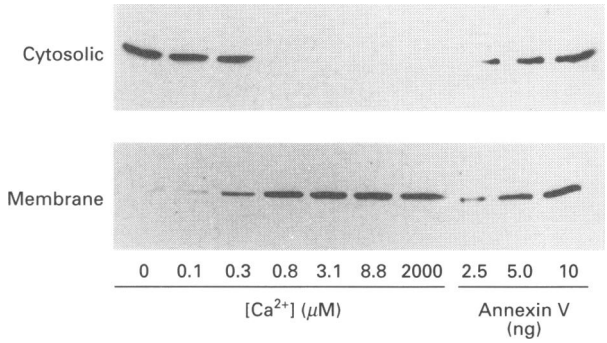

Figure 2 Calcium induces the relocation of annexin V from the cytosol to membranes

Platelets were lysed and subfractionated in solutions containing defined $[Ca^{2+}]$ as indicated. Following separation of membranes and cytosol the fractions were probed for annexin V by SDS/PAGE and Western blotting.

extraction. This finding confirms that calcium plays an important role, either directly or indirectly, in mediating the association of annexin V with membranes.

In platelets $[Ca^{2+}]_i$ is tightly regulated and the different platelet responses (shape change, phosphatidylserine expression, fibrinogen receptor expression and secretion) require different $[Ca^{2+}]_i$ for their initiation [48]. We therefore determined the $[Ca^{2+}]$ required for intracellular annexin V to bind to membranes. Platelet membranes were isolated from cells lysed in EGTA alone or in various concentrations of Ca^{2+} as described in the Materials and methods section. The distribution of annexin V between the cytosol and membranes was then determined. When platelets were lysed in the presence of EGTA or 0.1 μM Ca^{2+}, it was apparent that annexin V remained cytosolic in nature (Figure 2). However, when the $[Ca^{2+}]$ in the lysis buffer was increased to 0.3 μM, annexin V was seen to relocate from the cytosol to the membranes (Figure 2). When the $[Ca^{2+}]$ was increased to 0.8 μM, annexin V was no longer detected in the cytoplasm and reached a maximum level in the membrane fraction (Figure 2). Quanti-

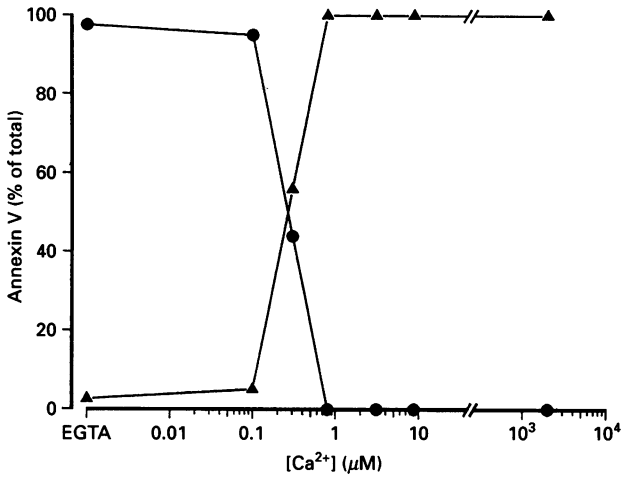

Figure 3 Quantification of the change in cytosolic and total membrane-associated annexin V with [Ca^{2+}]

The proportion of cytosolic (●) and membrane-bound (▲) annexin V was determined as described in the Materials and methods section.

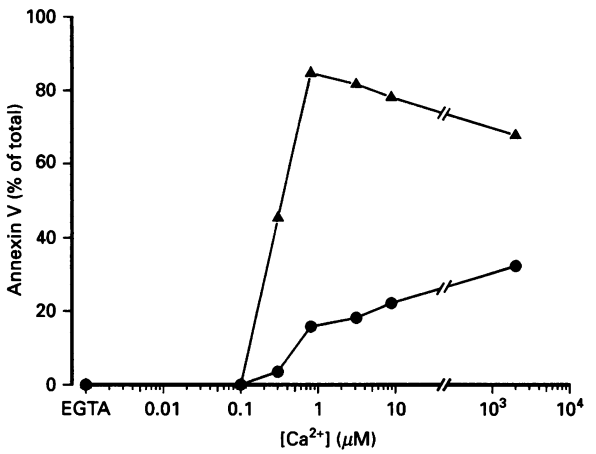

Figure 5 Quantification of the dependency on [Ca^{2+}] of the formation of the EGTA-elutable and EGTA-resistant annexin V

The percentage of annexin V in the EGTA-soluble (●) and EGTA-resistant (▲) membrane fractions was determined as described in Figure 4 and the Materials and methods section.

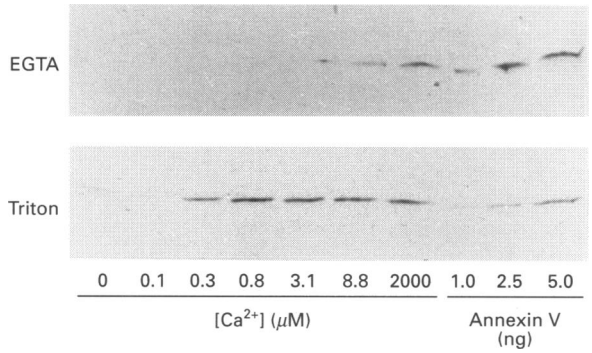

Figure 4 EGTA-elutable and EGTA-resistant forms of annexin V bind to membranes with different calcium dependencies

A representative experiment in which platelets were lysed in the presence of the indicated [Ca^{2+}]. Following subfractionation the membranes were isolated and the EGTA-elutable material separated from the EGTA-resistant material as described in Materials and methods section. The EGTA-elutable material (EGTA) and the EGTA-resistant material (Triton) were then analysed for annexin V by SDS/PAGE and Western blotting.

fication of the proportion of annexin V in the cytosol and membranes was carried out. The results from a representative experiment are shown in Figure 3. In the presence of EGTA alone 95% of the annexin V was located in the cytosol, whereas when the [Ca^{2+}] was increased to 0.8 μM Ca^{2+} 100% of the total annexin V was associated with membranes. Figure 3 also demonstrates that the [Ca^{2+}] required for half-maximal binding is approximately 0.35 μM. Measurements of $[Ca^{2+}]_i$ using fura-2 have previously demonstrated that cytoplasmic [Ca^{2+}] observed following thrombin-stimulation is approximately 1.5–2 μM [48]. Our data for annexin V association with membranes demonstrates that relocation can occur between the $[Ca^{2+}]_i$ found in the resting cell and that seen in stimulated platelets. It has also been shown that the threshold $[Ca^{2+}]_i$ for the onset of secretion is 0.8 μM [48], a [Ca^{2+}] identical to that required for maximum membrane annexin V association. These data correlating the calcium-dependency of annexin V relocation with the onset of secretion further support the hypothesis [15–18] that annexins play a role in exocytosis.

As shown previously, annexin V can associate with membranes in a manner which is reversed when the Ca^{2+} ions are chelated by EGTA and also in a manner that requires non-ionic detergent for its extraction [39]. Consequently, further studies were carried out to characterize the [Ca^{2+}] required to produce each membrane-associated form of annexin V. Membranes derived from platelets lysed in defined [Ca^{2+}] were prepared as described above. The EGTA-elutable material was then separated from the EGTA-resistant material. The EGTA-elutable annexin V and the EGTA-resistant annexin V bound at each [Ca^{2+}] was then determined by Western blotting (Figure 4). At low [Ca^{2+}], little EGTA-elutable material was bound; membrane association occurred only at [Ca^{2+}] above those seen in activated cells (Figure 4, EGTA panel). In contrast the form of annexin V which is resistant to extraction with EGTA, but is extracted with Triton X-100, was seen to bind at 0.3 μM Ca^{2+}, reaching a maximum at 0.8 μM (Figure 4, Triton panel). The [Ca^{2+}] required for half-maximal binding of the EGTA-resistant annexin V was then quantified (representative experiment shown in Figure 5). It is apparent that two distinct membrane binding curves exist (Figure 5). The EGTA-resistant annexin V reaches a half-maximal level of binding at 0.3 μM Ca^{2+}, and reached a maximum level of binding at 0.8 μM Ca^{2+}. At this [Ca^{2+}] approximately 85% of the total membrane-associated annexin V requires Triton X-100 for its extraction. In contrast to the EGTA-resistant annexin V, the EGTA-elutable annexin V was seen to require millimolar Ca^{2+} for maximal binding. Interestingly, at these non-physiological [Ca^{2+}] the proportion of annexin V which binds in an EGTA-elutable manner increases at the expense of the EGTA-resistant annexin V.

At physiological [Ca^{2+}] therefore, annexin V binds to the membranes in two distinct ways. The majority (85%) of the total annexin V is resistant to extraction with EGTA, the remaining (15%) annexin V can be eluted with EGTA. These data suggest that in platelets the form of annexin V which is resistant to extraction with EGTA is the physiologically important form of the protein. It is this form which is associating predominantly with the membrane at calcium concentrations equivalent to those

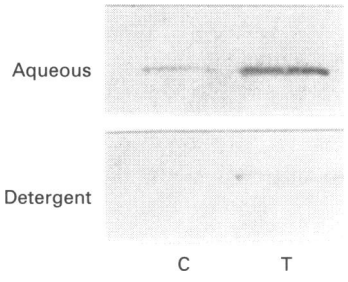

Figure 6 SDS/PAGE and Western-blot analysis of the aqueous and detergent-rich phases from the Triton X-114 extract

Platelet membranes prepared from control (C) or thrombin-stimulated platelets (T) were solubilized with Triton X-114 as described in the Materials and methods section. The aqueous and detergent phases were than analysed for annexin V by Western blotting.

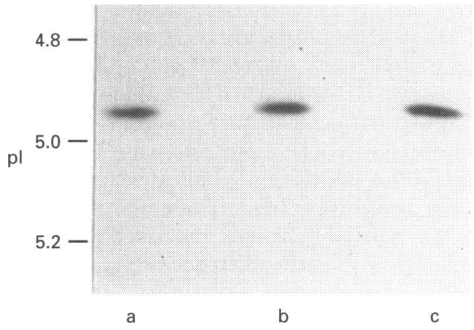

Figure 7 Determination of the isoelectric point of membrane-associated annexin V

Cytosolic material isolated from resting platelets (lane a), EGTA-resistant material (lane b) isolated from thrombin-stimulated platelets and a combination of both cytosolic and EGTA-resistant annexin V (lane c) were run on isoelectric focusing gels as described in the Materials and methods section.

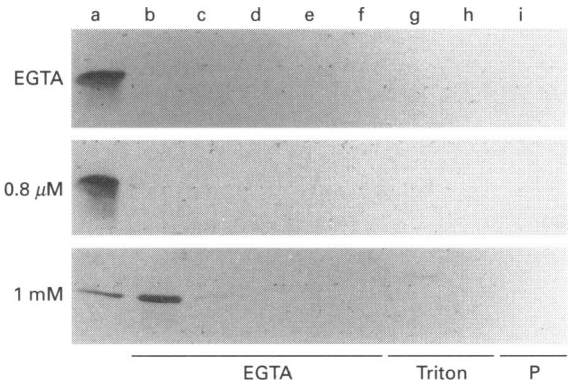

Figure 8 Binding of annexin V to liposomes

Pure annexin V (0.5 μg) was incubated with liposomes in the presence of EGTA, 0.8 μM free Ca^{2+} or 1 mM free Ca^{2+}. The liposomes were subfractionated as described in the Materials and methods section. The unbound annexin V (lane a), the EGTA-elutable annexin V (lanes b to f) and the Triton X-100-extractable annexin V (lanes g and h) were detected by SDS/PAGE and Western blotting. Lane (i), pellet after Triton X-100 extraction.

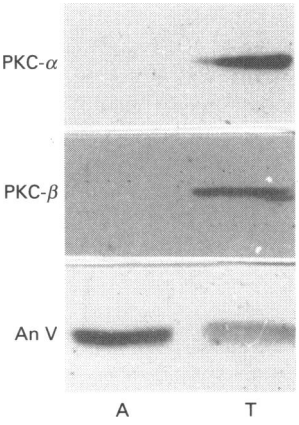

Figure 9 Evidence that protein kinase C is not involved in the binding of annexin V to the membrane

Membranes derived from thrombin-stimulated (T) or A23187-treated (A) platelets were prepared as described in the Materials and methods section. Triton X-100 extracts were then analysed for protein kinase C-α (PKC-α), protein kinase C-β (PKC-β) or annexin V (An V) by SDS/PAGE and Western blotting.

in stimulated platelets and it is therefore likely that this is the interaction which occurs *in vivo*. As described above, binding of the EGTA-elutable material was seen up to millimolar [Ca^{2+}]. This is similar to the binding of annexin V *in vitro* to liposomes containing acidic phospholipids [49]. The binding of annexin V which is reversed by EGTA is therefore less likely to be associated with physiological processes.

A series of experiments was then performed to investigate the nature of the mechanism by which the EGTA-resistant annexin V associates with the membrane. It has been previously suggested that a covalent modification of annexin V may enable it to insert into the membrane in a manner such that it behaves as an integral membrane protein [30]. To investigate this possibility we performed temperature-induced phase separation of EGTA-resistant membrane proteins solubilized in Triton X-114 as described in the Materials and methods section. Following phase separation, annexin V isolated from thrombin-stimulated platelets was found to partition into the aqueous phase (Figure 6). It is therefore apparent that the hydrophobicity of annexin V is not increased in such a way that it can insert directly into the phospholipid structure of the membrane.

It is, however, possible that the binding of annexin V to membranes is associated with a post-translational modification. Such a modification would result in a change in the isoelectric point of annexin V. Platelet-derived cytosolic material and EGTA-resistant membrane components were therefore subjected to isoelectric focusing as described in the Materials and methods section. Comparison of cytosolic annexin V (Figure 7, lane a) with EGTA-resistant membrane-associated annexin V (Figure 7, lane b) demonstrates that the isoelectric point of annexin V does not change following relocation to membranes (Figure 7, lane c). These studies suggest that a covalent modification of annexin V does not occur and that activation of platelets must result in interaction of annexin V with platelet membranes via modification of a membrane component. To determine whether Ca^{2+}, acidic phospholipid and annexin V alone are sufficient to generate EGTA-resistant annexin V a series of liposome binding experiments were performed. When the annexin V binding assay was carried out in the presence of EGTA (Figure 8, panel EGTA) or 0.8 μM free calcium (Figure 8, panel 0.8 μM) neither EGTA-

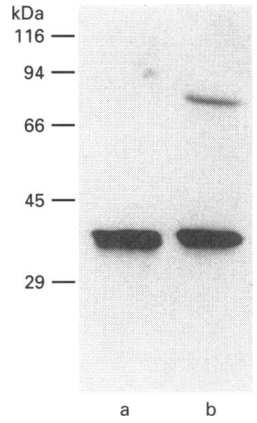

Figure 10 Cross-linking of annexin V to platelet membranes

Triton X-100-soluble fractions were prepared from control membranes (lane a) or membranes incubated with 5 mM DMS (lane b) as described in the Materials and methods section. The formation of high-molecular-mass complexes was visualized by SDS/PAGE and Western blotting against annexin V.

elutable (Figure 8, lanes b–f) nor EGTA-resistant (Figure 8, lanes g and h) binding was observed. In contrast, when the liposome binding assay was carried out in the presence of 1 mM Ca^{2+} annexin V was seen to bind to the liposomes. This form of annexin V was, however, found to be completely eluted from the membranes following a single EGTA wash (Figure 8, panel 1 mM, lane b). It is therefore apparent that phospholipids alone are not directly responsible for mediating the interaction of the Triton X-100-extractable form of annexin V with the membrane. These results suggest that there may be two physiological binding sites for annexin V: a phospholipid-binding site requiring a high $[Ca^{2+}]$, and a non-phospholipid-binding site activated by a lower $[Ca^{2+}]$.

Evidence from others suggests that annexin V can inhibit protein kinase C [50,51], and that annexin V may directly bind to protein kinase C [50]. We therefore investigated the possibility that protein kinase C may be involved in the relocation of annexin V to membranes. Membranes prepared from thrombin-stimulated platelets, or platelets treated with the calcium ionophore A23187, were isolated as described in the Materials and methods section. The Triton X-100-extractable material was probed for protein kinase C isoforms, which were identified as single immunoreactive species by Western blotting (Figure 9). In agreement with others [52] we found that thrombin stimulation induced sustained membrane association of protein kinase C-α (Figure 9, panel PKC-α, lane T), and protein kinase C-β (Figure 9, panel PKC-β, lane T). In contrast, treatment with A23187 did not result in any detectable association of protein kinase C with the membranes (Figure 9, panels PKC-α and PKC-β, lanes A). This indicates that an increase in $[Ca^{2+}]_i$ alone, a treatment which produces substantial annexin V relocation (Figure 9, panel An V), is not sufficient to induce protein kinase C to associate with membranes. This result therefore indicates that protein kinase C is not playing a role in the binding of annexin V to membranes. However, the possibility cannot be ruled out that the two proteins interact with each other once relocation to the membranes has occurred.

To investigate the possibility that annexin V may be binding to a membrane protein, cross-linking experiments were performed

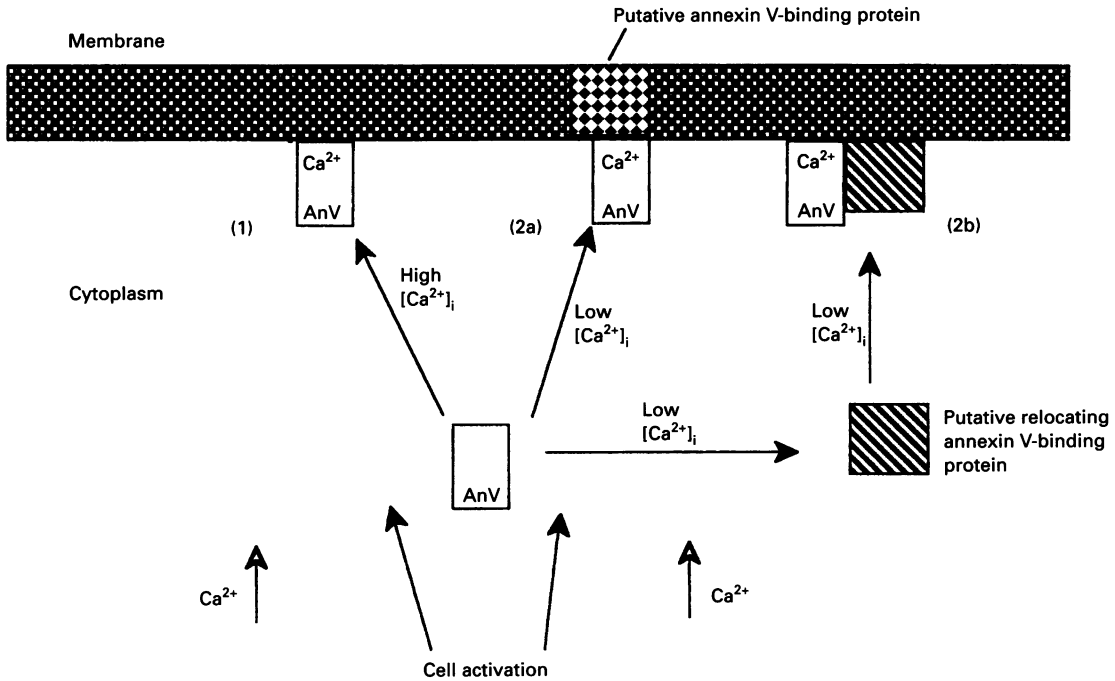

Figure 11 Proposed model for annexin V relocation to platelet membranes following physiological stimulation or exposure of intracellular annexin V to Ca^{2+}

Following cellular activation or exposure of membranes to Ca^{2+} and cytosol, annexin V associates with the membrane in two distinct ways; (1) in a manner where annexin V binds at high $[Ca^{2+}]$ and can be eluted with EGTA, and (2) in a manner which binds at low physiological $[Ca^{2+}]$ and requires Triton X-100 for its extraction. It is possible that the EGTA-resistant annexin V is binding to a non-phospholipid membrane component (2a) or another cytosolic component which relocates to the membrane following cellular activation (2b).

on isolated EGTA-washed membranes containing tightly associated annexin V. The bifunctional cross-linker DMS was added to isolated membranes and complex formation investigated by SDS/PAGE and Western blotting. A single high-molecular-mass immunoreactive band of 85 kDa was observed in the presence (Figure 10, lane b), but not the absence, of DMS (Figure 10, lane a). When the experiment was repeated using dithiobis(succinimidylpropionate) (DSP), an alternative bifunctional cross-linking reagent, a similar 85 kDa complex was observed (results not shown). Interestingly Giambanco et al. [53] used cross-linking reagents to observe cross-linked 85–90 kDa complexes when annexin V was added to crude cytoskeletal fractions isolated from bovine brain in the presence of high [Ca^{2+}]. Our results suggest that, following exposure to physiological [Ca^{2+}], annexin V relocates from the cytosol to the membranes and is linked to the membrane phospholipids via a 50 kDa annexin V-binding protein. There is growing evidence for other annexins binding to proteins. Annexin II exists as a heterotetramer of p36 and p11, a member of the S-100 family of proteins. In addition annexin II has been shown to bind to S-100 protein [54] and the glial fibrillary acidic protein [55], while annexin II, annexin VI [56] and annexin XI [57] can associate with calcyclin in a calcium-dependent manner.

A model summarizing the possible mechanisms of relocation is shown in Figure 11. Following stimulation with either physiological agonists or treatments which elevate $[Ca^{2+}]_i$ annexin V relocates from the cytosol to the membranes in one of two separate ways. First, in such a way that maximal binding occurs at relatively high calcium concentrations and is reversible by treatment with EGTA (Figure 11, 1). Secondly, annexin V interaction also occurs at lower calcium concentrations in a manner which is resistant to reversal with EGTA. This association could either represent direct binding of annexin V to a membrane component (Figure 11, 2a) or to another cytosolic protein relocating to the membrane (Figure 11, 2b). A third manner of association can also be envisaged. This would involve a covalent modification of annexin V, with the result that annexin V changes its conformation and inserts into the membrane. However, as discussed above, there is no evidence that such a modification occurs and it is therefore likely that other components are involved.

In summary we have shown that calcium plays a vital role in mediating the relocation of annexin V to membranes. We have demonstrated that the EGTA-resistant annexin V binds to membranes with a lower calcium-dependency than the EGTA-elutable annexin and that molecules in addition to phospholipids are required for this tight association with the membranes. Further studies will concentrate on elucidating the mechanism by which the EGTA-resistant annexin V associates with membranes.

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