Annexin V-Mediated Calcium Flux across Membranes Is Dependent on the Lipid Composition: Implications for Cartilage Mineralization†

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ABSTRACT: Annexin V is a major component of matrix vesicles and has a role in mediating the influx of Ca^{2+} into these vesicles, thus promoting the initiation of hypertrophic cartilage matrix mineralization. However, the mechanisms and factors regulating annexin V-mediated Ca^{2+} influx into these vesicles are not well understood. Since the lipid composition of matrix vesicles differs from that of the plasma membrane of chondrocytes and is rich in phosphatidylserine, we asked whether the lipid composition may regulate annexin V function. We prepared liposomes containing different concentrations of phosphatidylserine and determined how the lipid composition affected (a) the interactions between annexin V and liposomes and (b) annexin V-mediated Ca^{2+} influx into the liposomes. We found that annexin V was able to bind to every liposome tested. However, we observed the most prominent increases in tryptophan 187 emission intensity, a measure of the degree of interactions between annexin V and lipid bilayers, only with liposomes containing a high concentration of phosphatidylserine. In addition, a significant fraction of annexin V associated with phosphatidylserine-rich liposomes was not extractable by EDTA treatment but required a detergent, indicating that annexin V inserts into bilayers of these liposomes. Chemical cross-linking analysis revealed that matrix vesicles and phosphatidylserine-rich liposomes induced the formation of the annexin V hexamer. Interestingly, a significant Ca^{2+} influx in the presence of annexin V occurred only in liposomes containing a high phosphatidylserine content. Moreover, annexin V-mediated Ca^{2+} influx into these liposomes was inhibited (i) by anti-annexin V antibodies and (ii) by treatment with zinc and cadmium, indicating the essential role of the protein in Ca^{2+} influx. The results of this study indicate that phosphatidylserine-rich bilayers induce the formation of a hexameric annexin V, possibly leading to a Ca^{2+}-dependent insertion of annexin V into the bilayer and establishment of annexin V-mediated Ca^{2+} influx into matrix vesicles or liposomes. The phosphatidylserine-rich membrane of matrix vesicles *in vivo* may thus offer an ideal specialized environment in which the biological function of annexin V is optimized, leading to rapid Ca^{2+} influx, intralumenal crystal growth, and cartilage matrix mineralization.

The annexins are a family of proteins which bind to negatively charged phospholipids in a Ca^{2+}-dependent manner. Twelve different annexins have been isolated from a large variety of cell types [for review, see Geisow et al. (1988) and Crompton et al. (1988)]. These proteins are characterized by the presence of four repeats, with the exception of annexin VI which has eight repeats. The four or eight repeats in each annexin form a conserved core region which is responsible for the Ca^{2+}-dependent binding of the protein to phospholipid membranes. The sequence of the N-terminal region varies greatly among the annexins and may contribute to their different cellular functions in which annexins are believed to be involved (Geisow et al., 1986, 1988; Crompton et al., 1988; Creutz, 1992; Zaks & Creutz, 1990; Ali et al., 1989).

Recently, human (Huber et al., 1990), rat (Concha et al., 1993), and chicken (Bewley et al., 1993) annexin V have been crystallized. X-ray analysis revealed a symmetric, slightly curved molecule in which each of the four repeats folds into a compact domain consisting of five α-helices. The domains are arranged to form a hydrophilic pore (Huber et al., 1990; Concha et al., 1993; Bewley et al., 1993), consistent with the finding that annexin V, when inserted into planar lipid bilayers, forms voltage sensitive ion channels (Rojas et al., 1990). Interestingly, the annexin V monomer is water-soluble and too small to span a membrane; thus, the formation of ion channels by annexin V is likely to involve multimeric annexin V molecules. This hypothesis is supported by a recent study showing the crystal structure of an annexin XII hexamer; this annexin XII hexamer not only has the spatial dimensions sufficient to span the membrane but also has a central pore lined with charged residues (Luecke et al., 1995). On the basis of such an annexin XII hexamer structure, the authors proposed that an annexin V hexamer would also have a central pore lined with charged residues (Luecke et al., 1995). Additional

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studies have indicated that annexin V is involved not only in mediating Ca^{2+} flux in cell or vesicle membranes but also in other physiological events, such as phospholipid-dependent inhibition of blood coagulation (Hauptmann et al., 1989; Andree et al., 1992), modulation of protein kinase C (Schlaepfer et al., 1992), and inhibition of phospholipase A2 activity (Davidson et al., 1990).

Matrix vesicles, cell membrane-derived microstructures, appear to have a critical role in initiating mineralization of skeletal tissues. Studies of cartilage mineralization have demonstrated that during the initial steps of matrix vesicle-mediated calcification the first crystal phase forms and grows inside the vesicle lumen (Anderson, 1969, 1990; Bonucci, 1970). Since matrix vesicles are enclosed by a membrane, proteins are required to mediate influx of mineral ions into the vesicles and to promote intralumenal crystal growth. After these intralumenal crystals have reached a certain size and structure, they rupture the membrane and grow out into the matrix. There is evidence that annexin V, a major component of matrix vesicles, mediates Ca^{2+} influx into these particles (Genge et al., 1989, 1990, 1992; Rojas et al., 1992; Kirsch & Wuthier, 1994). First, Ca^{2+} uptake by matrix vesicles is highly protease sensitive, indicating that it is a protein-mediated process (Genge et al., 1988; Kirsch & Wuthier, 1994). Second, Ca^{2+} uptake is strongly inhibited by Zn^{2+}, an ion that has been shown to bind to annexins and inhibit the Ca^{2+} channel activity of annexin V in synthetic phosphatidylserine bilayer membranes (Sauer et al., 1989; Rojas et al., 1992; Wuthier, 1992). Thus, matrix vesicles appear to provide a specialized environment enabling annexin V to mediate Ca^{2+} influx into these particles. Even though matrix vesicles are derived from the plasma membrane of hypertrophic chondrocytes, the lipid composition of the matrix vesicle membrane is different from that of the chondrocyte plasma membrane and is enriched in phosphatidylserine (Wuthier, 1975). Thus, it is possible that such a specialized lipid environment may allow annexin V to specifically mediate Ca^{2+} influx into matrix vesicles. In the present study, we tested the hypothesis that the lipid composition may regulate annexin V-mediated Ca^{2+} influx into matrix vesicles. To test our hypothesis, we measured Ca^{2+} influx into fura-2-loaded liposomes containing various lipid compositions in the presence of annexin V. Our findings indicate that the lipid composition affects the structure of annexin V and its ability to mediate Ca^{2+} flux across membranes.

EXPERIMENTAL PROCEDURES

Preparation of Liposomes. Phospholipids were obtained from Avanti Polar Lipids (Birmingham, AL). Fura-2 was obtained from Molecular Probes (Eugene, OR). Large thin-walled liposomes were prepared using a dehydration/rehydration technique and were loaded with fura-2 as described previously (Reeves & Dowben, 1969; Berendes et al., 1993a). The liposomes contained phosphatidylserine, phosphatidic acid, phosphatidylglycerol or phosphatidylinositol, and phosphatidylethanolamine in molar ratios of 2.5:7.5, 5:5, 7.5:2.5, and 9:1; other liposomes used contained phosphatidylserine, phosphatidylcholine, and cholesterol in a molar ratio of 2:5:3. In addition, liposomes containing phosphatidylcholine or phosphatidylethanolamine were prepared. After the phospholipids were dried down from chloroform/methanol (2:1, v:v), the lipid film was rehydrated for 30 min in a stream of nitrogen saturated with water. The rehydrated phospholipids were overlaid with 180 μM EGTA, 162 mM sucrose, and 5 mM HEPES (pH 7.4) containing 100 μM fura-2. After incubation for 2 h at 37 °C, the suspension was washed four times with 200 μM EGTA, 180 mM sucrose, and 10 mM HEPES (pH 7.4), resuspended in the same buffer, and used for Ca^{2+} influx measurements within the next 24 h. Alternatively, a buffer containing 150 mM NaCl and 10 mM *N*-[tris(hydroxymethyl)methyl]-2-aminoethanesulfonic acid (TES) (pH 7.4) was used for the preparation of fura-2-loaded liposomes and Ca^{2+} influx measurements.

Measurement of Ca^{2+} Influx into Fura-2-Loaded Liposomes. The Ca^{2+} influx into fura-2-loaded liposomes was measured in the absence or presence of annexin V or ionophore A23187 (Sigma) in a fluorescence cuvette with a 1 cm path length, using a method previously described (Berendes et al., 1993a). Briefly, liposomes (final lipid concentration of 20 mM) were first suspended in 200 μM EGTA, 180 mM sucrose, and 10 mM HEPES (pH 7.4) or in 150 mM NaCl and 10 mM TES (pH 7.4). Ca^{2+} was then added to a final concentration of 600 μM. The suspensions were prewarmed to 37 °C. Ca^{2+} influx was initiated by adding annexin V isolated from cartilage, recombinant annexin V (both in nanomolar concentrations), or ionophore A23187 at a final concentration of 1 μM. The rate of Ca^{2+} influx into the fura-2-loaded liposomes was determined by measuring the fluorescence ratio at the two excitation wavelengths of 340 and 380 nm and the emission wavelength of fura-2 of 510 nm as a function of time. The excitation wavelength of the Ca^{2+}-bound form of fura-2 is 340 nm, while the excitation wavelength of the free form of fura-2 is 380 nm.

To study the effects of antibodies to annexin V (Mollenhauer et al., 1984) on Ca^{2+} influx into liposomes, Ca^{2+} (final concentration of 600 μM) and 200 nM annexin V were first added to liposome suspensions (phosphatidylserine/phosphatidylethanolamine in a molar ratio of 9:1) to allow annexin V to bind to the liposomes. Then anti-annexin V IgGs (0.1 μg/sample) were added to these suspensions. In control experiments, Ca^{2+} influx into liposomes was measured in the presence of the anti-annexin V IgG fraction and 1 μM ionophore A23187.

To study the effects of Zn^{2+} and other ions on Ca^{2+} influx into liposomes in the presence of annexin V, liposomes containing phosphatidylserine and phosphatidylethanolamine in a molar ratio of 9:1 were suspended in 200 μM EGTA, 180 mM sucrose, and 10 mM HEPES (pH 7.4) containing various concentrations (0−225 μM) of $ZnCl_2$, $CdCl_2$, $MgCl_2$, or $MnCl_2$. Ca^{2+} was then added to a final concentration of 600 μM; the suspensions were prewarmed at 37 °C, and Ca^{2+} influx was initiated by adding annexin V at a concentration of 200 nM.

Annexin V/Liposome Binding Studies. To test the binding of annexin V to the various liposomes, 200 nM annexin V isolated from cartilage or recombinant annexin V was incubated with liposomes (final lipid concentration of 20 mM) in 200 μM EGTA, 180 mM sucrose, and 10 mM HEPES (pH 7.4) in the presence of 600 μM Ca^{2+} (corresponding to a 400 μM free Ca^{2+} concentration) for 1 h at room temperature. After centrifugation at 200000*g* for 15 min, which allowed us to pellet the liposomes quantitatively, the pellets were washed twice and resuspended in 20 μL of the above buffer. Aliquots of these suspensions (5 μL) were

dotted onto nitrocellulose membranes. Liposome suspensions which were not incubated with annexin V were used as controls. After blocking with low-fat milk protein, the membranes were immunostained with a specific rabbit polyclonal antiserum to annexin V (Mollenhauer et al., 1984), followed by alkaline phosphatase-conjugated goat anti-rabbit IgG (Promega) in a 1:5000 dilution as secondary antibody. The optical density of the color reaction was determined using a densitometer. To ascertain the linearity of staining, different concentrations of annexin V were dotted onto nitrocellulose filters and immunostained as described above. Analysis of the optical density of the color reaction revealed a linear curve in the concentration range between 1 and 500 nM annexin V.

Extraction of Liposomes. Liposomes containing phosphatidylserine and phosphatidylethanolamine in various molar ratios were first incubated with 200 nM annexin V in the presence of Ca^{2+} (final concentration of 600 μM) in phosphate-buffered saline for 30 min at room temperature. After the liposomes were collected by centrifugation at 200000*g* for 15 min, they were washed with Ca^{2+} containing phosphate-buffered saline and then extracted with phosphate-buffered saline containing 10 mM EDTA for 1 h at room temperature, followed by centrifugation at 200000*g* for 15 min and extraction with phosphate-buffered saline containing 1% Triton X-100 for 1 h at room temperature. The extracts were precipitated with 3 volumes of ethanol and used for SDS gel electrophoresis.

Fluorescence Measurements. Fluorescence measurements were made using a Photon Technology Instruments fluorimeter. To measure the emission intensity of tryptophan 187, liposomes were incubated with 200 nM annexin V in 200 μM EGTA, 180 mM sucrose, and 10 mM HEPES (pH 7.4) or in 150 mM NaCl and 10 mM TES (pH 7.4). Tryptophan excitation was at 295 nm, while emission was at 350 nm. Fluorescence changes were induced by the addition of $CaCl_2$ in a concentration of 600 μM.

Chemical Cross-Linking Studies. For the cross-linking experiments, annexin V was incubated with liposomes, as described above for the binding studies, and then centrifuged. The liposome or matrix vesicle pellets containing annexin V were resuspended in 40 μL of 100 mM KCl, 1 mM $CaCl_2$, and 25 mM HEPES (pH 8.0), and 10 μL of 200 mM dimethylsuberimidate (DMS) in 100 mM KCl and 25 mM HEPES (pH 8.0) was added to give a final volume of 50 μL. The suspensions were incubated for 3 h at room temperature, and the reaction products were analyzed by SDS−PAGE and immunoblotting using antibodies against annexin V. The optical density of the color reaction was determined using a densitometer; in each lane, the optical density of the bands of monomeric and hexameric annexin V were determined and the percentage of staining of each band was calculated.

Proteins and Antibodies. Annexin V was isolated from sternal cartilage of 6−8-week-old broiler strain chickens and purified to homogeneity as described previously (Mollenhauer & von der Mark, 1983; Mollenhauer et al., 1984; von der Mark et al., 1991). Anti-annexin V IgG was prepared from rabbit antisera by chromatography on protein A-Superose (Pharmacia, Uppsala, Sweden) as described (Mollenhauer et al., 1984).

Recombinant annexin V was prepared using the pGEX expression vector (Pharmacia) following the manufacturer's instructions. Briefly, a full length chicken annexin V cDNA clone (Pfannmueller et al., 1993) was subcloned into the pGEX expression vector to create the new plasmid pGEX-AV. Subsequently, recombinant annexin V−GST fusion protein was expressed in *Escherichia coli* DH5αF′ and purified. The fusion protein was subjected to thrombin cleavage to release the annexin V molecules from the GST moiety. The reversible Ca^{2+}-dependent binding of annexin V to phosphatidylserine-containing liposomes was used as an affinity purification step to remove bacterial contaminants and thrombin. The annexin V-containing fractions were incubated with liposomes containing phosphatidylserine and phosphatidylethanolamine in a molar ratio of 2.5:7.5 in phosphate-buffered saline (pH 7.4) containing 1 mM $CaCl_2$. After centrifugation, the annexin V bound to the liposome pellet was released by incubation with phosphate-buffered saline containing 10 mM EDTA. The released protein was dialyzed against phosphate-buffered saline and then used for incubation with liposomes.

SDS−Polyacrylamide Gel Electrophoresis. Samples were dissolved in 3% SDS sample buffer (Laemmli, 1970) with dithiothreitol, denatured at 100 °C for 3 min, and analyzed by electrophoresis in 12% (w:v) SDS−polyacrylamide gels. Gels were stained with Coomassie Blue.

Immunoblotting. Samples were electroblotted onto nitrocellulose filters (Schleicher and Schuell) after electrophoresis on 12% (w:v) SDS−polyacrylamide gels as described previously (Kirsch & von der Mark, 1990). After blocking with a solution of low-fat milk protein, blotted proteins were immunostained with rabbit anti-annexin V IgG, using alkaline phosphatase-conjugated goat anti-rabbit IgG in a 1:5000 dilution as secondary antibody.

RESULTS

Ca^{2+} Influx into Liposomes. To study the role of annexin V in Ca^{2+} influx into matrix vesicles, we used *in vitro*-reconstituted vesicles as an experimental system. We prepared liposomes made of phosphatidylserine and phosphatidylethanolamine in a molar ratio of 9:1 and containing fura-2, a Ca^{2+} sensitive dye. We also isolated and purified annexin V to homogeneity (Figure 1b) from chicken sternal cartilage (Berendes et al., 1993a; Mollenhauer & von der Mark, 1983; Mollenhauer et al., 1984; von der Mark et al., 1991). We then studied the effects of annexin V on Ca^{2+} influx into fura-2-loaded liposomes (Figure 2). When the fura-2-loaded liposomes were first exposed to Ca^{2+} and warmed to 37 °C in the absence of annexin V, we observed increases in the fluorescence ratio (see in Figure 2 the arrows), indicating the presence of trace amounts of fura-2 in the suspension medium. Addition of 200 nM annexin V led to a significant further increase in the fluorescence ratio (Figure 2, open squares), whereas addition of buffer alone did not further increase the ratio (Figure 2, open circles). The rate of Ca^{2+} influx into the liposomes was dependent on the concentration of annexin V (Figure 3). The highest influx rate was observed in the presence of 400 nM annexin V; interestingly, in the presence of 800 nM annexin, the rate of Ca^{2+} influx declined. Annexin V denatured by heating to 100 °C for 10 min mediated very little Ca^{2+} influx (Figure 3, den. AV).

To exclude the possibility that contaminating proteins could affect the results, we produced recombinant chicken

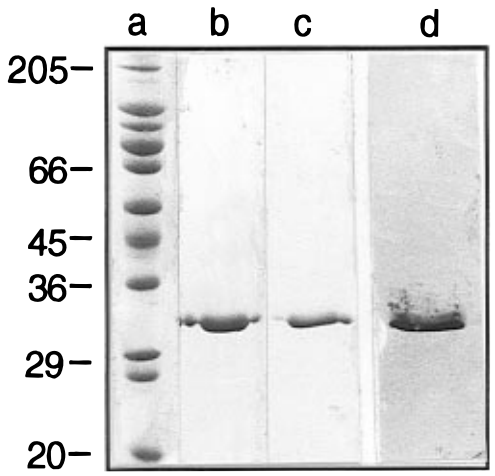

FIGURE 1: SDS−polyacrylamide gel electrophoresis and immunoblotting of recombinant chicken annexin V. Protein samples were applied to 12% (w:v) SDS−polyacrylamide gels after reduction with dithiothreitol. Gels were stained with Coomassie Blue: lane a, molecular weight markers. lane b, annexin V isolated and purified from chicken sternal cartilage. lane c, purified recombinant chicken annexin V. lane d, immunoblotting of the contents of lane c with a rabbit antiserum against chicken annexin V.

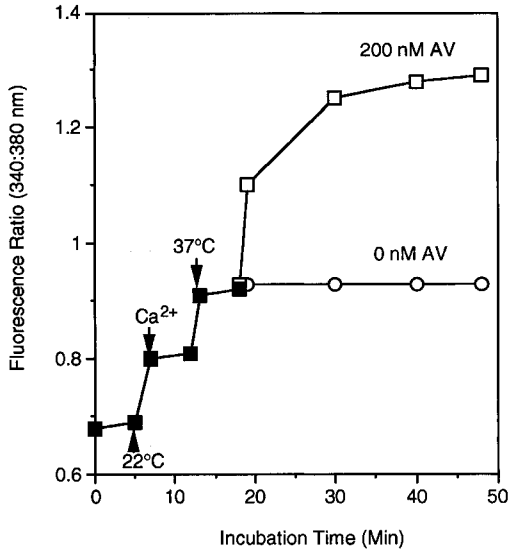

FIGURE 2: Ca^{2+} influx into liposomes in the presence of cartilage-derived annexin V. Fura-2-loaded liposomes containing phosphatidylserine and phosphatidylethanolamine in a molar ratio of 9:1 were resuspended in 200 μM EGTA, 180 mM sucrose, and 10 mM HEPES (pH 7.4). $CaCl_2$ (600 μM) (free Ca^{2+} concentration is 400 μM) was added to the liposome suspension at the time labeled Ca^{2+}; then the suspension was prewarmed to 37 °C (labeled 37 °C). Annexin V (dissolved in phosphate-buffered saline at pH 7.4) was in a concentration of 200 nM (open squares). Buffer (phosphate-buffered saline at pH 7.4) with 0 nM annexin V was used as a control (open circles). The rate of Ca^{2+} influx into the liposomes was determined by measuring the fluorescence ratio at the two excitation wavelengths of fura-2 of 340 and 380 nm. Note the annexin V-mediated Ca^{2+} influx into liposomes containing phosphatidylserine and phosphatidylethanolamine in a molar ratio of 9:1.

annexin V in *E. coli* using the pGEX expression vector. The recombinant protein migrated as a single band in SDS−polyacrylamide gels (Figure 1c), had the same molecular weight as cartilage-derived annexin V (Figure 1b), and specifically reacted with an antiserum to annexin V (Figure 1d). In the presence of recombinant annexin V, the rates of Ca^{2+} influx into fura-2-loaded liposomes were very similar

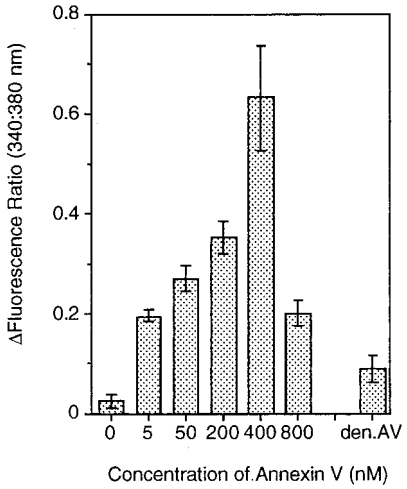

FIGURE 3: Dependence of Ca^{2+} influx into liposomes on the concentration of annexin V. Various concentrations of annexin V (0, 5, 25, 50, 200, 400, and 800 nM) were added to liposome suspensions (phosphatidylserine and phosphatidylethanolamine in a molar ratio of 9:1) in the presence of 600 μM Ca^{2+} (free Ca^{2+} concentration is 400 μM) as described in Figure 2. The change in the fluorescence ratio (340 nm:380 nm) was measured for 30 min. The bars express the difference between the fluorescence ratios (340 nm:380 nm) before and after addition of annexin V. Data were obtained from three different experiments; values are means + SE. Note that the highest Ca^{2+} influx into liposomes was observed in the presence of 400 nM annexin V; the addition of 200 nM denatured annexin V (den. AV) to liposomes resulted in little Ca^{2+} influx.

to those obtained with cartilage-derived annexin V (see Figure 8 below).

Binding of Annexin V to Liposomes Containing Various Lipid Compositions. To test whether binding of annexin V to liposomes is affected by the lipid composition, we prepared liposomes containing the following lipid mixtures: phosphatidylcholine, phosphatidylethanolamine, phosphatidylserine, and phosphatidylethanolamine in molar ratios of 2.5:7.5, 5:5, 7.5:2.5, or 9:1 and phosphatidylserine, phosphatidylcholine, and cholesterol in a molar ratio of 2:5:3. It should be noted that phosphatidylserine, phosphatidylcholine, and cholesterol are the major lipid components in the plasma membrane of chondrocytes (Wuthier, 1975). Binding of cartilage-derived or recombinant annexin V to the liposomes was determined using a dot blot assay. We found that both cartilage-derived and recombinant annexin V bound to liposomes containing various amounts of phosphatidylserine at comparable levels (Figure 4). No significant binding of annexin V in the presence of Ca^{2+} was obtained to liposomes containing only phosphatidylcholine or phosphatidylethanolamine and no phosphatidylserine (Figure 4).

It has been previously shown that binding of annexin V to phospholipids leads to a conformational change enabling tryptophan 187 to come into contact with the lipid bilayer; this contact leads to an increase in emission intensity (Concha et al., 1993; Meers & Mealy, 1994). Therefore, we measured the changes in emission intensity when annexin V binds to liposomes containing various molar ratios of phosphatidylserine and phosphatidylethanolamine. Interestingly, the most prominent increase in the emission intensity was observed with liposomes containing phosphatidylserine and phosphatidylethanolamine in a molar ratio of 9:1, suggesting that the most prominent changes in the conformation of annexin V occur in phosphatidylserine-rich liposomes leading

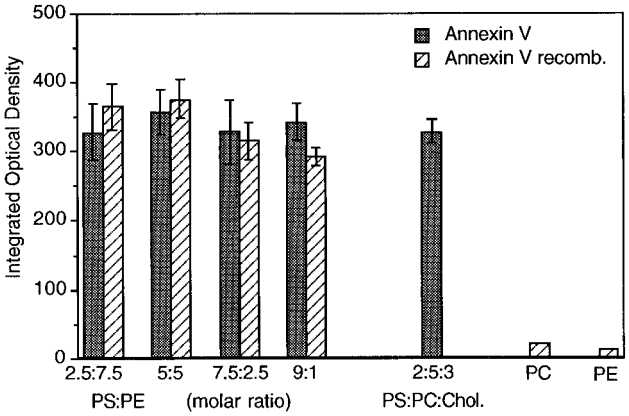

FIGURE 4: Binding of annexin V to liposomes containing various lipid compositions. Annexin V isolated from cartilage or recombinant annexin V (200 nM) was incubated with liposomes containing various lipid compositions as described in Experimental Procedures. After centrifugation and washing, aliquots of the liposome suspensions were dotted onto nitrocellulose filters. Bound annexin was immunostained using a specific antibody to annexin V. The intensity of the bands was analyzed by densitometry. Data are expressed as integrated optical density for each band. Background staining of liposome suspensions without annexin V was subtracted. Data were obtained from three different experiments; values are means + SE. Note that annexin V isolated from cartilage (Annexin V) was able to bind to liposomes containing various concentrations of phosphatidylserine (PS); no significant binding was observed to liposomes containing only phosphatidylcholine (PC) or phosphatidylethanolamine (PE). Recombinant annexin V (Annexin V recomb.) showed the same binding pattern. The binding to liposomes containing phosphatidylserine, phosphatidylcholine, and cholesterol in a molar ratio of 2:5:3 was only tested with annexin V isolated from cartilage; the binding to liposomes containing phosphatidylcholine or phosphatidylethanolamine was tested only with recombinant annexin V.

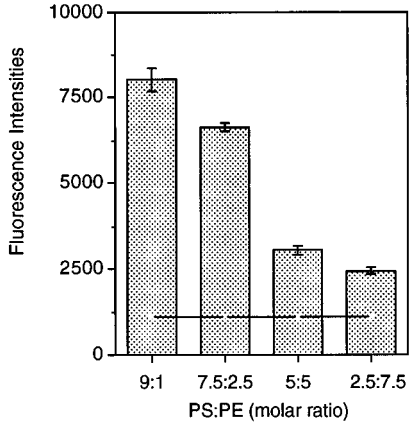

FIGURE 5: Binding of annexin V to liposomes containing phosphatidylserine and phosphatidylethanolamine in different molar ratios as measured by tryptophan fluorescence. The bars show the fluorescence increase in tryptophan upon annexin V binding to liposomes containing phosphatidylserine and phosphatidylethanolamine in molar ratios of 9:1, 7.5:2.5, 5:5, or 2.5:7.5 induced by 400 μM free Ca^{2+}. The annexin V concentration in all experiments was 200 nM. All experiments were performed at 22 °C in 200 μM EGTA, 180 mM sucrose, and 10 mM HEPES (pH 7.4). Data were obtained from three different experiments; values are means + SE. The line on the graph represents the increase due to Ca^{2+} addition in the absence of liposomes.

to an exposure of tryptophan 187 in a position where it can interact with the phospholipids in an optimal manner (Figure 5). To test the possibility that increases in the emission intensity may be related to the low-salt sucrose buffer that was used, the experiments were repeated in TES buffer containing 150 mM NaCl; similar results were obtained with

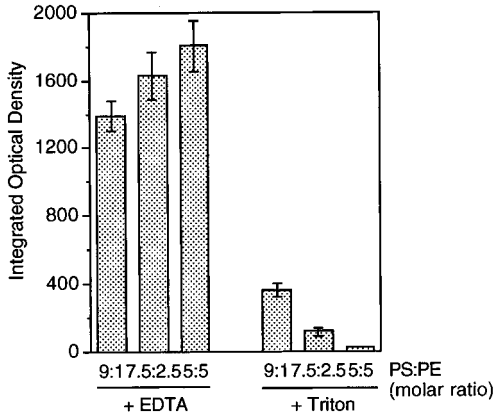

FIGURE 6: Extraction of annexin V bound to liposomes containing different molar ratios of phosphatidylserine/phosphatidylethanolamine with EDTA and Triton X-100. Annexin V bound to liposomes containing phosphatidylserine and phosphatidylethanolamine in different molar concentrations (9:1, 7.5:2.5, or 5:5) was extracted with 10 mM EDTA followed by 1% Triton X-100. While most of annexin V was extractable with EDTA from liposomes containing phosphatidylserine and phosphatidylethanolamine in molar ratios of 7.5:2.5 and 5:5, about 20−25% of annexin V was not extractable with EDTA from liposomes containing phosphatidylserine and phosphatidylethanolamine in a molar ratio of 9:1 but required 1% Triton X-100.

the most prominent increase in the emission intensity with liposomes containing a high phosphatidylserine concentration in the presence of TES buffer containing 150 mM NaCl (data not shown), indicating that changes in the emission intensities were not related to the low-salt sucrose buffer.

We next incubated liposomes containing phosphatidylserine and phosphatidylethanolamine in different molar ratios with annexin V in the presence of Ca^{2+} to allow binding of annexin V to the liposomes. We then incubated these annexin V-containing liposomes with 10 mM EDTA followed by Triton X-100. While most of annexin V was extracted by EDTA treatment from liposomes containing phosphatidylserine and phosphatidylethanolamine in molar ratios of 7.5:2.5 or 5:5, about 20−25% of annexin V was not extractable with EDTA from liposomes containing phosphatidylserine and phosphatidylethanolamine in a molar ratio of 9:1, but required Triton X-100 (Figure 6).

The results above indicated that annexin V establishes close and tight interactions with phosphatidylserine-rich bilayers, and significant amounts of annexin V may become inserted at least partially into the membrane such that the protein cannot be extracted with EDTA. Therefore, we next asked whether phosphatidylserine-rich bilayers or matrix vesicle membranes promote the formation of oligomeric states of annexin V which could allow the protein to insert into and possibly span the membrane. To investigate this question, chemical cross-linking studies using the bifunctional reagent, dimethylsuberimidate, were performed. After cross-linking of matrix vesicles isolated from calcifying cartilage or liposomes containing various phosphatidylserine concentrations and annexin V, immunoblot analyses using antibodies against annexin V revealed bands with molecular masses of approximately 33 and 205 kDA in matrix vesicles (Figure 7, lane a) and liposomes containing phosphatiylserine and phosphatidylethanolamine in a molar ratio of 9:1 (Figure 7, lane b), corresponding to monomeric and hexameric forms of annexin V. In contrast, only the monomeric form of annexin V was detectable in liposomes containing less

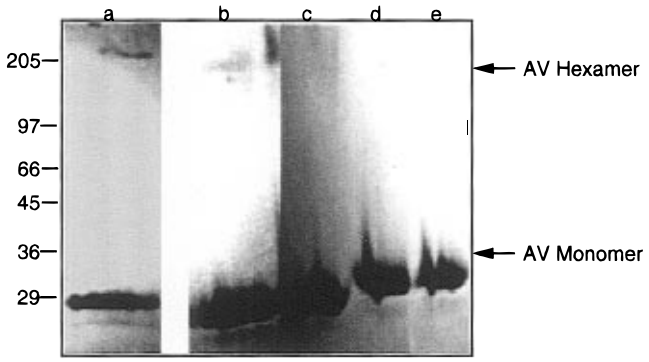

FIGURE 7: Chemical cross-linking studies of annexin V associated with matrix vesicles or liposomes containing phosphatidylserine and phosphatidylethanolamine in different molar ratios: lane a, annexin V cross-linked by dimethylsuberimidate (DMS) in matrix vesicles isolated from mineralizing cartilage. lanes b−e, annexin V cross-linked by DMS in the presence of 1 mM $CaCl_2$ and liposomes containing phosphatidylserine and phosphatidylethanolamine in molar ratios of 9:1 (lane b), 7.5:2.5 (lane c), 5:5 (lane d), and 2.5:7.5 (lane e). Note that the annexin V hexamer was only detected in matrix vesicles and liposomes containing a 9:1 molar ratio of phosphatidylserine/phosphatidylethanolamine.

phosphatidylserine (Figure 7, lanes c−e). Densitometric analysis revealed that about 20−25% of annexin molecules in matrix vesicles isolated from calcifying cartilage formed cross-linked hexamers (Figure 7, lane a); likewise, about 15% of annexin V molecules formed hexamers in liposomes containing phosphatidylserine and phosphatidylethanolamine in a molar ratio of 9:1 (Figure 7, lane b).

Ca^{2+} Influx into Liposomes Containing Various lipid Compositions. The results above suggested that a high concentration of phosphatidylserine induces the formation of annexin V hexamers which may insert into the bilayer and become inextractable with EDTA. We next asked whether the phosphatidylserine concentration also affects annexin V-mediated Ca^{2+} influx into liposomes. We prepared fura-2-loaded liposomes of different lipid compositions as above. We found that cartilage-derived annexin V elicited a significant Ca^{2+} influx only into liposomes containing phosphatidylserine/phosphatidylethanolamine at a molar ratio of 9:1 (Figure 8, Annexin V). Recombinant annexin V showed a similar behavior; maximal Ca^{2+} influx was still observed at a molar ratio of 9:1, although some Ca^{2+} influx occurred with phosphatidylserine/phosphatidylethanolamine at a ratio of 7.5:2.5 (Figure 8, Annexin V recomb.). This observation could reflect the lack of some post-translational modifications in the recombinant protein; for example, it is known that the N terminus of cartilage-derived annexin V is blocked (Genge et al., 1991). In the presence of ionophore A23187, Ca^{2+} influx was observed regardless of the lipid content of the liposomes (data not shown). Similar results were obtained when liposomes were prepared in TES buffer containing 150 mM NaCl instead of the low-salt sucrose buffer (Figure 8, Annexin V recomb./150 mM NaCl).

Annexin V-mediated Ca^{2+} influx into liposomes (phosphatidylserine and phosphatidylethanolamine in a molar ratio of 9:1) (Figure 9a) was specifically inhibited by an anti-annexin V IgG fraction (Figure 9c), while a nonimmune IgG fraction had no effect (Figure 9b). The anti-annexin V IgG fraction had no effect on Ca^{2+} influx into liposomes in the presence of ionophore A23187 (Figure 9d,e).

Ca^{2+} Influx into Liposomes Containing Other Negatively Charged Phospholipids. We next asked whether the annexin

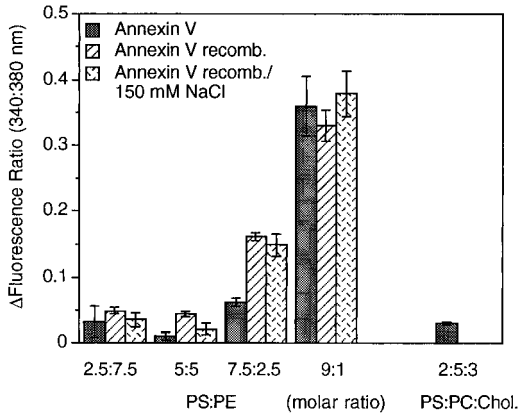

FIGURE 8: Ca^{2+} influx into fura-2-loaded liposomes containing phosphatidylserine and phosphatidylethanolamine in different molar ratios in the presence of cartilage-derived annexin V (Annexin V) or recombinant annexin V (Annexin V recomb.) Ca^{2+} influx into fura-2-loaded liposomes containing various lipid compositions was measured in the presence of annexin V isolated from cartilage (Annexin V; 200 nM) or recombinant annexin V (Annexin V recomb.; 200 nM) as described in Experimental Procedures. Experiments were performed either in low-salt sucrose buffer (Annexin V and Annexin V recomb.) or in TES buffer containing 150 mM NaCl (Annexin V recomb./150 mM NaCl). Data were obtained from three different experiments; values are means + SE. Note that significant Ca^{2+} influx in the presence of annexin V isolated from cartilage only occurred into liposomes containing phosphatidylserine and phosphatidylethanolamine in a molar ratio of 9:1; in the presence of recombinant annexin V, the highest Ca^{2+} influx rate was observed into liposomes containing phosphatidylserine and phosphatidylethanolamine in a molar ratio of 9:1, while some Ca^{2+} influx was measured into liposomes containing phosphatidylserine and phosphatidylethanolamine in a molar ratio of 7.5:2.5.

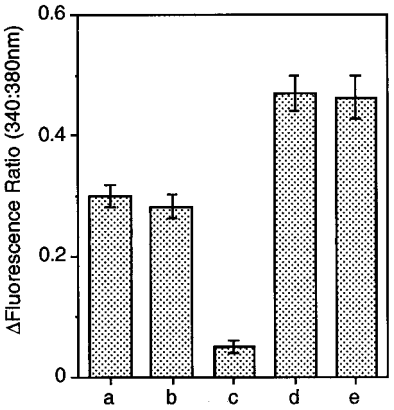

FIGURE 9: Inhibition of annexin V-mediated Ca^{2+} influx into liposomes in the presence of an anti-annexin V IgG fraction. Ca^{2+} influx into fura-2-loaded liposomes (9:1 phosphatidylserine/phosphatidylethanolamine) was measured in the presence of (a) annexin V (AV), (b) AV and nonimmune IgG, (c) AV and anti-AV IgG, (d) ionophore A23187, or (e) ionophore and anti-AV IgG as described in Experimental Procedures.

V-mediated Ca^{2+} influx into liposomes requires only a high concentration of negative charges or also requires specific phospholipid head groups. Therefore, liposomes containing different negatively charged phospholipids, such as phosphatidic acid, phosphatidylglycerol, phosphatidylinositol or phosphatidylserine, and phosphatidylethanolamine, in various molar ratios were prepared and loaded with fura-2. To verify that all liposomes were loaded with fura-2, liposome suspensions were solubilized with Triton X-100 (data not shown). While some Ca^{2+} influx in the presence of recombinant annexin V was observed into liposomes containing phos-

Table 1: Ca^{2+} Influx into Fura-2-Loaded Liposomes Containing Different Lipid Compositions in the Presence of Recombinant Annexin V[a]

	2.5:7.5 molar ratio	5:5 molar ratio	7.5:2.5 molar ratio	9:1 molar ratio
PA/PE	0	0	0.019 + 0.003	0.078 + 0.014
PG/PE	0	0	0	0
PI/PE	0	0	0	0.008 + 0.002
PS/PE	0.009 + 0.002	0.011 + 0.004	0.075 + 0.007	0.175 + 0.015

[a] Data express the difference between the fluorescence ratios (340 nm:380 nm) before and after addition of recombinant annexin V (200 nM) and were obtained from three different experiments. Values are means + SE. The abbreviations are as follows: PA, phosphatidic acid; PE, phosphatidylethanolamine; PG, phosphatidylglycerol; PI, phosphatidylinositol; PS, phosphatidylserine.

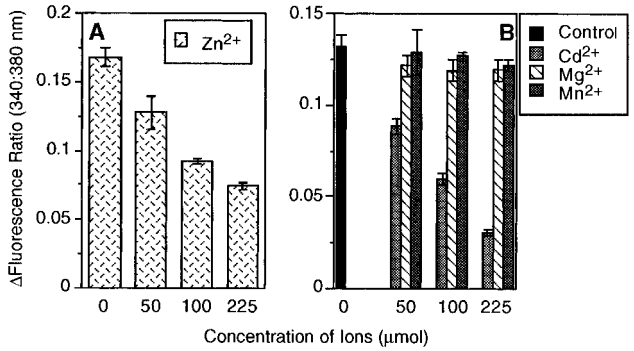

FIGURE 10: Ca^{2+} influx into liposomes in the presence of annexin V and Zn^{2+} (A), and Cd^{2+}, Mg^{2+}, or Mn^{2+} (B). Ca^{2+} influx into fura-2-loaded liposomes (phosphatidylserine/phosphatidylethanolamine at a molar ratio of 9:1) was measured in the presence of Zn^{2+} (A) and Cd^{2+}, Mg^{2+}, or Mn^{2+} (B) in different concentrations (0, 50, 100, and 225 μmol) and annexin V (200 nM) as described in Experimental Procedures. While Mg^{2+} and Mn^{2+} did not affect annexin V-mediated Ca^{2+} influx into liposomes, Zn^{2+} and Cd^{2+} inhibited annexin V-mediated Ca^{2+} influx in a dose-dependent manner.

phatidic acid and phosphatidylethanolamine in a molar ratio of 9:1, the highest influx occurred into liposomes containing phosphatidylserine and phosphatidylethanolamine in a molar ratio of 9:1 (Table 1). No Ca^{2+} influx was detected into liposomes containing lower concentrations of phosphatidic acid or containing phosphatidylglycerol or phosphatidylinositol even in high concentrations (Table 1).

Effects of Other Ions on the Ca^{2+} Influx into Liposomes. To gain further insights into the mechanisms by which annexin V mediates Ca^{2+} influx into liposomes, we tested whether other ions, such as zinc, cadmium, magnesium, and manganese, affect Ca^{2+} influx. We should point out that Zn^{2+}, Mg^{2+}, and Mn^{2+} are present at relatively high concentrations in mineralized tissues and may be essential for the normal mineralization process, but their exact role remains unclear (Guggenheim & Gaster, 1973; Hurley & Asling, 1963; Bird & Thomas, 1963; Sauer et al., 1989; Wuthier, 1992). We measured the Ca^{2+} influx into liposomes containing phosphatidylserine and phosphatidylethanolamine in a molar ratio of 9:1 in the presence of annexin V and various concentrations of Zn^{2+}, Cd^{2+}, Mn^{2+}, or Mg^{2+}. While Zn^{2+} did not affect the binding of annexin V to liposomes in the presence of Ca^{2+} (data not shown), it decreased the annexin V-mediated Ca^{2+} influx into liposomes in a dose-dependent manner (Figure 10A). However, Zn^{2+} had no effect on Ca^{2+} influx into liposomes in the presence of ionophore A23187 (data not shown). Cd^{2+} also led to a significant dose-dependent decrease of Ca^{2+} influx (Figure 10B), while Mn^{2+} or Mg^{2+} had no effect (Figure 10B).

DISCUSSION

The results of our study show that annexin V isolated from chicken sternal cartilage or recombinant annexin V is able to mediate a rapid Ca^{2+} influx into artificial liposomes. The maximal Ca^{2+} influx, the highest increase in tryptophan 187 emission intensity, and the requirement of detergent solubilization are all observed when cartilage-derived or recombinant annexin V is associated with liposomes containing a high concentration of phosphatidylserine. High phosphatidylserine content induces the formation of the annexin V hexamer; the hexameric form of annexin V also exists in matrix vesicles. The findings suggest that the formation of hexameric annexin V molecules in a high phosphatidylserine environment may enable the molecules to become more firmly and deeply inserted into the membrane; this may be causally related to the ability of the protein to mediate the flux of Ca^{2+} across the membrane. The data lend support to our hypothesis that by being phosphatidylserine-rich, the membrane of matrix vesicles *in vivo* may offer an ideal environment for optimal protein−protein and protein−lipid interactions and for optimal function of annexin V in Ca^{2+} influx and cartilage matrix mineralization.

Our conclusion that annexin V may form functionally important hexamers correlates well with the recent crystallographic study on annexin XII (Luecke et al., 1995). The authors proposed a model in which the disc-shaped hexamer structure of annexin XII is stabilized by electrostatic bonding and Ca^{2+} ions. They also proposed a model of membrane integration in which 18 Ca^{2+} ions situated on the perimeter of the annexin XII hexamer interact with the phospholipid head groups (Luecke et al., 1995). We now demonstrate that liposomes containing a high concentration of phosphatidylserine induce the formation of the annexin V hexamer. In addition, we find the highest increase in the tryptophan 187 emission intensity in phosphatidylserine-rich liposomes, suggesting the most prominent change in the conformation of annexin V in the presence of a high phosphatidylserine concentration; this rearrangement moves the tryptophan residue near the convex surface of the protein where it might interact with the phospholipids in an optimal manner. Further, significant amounts of annexin V become EDTA-resistant in phosphatidylserine-rich liposomes, in agreement with the observation that membranes from lung, heart, and other tissues contain EGTA-resistant detergent-soluble annexin V (Bianchi et al., 1992; Trotter et al., 1995). The hexameric form of annexin V also exists in matrix vesicles, particles which are enriched in phosphatidylserine (Wuthier, 1975). Thus, it is reasonable to conclude that the hexameric form of annexin V is able to insert into the phosphatidylserine-rich membrane.

Previous studies have shown that annexin V binds to phospholipids through interactions with specific phospholipid binding sites and nonspecific associations with negative charge (Meers & Mealy, 1993, 1994). In addition, it has been demonstrated that highly specific interactions exist between annexin V and the serine head group; the Ca^{2+} binding loops of annexin V appear to designed specifically to fit the serine head group (Swairjo et al., 1995). Thus, it is plausible that these specific interactions between annexin

V, Ca^{2+}, and the serine head group are required for the formation and stabilization of the hexameric form of annexin V, while other negatively charged phospholipids cannot promote and stabilize the formation of the annexin V hexamer; thus, no Ca^{2+} influx occurs into these liposomes.

Annexin V is produced by many cell types in which it is believed to exert a variety of functions (Creutz, 1992; Geisow et al., 1986; Zaks & Creutz, 1990; Ali et al., 1989; Rojas et al., 1990, 1992; Kirsch & Wuthier, 1994; Hauptmann et al., 1989; Andree et al., 1992; Schlaepfer et al., 1992; Davidson et al., 1990). Our data clearly indicate that the lipid composition of the membrane is an important factor controlling annexin V functions and that phosphatidylserine appears to serve as an activator of annexin V function. Interestingly, phosphatidylserine was shown to serve as an activator for other Ca^{2+}/lipid binding proteins. For example, protein kinase C binds various phospholipids, but only phosphatidylserine influences its catalytic activity (Gavrilova & Petkova, 1995; Yang & Glaser, 1995; Orr & Newton, 1992). Thus, it is reasonable to conclude that the degree and extent of association of annexin V with different lipid bilayers may control annexin V function in different tissues and cells.

Our findings demonstrate that annexin V is able to modulate Ca^{2+} permeability in phosphatidylserine-rich membranes and that annexin V-mediated influx of Ca^{2+} is highly specific and is not simply due to leakiness of the membrane caused by annexin V. To begin with, we find that the Ca^{2+} influx is inhibited by antibodies to annexin V, clearly invoking an essential and specific protein participation in the influx process. In addition, we find that Zn^{2+} and Cd^{2+} inhibit annexin V-mediated Ca^{2+} influx into liposomes as indicated by decreases in fura-2 emission. Both Zn^{2+} and Cd^{2+} have been shown to bind with high affinity to fura-2 and produce excitation and emission spectra similar to those produced by Ca^{2+} (Hinkle et al., 1992; Hinkle & Osborne, 1994). Thus, if annexin V were to merely render the membrane leaky (allowing Zn^{2+} and Cd^{2+} to flow inside or fura-2 to flow outside), we should observe an increase in fura-2 emission and not a decrease. On the basis of these findings and patch-clamp studies demonstrating a single-channel activity of annexin V in artificial lipid bilayers (Rojas et al., 1990, 1992; Berendes et al., 1993b; Burger et al., 1994), it is reasonable to conclude that annexin V can act as an ion channel protein. This conclusion is also supported by crystallography studies showing a hydrophilic pore through the protein, which suggests an ion channel (Huber et al., 1990; Bewley et al., 1993). Our findings demonstrate that annexin V is only able to mediate Ca^{2+} influx into phosphatidylserine-rich liposomes; these liposomes also promote the formation of the annexin V hexamer, and detergents are needed to extract significant amounts of annexin V from these liposomes. Thus, it is very likely that the hexameric form of annexin V which has the spatial dimension to span the membrane can insert into the membrane and mediate ion flux across the membrane. Ions such as Zn^{2+} and Cd^{2+} may block the central channel pore of the annexin V hexamer and hinder the passage of Ca^{2+} ions through this pore and into liposomes.

Previous studies have shown that Zn^{2+} inhibits Ca^{2+} influx into matrix vesicles (Sauer et al., 1989) and the transition from a noncrystalline to a crystalline mineral phase (Legeros et al., 1977; Taylor et al., 1990). In addition, tibiae from zinc-deficient rat fetuses show irregular and extensive calcification of cartilage (Da Cunha Ferreira et al., 1991), while elevated levels of Zn^{2+} are associated with osteochondrosis, a disease characterized by a near lack of mineralization of growth plate cartilage (Gunson et al., 1982). Interestingly, our data show that Zn^{2+} inhibits Ca^{2+} influx into phosphatidylserine-rich liposomes mediated by annexin V. It appears therefore that Zn^{2+} may have a direct effect on the function of annexin V in matrix vesicles *in vivo* and may control the initial stages of matrix vesicle-dependent calcification.

As summarized above, annexin V mediates Ca^{2+} influx into matrix vesicles, particles enriched in phosphatidylserine compared to the plasma membrane of chondrocytes (Wuthier, 1975). Interestingly, we found that the hexameric structure of annexin V also exists in these particles. However, the phosphatidylserine content in matrix vesicles is not as high as that required for maximal channel activity by annexin V in artificial liposomes. One way to reconcile this difference is to propose that clustering of phosphatidylserine may occur in the membrane of matrix vesicles, leading to a local high concentration of phosphatidylserine surrounding annexin V molecules. Indeed, protein kinase C, which also binds in a Ca^{2+}-dependent manner to phospholipids, induces extensive clustering of anionic phospholipids in membranes (Bazzi & Nelsestuen, 1991). Alternatively, other matrix vesicle factors in addition to annexin V may support Ca^{2+} influx into these particles.

In conclusion, this study provides evidence that phosphatidylserine promotes a conformational change upon annexin V binding, which enables the protein to form hexamers and to mediate Ca^{2+} influx into liposomes and matrix vesicles. In addition, our findings provide the first insights into the mechanisms by which Zn^{2+} controls annexin V-mediated Ca^{2+} influx into matrix vesicles *in vivo*. Annexins have been shown to be involved in membrane budding and exocytosis (Creutz, 1992; Zaks & Creutz, 1990; Ali et al., 1989; Lin et al., 1992). Thus, it is possible that, in the presence of elevated intracellular Ca^{2+} concentrations found in hypertrophic chondrocytes (Kirsch et al., 1992; Iannotti & Brighton, 1989; Gunter et al., 1990), annexin V may promote the release of phosphatidylserine-rich matrix vesicles from the plasma membrane of hypertrophic chondrocytes. These phosphatidylserine-rich particles would then provide a specialized environment for maximizing annexin V functions, leading to the rapid influx of Ca^{2+} required for intralumenal crystal growth during the initial calcification phase.

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