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Changes in Annexin (Lipocortin) Content in Human Amnion and Chorion at Parturition

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Abstract Arachidonic acid is mobilized from fetal membrane phospholipids at parturition leading to increased production of oxytocic prostaglandins which may initiate or maintain myometrial contractions. Phospholipid mobilization requires activation of phospholipase A_2 or C, both of which require calcium for activity. The annexins (lipocortins) are a superfamily of proteins which bind to calcium and phospholipids and thereby may alter phospholipase activity through two mechanisms: modulation of intracellular free Ca^{2+} concentrations or regulation of the accessibility of phospholipids to hydrolyzing enzymes. Using Western immunoblotting with monospecific polyclonal antibodies, annexins I–VI were identified in human amnion and chorion/decidua at term in tissues obtained from patients in labor or not in labor. Each annexin was present in two distinct pools: a pool which only associated with the membrane in the presence of calcium (calcium-dependent pool) and a calcium-independent pool that remained membrane bound in the presence of calcium chelators. Annexin I was present as two species, resolving at 36 kDa and 68 kDa. The total concentration of annexin I in both amnion and chorion/decidua was significantly decreased with labor, while the total concentration of annexin V in chorion significantly increased with labor. The size of individual pools of annexins also changed with labor: the calcium-dependent pool of annexins I and II in both amnion and chorion significantly decreased; the calcium-dependent pool of annexin V increased in chorion; and calcium-independent pools of annexin I in amnion and annexins I, II, and V in chorion significantly decreased with labor. The decrease in total annexin I concentration with labor in amnion reflects a substantial decrease (80–90%) in the pool tightly bound to the membrane in a calcium-independent manner. This striking change distinguishes annexin I as a potential candidate inhibitor which is specifically downregulated at parturition, potentially leading to increased access of phospholipases to substrate phospholipids and increased prostaglandin production at labor.

Key words: annexin, lipocortin, prostaglandin metabolism, phospholipase A_2, membrane-protein interaction, calcium-phospholipid binding, amnion, chorion

A superfamily of proteins, termed annexins or lipocortins, which bind with high affinity to calcium and phospholipids, have recently been cloned and sequenced [Pepinsky et al., 1988; Moss and Crumpton, 1990]. The annexin family includes a diverse group of proteins which have previously been described as anti-inflammatory proteins [Pepinsky et al., 1988], calcium mediators [Moore et al., 1984; Mathew et al., 1986], calcium and phospholipid binding proteins involved in exocytosis and signal transduction [Creutz et al., 1987], or as anticoagulants [Maurer-Fogy et al., 1988; Tait et al., 1988; Römisch et al., 1990]. The annexins appear to be central to a number of signal transduction pathways by virtue of their intracellular localization, their high affinity interaction with calcium and phospholipids, and their potential involvement in phosphorylation cascades. The affinity of annexins for calcium and phospholipid binding is altered by phosphorylation by both tyrosine and serine/threonine kinases [Glenney, 1986; Sheets et al., 1987; Creutz et al., 1987; Varticovski et al., 1988], suggesting that their interactions with calcium and phospholipids are under physiological control.

The fetal membranes are thought to be an important target in a paracrine signaling pathway from the fetus to the myometrium initiating a cascade of events leading to myometrial

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contractions [Challis, 1985]. The mobilization of arachidonic acid from anionic phospholipids, predominately phosphatidylethanolamine, appears to be a crucial step in prostaglandin production in human fetal membranes during labor [Okazaki et al., 1981]. The specific biochemical mechanisms and the regulatory points in the metabolic pathway are poorly understood. The activity of phospholipase measured in homogenates of fetal membranes, using synthetic substrates in vitro, appears to be elevated during the third trimester of pregnancy, although arachidonic acid release and prostaglandin production in vivo are only activated at parturition [Okazaki et al., 1982], suggesting that an inhibitory mechanism regulates the release in vivo. The inhibitory mechanism could be acting directly on the enzyme or indirectly, e.g., through sequestration of the substrate prior to parturition. The role of annexins in inhibition of phospholipase activity in vivo is still controversial. However, in vitro studies have shown that annexins can act via the sequestration of membrane phospholipids [Ahn et al., 1987; Machoczek et al., 1989], a mechanism termed the *substrate depletion* model [Davidson et al., 1990]. Of the annexins, annexins I and II show the lowest dependence on calcium and the highest affinity for binding to anionic phospholipids [Blackwood and Ernst, 1990]. These two annexins are therefore candidates as inhibitors of phospholipase A_2 [Pepinsky et al., 1988] and phospholipase C activity [Machoczek et al., 1989] and hence inhibition of arachidonic acid release in vivo.

Annexins are potent anti-inflammatory compounds in in vitro model systems in which the inflammatory response is coupled to production of prostaglandins. Recombinant annexin I inhibits prostaglandin production mediated specifically by phospholipase A_2 in three animal models of inflammation: the perfused guinea pig lung, the rat paw stimulated with carrageenin, and in rat brain subjected to ischemia and reperfusion [Cirino et al., 1987, 1989; Relton et al., 1991]. In all three systems, one of the prostaglandins that is inhibited by recombinant lipocortin I is prostaglandin E_2 (PGE_2), the principal prostaglandin produced by amnion tissue in vivo at labor [Challis, 1985] and in vitro in response to agents which stimulate prostaglandin production [Casey et al., 1988; Sander and Myatt, 1990]. These studies were undertaken to examine changes in annexin concentrations which occur in vivo at parturition in human fetal membranes and to identify those annexins which may be important regulators of prostaglandin production in the fetal membranes.

MATERIALS AND METHODS

Tissue Acquisition and Processing

Human fetal membranes and placenta were obtained under an IRB-approved protocol following spontaneous vaginal delivery or elective cesarean section at term. Patients were not subjected to any procedure which would not normally occur. Amnion and chorio/decidual membranes were physically separated from the placenta, transported back to the laboratory in ice-cold phosphate buffered saline (PBS), rinsed 2–3 times in 10 volumes of ice-cold PBS (to remove blood), and quickly frozen. Placenta was chopped into cubes, washed in PBS, and quickly frozen. In initial experiments, portions of the tissues were processed immediately after delivery without freezing and compared with tissue which had been frozen. The process of freezing the tissue prior to homogenization did not alter either recovery or distribution of annexins into the calcium-dependent and calcium-independent pools. Subsequently, all tissues were quickly frozen and stored at −80°C until processed.

Subcellular Fractionation of Annexins

Initial fractionation procedures were worked out using placental tissues. Frozen tissue was weighed and then homogenized in buffer A (200 mM sucrose, 25 mM Tris-HCl, pH 7.5, 1 mM DTT) containing protease inhibitors (2 mM EGTA, 10 mM PMSF, 0.4 mg/l leupeptin, 4 mg/l pepstatin, 35 mg/l TPCK, 35 mg/L TLCK, and 2 mg/l chymostatin) and phosphatase inhibitors (50 mM NaF, 10 mM Na_2 EDTA, 5 mM $ZnCl_2$) in a Waring miniblender for 2 × 30 s. We have previously used this mixture of protease inhibitors to obtain undegraded initiation factors during protein purification from liver, a tissue with high proteolytic activity [Kimball et al., 1987]. We also included inhibitors of protein phosphatases [Foulkes et al., 1982] to prevent changes in the phosphorylation state of annexins since it has been reported that changes in annexin phosphorylation alter membrane association or interaction [Sheets et al., 1987]. Aliquots of the homogenate were taken for measurements of protein content for normalization of the samples. The recovery of annexins in each fraction during the following procedures was

carefully monitored. Following homogenization, cellular debris was removed by centrifugation at 1,000*g* for 10 min at 4°C. The supernatant was again centrifuged at 9,000*g* for 20 min at 4°C to remove mitochondria and lysosomes. In the postlysosomal supernatant recovery was greater than 90% and the loss of annexins corresponded to volume loss. A membrane pellet was then obtained in the presence of 5 mM $CaCl_2$ by ultracentrifugation at 100,000*g* for 1 h. Greater than 95% of the annexins were recovered in the membrane pellet. The pellet was resuspended in ⅕ to $\frac{1}{10}$th of the original volume in buffer A containing 10 mM EGTA. The pellet was resuspended until no particulate material was visible by light microscopy (200×) using a glass on glass dounce homogenizer (Kontes, Vineland, NJ). Subsequent recentrifugation (in the presence of millimolar excess EGTA and the absence of calcium) separated annexins into two fractions, one extractable with EGTA and one which remained associated with the membrane pellet, the latter containing between 25 and 75% of each individual annexin. The process of resuspension and recentrifugation of the pellet was repeated for a total of four extractions. The final pellets were resuspended in buffer A and aliquots of each pellet and supernatant were saved until assayed. Volumes at each step during fractionation procedures were recorded to enable calculation of recovery and distribution of annexins.

To further examine the nature of the association of annexins with the membrane, membrane pellets from one placenta were obtained following centrifugation in the presence of 5 mM $CaCl_2$, resuspended in buffer A, and divided into five aliquots. The first three aliquots were subjected to four sequential suspension/centrifugation cycles with buffer A containing one of the following additions: EGTA alone (10 mM); Triton X-100 (0.5%) and 5 mM $CaCl_2$; or Triton X-100 (0.5%) and 10 mM EGTA. The remaining two aliquots were carried through four sequential extractions with buffer A containing either EGTA alone or Triton X-100 (0.5%) and 5 mM $CaCl_2$, followed by two additional extraction cycles with buffer A containing both Triton X-100 (0.5%) and 10 mM EGTA. Aliquots from each pellet and supernatant were saved for immunoblotting. At every fractionation step, volumes were recorded so that samples could be matched for examination of the distribution of annexins in individual fractions.

Identification and Quantitation of Annexins by Western Immunoblotting

Prior to electrophoresis, sample volumes were adjusted to account for volume changes at each step of the fractionation procedure, and matched to the protein content of the original homogenate, so that 10 μl of sample represented material in the fraction corresponding to the equivalent of 50 μg of original homogenate protein. Therefore, samples are directly comparable across the fractionation procedure and between individual tissues. Samples were diluted by addition of an equal volume of 2× gel sample buffer (2% SDS, 40 mM Tris/Cl, pH 6.8, 10% 2-mercaptoethanol, 20% glycerol, and 0.02% bromophenol blue), boiled for 5 min, and centrifuged in a microfuge at 10,000*g* for 2 min. Samples (10 μl) were loaded in individual lanes above a stacking gel (5% acrylamide in stacking gel buffer, 25 mM Tris-HCl, pH 6.8) overlayed on 8–~23% acrylamide gradient gels in Tris-HCl, pH 8.8, and separated by electrophoresis at 100–300 V for ~1 h in a Daiichi minigel apparatus according to the method of Laemmli [1970]. Following separation, samples were transferred to a membrane (Nitroplus 2000, Magnagraph, Inc., Westboro, MA) by electroblotting in a semidry apparatus at approximately 1 mA/cm^2 for 2 h. Prestained standards (high and low molecular weight, Biorad, Richmond, CA) were included in separate lanes in each gel to monitor transfer efficiency, which was greater than 90% in all experiments. Following transfer, the membranes were blocked and incubated with specific antibodies as follows: Tris-buffered saline (TBS: 50 mM Tris-HCl, pH 7.5, 500 mM NaCl) containing 5% nonfat dry milk, 1 h; TBS, 2 changes, 5 min each; rabbit anti-human annexin I, II, IV, or V (provided by R.B. Pepinsky, Biogen Corp., Cambridge, MA) diluted 1:2,000 in TBS or purified rabbit IgG against anti-human annexin VI (p68, provided by S. Moss, ICRF, London) diluted 1:1,000 or monoclonal anti-bovine annexins I, II, IV, or VI (Zymed Laboratories, San Francisco, CA) diluted 1:2,000 in TBS, 12–18 h; TBS or TTBS (TBS containing 0.5% Tween-20), 3 changes, 20 min each; goat anti-rabbit IgG conjugated to alkaline phosphatase (Promega, Madison, WI) diluted 1:10,000 in TBS, 2 h; TBS or TTBS, 3 changes, 20 min each. Immunoreactive bands were visualized by addition of substrate (nitroblue tetrazolium, 100 μg/ml, 5-bromo,4-chloro,3-indoyl phosphate, 50 μg/ml) in develop-

ing buffer (50 mM Tris-HCl, pH 10.4, 100 mM NaCl, 3 mM $MgCl_2$). Quantitation was carried out with a Hoeffer densitometer connected to an IBM-PC computer.

The procedure for Western immunoblotting was carefully developed to ensure that the concentration and distribution of an individual annexin could be compared in one set of gels. For each antibody, titer sensitivity and linearity of densitometry were determined with serial dilutions of partially purified annexins from placental tissue. The log of the sample concentration was plotted against the integrated optical density and conditions were selected which led to linear changes in density over the range of annexin concentrations in the fetal membrane samples. A 20% decrease in the integrated optical density corresponded to approximately a 40–60% decrease in sample concentration in the range of dilutions represented in the figures. Western immunoblotting is subject to variability, particularly in the steps involving antibody binding and visualization with substrates. When determining changes in the distribution of annexins in different physiologic states, all samples (up to 120) were loaded, electrophoresed, transferred, and processed within a single batch of minigels. Distribution in calcium-dependent vs. calcium-independent pools was determined by separation into EGTA-resistant membrane and EGTA-extractable supernatant fractions. Total annexin concentrations were determined by measurements of annexins in the first 100,000*g* pellet obtained in the presence of buffer A containing 5 mM $CaCl_2$. For each experimental run, preliminary blots were performed to determine the dilutions of samples which were necessary to give densitometric readings in the linear range determined for each antibody.

For annexins IV and VI, the concentrations in some samples were below the limit of detection of the scanner, therefore we only quantitated annexins I, II, and V. For each annexin, the mean integrated optical density of the immunoreactive band(s) in total, calcium-dependent, or calcium-independent membrane-binding pools both in labor and nonlabor conditions was independently quantitated. Means and standard errors were calculated. Significance of differences between labor and nonlabor samples was determined by Student's t-test. For graphical representation, the integrated optical density values for each condition (calcium-dependent or -independent pool, labor or nonlabor tissue) were expressed as a percentage of the mean value for annexin I, II, or V in the nonlabor, calcium-dependent pool. For annexin I, since there were two forms, the monomeric form was chosen for normalization.

RESULTS

We have identified annexins I–VI in amnion and chorio/decidual membranes by Western immunoblotting (Fig. 1). We have confirmed the specificity of annexin I, II, IV, and VI polyclonal antibodies by comparison with monoclonal anti-annexin antibodies against annexin I (Zymed Laboratories and Dr. Pepinsky, Biogen Corp.) and annexins II, IV, and VI antibodies (Zymed Laboratories) which are known to be monospecific, and by examination of chromatographic fractions and other tissues and cells (results not shown). Annexin I was identified at approximately 35 kDA. Anti-annexin I antibody also identified annexin III, which resolved at approximately 32 kDa, and a protein of approximately 68 kDa, which probably corresponds to the dimeric form of annexin I in placenta which is covalently crosslinked by transglutaminase [Pepinsky et al., 1989]. Annexin II resolved slightly above annexin I at approximately 36 kDa. Annexins IV and V resolved at the same apparent molecular weight as annexin III, approximately 32 kDa. Some faint bands were also

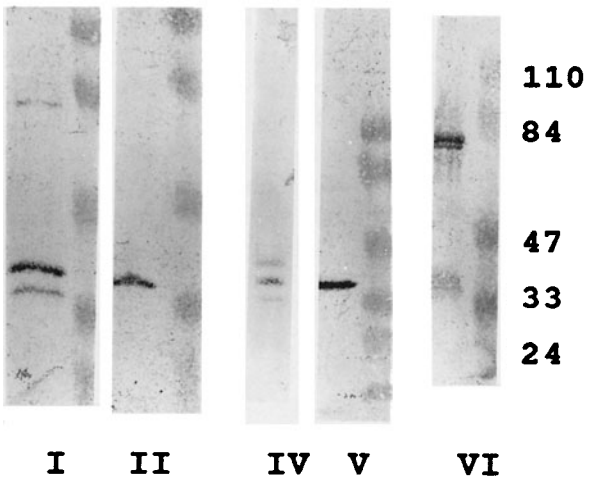

Fig. 1. Western immunoblotting of annexins I–VI in placental tissue. Membrane pellets from placenta were analyzed by Western immunoblotting as described in Materials and Methods. Individual gels were run for each annexin. All antibodies were monospecific, with the exception of annexin I, which also recognized annexin III. The approximate molecular weights of the prestained standards are shown on the right. Blots were aligned by the 33 kDa standard.

apparent with polyclonal anti-annexin IV antibody, although the principal band, at 32 kDa, was the only band detected with the monoclonal anti-annexin IV antibody. Annexin VI resolved as a doublet at approximately 65 kDa. Since the electrophoretic mobility of some of the annexins is very close or identical on one-dimensional sodium dodecyl sulfate-polyacrylamide gel electrophoresis (1-D SDS-PAGE), we examined the cross-reactivity of the antibodies we used for immunoblotting to ensure that we were detecting only a single annexin under the conditions employed. We observed no cross-reactivity except with the polyclonal antibody for annexin I, which cross-reacts with annexin III, as previously reported [Pepinsky et al., 1988]. The annexin III band was not detected with the monoclonal anti-annexin I antibody (results not shown). Although we could readily separate annexins I and III on SDS-PAGE, the width of the light beam in the densitometer did not allow resolution of the individual integrated optical densities of the two bands, so the value presented as annexin I represents the sum of both bands. We estimate that annexin III constitutes less than 25% of the total immunoreactive signal in the summed integrated optical density.

To evaluate the potential involvement of annexins in regulation of phospholipase A_2 activity, we measured the content of annexins in amnion and chorio/decidual membranes obtained from patients before the onset of labor compared to the content in tissues obtained from patients in labor. We initially utilized the property of calcium-dependent, reversible binding of annexins to membrane phospholipids [Creutz et al., 1987; Geisow, 1986] to optimize isolation and quantitation procedures (Fig. 2). EGTA extraction led to recovery of annexins in the postmicrosomal supernatant (Fig. 2, lane D), however, a significant proportion of each of the annexins remained membrane-associated (lane H). This fraction could not be removed by sequential extractions of the membrane pellet with 50 volumes of buffer containing 10 mM EGTA (Fig. 2, lanes D–G). The annexin which remained associated with the particulate fraction partially partitioned into organic solvents (results not shown) indicating a high affinity association with the membrane lipids. Further extraction procedures were then employed based on methods which lead to isolation of cytoskeletal-associated proteins [e.g., Glenney et al., 1987]. A portion of the annexins was extracted with the

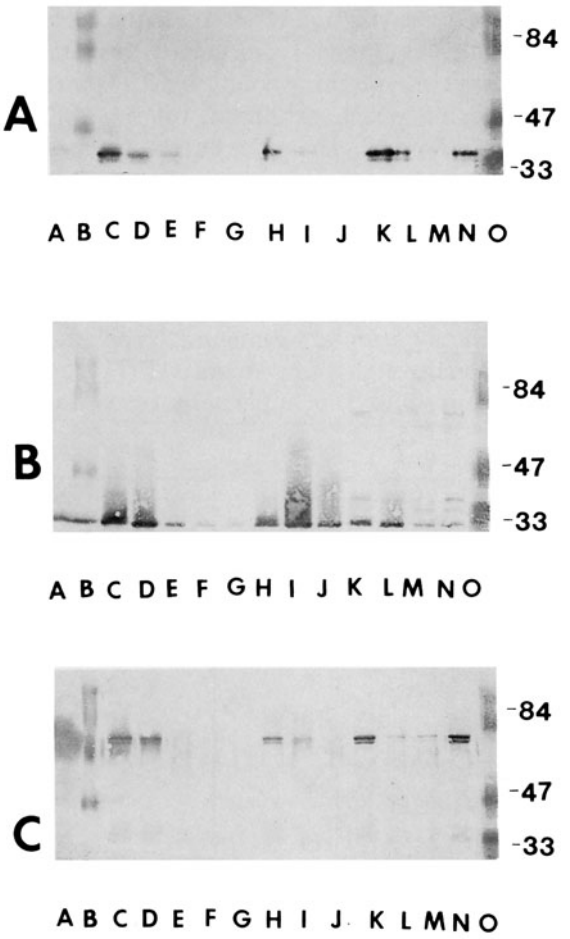

Fig. 2. Extraction of annexins from placenta with EGTA and nonionic detergents. Placenta was homogenized, fractionated by centrifugation in the presence or absence of calcium, EGTA, and Triton X-100, and immunoblotted with (**A**) anti-annexin II, (**B**) anti-annexin V, or (**C**) anti-annexin VI as described in Materials and Methods. **Lanes B,O:** Prestained molecular weight standards; approximate molecular weights are shown on the right. **Lanes A,C:** Post 100,000*g* supernatant (A) and pellet (C) in the presence of 5 mM $CaCl_2$. **Lanes D–G:** Four sequential extractions of the pellet from C with buffer A containing 10 mM EGTA. **Lane H:** Remaining annexins in the pellet after extractions with EGTA in D–G. **Lanes I,J:** Two sequential extractions of the pellet from C with buffer A containing 5 mM $CaCl_2$ and 0.5% Triton X-100. **Lane K:** The pellet after four extractions with buffer A containing 5 mM $CaCl_2$ and 0.5% Triton X-100. **Lanes L,M:** Supernatant fractions after extraction of pellet in C with buffer A containing 10 mM EGTA and 0.5% Triton X-100. **Lane N:** The residual annexins in the pellet after extractions with EGTA and Triton X-100 (in L and M).

membrane lipids in the presence of calcium (Fig. 2, supernatant in lanes I and J), with a portion remaining associated in a calcium-dependent manner with the detergent-extracted cytoskeleton (lane K). Extraction with both EGTA and Triton X-100, either sequentially (lanes L and M) or together (results not shown), did not lead

to complete extraction of all annexins from the residual pellet (lane N). Since we were primarily interested in examining changes in the two pools of annexins which exhibited different affinity interactions with the membrane, we isolated and separated annexins into these two pools to determine whether there were specific changes which occurred with labor.

A representative separation of annexins I and II in amnion and chorion is shown in Figure 3. In all fractionation experiments, the pellets were processed through four sequential EGTA extraction steps, and the supernatants containing

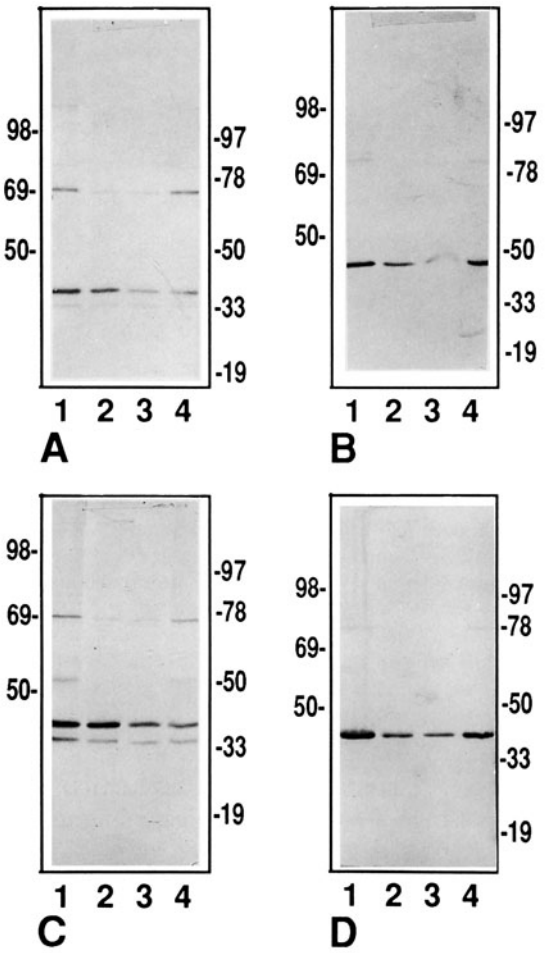

Fig. 3. Identification of annexins I and II in calcium-dependent and calcium-independent membrane-bound pools from amnion (A and B) and chorion/decidua (C and D) by Western immunoblotting. **A, C:** Polyclonal anti-annexin I antibody. **B, D:** Polyclonal anti-annexin II antibody. **Lane 1:** Total annexins in the membrane pellet in buffer A containing 5 mM $CaCl_2$. **Lanes 2, 3:** EGTA-extractable annexins (representing the calcium-dependent pool) in two sequential supernatants after centrifugation in buffer A containing 10 mM EGTA. **Lane 4:** Membrane-associated annexins (representing the calcium-independent pool) after EGTA extraction.

EGTA-extractable annexins were pooled prior to quantitative immunoblotting. Greater than 95% of the EGTA-extractable annexins were extracted in a single step in some experiments (e.g., Fig. 2, cf. lanes E–H), while a second extraction was required to remove 95% of the extractable annexin in other experiments (e.g., Fig. 3, cf., lanes 2 and 3). Using the annexin I antibody, a 68 kDa immunoreactive protein was detected in both amnion and chorion/decidual membranes, representing up to 50% of the total annexin I in amnion (prior to labor). The electrophoretic mobility on both 1-D and 2-D gels of this form of annexin I was identical in amnion, chorion, and placenta (results not shown), suggesting that this form corresponds to the crosslinked dimer previously identified in placental tissue [Pepinsky et al., 1989].

Changes in total content of annexins with labor are shown in Figure 4. The mean integrated optical density of annexin I significantly decreased by 51% with labor in amnion ($P < 0.05$) and by 45% in chorion/decidua ($P < 0.01$), while the integrated optical density of annexin II remained unchanged in both amnion and chorion/decidua. The integrated optical density of annexin V remained unchanged in amnion but increased significantly by 45% with labor in the chorion/decidua ($P < 0.05$). Although we could detect annexins IV and VI in amnion and chorion/decidua, we were unable to quantitate the immunoreactive bands.

The changes occurring in the distribution of the calcium-dependent and calcium-independent membrane-associated pools of annexins I, II, and V in amnion with labor are shown in Figure 5. The calcium-dependent pools decreased for both annexin I monomer (21%, $P < 0.025$) and annexin II (33%, $P < 0.05$) while the calcium-independent pools decreased significantly and markedly for annexin I, for both the monomeric (73%, $P < 0.025$) and the dimeric forms (91%, $P < 0.025$). Both pools of annexin V and the calcium-independent pool of annexin II were unchanged with labor.

The changes occurring with labor in the distribution of the calcium-dependent and calcium-independent pools of annexins in chorion/decidua are shown in Figure 6. The mean integrated optical density of the calcium-dependent pools of annexin I monomer decreased 76% ($P < 0.025$) as did annexin I dimer (60%, $P < 0.005$), while the calcium-dependent pool of annexin V markedly increased (200%, $P < 0.025$). De-

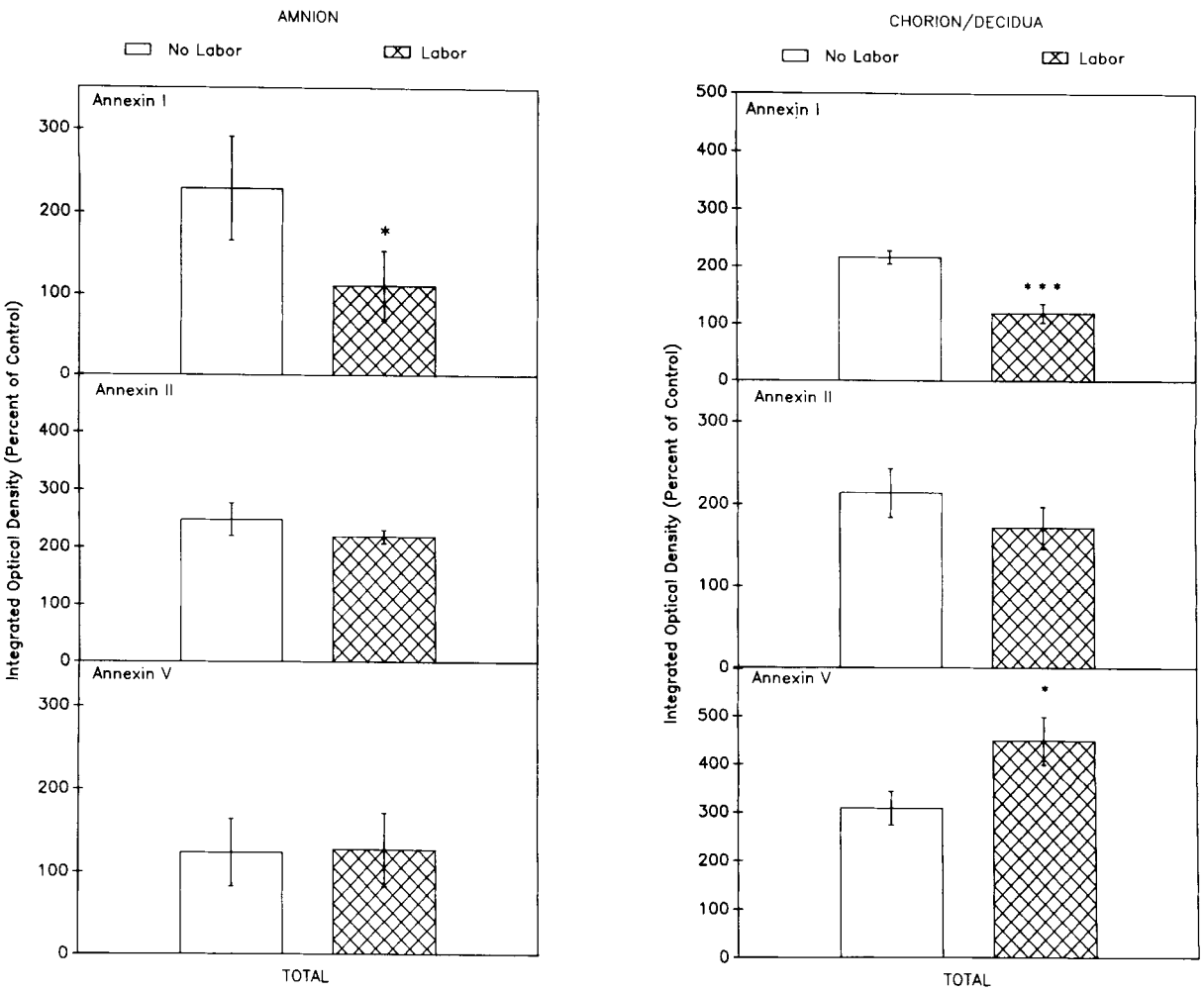

Fig. 4. Changes in total annexin concentrations in amnion and chorion/decidua with labor. Total annexins were isolated and immunoblotted with specific antibodies for annexins I, II, and V. Values are expressed as percent of the mean value of the integrated optical density of the annexin in the nonlabor, calcium-dependent pool. Significant changes were determined from integrated optical densities in samples from labor and nonlabor: $*P < 0.05$; $***P < 0.01$ (Student's t-test).

creases in the calcium-independent pools of annexin I monomer (26%, $P < 0.05$), annexin II (30%, $P < 0.25$), and annexin V (33%, $P < 0.025$) occurred with labor. The calcium-dependent pool of annexin II and the calcium-independent pool of annexin I dimer were unchanged with labor.

DISCUSSION

Although annexins have been universally characterized as calcium-dependent proteins, the nature of their interaction with cellular components has not been thoroughly characterized. Annexins were initially identified by several laboratories on the basis of the calcium dependency (EGTA extractability) of their interaction with the membrane. However, annexins exhibit promiscuous calcium-dependent interactions with a wide range of macromolecules, ranging from fatty acids and phospholipids to individual cytoskeletal proteins [Moore et al., 1984; Creutz et al., 1987; Pepinsky et al., 1988]. There have been a number of distinct methods used to isolate annexins, based on the particular function of annexins which was under investigation. Two of the most widely used methods have isolated annexins based on calcium-dependent interactions with membrane phospholipids (in the absence of detergents) or cytoskeletal elements (in the presence of detergents), employing centrifugation to isolate annexins from the bulk of other proteins. We utilized EGTA extractions of mem-

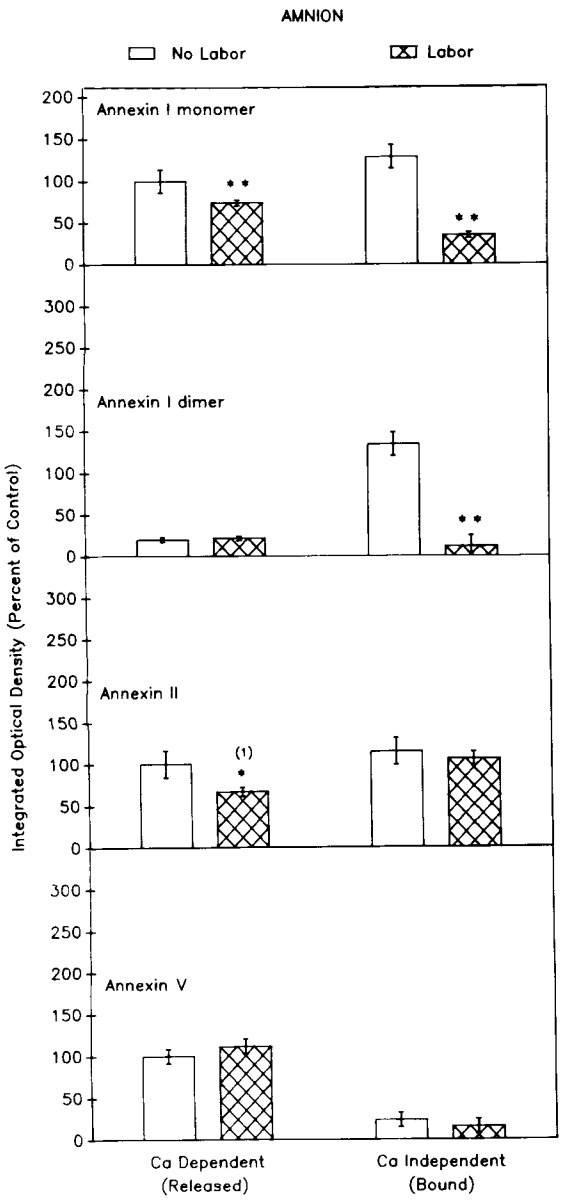

Fig. 5. Changes in the distribution of annexins in amnion with labor. Annexins were separated into calcium-dependent and calcium-independent membrane-bound pools as described in Materials and Methods. Values are expressed as the percent of the mean integrated optical density of the annexin in the nonlabor, calcium-dependent pool. Significant differences were determined from the integrated optical densities of samples from labor and nonlabor: $*P < 0.05$; $**P < 0.025$ (Student's t-test).

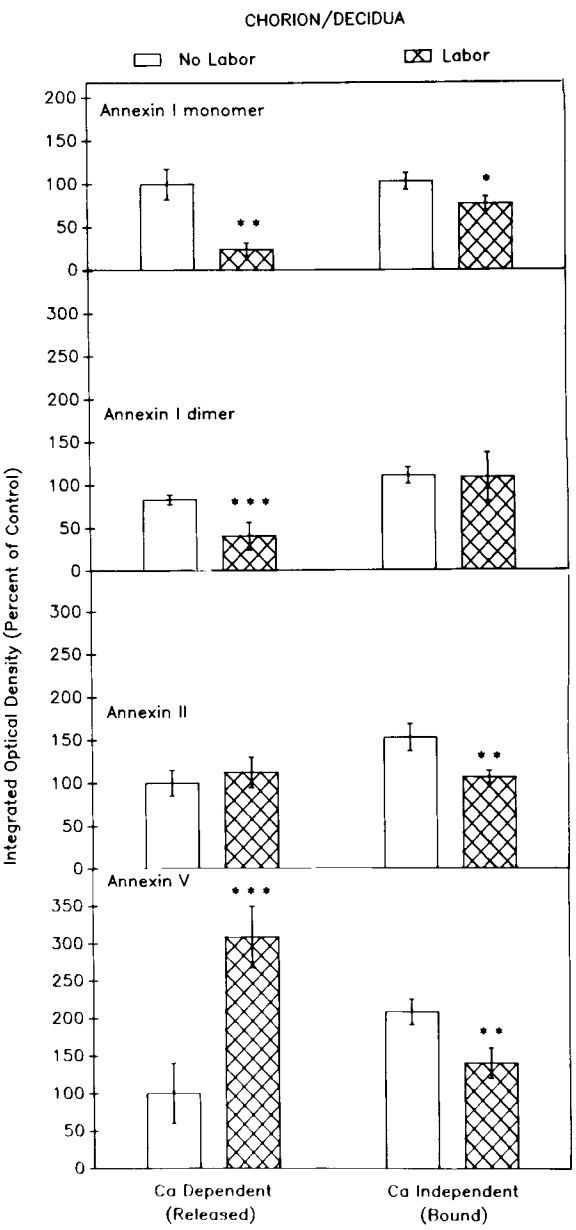

Fig. 6. Changes in the distribution of annexins in chorion/decidua with labor. Annexins were separated into calcium-dependent and calcium-independent membrane-bound pools as described in Figure 5. Significant differences between labor and nonlabor: $*P < 0.05$; $**P < 0.025$; $***P < 0.01$ (Student's t-test).

brane preparations *in the absence of detergents* to separate annexins based on the calcium dependence of their association with membranes which contain phospholipids, since our interest was in determining whether there was a change in the sequestration of phospholipids by annexins. Experimental protocols developed to investigate the calcium-dependent interaction of annexins with the cytoskeleton have used nonionic detergents to remove lipids which would be unsuitable for our studies, since a large fraction of the annexins would be discarded in the initial detergent extract. Two of the fractions isolated during these procedures have not been previously described or characterized. The first is the frac-

tion which is extractable with detergents in the presence of calcium, presumably representing annexins which have high affinity interactions with membrane lipids, and the second is the fraction which remains associated with the cytoskeleton following sequential or combined extractions with detergent and EGTA. The fraction exhibiting high affinity interaction, which may be the most important pool with respect to phospholipase inhibition, has only rarely been examined or described [Sheets et al., 1987; Varticovski et al., 1988]. Consequently, much of the literature must be critically reevaluated in light of these findings.

In these studies, we have shown that annexins I–VI can be separated into a number of discrete intracellular pools based on their extractability with combinations of EGTA and nonionic detergents. Since hydrolysis of arachidonic acid by phospholipases is a function specifically dependent on membrane phospholipids, we developed a method to examine the interaction of annexins with the membrane, to determine whether there were changes in annexin associations with the membrane at labor, when phospholipase hydrolysis of phospholipids is specifically increased. We have confirmed and extended previous reports which identified calcium-independent forms of annexin I in placenta [Sheets et al., 1987]. However, in both amnion and chorion the calcium-independent fraction of each annexin constitutes a significant fraction (Figs. 2, 5, and 6), representing between 25 and 75% of the total cellular content. This calcium-independent form may represent a novel form of annexins with distinct properties or functions in the cell. This form which remains in tight association with the membrane is likely to represent an important form of annexin with respect to the substrate depletion model of phospholipase inhibition.

The decreases in concentrations of specific forms and pools of annexin which occur with labor identify annexin I as a potential candidate for regulation of prostaglandin production in human fetal membranes at parturition. In a typical fibroblast cell, Schlaepfer and Haigler [1987] calculated that annexin I is sufficiently abundant to coat the entire inner surface of the phospholipid bilayer. Recently the high resolution crystal structure of annexin V has been identified and compared to structures obtained at lower resolution of annexins II and VI which appear to share similar gross characteristics [Huber et al., 1990]. The rough dimensions of the molecule (approximately 40×40 Å) would allow a single annexin molecule to interact with or sterically block approximately 40 phospholipids (assuming a unit area/phospholipid molecule of ~ 40 Å^2). Therefore, the marked decreases in the calcium-independent, tightly associated form of annexin I in the amnion may have a major impact on the access of phospholipases to substrates at parturition. Annexin I binds with highest affinity to phosphatidyl inositol (PI) and phosphatidyl ethanolamine (PE) [Blackwood and Ernst, 1990]. In human amnion, PI and PE were the only phospholipids to show significant decreases in arachidonic acid content with labor [Okita et al., 1982], suggesting that there is preferential utilization of these phospholipid substrates for hydrolysis and release of arachidonic acid. The decrease observed in annexin I at labor is consistent with the binding characteristics of annexin I for these two phospholipids, and with a potential specific role in substrate sequestration and inhibition of prostaglandin production in amnion prior to labor.

The nature of the interaction of the calcium-independent form with the particulate fraction remains to be determined. One possibility is that a large proportion of the annexins remains associated with the particulate fraction due to their interaction with cytoskeletal proteins such as actin [Glenney, 1986], spectrin, or other proteins in the membrane or cytoskeleton [Cheney and Willard, 1989]. Alternatively, covalent modifications may occur which alter the binding characteristics of annexins to the membrane. A number of agents which trigger prostaglandin production in amnion activate signaling pathways which alter annexin binding to phospholipids [e.g., EGF: Sheets et al., 1987; phorbol esters: Creutz et al., 1987]. A number of post-translational modifications of annexins have been shown to alter their affinity for phospholipids. Phosphorylation of annexin I was suggested to be responsible for a change in the calcium dependence of the membrane interaction in placental tissue [Sheets et al., 1987]. Acylation of proteins is another modification which can alter membrane interactions, and annexin II has been reported to be covalently modified by myristate or a metabolite of myristate [Soric and Gordon, 1985]. Crosslinked annexin I exhibits much higher affinity binding to membrane lipids [Pepinsky et al., 1989]. Indeed, the majority of dimerized annexin I in amnion is present in the tightly

associated pool. The abundance of the dimeric form and its specific loss at parturition in amnion emphasize the potential importance of the high affinity binding forms of annexin I in amnion to phospholipase inhibition.

We have separated the annexins into two pools based on the calcium dependency of the interaction with the microsomal fraction, and have shown changes in the concentration of several of these pools in amnion and chorio/decidua at parturition. The abundance of annexins, the presence of multiple annexins in individual cells and tissues, and the complex nature of changes which occur with labor suggest that these proteins may be part of a multicomponent transduction system which regulates the coordinated response of a cell or tissue to a changing environment. A full understanding of the role of annexins in fetal membranes will require evaluation of their individual roles as well as potential overlapping interactions with cellular components.

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