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Membrane-bound annexin V isoforms (CaBP33 and CaBP37) and annexin VI in bovine tissues behave like integral membrane proteins

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The distribution of annexin V isoforms (CaBP33 and CaBP37) and of annexin VI in bovine lung, heart, and brain subfractions was investigated with special reference to the fractions of these proteins which are membrane-bound. In addition to EGTA-extractable pools of the above proteins, membranes from lung, heart, and brain contain EGTA-resistant annexins V and VI which can be solubilized with detergents (Triton X-100 or Triton X-114). A strong base like Na_2CO_3, which is usually effective in extracting peripheral membrane proteins, only partially solubilizes the membrane-bound, EGTA-resistant annexins analyzed here. Also, only 50–60% of the Triton X-114-soluble annexins partition in the aqueous phase, the remaining fractions being recovered in the detergent-rich phase. Altogether, these findings suggest that, by an as yet unknown mechanism, following Ca^{2+}-dependent association of annexin V isoforms and annexin VI with membranes, substantial fractions of these proteins remain bound to membranes in a Ca^{2+}-independent way and behave like integral membrane proteins. These results further support the possibility that the above annexins might play a role in membrane trafficking and/or in the regulation of the structural organization of membranes.

Annexin V (CaBP33 and CaBP37); Annexin VI; Membrane; Binding; Calcium; Lung; Heart; Brain

1. INTRODUCTION

Proteins of the annexin family share the ability to bind to acidic phospholipids and to natural membranes in the presence of Ca^{2+} (for reviews see [1–3]). Each annexin is made of an N-terminal tail of variable length and unique to individual annexins, which is supposed to play a role in the diversification of the biological functions of single species, and of a core. This latter is made of four, in the case of the 32–37 kDa annexins, or eight, in the case of the 67–73 kDa annexins, internal repeats 70 residues in length, each of which contains a highly conserved consensus sequence, the endonexin fold, which is suggested to take part in the coordination of binding of both Ca^{2+} and phospholipids [3–5]. Unlike the Ca^{2+}-binding proteins of the EF-hand type, Ca^{2+}-binding to annexins does not induce the exposure of hydrophobic domains; rather, Ca^{2+} would cross-bridge any annexin to the negatively charged headgroups of acidic phospholipids and/or certain annexins to target proteins [1–3]. Given the above model, it is expected that chelation of Ca^{2+} will result in the complete reversal of annexin binding. While this has been proven true whenever annexin binding to liposomes or proteins had been investigated in reconstitution experiments in vitro [6–15], different results were obtained with certain annexins whenever tissue or cell subfractionation was used to investigate the binding of endogenous annexins to natural membranes. So, fractions of annexins I, II, IV–VII were reported to exist in several cell types in a membrane-bound form, to resist extraction with EGTA, and to require detergents for their solubilization [10,16–20]. Thus some annexins behave like integral membrane proteins once bound to natural membranes.

We have previously shown that annexin V (CaBP33) and CABP37 (an annexin that is an isoform of annexin V by amino acid sequence analysis (Learmonth et al., submitted for publication)) in bovine brain, and annexins V (CaBP33) and VI in porcine heart (CaBP37 is not expressed in porcine tissues [19]) exist in a membrane-bound form, and that Triton X-100 is required for the solubilization of these proteins from membranes prepared in the presence of and extensively washed in 5 mM EGTA [10,19]. To answer the question of whether or not annexin V isoforms and annexin VI (an annexin that is usually found associated with membranes [20,21]) behave like integral membrane proteins, post-nuclear membrane fractions from bovine tissues were subjected to extraction with Triton X-100, Triton X-114, or alkali, and the presence of the above annexins in these extracts was investigated by immunoblotting. We will show that the membrane-bound, EGTA-resistant fractions of these proteins can be solubilized with the detergents used, resist in part extraction with alkali, and do not completely partition in the detergent-rich phase after solubilization with Triton X-114.

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2. MATERIALS AND METHODS

2.1. *Protein purification and characterization of antisera*

The annexin V isoforms were purified from bovine brain as in [22]. In some experiments, to speed up the preparation and to increase the yield, the two proteins were purified from bovine lung as in [23]. The latter procedure was used to purify annexin VI. In fact, the EGTA-extracted material from the last Ca^{2+}-precipitate in this procedure contains polypeptides of 32–37 kDa and of 67–70 kDa in large amounts plus minor contaminants (also see [19]). By sequential chromatography on DEAE-Sephacel and AcA54 it is possible to obtain the annexin V isoforms and annexin VI in pure forms. The annexin V isoforms were separated from one another by chromatography on DEAE-Sephacel as in [10]. Antisera against the unfractionated mixture of annexin V isoforms or individual isoforms were raised in rabbits and characterized by immunoblotting as in [10]. Strong immunological cross-reactivity was observed between individual annexin V isoforms. However, none of these antisera bound to other annexins or to unrelated proteins [10,19]. Electrophoretically pure annexin VI was used to immunize rabbits by multisite injections as in [10]. The anti-annexin VI antiserum used in the present experiments also demonstrated no immunological cross-reactivity with other annexins or with unrelated proteins by immunoblotting (see section 3). In the experiments to be described, the anti-annexin V antiserum was used at 1:1000 and the anti-annexin VI antiserum at 1:1500. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblotting were done as in [24,25], respectively.

2.2. *Fractionation and subfractionation of bovine tissues*

All operations were done at 4°C unless stated otherwise. Frozen bovine lung, heart, or brain (150 g) were thawed, homogenized in 750 ml of 10 mM Tris–HCl, pH 7.5, 150 mM NaCl, 5 mM EGTA, 0.25 mM PMSF (buffer A), and centrifuged at 750 × *g* for 15 min. The resultant supernatants were saved and the pellets re-homogenized and centrifuged as above. The supernatants from individual tissues were combined, made 6 mM with a stock solution of $CaCl_2$, stirred for 15 min, and centrifuged at 60,000 × *g* in a Kontron (Centrikon H-501) centrifuge for 30 min. The resultant pellets were treated as in [23]. Briefly, they were resuspended each in 300 ml of buffer A containing 1 mM $CaCl_2$ instead of EGTA (buffer B) and centrifuged as above. The supernatants were discarded and the pellets resuspended in 300 ml of buffer B minus NaCl and centrifuged again. Individual final pellets were resuspended in 120 ml of 20 mM Tris-HCl, pH 7.5, 10 mM EGTA (buffer C), stirred for 15 min, and centrifuged at 200 000 × *g* in a Kontron (Centrikon T-1055) centrifuge for 60 min. The resultant supernatants, hereafter referred to as the EGTA-extracts, were saved, whilst the pellets were washed five times in buffer C by cycles of resuspension and centrifugation. Individual final pellets were divided in four aliquots. One of these was resuspended in 30 ml of buffer C, the second aliquot in 30 ml of buffer C containing 1% (v/v) Triton X-100, the third aliquot in 30 ml of buffer C containing 1% (v/v) Triton X-114, and the fourth one in 30 ml of 0.1 M Na_2CO_3, pH 11. After 60 min under agitation, the suspensions were centrifuged at 200,000 × *g* for 2 h. The resultant supernatants, hereafter referred to as the last EGTA-wash, the Triton X-100-extract, the Triton X-114-extract, and the Na_2CO_3-extract, respectively, were saved, and the pellets resuspended each in 30 ml of 20 mM Tris-HCl, pH 7.5. The Na_2CO_3-extracts and residues were brought to pH 7.5 with acetic acid before further processing. The three EGTA-extracts, the last EGTA-washes, the Triton X-100-, Triton X-114-, and Na_2CO_3-extracts, and the resuspended pellets were subjected to SDS-PAGE (10% acrylamide). Electrophoretically separated proteins were either stained with Coomassie blue or transblotted onto nitrocellulose paper for immunostaining with the anti-annexin V or the anti-annexin VI antiserum. Individual Triton X-114-extracts were further processed as in [26]. Briefly, they were incubated at 30°C for 5 min until solutions became cloudy and centrifuged at 600 × *g* at room temperature to obtain upper (aqueous) and lower (detergent-rich) phases, which were separated from one another by aspiration. Individual aqueous phases were saved, whilst individual detergent-rich phases were brought to 30 ml with 20 mM Tris-HCl, pH 7.5, 1% Triton X-114, incubated at 30°C for 5 min, and centrifuged as above. The aqueous phases were discarded, and the detergent-rich phases were each brought to 30 ml with cold 20 mM Tris-HCl, pH 7.5. The aqueous and detergent-rich phases were subjected to SDS-PAGE and immunoblotting as above.

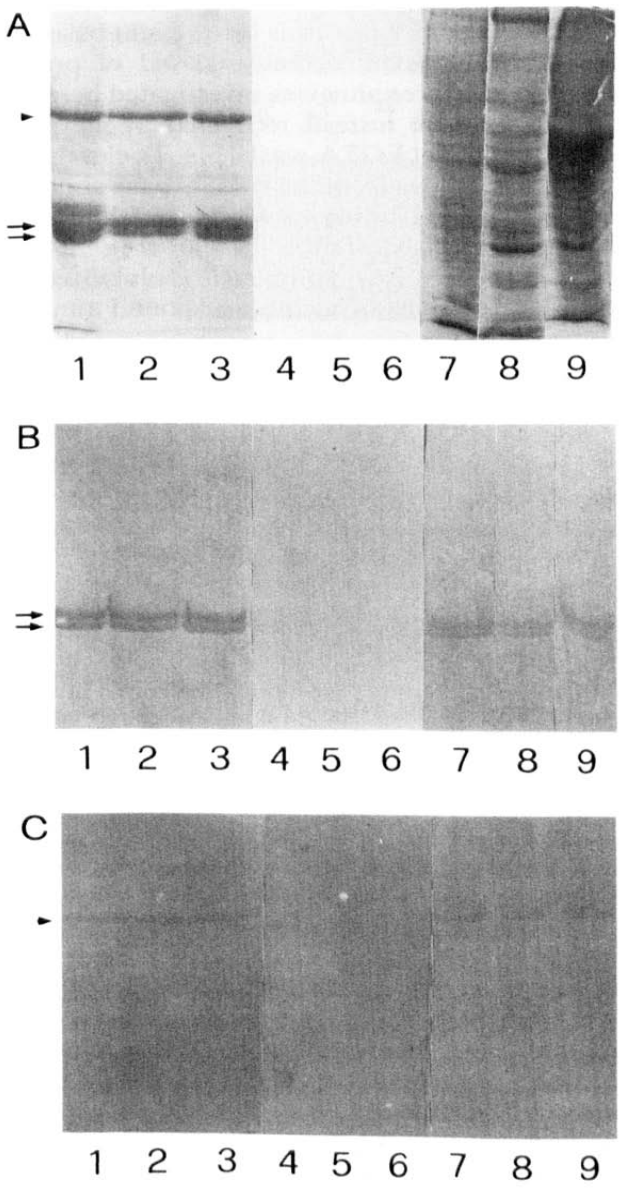

Fig. 1. SDS-PAGE and Western blot analysis of EGTA-extracts and residues from bovine tissues. The EGTA-extracts from lung (lanes A1, B1, and C1), heart (lanes A2, B2, and C2), and brain (lanes A3, B3, and C3), the last EGTA-washes from the same tissues (lanes 4–6, respectively, in A–C), and the EGTA-resistant residues (lanes 7–9, respectively, in A–C) were subjected to SDS-PAGE. Gels were either stained with Coomassie blue (panel A) or transblotted onto nitrocellulose paper for immunostaining with the anti-annexin V (CaBP33) antiserum (panel B) or the anti-annexin VI antiserum (panel C). The double arrow points to the position of the two annexin V isoforms, and the arrowhead to the position of annexin VI. Note that the three annexins are recovered in the EGTA-extracts and residues, but not in the last EGTA-washes.

3. RESULTS AND DISCUSSION

The EGTA-extracts obtained from bovine lung, heart, and brain as described in section 2 contained the annexin V isoforms and annexin VI as revealed by Coomassie blue staining of SDS gels and immunostaining of transblotted proteins, irrespective of the tissue considered (Fig. 1). By far, bovine lung was the richest source of the annexin V isoforms on a weight basis. The last EGTA-washes were virtually devoid of proteins and, hence, of the three annexins investigated here (Fig. 1). These latter were instead recovered in the pellets obtained after the last EGTA-wash (Fig. 1), clearly indicating that membranes from the three organs contained fractions of individual annexins which were bound in a Ca^{2+}-independent way. Triton X-100 (Fig. 2A) or Triton X-114 (Fig. 3A) completely solubilized the tightly bound fractions of membrane-bound annexins,

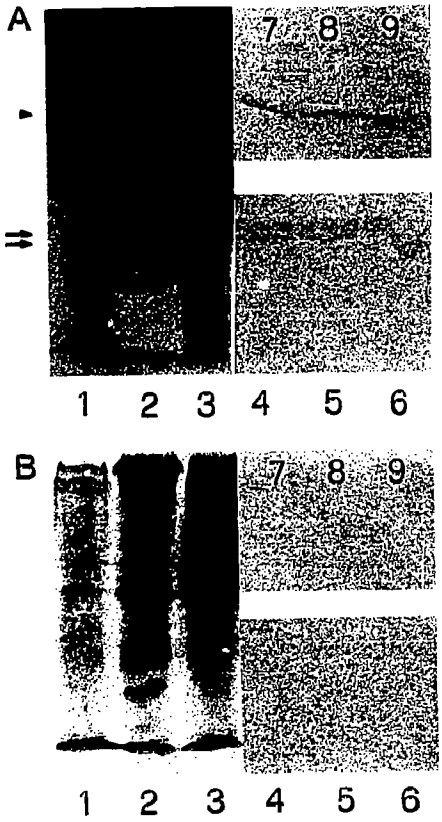

Fig. 2. SDS-PAGE and Western blot analysis of Triton X-100-extracts and residues from bovine tissues. The Triton X-100 extracts from lung (lanes 1, 4, and 7 in panel A), heart (lanes 2, 5, and 8 in panel A), and brain (lanes 3, 6, and 9 in panel A), and the Triton X-100-residues from the same tissues (panel B, same lanes) were subjected to SDS-PAGE. Gels were either stained with Coomassie blue (lanes 1–3 in panels A and B, respectively) or transblotted onto nitrocellulose paper for immunostaining with the anti-annexin V (CaBP33) antiserum (lanes 4–6 in panels A and B) or the anti-annexin VI antiserum (lanes 7–9 in panels A and B). The double arrow points to the position of the two annexin V isoforms, and the arrowhead to the position of annexin VI. Note that Triton X-100 completely solubilizes the membrane-bound, EGTA-resistant fractions of the three annexins.

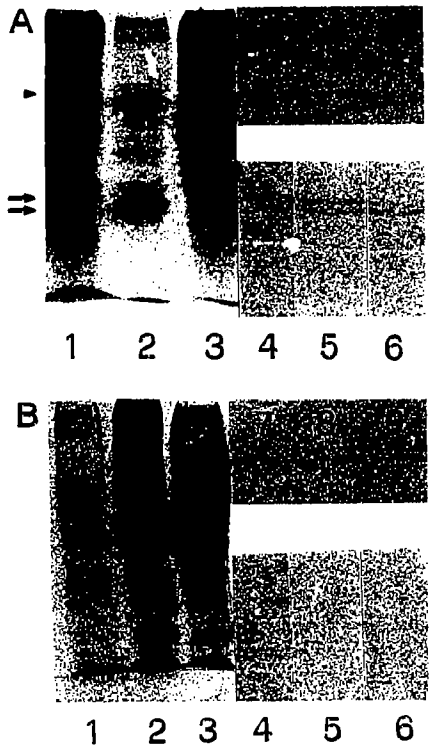

Fig. 3. SDS-PAGE and Western blot analysis of Triton X-114-extracts and residues from bovine tissues. Conditions were as described in Fig. 2. The double arrow points to the position of the two annexin V isoforms, and the arrowhead to the position of annexin VI. Note that Triton X-114 completely solubilizes the membrane-bound, EGTA-resistant fractions of the three annexins.

with no residual immunostaining of the detergent-resistant pellets (Fig. 2B and 3B, respectively). When the EGTA-resistant pellets were extracted with Na_2CO_3, about 50% of the annexin V isoforms and about 70% of annexin VI were solubilized (Fig. 4A), whilst the remaining fractions remained in the Na_2CO_3-resistant pellets (Fig. 4B). Thus a strong base failed to completely solubilize the EGTA-resistant fractions of the three annexins analyzed. Treatment of the Na_2CO_3-resistant pellets with Triton X-100 resulted in the solubilization of Na_2CO_3-resistant annexins (not shown). These data were consistent with the possibility that at least fractions of the membrane-bound, EGTA-resistant annexins behave like integral membrane proteins. To further probe this conclusion, individual Triton X-114 extracts were processed as in [26] to obtain aqueous and detergent-rich phases. By immunoblotting 30-50% of individual annexins were recovered in the detergent-rich phases (Fig. 5) thus supporting the above conclusion.

The mechanism responsible for Ca^{2+}-independent association of annexin V isoforms and annexin VI with membranes is not known. Almost certainly, Ca^{2+} is essential for binding, but association of some annexins with membranes becomes Ca^{2+}-independent thereafter. This has been proven true with protein kinase C also [27–29], although Ca^{2+}-independent penetration of pro-

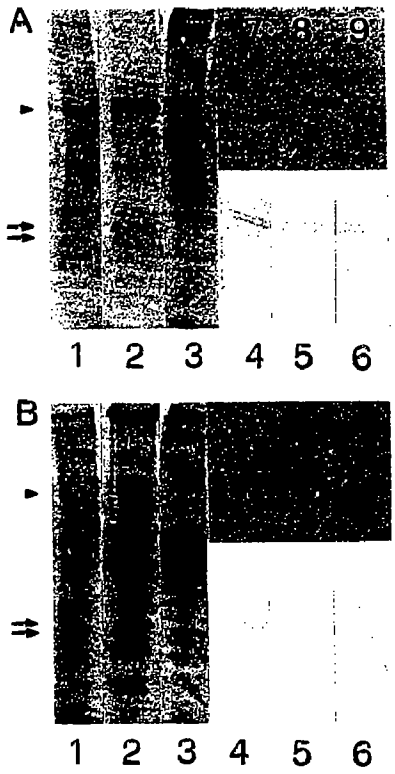

Fig. 4. SDS-PAGE and Western blot analysis of Na_2CO_3-extracts and residues from bovine tissues. The Na_2CO_3-extracts from lung (lanes 1, 4 and 7 in panel A), heart (lanes 2, 5 and 8 in panel A), and brain (lanes 3, 6 and 9 in panel A), and the Na_2CO_3-residues from the same tissues (panel B, same lanes) were subjected to SDS-PAGE. Gels were either stained with Coomassie blue (lanes 1–3 in panels A and B, respectively) or transblotted onto nitrocellulose paper for immunostaining with the anti-annexin V (CaBP33) antiserum (lanes 4–6 in panels A and B) or the anti-annexin VI antiserum (lanes 7–9 in panels A and B). The double arrow points to the position of the two annexin V isoforms, and the arrowhead to the position of annexin VI. Note that Na_2CO_3 does not completely solubilize the membrane-bound, EGTA-resistant fractions of the three annexins.

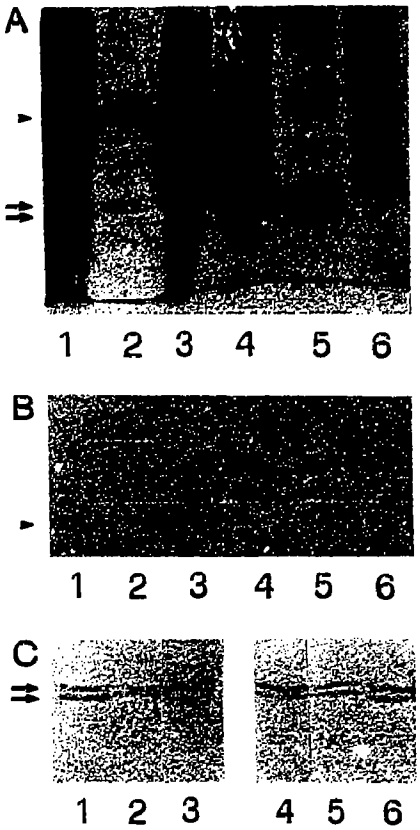

Fig. 5. SDS-PAGE and Western blot analysis of the aqueous and detergent-rich phases from the Triton X-114-extracts from bovine tissues. The aqueous phases from lung (lane 1 in panels A–C), heart (lane 2 in panels A–C), and brain (lane 3 in panels A–C), and the detergent-rich phases from lung (lane 4 in panels A–C), heart (lane 5 in panels A–C), and brain (lane 6 in panels A–C) were subjected to SDS-PAGE. Gels were either stained with Coomassie blue (panel A) or transblotted onto nitrocellulose paper for immunostaining with the anti-annexin VI antiserum (panel B) or the anti-annexin V (CaBP33) antiserum (panel C). The double arrow points to the position of the two annexin V isoforms, and the arrowhead to the position of annexin VI. Note that the three annexins do not completely partition in the aqueous phases.

tein kinase C into lipid bilayers has been reported [30]. There is no evidence presented for post-translational modifications of these proteins, such as myristoylation or palmitoylation, that would justify penetration into the membrane lipid bilayer. Based on phospholipid/Ca^{2+}-dependent changes in the intrinsic (Trp) fluorescence of annexins V and VI, penetration of these annexins into the lipid bilayer of liposomes was considered unlikely, although not conclusively excluded [14]. On the other hand, annexin VII (synexin), which is unique in the annexin family because of its unusually long N-terminal tail, was reported to penetrate partway into the membrane lipid bilayer and to display Ca^{2+} channel activities once bound to planar lipid membranes [31]. Similar Ca^{2+} channel activities were recently reported for annexin V [32]. Data from the analysis of the crystal structure of human annexin V support the hypothesis that this protein might have Ca^{2+} channel activities [33]. Whether there is any relationship between the Ca^{2+}-dependent and Ca^{2+}-independent associations of annexin V with membranes and its supposed Ca^{2+} channel activity remains to be investigated.

Immunocytochemical studies indicated that annexins V and VI exist in a membrane-associated form in vivo [21,34]. Of course, immunocytochemistry cannot discriminate between loosely and tightly bound proteins. Biochemical analyses would better discriminate. In line with this, the present report shows that 40-50% of the membrane-bound, EGTA-resistant annexin V isoforms and annexin VI behave like integral membrane proteins because these fractions cannot be solubilized by Na_2CO_3 and partition in the detergent-rich phase after extraction with Triton X-114. The above percentages appear relatively high and it is unlikely that they represent the result of cross-contamination. Treatment of adrenal medulla membranes with Na_2CO_3 in exactly the same way as in the present report resulted in the complete solubilization of membrane-bound, EGTA-resis-

tant annexin II [20]. Also, membrane-bound annexin II was observed to completely partition in the aqueous phase after extraction with Triton X-114 [20]. At present, there is no explanation for only 40–50% of membrane-bound, EGTA-resistant annexin V isoforms and annexin VI behaving like integral membrane proteins. Recent observations on matrix vesicle annexins reveal that annexins possess classical proteolipid-like properties and that under acidic conditions annexins undergo a conformational change which determines a marked increase in the hydrophobicity of the molecules [35]. We also observed large conformational changes in and strong binding of the annexins analyzed in the present report to PS/PC liposomes with decreasing pH values (unpublished data). Lastly, while the Ca^{2+}-dependent interaction of annexins V and VI to liposomes was recently demonstrated to be largely ionic in nature, hydrophobic interactions were not excluded on the basis of lack of headgroup specificity [15]. It is thus possible that, following Ca^{2+}-dependent binding of annexin V isoforms and annexin VI to membranes, fractions of these proteins become tightly bound to phospholipids in a Ca^{2+}-independent way because of local changes in the lipid bilayer and/or changes in the conformation of individual proteins. The physiological relevance of these data remains to be established, yet they seem to be in line with the possibility that these proteins might have a role in membrane trafficking. The above-mentioned Ca^{2+} channel activity of annexin V [32] and the reported effect of annexin VI on Ca^{2+}-induced Ca^{2+} release in skeletal muscle cells [36] appear to support that possibility. Alternatively, these proteins may be involved in the regulation of the structural organization of membranes.

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