ANNEXINS FROM BOVINE ADRENAL CORTEX EXHIBIT SPECIFIC CYTOSOL/MEMBRANE SOLVATION

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Cytosol/membrane localization of annexins I to VI was analyzed in tissue extracts from bovine adrenal cortex. Based on their solubility in either aqueous or detergents solutions, they were subfractionated in three groups named cytosolic (C), membrane-bound (MB) and membrane-inserted (MI). Less than 1% of the total annexins present in the tissue were recovered in the C fraction when as much as 76.5 and 22.5% were obtained respectively in the MB and the MI fractions.

By immunoblotting after SDS-PAGE, it was shown that the various members of the annexin family were not equally recovered in the different fractions. A-V and A-VI were found present in the three fractions whereas the distribution of A-I, A-II, A-III and A-IV was distinct, suggesting different cellular functions.   © 1993 Academic Press, Inc.

Annexins belong to a family of structurally related proteins detected in diverse eukaryotic organisms from mammalian to sponge, slime molds and higher plants. They differ slightly across species (1). They possess a core domain composed of a conserved segment of 70-80 amino-acids which is repeated four times in annexins with masses in the 30-50 kDa range and eight times in the 70 kDa annexin (2). Each repeat contains a consensus sequence of 17 amino-acids termed endonexin fold (3). Annexins reversibly bind acidic phospholipids in a calcium dependent manner, which differ with respect to the concentration of Ca^{2+} (4,5). They coexist in a wide variety of tissues but differ in their relative concentration (6) and in their cellular localization. For example, in endothelial cells, using immunofluorescence and electron microscopy after immunogold labeling, it was shown that A-I was located both in the cytoplasm close to the plasma membrane and inside the nucleus when A-II, A-V and A-VI were detected in these cells almost exclusively in the cytoplasm (7). At the opposite, different members of the annexin family

were shown to behave as integral proteins. They require detergent (8-12) or acidic chloroform-methanol (13) for their solubilization and appears as good candidates for the orchestration of membrane events of the cellular traffic (14,15).

As a first step to precise in the adrenal cortex, the possible participation of annexins in the sorting of LDL-receptor complexes, we analyzed the annexin content of the tissue. The respective amount of cytosolic and membrane-associated annexins and the forces which govern these locations were precised. Based on their partitioon in either ionic or detergents solutions, annexins were subfractionned in three groups named C for cytosolic, MB for membrane-bound and MI for membrane-inserted. In the present study we show that the various annexins have caracteristic cytosol/membrane repartitions, suggesting that these proteins may be involved in different cellular processes and that hydrophobic interactions are an important aspect of the annexin function(s).

MATERIAL AND METHODS

All reagents were of analytical grade.

Extraction of annexins.

150 g of bovine adreno cortical tissue were scrapped off from 10-15 adrenal glands of freshly slaughtered animals. Homogeneization was performed in 3 volumes (450 ml) of buffer I (10 mM Hepes, 1 mM NaN_3, 2 mM PMSF, 1 µg/ml leupeptin, 1 µg/ml pepstatin, pH 7.4) using a Waring blender (3 x 15 seconds). EDTA was omitted in this extraction buffer.
Debris and nuclei were pelleted and removed by centrifugation at 3,000 g for 10 minutes. The fractions named C, MB_1, MB_2, MB_3 and MI were recovered from P1, P2, P3 and P4 (fig.1). These pellets were resuspended with a dounce homogeneizer (pestle B) in buffer II (buffer I + 5 mM EDTA), buffer III (buffer II + 150 mM NaCl), buffer IV (buffer II + 500 mM NaCl) and buffer V (buffer II + 2 % Triton-X100 + 0.25 mM octylglucoside) respectively. After overnight incubation, the suspensions were centrifuged at 105 000g for one hour (twice). The supernatants (S0, S1, S2, S3 and S4) were processed for annexin enrichment by addition of $CaCl_2$ to a final molar concentration of 3 mM. After overnight incubation the suspensions were centrifuged at 40,000g for 30 minutes. Pellets were washed twice with (buffer I + 150 mM NaCl + $CaCl_2$ 5mM) and resuspended with 10 ml of (buffer I + 10mM EGTA). After overnight incubation, the suspensions were centrifuged at 105,000g for one hour. The supernatants were respectively named C, MB_1,MB_2, MB_3 and MI.

Identification of annexins.

The different fractions (C, MB_1, MB_2, MB_3 and MI), were analyzed by SDS-PAGE (12% acrylamide) according to Laemmli (16) and transferred by the semi-dry procedure (10 mA per cm^2 for 45 minutes) onto a nitrocellulose sheet. Rabbit polyclonal antibodies against A-I, A-III, A-V and A-VI purified from human placenta, were prepared by popliteal lymph mode injection according to Goudie et al (17). The anti-A-II serum was a gift from J.C Cavadore (Montpellier). The anti-A-IV serum was kindly provided by C. Creutz (Charlottesville, Va USA). The different anti-sera were used at a dilution 1:2000. The proteins were visualized using horseradish peroxidase-conjugated goat anti-rabbit IgG and 4-chloro-1-naphtol as substrate.
In all fractions, the major polypeptides detected by Coomassie blue staining were identified as members of the annexin family. The protein content of each fraction - determined by the method of Peterson (18) - was thus considered as corresponding to the annexin content. It was expressed

in % using 100% as the amount of annexins present in $(C+Mb_1+Mb_2+Mb_3+MI)$.

The 2-dimensional (2-D) electrophoretic analysis was performed according to O'Farrell (19).

All the experiments were performed in triplicate.

RESULTS AND DISCUSSION

Cytosol-Annexins interactions. (C fraction).

The cytosolic fraction (so-called C fraction) was recovered after tissue homogeneization in buffer I, which contains neither EDTA nor NaCl (fig. 1). Few polypeptides were detected by Coomassie blue staining (fig. 2A). The immunoblotting data presented in figure 2B indicate that A-V and A-VI are the two members of the annexin family present in this fraction. The annexin content of the C fraction was found close to 150 μg i.e less than 1% of the annexins recovered from the adrenal cortex. From immunocytochemical data, such a cytosolic localization of annexins was often
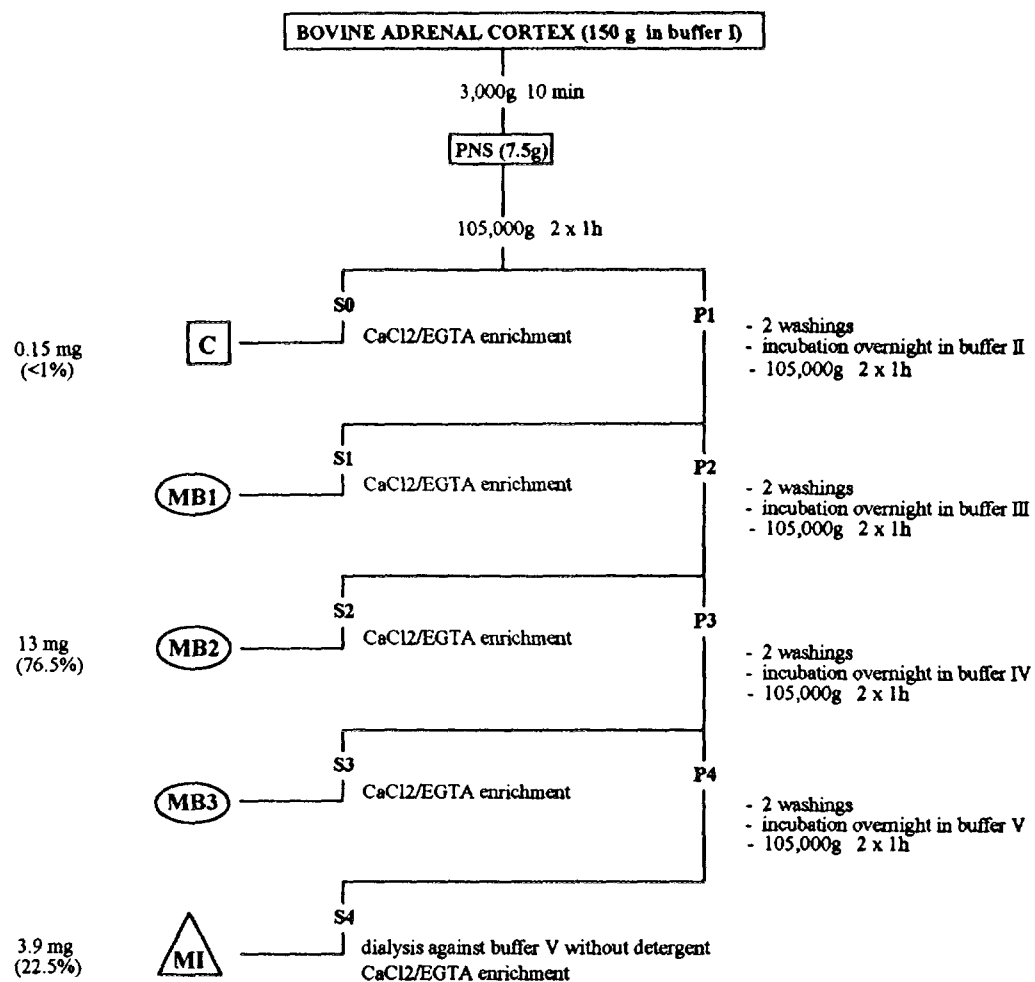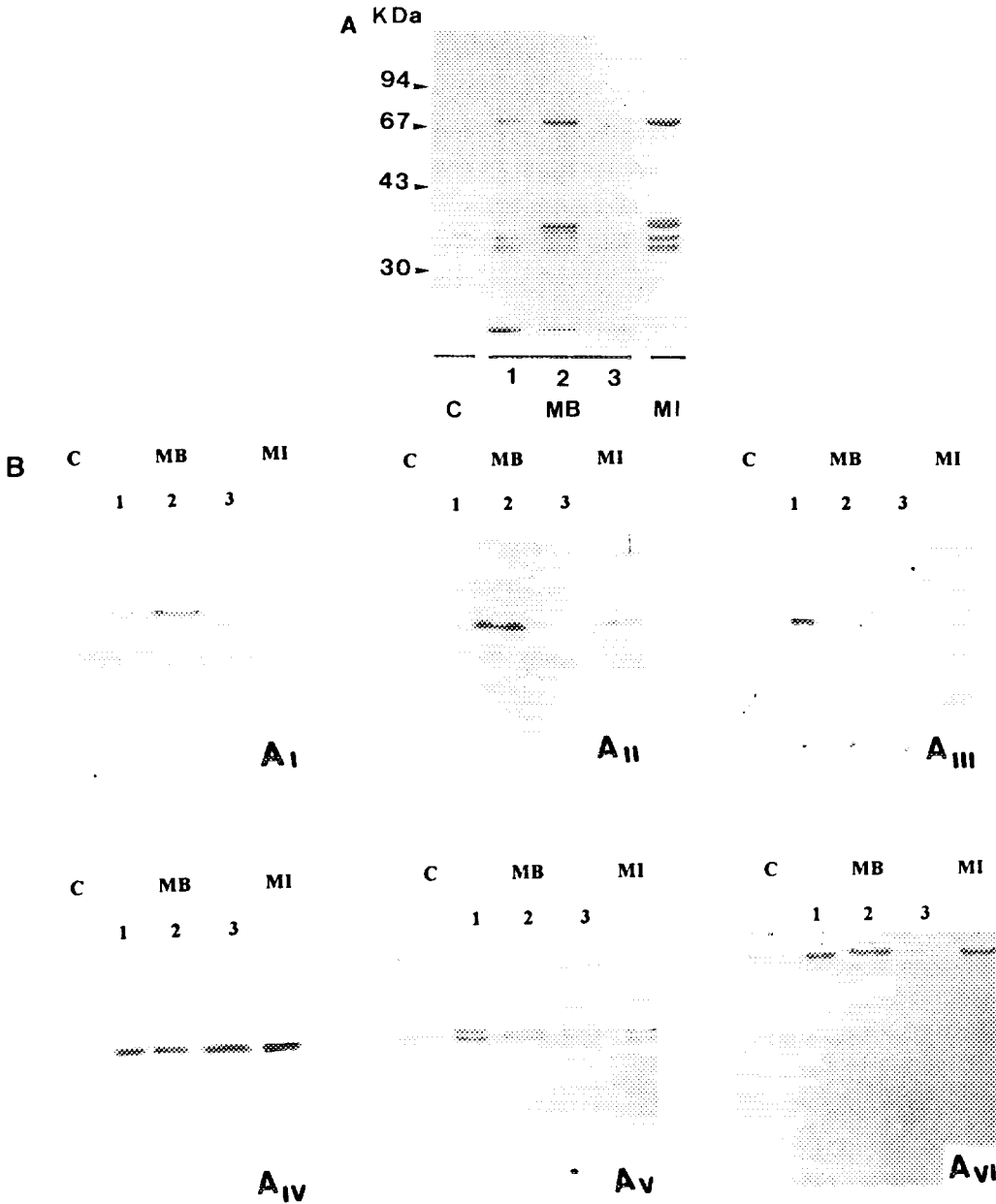

Figure 1. Illustration of the annexins extraction and enrichment.

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Figure 2. (A): Coomassie Brilliant blue-stained SDS-PAGE of the different annexin-enriched fractions (2 to 5 μg per well). (B): Corresponding Western blots using antibodies to the different annexins A-I to A-VI. C: annexins of the cytosolic compartment, MB: annexins interacting with membranes via electrostatic interactions, MI: annexins interacting with membranes via hydrophobic interactions.

reported. Using monoclonal antibodies raised against A-IV, Massey *et al* (20) observed an homogeneous labeling in intestinal mucous secreting cells and in centro acinar pancreatic cells. In endothelial cells, antibodies raised against A-II, A-V and A-VI detected these proteins almost exclusively in the cytoplasm (7).

Lipid head groups-Annexins interactions. (MB fraction).

As shown on figure 1, we sequentially solvated the annexins bound to the membranes via Ca^{2+} (MB_1 subfraction) and via other ions (MB_2 and MB_3 subfractions). Altogether they contribute to 76.5% (13 mg) of the annexins recovered in the adrenal cortex homogenate.

The electrophoretic patterns presented in figure 2A reveal in the MB_1 subfraction the presence of several polypeptides in the 30-50 kDa range and a 70 kDa signal. Figure 2B shows that MB_1 contains all the annexins searched for (A-I to A-VI). Special attention has to be paid to A-III which is only detected in this subgroup, indicating that Ca^{2+} is the major cation involved in the membrane-A-III interacting process. The annexin content of this subfraction is found equal to 70% of the annexins isolated from the adrenal cortex homogenate and 92% of the total MB annexins content. Their recovery is made possible by conducting the experiments in the presence of EDTA but in the absence of NaCl, an uncommon experimental procedure. The Ca^{2+}-dependent interacting process between annexins and membranes is not yet precised. It is not known how the interaction with other proteins (p10 light chains, calcyclin) and the lipid composition of the membranes may influence the calcium requirement of the annexins. Up to now, the calcium effect has been mainly related to conformational changes underwent by annexins (21,22), when it is well known that Ca^{2+} affects considerably the dynamics and phase behaviour of anionic lipids by sequestering H_2O molecules involved in lipid head group hydration (23,24).

Increasing the ionic strength of buffer II led to the disruption of electrostatic interactions not involving Ca^{2+}. In MB_2 which contains the annexins loosely bound to membranes, A-III is the only member of the annexin family search for which is not detected under the present experimental conditions. In MB_3, which contains the annexins firmly interacting with the membranes, only A-IV, A-V and A-VI are recovered. Taking into account the procedure used in the present work for annexins extraction (see fig.1), the presence of annexins in MB_2 and MB_3 subfractions indicates that, in contrast with the data reported in the literature, a significant amount of annexins have the ability to interact with membranes in the absence of calcium. It corresponds to 9.5% of the annexins present in the adrenal cortex homogenate.

Lipid hydrophobic tails-Annexins interactions. (MI fraction).

At this step of the procedure, the annexins still present in the P_4 pellet were considered as interacting with the hydrophobic domain of the membrane (MI fraction). They were recovered by addition of (2% Triton X-100 + 25 mM octylglucoside) to buffer II. Figure 2B indicates that MI contains A-I, A-II, A-IV, A-V and A-VI. Only A-III is not detected. Protein quantification indicates that the annexins of this fraction represent as much as 22.5% of the adrenal cortex annexins.

Rather few reports concern the partition of annexins within the hydrophobic core of the membrane. To our knowlegdge, only A-I, A-V and A-VI were reported to be recovered after membrane solubilization by detergents (8-12, 25). In matrix vesicles implicated in mineral deposition in cartilage, A-V and A-VI were shown to selectively partition in an acidic organic phase revealing unsuspected proteolipid-like properties (13). This unusual solvation property raises the question of the existence of stable annexin-lipid complexes.
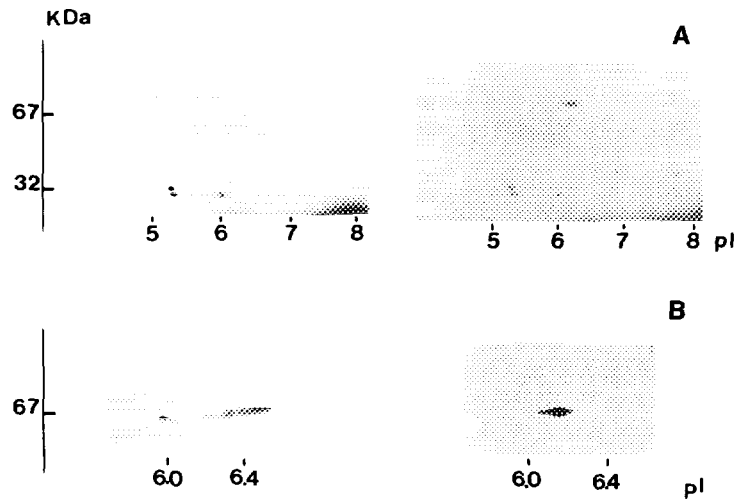
Figure 3. Two dimensional electrophoresis of MB₁ (left) and MI (right) fractions. 8 μg of each sample was layered on the gel. (A): Coomassie blue staining; (B): corresponding Western blot using anti-A-VI antibodies.

In conclusion, it appears that the adrenal cortex contains a large amount of annexins: 17 mg are recovered starting from 150 g of cortex tissue (7.5 g of proteins in the PNS). By using an original experimental procedure of extraction, we identified the annexin content of both the cytosol and the membrane compartments. Quite interestingly, A-V and A-VI are detected in the C, MB and MI fractions. Such different solvation properties could either reflect an heterogeneity at the molecular level or in its three dimensional structure. A first sets of experiments, performed by 2D electrophoresis and immunoblotting on MB and MI, clearly showed that the spot attributed to A-VI largely differed depending on the solvation medium (fig.3A and 3B). The population of A-VI solvated by the hydrophobic domain of the membranes (MI fraction) appeared more homogeneous than the corresponding molecules interacting with the membranes via electrostatic forces (MB₁ fraction). This is consistent with structural differences possibly related to post-traductional events that await further studies.

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