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Annexin 1 is present in different molecular forms in rat cerebral cortex

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Previously we have purified annexin 1 [J. Neurochem. 56 (1991) 1985–1986] from pig cerebral cortex as a monomeric protein of 37 kDa. Here, the localization of annexin 1 was investigated in subcellular fractionations of rat cerebral cortex using immunodetection by a specific antibody. In contrast to synaptophysin, a specific synaptic vesicle integral membrane protein, annexin 1 is located in the synaptic plasma membrane fraction where it appears on SDS-PAGE as a polypeptide of 74 kDa Annexin 1 is extracted also as a 74 kDa polypeptide from the purified synaptic plasma membranes. These results suggest for the 74 kDa molecular form an enzymatic dimerization of annexin 1 when associated to the membrane

Annexin 1; Lipocortin 1; Calpactin 2; Calcium-binding protein; Phospholipid-binding protein; Synaptic plasma membrane, Transglutamination

1. INTRODUCTION

Annexins are a family of proteins that have in common the property to bind to anionic phospholipids in the presence of calcium. This property resides in a particular primary structure consisting of two domains: a small N-terminal domain specific for each protein and a common C-terminal core. The core, formed of 4 or 8 repetitive units of approximately 70 amino acids each, is the domain responsible for calcium and phospholipid binding [1–3].

Whereas annexin 1 is always found as a monomeric protein of 35–37 kDa [4,5], annexin 2 is present in most tissues and cells in two forms, a monomer of 36 kDa and a heterotetramer of 89 kDa composed of two subunits of 36 kDa and two polypeptide chains of 11 kDa [6]. Annexin 6 as a monomer of 67 kDa appears to be derived from a duplication of the four repeats [7].

Annexins 1 and 2 were shown to be present in pig cerebral cortex and were purified from this tissue as monomers of respectively 37 and 36 kDa together with annexin 4 (32 kDa) and annexin 6 (67 kDa) [8]. Specific polyclonal antibodies directed against the different annexins were obtained [8].

In the present study, we provide evidence for the localization in the rat brain synaptic plasma membrane of annexin 1, exclusively as a 74 kDa molecular form.

2. MATERIALS AND METHODS

Subcellular fractions of rat brain cortex were isolated in 0.32 M sucrose by successive centrifugations without adding either calcium or EGTA as described [9,10]. Purified synaptic plasma membranes were obtained according to [11]. Partially purified annexins from rat brain as well as annexin 1 from synaptic plasma membranes were extracted according to the protocol described in [12]. Briefly, membranes were extracted with 25 mM Tris buffer pH 7.4 containing 5 mM $MgCl_2$, 0.5 mM DTT, 10 mM EGTA, 1 mM PMSF, 1 mM benzamidine, 1 mM DIFP, 20 μg/ml trypsin inhibitor, 5 μg/ml leupeptin and pepstatin, for 30 min at 4°C and centrifuged for 30 min at 100,000 × *g*. The supernatant adjusted to a final concentration of 2 mM $CaCl_2$ pH 7.4 was centrifuged for 30 min at 100,000 × *g*. The pellet was resuspended and homogenized in 10 mM imidazole buffer pH 7, 10 mM EGTA, 0.5 M NaCl with the protease inhibitors mentioned above (Protocol 1).

A second protocol of extraction of annexin 1 has been tested. The membranes were treated by 10 mM imidazole buffer pH 7 containing 75 mM KCl, 2 mM $MgCl^2$, 0.5 mM $CaCl^2$, 0.5% Triton X-114, the proteases inhibitors, centrifuged for 30 min at 100,000 × *g*. The pellet subsequently extracted by 2 mM EDTA, 0.5% SDS, centrifuged, represents the Triton X-114-insoluble cytoskeletal proteins. The Triton X-114 supernatant laid on a 6% w/v sucrose cushion containing 10 mM imidazole buffer pH 7, 75 mM KCl, 2 mM $MgCl_2$, 0.5 mM $CaCl_2$, 1 mM sodium azide, 0.06% Triton X-114 was incubated for 5 min at 30°C, the opalescent solution obtained was centrifuged for 3 min at 12,000 × *g* in a microfuge. The oil drop (detergent phase) at the bottom of the tube and the supernatant (aqueous phase) were kept (Protocol 2).

Rabbit annexin 1 antiserum was obtained as described [8]. Monoclonal antisynaptophysin (clone SV-38) was from Sigma Immuno Chemicals.

To identify annexin 1, samples were preparared and separated on SDS-PAGE using essentially a 12% acrylamide gel. The gel was subjected to transblotting by electrophoretic transfer to nitrocellulose membranes using the transfer buffer Tris-glycine pH 9 containing 25% methanol The analysis on immunoblot was performed with rabbit anti-annexin 1 antiserum (dilution 1/1000) and alkaline phosphatase conjugated sheep anti rabbit IgG secondary antibodies.

3. RESULTS

Two steps of the purification of annexins from rat brain according to the Protocole 1 are shown on Fig. 1. The pattern on SDS-PAGE shows that in the first

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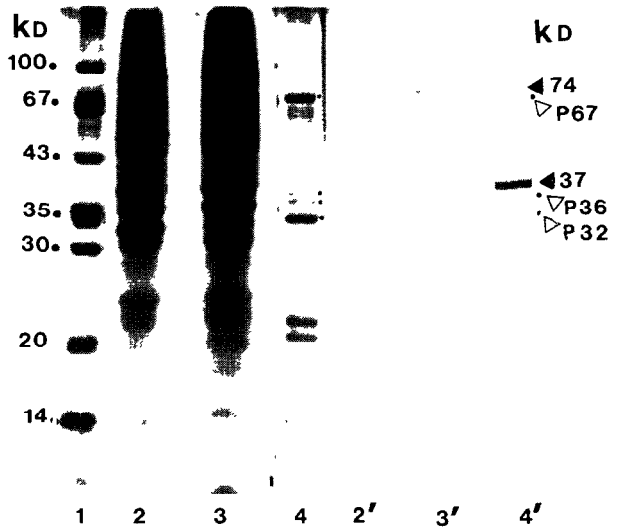

Fig. 1. Identification of the two molecular forms of annexin 1 in rat cerebral cortex Lane 1, molecular weight markers; lane 2, 10 mM EGTA extract from rat brain tissue. supernatant; lane 3, 10 mM EGTA + 1% Triton X-100 extract: supernatant; lane 4, partially purified annexins following several $CaCl_2$ precipitations. 12% SDS-PAGE. Coomassie blue staining· lanes 1,2,3,4. Immunoblot· antiannexin 1, lanes 2′,3′,4′).

extraction of the brain tissues by the EGTA buffer, two bands of 74 and 37 kDa are well visible on the immunoblot with antiannexin 1. If this extraction is followed by a second one with the same buffer but containing 0.5% Triton X-100, the 74 kDa band is reinforced (Fig. 1 lanes 2,2′ and 3,3′). The annexins partially purified following calcium precipitation, centrifugation and extraction of the pellet by the EGTA buffer were clearly resolved on the gel, annexin 1 (P37), annexin 2 (P36), annexin 4 (P32), annexin 6 (P67). The corresponding immunoblot with antiannexin 1 shows the presence of one concentrated band of 37 kDa (Fig. 1, lanes 4,4′).

To determine the subcellular distribution in rat cerebral cortex of annexin 1 and synaptophysin, an integral membrane protein specific of synaptic vesicles, the extracts of each subcellular fraction obtained by differential centrifugations were loaded in an equal protein basis and analyzed by SDS PAGE followed by protein staining and immunodetection using antibodies against annexin 1 and synaptophysin (Fig. 2). We have found that annexin 1 is present in all the particulate fractions but in very low amounts (Fig. 2B), except in the crude synaptic fraction (lane 3). In the particulate fraction recovered at low speed centrifugation (lane 1), in the microsomal (lane 2) and in the crude synaptic vesicle fraction (lane 4), one doublet of 38–39 kDa and one 74 kDa band are visible. In the cytosol (lane 5), as well as in the supernatant fraction (lane 7) obtained upon fractionation of the crude synaptic vesicle fraction (lane 4) by sucrose density gradient centrifugation, one 38 kDa band is only present. Analysis of the other fractions of the gradient reveals that the 74 kDa form is the only one

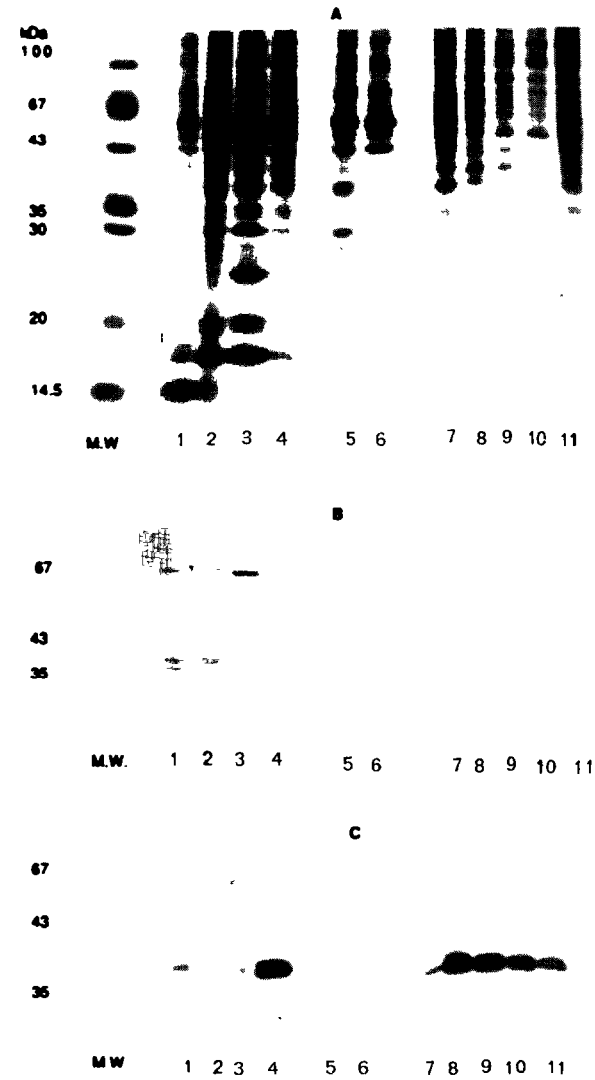

Fig. 2. Analysis of annexin 1 distribution in rat brain particulate and soluble fractions. (A) Coomassie blue staining. (B) Immunoblot. antiannexin 1. (C) Immunoblot: anti-synaptophysin. Rat brain homogenate was centrifuged at low speed ($3,000 \times g$/10 min). The resulting pellet (lane 1) was electrophoresed. The low speed supernatant fraction was centrifuged at medium speed ($9,200 \times g$/15 min) and the supernatant was removed. The pellet was washed by resuspending in isolation buffer and recentrifuging ($10,500 \times g$/10 min) From this, the supernatant S′2 and S2 were pooled and centrifuged at $120,000 \times g$/1 h. The resulting pellet (lane 2, or microsomal fraction) and supernatant (lane 5, or cytosolic fraction) were electrophoresed. Meanwhile, the resulting $10,500 \times g$ centrifuged pellet was lysed and centrifuged ($25,000 \times g$/20 min) to yield a pellet (lane 3 or crude synaptic plasma membranes) that was electrophoresed, and a supernatant that was centrifuged ($165,000 \times g$/2 h). The supernatant (lane 6) was electrophoresed and the pellet (lane 4, or crude synaptic vesicle fraction) was resuspended and layered on top of a linear sucrose gradient. After centrifugation ($65,000 \times g$/2 h) fractions were collected (lanes 7 to 11) and electrophoresed. Samples of each fraction (15 μg) were separated on 12% SDS-PAGE and transferred to nitrocellulose membranes. The nitrocellulose membranes were incubated with antisera (antiannexin 1 dilution 1/1000 and antisynaptophysin 1/200) and visualized by the alkaline phosphatase procedure.

present (lane 8 to 11). Thus, these results suggest that the 74 kDa annexin 1 is associated with the brain particulate fractions and mainly concentrated in the crude synaptic plasma membranes. In the cytosol fractions, the 38 kDa monomeric form is only present. Furthermore, analysis of the fractions demonstrate that the 74 kDa form is concentrated in the crude synaptic plasma membrane in contrast to synaptophysin which is found highly concentrated in the synaptic vesicles fractions (Fig. 2C).

To further explore the association of the 74 kDa annexin 1 with the synaptic plasma membranes we analyzed the molecular properties of this protein in a highly purified fraction obtained by a Percol gradient procedure [11]. Fig. 3A shows that annexin 1 is present exclusively in a 74 kDa molecular from in the EGTA extract as well as in the calcium precipitate. When synaptic plasma membranes are extracted with a buffer containing 0.5% Triton X-114 (Fig. 3B), annexin 1 is also present in a 74 kDa molecular form in the detergent and in the aqueous phases but is absent from the cytoskeletal fraction. In the detergent phase the 74 kDa form is concentrated since the amount of proteins is very low and not well visible in the gel stained by Coomassie blue.

The serum antibody we used in this study has been prepared from the highly purified 37 kDa protein electrophoresed on SDS-PAGE, the corresponding bands of which have been cut and extracted to inject into the rabbits. This antibody reacts specifically with the 37 kDa protein which corresponds to annexin 1 as seen in Fig. 1 lanes 4 and 4'. In the preparations or extracts of tissues it reacts also with a band of 34 kDa which has been shown to be the proteolysis degradation product of annexin 1 by calpain a calcium dependent protease highly active in brain tissues.

In order to demonstrate that the 74 kDa form originated from the 37 kDa annexin 1 and that the 74 kDa form was not a different protein which cross-reacts with anti annexin 1, the serum was depleted from 37 kDa immunoreactivity by absorption on a purified monomeric 37 kDa bound-affinity column. The effluent of the column was tested and did not react at all with the purified 37 kDa monomer, in contrast the antibody bound to the column and eluted, reacted strongly at 1 μg/ml with the 37 kDa protein. The serum depleted from the 37 kDa protein does not cross react at all with the 74 kDa present in the crude synaptic plasma membrane fraction nor in the EGTA or the Triton X-100 extracts from this fraction (Fig. 4).

4. DISCUSSION

The results reported here represent the first experimental evidence of the presence of a protein with a synaptic plasma membrane localization cross-reacting with an anti annexin 1. This protein is a 74 kDa peptide and not the 37 kDa annexin, already extracted and purified from rat brain [8].

One can suggest that under the conditions used for the purification of annexins from brain tissue, annexin 1 is recovered under its monomeric form in the EGTA supernatant whereas the major part of the 74 kDa form remains bound to the particulate fraction, especially when the extraction buffer does not contain Triton X-100 [13]. In contrast, under the conditions of the subcellular fractionations of rat brain (sucrose solution without no Ca^{2+}, Mg^{2+} or EGTA) according to the procedure of Jahn et al. [10], it is remarkable that the dimeric form of annexin 1 is concentrated in the synaptic plasma membrane fraction.

It is shown that the 74 kDa form originates from the 37 kDa form since the antibody which is depleted from

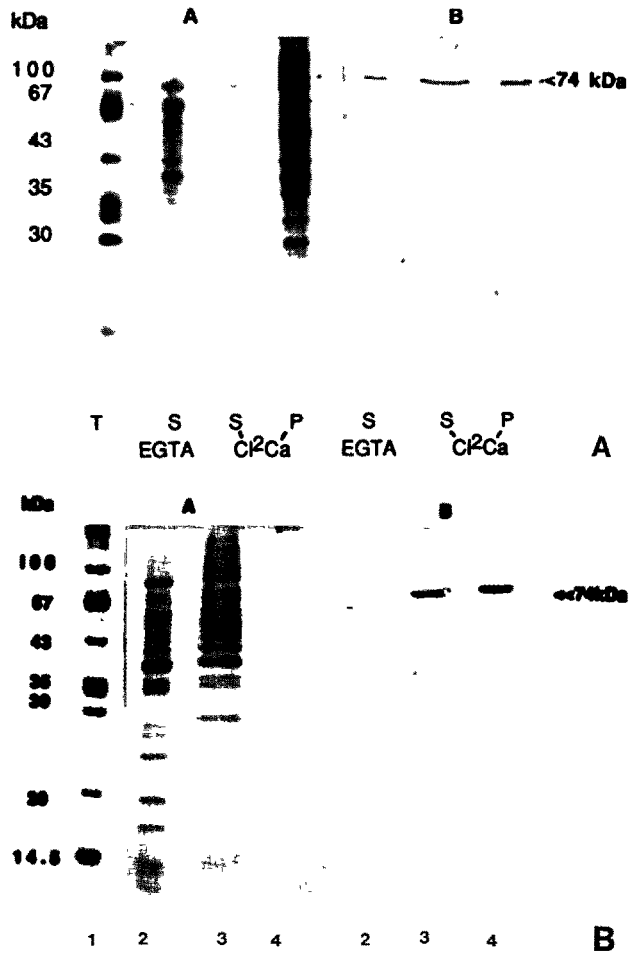

Fig. 3 (A). Identification of annexin 1 in purified synaptic plasma membranes. 12% SDS-PAGE. A. Coomassie blue staining. B. Immunoblot. antiannexin1. EGTA S: EGTA supernatant. $CaCl_2$ S: $CaCl_2$ supernatant. $CaCl_2$ P: $CaCl_2$ pellet. (see section 2, protocol 2). Fig. 3 (B). Purified synaptic plasma membranes extracted by Triton X-114, 1 mM calcium. 12% SDS-PAGE. A. Coomassie blue staining. B. Immunoblot: anti annexin 1. 1. Protein markers. 2. Triton X-114-insoluble fraction. 3. Triton X-114-soluble fraction, aqueous phase. 4. Triton X-114-soluble fraction, detergent phase (see section 2, protocol 1).

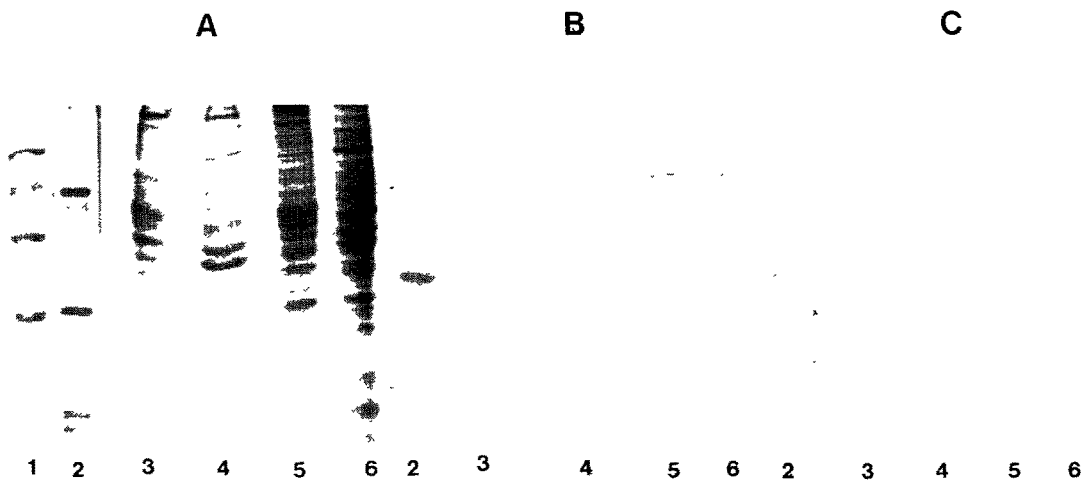

Fig. 4. Blocking the immunostaining of the 74 kDa protein by absorption of the antiserum by the annexin 1 monomer. 12% SDS-PAGE. A. Coomassie blue staining. B. Immunoblot: anti annexin 1, native serum. C. anti annexin 1 serum effluent after immunoabsorption on an annexin 1 affinity column. Lane 1, protein markers; lane 2, partially purified annexins from rat brain; lane 3, crude synaptic plasma membranes LP1; lane 4, EGTA extract from LP1, lane 5, Triton X-100-insoluble in 1 mM EGTA buffer; lane 6, Triton X-100-soluble fraction in 1 mM EGTA buffer.

its immunoreactivity towards the 37 kDa protein does not react at all towards the 74 kDa form. These data suggest that the 74 kDa form is likely to be a covalently linked homodimer of the 37 kDa monomer since it is not dissociated by SDS and could be obtained in a soluble form by a 10 mM EGTA extraction.

Experimental evidence for the existence of a dimerized form of annexin 1 has already been reported in human placenta [14] and in A431 cells [15]. These studies demonstrated that dimerization is the result of a calcium-dependent transglutaminase activity, that catalyzes an acyl-transfer reaction between peptide-bound glutamine residues and the ε amino groups of lysine residues. It has been suggested that the cross-linking site may be located in the 3 kDa N-terminal tail region of the protein and that the cross-linked annexin 1 has higher sensitivity to phospholipid binding and probably sticks more tightly to the membranes [16]. These observations are consistent with the results reported here that show that the 74 kDa form is only present in the particulate fraction and is partitioned in the aqueous and in the detergent phase of Triton X-114. Its presence in the detergent phase suggests that dimeric annexin 1 might contain a large hydrophobic domain tightly associated to the membrane in the presence of calcium. This hydrophobicity may be related to the physiological properties of the protein. Several observations support the dimer being a physiological form in synaptic plasma membrane. The protein is prominent in crude synaptic fractions and thus is not created artifically during purification, on an other hand, Pollard et al. found that annexin 1 has fusion potency [17]. In this perspective, it might be involved in the exocytosis of the neurotransmitters. A study of annexin 1 in stimulated synaptosomes would be of a great interest.

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REFERENCES

[1] Geisow, M.J., Walker, J.H., Boustead, C. and Taylor, W. (1987) Biosci. Rep. 7, 289–298.
[2] Burgoyne, R.D and Geisow, M J (1989) Cell Calcium 10, 1–10.
[3] Pepinsky, R.B., Tizard, R., Mattaliano, R.J., Sinclair, L.K., Miller, G.T., Browning, J.L., Chow, E.P., Burne, C., Huang, K.S., Pratt, D., Wachter, L., Hession, C., Frey, A.Z. and Wallner, B.P. (1988) J. Biol. Chem. 263, 10799–10811.
[4] De Be, K., Misono, K.S., Lukas, T.J., Moczkowski, B. and Cohen, S (1986) J. Biol Chem. 261, 13784–13792.
[5] Glenney, J.R. (1986) Proc. Natl. Acad. Sci. USA 83, 4258–4262
[6] Gerke, V. and Weber, K. (1985) EMBO J. 3, 227–233.
[7] Crompton, M.R., Owens, R.J , Totty, N.F., Moss, S.E., Waterfield, M.D. and Crumpton, M.J. (1988) EMBO J. 7, 21–27.
[8] Regnouf, F., Rendon, A. and Pradel, L.A (1991) J. Neurochem. 56, 1985–1996.
[9] Huttner, W B., Schiebler, W., Greengard, P and de Camilli, P. (1983) J. Cell. Biol. 96, 1374–1388.
[10] Jahn, R., Schiebler, W , Ouimet, C. and Greengard, P. (1985) Proc. Natl Acad. Sci USA 82, 4137–4141.
[11] Rendon, A and Masmoudi, A. (1985) J. Neurosci. Methods 14, 41–51.
[12] Südhof, T.C., Ebbecke, M., Walker, J.H., Fritsche, U. and Boustead, C. (1984) Biochemistry 23, 1103–1109.
[13] Gregory Moore, K., Goulet, F. and Sartorelli, A C. (1992) Protein Expression and Purification 3, 1–7.
[14] Pepinski, R B., Sinclair, L.K., Chow, E.P and O'Brine-Greco, B. (1989) Biochem. J. 263, 97–103.
[15] Ando, Y., Imamura, S., Owada, K.M and Kannagi, R. (1991) J. Biol. Chem. 266, 1101–1108.
[16] Ando, Y., Imamura, S , Owada, M.K., Kakunaga, T. and Kanagi, R (1989) Biochem. Biophys Res. Commun. 163, 944–951.
[17] Pollard, H.B , Guy, H.R., Arispe, N., de la Fuente, M., Lee, G., Rojas, E.M., Pollard, J.R., Srivastava, M., Zhang-Keck, Z.K., Merezhinskaya, N., Caohuy, H., Burns, A.L. and Rojas, E. (1992) Biophys. J. 62, 15–18.