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EVIDENCE FOR A SPECTRIN-LIKE PROTEIN AS A MAJOR COMPONENT OF THE SYNAPTOSOMAL MEMBRANE CYTOSKELETON

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SUMMARY: After treatment of synaptosomes with Nonidet-containing buffers, a proportion of the proteins remained insoluble. The major component (50%) of the residue was identified as a spectrin-like protein by immunodetection after mono- and bi-dimensional gel electrophoresis and transfer to nitrocellulose paper. Actin was also present.

INTRODUCTION: The plasma membrane interacts with intracellular cytoskeletal structures and there is evidence for the linking of actin to transmembrane proteins both in the erythrocyte(1) and lymphocyte cell membranes(2). In the erythrocyte there is a complex network of cytoskeletal proteins underlying the cytoplasmic face of the cell membrane; the major components of this system are spectrin, actin and ankyrin(1). In other cells the situation is less clear. Although actin-containing matrices(3) and actin-membrane protein complexes(2) have been isolated from other cell membranes there is no evidence for a role of spectrin in these cases. Recently however spectrin has been shown not to be unique to erythrocytes, but also to be found in other cell types, notably brain(4,5). It is possible therefore that a submembranous spectrin-actin network is a general feature of cell membranes. It has also been proposed(2,6) that actin-containing microfilaments are linked to membrane proteins and such a link could play an important role in the response of the cell of external stimuli. There is no evidence for a role of spectrin in such links.

Cytoskeletal proteins associated with membranes can be separated from other membrane components by extraction with non-ionic detergents(2,3)

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and this experimental approach has been used in the present work to isolate the cytoskeleton from synaptosomes. Synaptosomes are pinched-off nerve--endings prepared from brain by differential and gradient centrifugation(7) and they have been much used for the study of neurotransmitter storage and release.

MATERIALS AND METHODS: Preparation of synaptosomes and cytoskeleton extraction: Synaptosomes were prepared from cerebral cortex of male rats (200 g) by the method of Whittaker and Barker(8) as described by Hesketh et al.(9), but without the lysis step. All operations were carried out at 0-4°C. Synaptosomes were collected from the interface between the layers of 0.8 and 1.2 M sucrose, diluted two-fold with 0.32 M sucrose + 1 mM $MgCl_2$ and centrifuged at 20,000 g x 30 min. The pellet of synaptosomes was resuspended in 0.32 M sucrose + 1 mM $MgCl_2$ and the protein content measured using the method of Lowry et al.(19). Extraction of the cytoskeleton was carried out using 10 mM Tris, 7.5, 0.15 M NaCl, 1% Nonidet P40, 1 mM $MgCl_2$ containing either 1 mM EGTA or 1 mM $CaCl_2$. Extraction was at 0-4°C for 30 min using a final protein concentration of 1 mg/ml and a final concentration of phenylmethyl sulphonyl fluoride of 0.5 mM. Following extraction, the samples were centrifuged at 35,000 x 30 min at 4°C and the pellet of insoluble cytoskeletal proteins was solubilized in either 10 mM Tris, pH 7.5, 1% SDS (for SDS gel electrophoresis) or in 2% Nonidet P40, 9.5 M urea, 5% mercaptoethanol, 2% ampholine pH 2-10 (for two-dimensional electrophoresis).

Electrophoresis: Samples for monodimensional SDS electrophoresis were heated at 100°C for 5 min in the presence of 1% SDS and 10% mercaptoethanol. Electrophoresis was carried out following the procedure of Laemmli(11). Samples for two-dimensional electrophoresis were solubilized by heating at 37°C for 60 min in the solubilization solution given above and analysed as described previously(12).

Electrophoretic blotting and immunological detection: After protein separation by mono- or two-dimensional gel electrophoresis, proteins were transferred to nitrocellulose sheets following the procedure of Towbin et al. (13) and reaction with antibodies carried out as described previously(14).

Anti-spectrin antibodies, raised in rabbits against human spectrin prepared from red blood cell membranes were used at a 1->500 dilution; they were a gift from Drs L. A. Pradel and R. Cassoly, Institut de Biologie Physico-Chimique, Paris.

RESULTS: After treatment of synaptosomes with Nonidet-containing buffers, a certain proportion of the proteins remained insoluble. These were collected by centrifugation and analysed by monodimensional SDS polyacrylamide gel electrophoresis. The results (Figure 1) showed the presence of seven major bands, the approximate molecular weights of which were calculated from their mobilities in SDS gels; 225,000, 158,000, 78,000, 70,000, 56,000, 49,000 and 45,000. Minor bands of approximate molecular weights 200,000 and 120,000 daltons were also observed together with two very high molecular weight bands

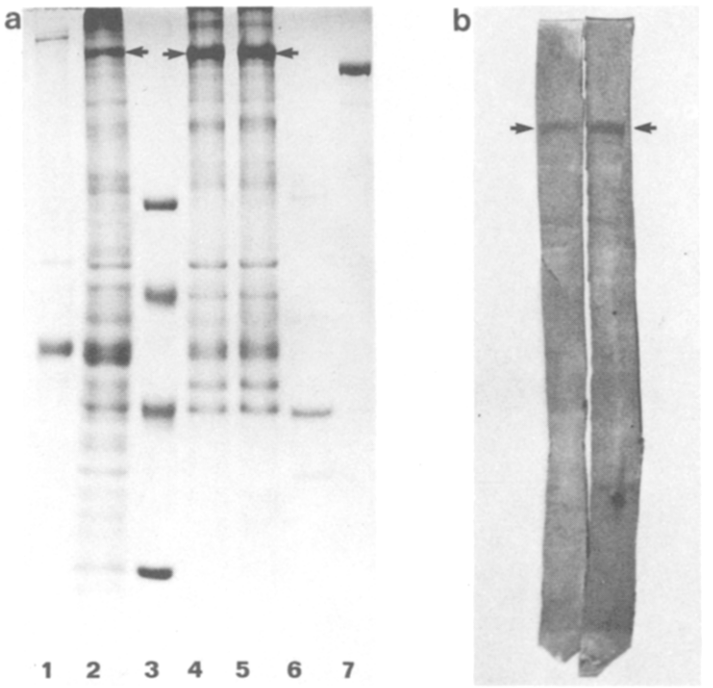

Figure 1: SDS-polyacrylamide gel electrophoresis of Nonidet-extracted synaptosomes and identification of spectrin. (a) 7.5% polyacrylamide gel. Lane 1. microtubule protein from rat brain showing tubulin, MAP 1 and MAP 2. Lane 2. synaptosomes. Lane 3. standards; phosphorylase b, 97,400 daltons, bovine serum albumin, 66,000, ovalbumin, 43,000 and carbonic anhydrase, 29,000. Lane 4. synaptosome residue after Nonidet extraction in presence of 1 mM EGTA. Lane 5. synaptosome residue after Nonidet extraction in presence of 1 mM $CaCl_2$. Lane 6. rabbit muscle actin. Lane 7. rabbit muscle myosin. Gel was stained with Coomassie Brilliant Blue. (b) Synaptosome residues after Nonidet extraction with EGTA (right) or $CaCl_2$ (left) were subjected to electrophoresis and then transferred to nitrocellulose sheets for immunochemical detection with anti-spectrin antibodies. (Arrows indicate spectrin-like protein).

(MW > 300,000). Extraction with buffer containing either 1 mM EGTA or 1 mM $CaCl_2$ gave cytoskeletal complexes showing a very similar polypeptide composition on SDS polyacrylamide gels (Figure 1).

The high molecular weight component (225,000) represented the major polypeptide protein component of the residue; densitometry showed it to comprise about 50% of the protein content. Its molecular weight suggested that it could be a spectrin-like protein. Following electrophoresis, transfer of the protein to nitrocellulose sheets and incubation with antibodies raised against spectrin showed that this high molecular weight band did contain protein which reacted with anti-spectrin antibodies (Figure 1). Thus

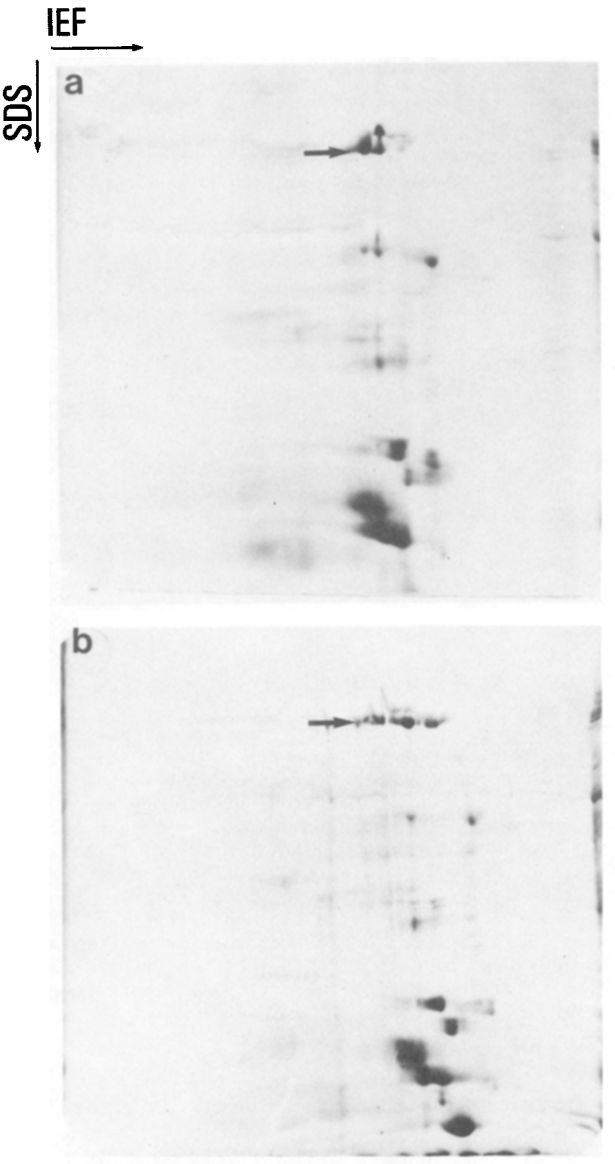

Figure 2: Two dimensional gel electrophoresis of Nonidet-extracted synaptosomes. Nonidet-insoluble material (1 mg protein) subjected to isoelectric focussing in the first dimension and SDS-polyacrylamide gel electrophoresis on a 6% polyacrylamide gel in the 2nd dimension. Extraction was with Nonidet-containing buffer with 1 mM EGTA (upper) or 1 mM $CaCl_2$ (lower). (——>) marks position of spectrin.

the major component of the cytoskeletal extract appeared to be a spectrin--like protein. Confirmation of this was achieved by two-dimensional electrophoresis; this showed the high molecular weight component to have an isoelectric point of approximately 5.5 and to exhibit many isoforms (Figure

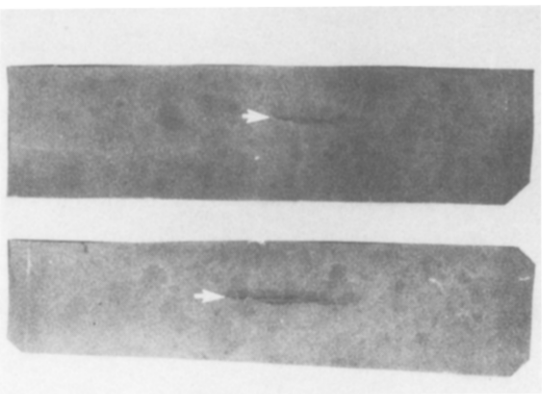

Figure 3: Immunochemical detection of spectrin following two-dimensional gel electrophoresis of Nonidet-extracted synaptosomes. Following extraction with Nonidet-containing buffer with 1 mM EGTA (upper) or 1 mM $CaCl_2$ (lower) the insoluble synaptosomal residue (0.5 mg protein) was submitted to 2 dimensional electrophoresis and protein then transferred to nitrocellulose sheets for immunochemical detection with anti-spectrin antibodies. Only the top of the nitrocellulose paper was submitted to immunodetection with rabbit anti-spectrin antibodies.

2). The isoelectric point is similar to that found for erythrocyte spectrin (15). Transfer of protein to nitrocellulose after two-dimensional electrophoresis and subsequent incubation with anti-spectrin antibodies again showed the high molecular weight component to react with anti-spectrin antibodies; immunochemical detection, as with Coomassie Blue staining, showed several spectrin isoforms (Figure 3). Two-dimensional electrophoresis showed no difference in polypeptide composition between extracts made with buffer with EGTA or $CaCl_2$.

Besides spectrin, the polypeptides of estimated molecular weight 56,000 and 45,000 were the next most significant components, in terms of their contribution to the total protein content. The band of molecular weight 45,000 comigrated with purified muscle actin and was identified as actin on this basis. For similar reasons the 56,000 molecular weight component was identified as tubulin. In comparison with total synaptosomal protein, the Nonidet insoluble proteins were much enriched in spectrin, not enriched in actin and they showed marked loss of tubulin.

DISCUSSION: As has been found with other cell membranes(2,3), treatment of synaptosomes with 1% Nonidet produced a residue of insoluble protein

which appeared to represent a plasma membrane-associated cytoskeleton. SDS-gel electrophoresis showed that the major component of this complex had a molecular weight of 225,000 and reaction with anti-spectrin antibodies after transfer to nitrocellulose paper showed this component to be a spectrin-like molecule. Two-dimensional electrophoresis confirmed that this major component was a spectrin-like protein. The occurrence of considerable amounts of spectrin in the residue suggests that the synaptosome has a membrane-associated spectrin cytoskeleton analogous to that found in the erythrocyte(1); actin is also present. A spectrin-like protein (also called fodrin) had been isolated from brain(4,5), however there has been no previous demonstration of spectrin associated with a purified brain membrane preparation. Also, although synaptosomes contain some minor glial contamination they are largely neuronal in origin(16,17); thus the present results, showing spectrin to be a major component of the synaptosome not only support previous observations(4,5) that brain contains a spectrin-like molecule but also extends them to show that spectrin is a component of the neuronal membrane. The monodimensional SDS gel electrophoresis showed spectrin to be a major component not only of the Nonidet-insoluble residue but also of the intact synaptosomal membrane. In contrast to the failure of Nonidet extraction of certain cell membranes to show the presence of spectrin(3), the present results support the hypothesis(4,5) that a spectrin-actin membrane-associated cytoskeleton is present in many cell types.

Two other components of the Nonidet-insoluble synaptosomal residue were tentatively identified as actin and tubulin. The presence of actin and the low quantity of tubulin present distinguishes the residue from postsynaptic densities isolated with detergents such as Triton x-100 or deoxycholate, since these are very rich in tubulin(17,18,19). The other components of the cytoskeletal residue remain to be identified and their interactions clarified. The component corresponding to a 200,000 daltons protein might be myosin heavy chains or perhaps an ankyrin-like molecule

since this erythrocyte cytoskeletal protein has been reported in brain(5,20). The presence of tubulin, spectrin and a 200,000 daltons component in the synaptosomal cytoskeleton is of some interest since ankyrin has been found to bind to brain tubulin(29). Other components may be transmembrane proteins linked to the cytoskeleton, as found in lymphocytes and the erythrocyte(1,2).

A spectrin-actin meshwork underlying the pre-synaptic plasma membrane could be involved in trapping synaptic vesicles in the cytoplasm (or attached to microtubules) and pulling them towards the plasma membrane; alternatively a spectrin containing cytoskeleton might have a structural role in the synaptic membrane.

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REFERENCES

1. Branton, D., Cohen, C. M. and Tyler, J. (1981). Cell 24, 24-32.
2. Koch, G. L. E. and Smith, M. J. (1978). Nature 273, 274-278.
3. Mescher, M. F., Jose, M. J. L. and Balk, S. P. (1981). Nature 289, 139-144.
4. Burridge, K., Kelly, T. and Mangeat, P. (1982). J. Cell Biol. 95, 478-486.
5. Bennett, V., Davis, J. and Fowler, W. E. (1982). Nature 299, 126-131.
6. Weihing, R. B. (1979). Methods Achiev. Exp. Path. 8, 42-109.
7. Whittaker, V. P. (1969). in Handbook of Neurochemistry (Ed. Lajatha, A.) Volume 2, pp 327-364. Plenum Press, New York.
8. Whittaker, V. P. and Barker, L. A. (1972). Methods Neurochem. 2, 1-52.
9. Hesketh, J. E., Kinloch, N. and Reading, H. W. (1977). J. Neurochem. 29, 883-884.
10. Lowry, O. H., Rosebrough, N. J., Farr, A. L. and Randall, R. J. (1951). J. Biol. Chem. 193, 265-275.
11. Laemmli, U. K. (1970). Nature 227, 680-685.
12. Aunis, D., Guerold, B., Bader, M. F. and Ciesielski-Treska, J. (1980). Neuroscience 5, 2261-2277.
13. Towbin, M., Staehelin, T. and Gordon, J. (1979). Proc. Natl. Acad. Sci. U.S.A. 76, 4350-4354.
14. Bader, M. F. and Aunis, D. (1983). Neuroscience 8, 165-181.
15. Blikstad, I. and Lazarides, E. (1983). Proc. Natl. Acad. Sci. U.S.A. 80, 2637-2641.
16. Fritz, A. H., Anderson, D. J. and Rustad, D. G. (1976). Brain Res. 101, 341-345.
17. Wang, Y-J. and Mahler, H. R. (1976). J. Cell Biol. 71, 639-658.
18. Cotman, C. W., Levy, W., Banker, G. and Taylor, D. (1971). Biochim. Biophys. Acta 249, 406-418.
19. Banker, G., Churchill, L. and Cotman, C. W. (1974). J. Cell Biol. 63, 456-465.
20. Bennett, V. and Davis, J. (1981). Proc. Natl. Acad. Sci. U.S.A. 78, 7550-7554.