LOCALIZATION OF SYNAPTIN ON SYNAPTIC VESICLE MEMBRANES, SYNAPTOSOMAL PLASMA MEMBRANES AND CHROMAFFIN GRANULE MEMBRANES

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1. Introduction

Synaptin is a membrane protein specific to the nervous system [1]. It is extremely enriched in synaptic vesicle fractions compared to whole brain, but also present in synaptosomal plasma membranes in rather large amounts [2]. No synaptin can be demonstrated in primary astroglial cell cultures [1]. In sodium dodecylsulphate (SDS) polyacrylamide gel electrophoresis synaptin has been shown to be a polypeptide with an app. mol. wt 45 000 (Bock, Norrild and Bhakdi, unpublished data). A number of studies have suggested that, during synaptic transmission, synaptic vesicles of the nerve terminal fuse with the presynaptic membrane and release their neurotransmitter content directly into the synaptic cleft [3–6]. Ca^{2+} ions as well as a metabolic source of energy [7–9] are required to couple the stimulus to the secretory response. After releasing their contents, the membranes of the vesicles are retrieved [6,10]. Little is known of the molecular events which occur during this complex process, but it seems reasonable to assume that specific membrane proteins are involved. In order to understand the function of the synaptic vesicles it is necessary to describe their molecular composition and organization. We here report the first demonstration of synaptin as a constituent of chromaffin granules isolated from ox adrenal medulla. The molecular weight of synaptin determined by SDS–polyacrylamide gel electrophoresis indicates that it differs from other chromaffin granule membrane proteins such as dopamin β-hydroxylase (EC 1.14.17.1), chromogranin A and chromomembrin b [11,12]. Furthermore, we have determined the topographical distribution of synaptin in membranes of chromaffin granules, brain synaptic vesicles, and brain synaptosomal plasma membranes.

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2. Materials and methods

Synaptosomes were prepared from homogenates of ox forebrain cortex by a modified Gray and Whittaker procedure [13]. In all media 5 mM sodium phosphate buffer, pH 7.4, was incorporated. The differential centrifugation was performed as described previously [1]. The density gradient consisted of two layers of sucrose containing 1.0 M and 1.2 M sucrose, respectively. After centrifugation for 80 min with approx. 55 000 × g at the sucrose interphase, the synaptosomes were collected at the interphase. For experiments with whole synaptosomes, 2 vol. isoosmotic sodium phosphate buffer, pH 7.4, 4°C was added dropwise to the synaptosomal fraction over a period of 30 min. The sample was thereafter

centrifuged for 10 min, 10 000 × *g*, and the pellet was resuspended in 0.32 M sucrose, 5 mM sodium phosphate, pH 7.4, and brought to room temperature (20–22°C). Electron microscopy showed synaptosomes contaminated with mitochondria and unidentified membranes. Synaptic vesicles and synaptosomal plasma membranes were prepared from synaptosomes which were osmolysed in 10 vol. icecold 1 mM sodium phosphate, pH 7.4, for 1 h [1].

Chromaffin granules and chromaffin granule membranes were prepared as previously described [14]. Adrenal medulla was homogenized in 0.3 M sucrose, 100 KIE/ml aprotinin (protease inhibitor), 10^{-5} M pargylin (*N*-methyl-*N*-2-propynyl-benzenemethanamine, monoamine oxidase inhibitor) and centrifuged 600 × *g* for 10 min. The supernatant was referred to as homogenate. Whole granules were obtained in 1.6 M sucrose, 100 KIE/ml aprotinin, 10^{-5} M pargylin. The granule membrane fraction was obtained after lysis by dialysis against 2 × 5 litres 50 mM sodium succinate, pH 6.0. After 72 min 100 000 × *g* centrifugation the first supernatant was taken as the matrix proteins and the remaining water insoluble sediment was washed twice in 10 vol. succinate buffer. The final pellet was used as source of granule membranes.

Quantitation of synaptin was performed by crossed immunoelectrophoresis [1,2,15,16]. The area enclosed by the synaptin-immunoprecipitate of a test sample was measured by a semiautomatic planimeter. A standard curve was obtained by area measurement of the synaptin-immunoprecipitates developed by crossed immunoelectrophoresis of varying amounts of a homogenate of ox forebrain cortical matter (reference). The concentration of synaptin was expressed in specific concentrations relative to the total protein content, 1.0 arbitrary unit (a.u.) being the amount of synaptin per gram total protein of the reference. Thus, if x μg protein of a test sample contained an amount of synaptin equal to the synaptin content in y μg protein of the reference as determined by interpolation on the standard curve, the test sample was said to contain y/x a.u. synaptin. For details see ref. [16]. The membrane topography of synaptin was determined by immunoabsorption [17,18]. In principle intact and lysed structures are added to an antiserum containing antibodies against one or several membrane proteins. Membrane proteins on the surface of the structures will be easily accessible to the corresponding antibodies, whereas proteins located on the internal side of the membrane only can react with their corresponding antibodies, when the structures are lysed. The decrease in antibody titres can thereafter be determined by quantitative immunoelectrophoretic techniques as outlined by Axelsen and Bock [19].

In the actual study the following subcellular fractions were prepared: whole synaptosomes, synaptosomal plasma membranes, synaptic vesicles, whole chromaffin granules and lysed chromaffin granules. Care was taken to prevent sudden changes in osmolarity and temperature to avoid lysis of the whole closed structures. A 300 μl subcellular fraction containing synaptin concentrations in the range of 0.3–11.1 a.u./g total protein was added to 20 μl antiserum. After incubation for exactly 30 min at room temperature (20–22°C) the subcellular fractions were removed by centrifugation at 180 000 × *g* for 45 min, and the synaptin titre of the absorbed antiserum was determined by crossed immunoelectrophoresis with intermediate gel [20].

3. Results

In table 1 the amount of synaptin in whole chromaffin granules, granule membranes and soluble granule proteins is shown. Synaptin was more than eight-times enriched in chromaffin granule membranes compared to homogenate of adrenal medulla, demonstrating that this protein is a constituent of the granule membrane. No synaptin could be demonstrated in the granule matrix.

Table 1
Determination of synaptin in chromaffin granules

	Synaptin (a.u.)
Homogenate of adrenal medulla	0.2
Whole granules	0.3
Granule membranes	1.7
Granule matrix	not detectable

The synaptin determinations were performed on separate samples from two independent fractionations. The results in the two experiments were identical for all fractions. 1.0. a.u. is the amount of synaptin per gram total protein in a homogenate of ox brain cortical matter. For further explanation see text.

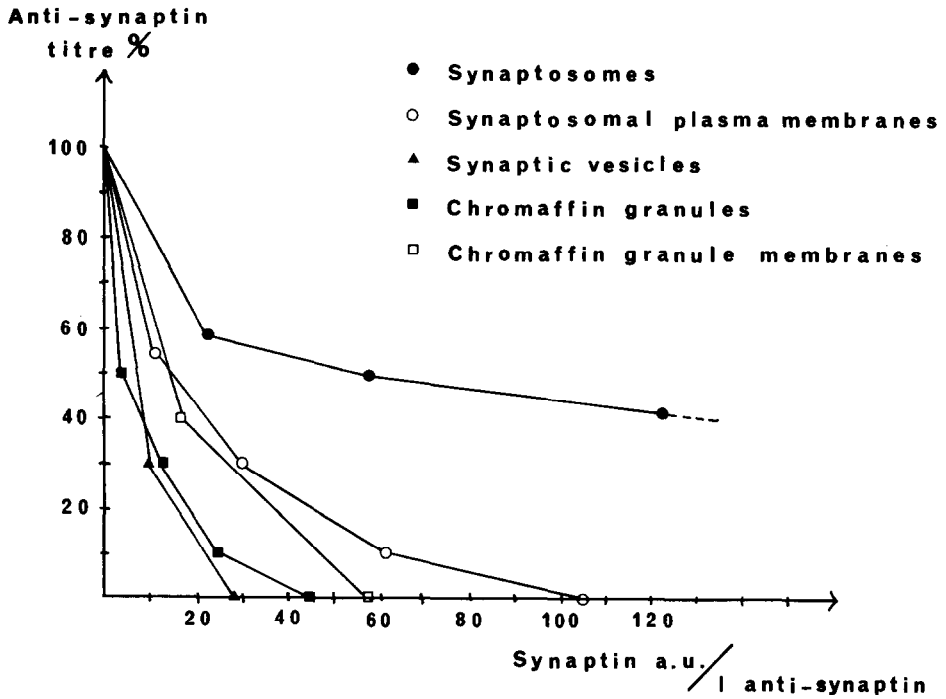

Fig.1. Immuno-absorption of anti-synaptin with various subcellular fractions. The abscissa indicates the amount of synaptin in arbitrary units (a.u.) in the individual subcellular fractions, which was added to a fixed volume of anti-synaptin (a.u./litre antiserum). The ordinate indicates the titre of the anti-synaptin after absorption, 100% being the titre in the control, containing antiserum, but no subcellular fraction. For experimental procedure and interpretation of results, see text.

In fig.1 the immunoabsorption experiment for the determination of the localization of the synaptin is shown. The less amount of synaptin in a specific subcellular fraction necessary to absorb the anti-synaptin activity from the antiserum, the more exposed was the antigenic determinants of synaptin in the respective fraction. From the figure it appears that the protein was easily accessible to the antibody when synaptic vesicles and whole chromaffin granules were used for absorption. Synaptin seemed to be a little less accessible on granule membranes and synaptosomal plasma membranes, probably due to resealing of these structures. Finally it can be seen that synaptin in whole brain synaptosomes was rather unaccessible to the antibody. Even after addition of 600 a.u. synaptin/litre antibody, 10% of the anti-synaptin titre was present (not shown on the figure). The absorbing capacity of the whole synaptosomes was assumed to be due to the free membranes which contaminated this fraction. From the immunoabsorption experiment it is concluded that synaptin is located on the outside of synaptic vesicles and chromaffin granules, and on the inside of synaptosomal plasma membranes.

4. Discussion

Little is known about protein composition of brain synaptic vesicles. The pattern in SDS– polyacrylamide gel electrophoresis is rather similar to that of synaptosomal plasma membranes with regard to proteins and glycoproteins, although synaptic vesicle membranes seemed to contain less polypeptides [21]. The topographical distribution of proteins on the synaptic vesicle has until now been unknown. The present demonstration of the localization of synaptin provides the first evidence of a specific protein located on the

outer surface of brain synaptic vesicles. More is known about the composition and molecular organization of chromaffin granule membranes [11,12]. The molecular weights of the proteins dopamin β-hydroxylase (subunits 75 000), chromogranin A (subunits 32 000–40 000) and chromomembrin b (25 000–30 000) indicate that these proteins are different from synaptin. The arrangement of dopamin β-hydroxylase and chromomembrin b in the membrane has been determined [22] and chromomembrin b has been shown to be present on the outer surface of these organelles, whereas dopamin β-hydroxylase faced the inside of the organelles. If a synaptic vesicle during exocytosis fuse with the presynaptic membrane, proteins on the vesicle surface must be expected to be incorporated in the presynaptic plasma membrane, located on the inside of the plasma membrane. Synaptin was distributed on the membranes in this way. The demonstration of synaptin on both brain synaptic vesicles and chromaffin granules raises the question of whether synaptin is restricted to catecholamine storing organelles or present on synaptic vesicles containing other neurotransmitters too. Recent studies on secretory granules from bovine neurohypophysis indicate that synaptin is present on these organelles (Bock, Torp-Pedersen, Treiman and Thorn, unpublished observation). Therefore it may be reasonable to assume that synaptin is a general marker protein of synaptic vesicles and presynaptic plasma membranes. In the search for components which might be involved in the attachment of neurotransmitter storing organelles to the plasma membranes during the exocytosis process, synaptin is a candidate due to its localization on the outer surface of brain synaptic vesicles and chromaffin granules and on the inside of synaptosomal plasma membranes.

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