Immunocytochemical Localization of Synaptophysin on the Smooth-Surfaced Tubular Membranes Present in Nerve Terminal and Preterminal Areas in the Rat Cerebellar Cortex*

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Summary. The distribution of synaptophysin, a major protein of the synaptic vesicle membrane, was immunocytochemically examined in the rat cerebellar cortex. A monoclonal antibody against synaptophysin recognized the epitope to be present in the presynaptic membranous structures including synaptic vesicles, presynaptic membrane, coated vesicles, and vacuoles of endocytotic origin. In the nerve terminal as well as preterminal areas, the antibody labeled the smooth-surfaced tubular membranes which were located in the relatively interior parts of these areas and consistent in size and appearance with the short tubules comprising the thinner parts of the axonal reticulum. However, the antibody did not stain the short tubular membranes, though similar in appearance to the above, which existed right below the axolemma in the preterminal and nerve terminal areas. The results are discussed with special reference to the precursor membrane compartments of synaptic vesicles.

Synaptic vesicles play a key role in neurotransmission and in membrane movements in the nerve terminal. It has been suggested that neurotransmitters stocked in them are released by exocytosis, leading to presynaptic membrane movements involving exocytosis-endocytosis coupling and the axonal smooth endoplasmic reticulum (ZIMMERMANN, 1979; HOLTZMAN, 1981). Recently, several research groups have prepared monoclonal antibodies against the component proteins of the synaptic vesicle membrane, synaptophysin, p65 and SV2, with molecular weights of 38, 65 and 100 KD, respectively (CARLSON and

KELLY, 1980; MATTHEW et al., 1981; WIEDENMANN and FRANKE, 1985; NAVONE et al., 1986; OBATA et al., 1987). BUCKLEY and KELLY (1985) stained synaptic vesicles and presynaptic membrane with an antibody to synaptophysin, thereby presenting evidence supporting the exocytosis of synaptic vesicles followed by incorporation of the vesicle membrane into the presynaptic membrane. Although the nerve endings have been well examined with the antibodies, the axon and the preterminal area remain to be precisely studied by immunocytochemistry for identifying the membrane structures with corresponding epitopes.

Earlier cytochemical works with heavy metals have suggested that the thin parts, short in length and tubulosaccular in appearance, of the smooth endoplasmic reticulum (SER) in the axon, pass by the preterminal area, enter the nerve terminal, and serve there as a major source of synaptic vesicles by budding off the vesicular structures (DROZ et al., 1975; REINICKE and WALTHER, 1978; HOLTZMAN and MERCURIO, 1980; KADOTA and KADOTA, 1985). However, it has not been determined yet whether the axonal SER contains the components of synaptic vesicle membrane. The ultrastructure and biochemical nature of the axonal SER remained to be characterized. The present work aims to examine immunocytochemically the details of the association of synaptophysin with the axonal SER, which until now have been cytochemically observed as smooth-surfaced tubular membranes in the preterminal and nerve terminal areas.

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MATERIALS AND METHODS

Monoclonal antibody

A monoclonal antibody to synaptophysin was prepared. A purified synaptic vesicle fraction was prepared as the antigen material from whole rat brains with modifications of our previous procedures (KADOTA et al., 1976). Briefly, synaptic vesicles were extracted from crude synaptosomes by hypo-osmotic treatment and then subjected to differential and sucrose density gradient centrifugation for purification. The synaptic vesicle fraction (S_5P) was suspended in a buffer solution of 10 mM 2-(N-porpholino) ethansulfonic acid (MES) at pH 6.0 (ml/5 brains), and stored at -70°C for 1–4 days. The specimens were then thawed, and 4 ml was layered on a sucrose density gradient consisting of 8 ml of 16% and 26 ml of 24% sucrose in MES in 40 ml tubes in a Hitachi RPS 27-2 swing bucket rotor. The tubes were centrifuged at 60,000 g for 2 h. The 16% layer, excluding the lowest part in contact with the 24% layer, was collected as a purified synaptic vesicle fraction, negatively stained, and examined by electron microscopy. The obtained fraction was of a very high purity. The materials were diluted two times with the addition of equal amounts of MES and centrifuged at 180,000 g for 1 h. The precipitate was resuspended in a minimum volume of MES to then serve as the antigen material. If further purification was needed, 10 ml of the double-diluted synaptic vesicle preparation (the 16% layer) was layered on a sucrose density gradient consisting of 9 ml each of 16%, 24% and 40% sucrose in MES. The density was spun at 60,000 g for 16 h and the interface band between 24% and 40% was harvested as a further purified synaptic vesicle fraction.

BALB/c mice were immunized according to methods by MISHELL and SHIIGI (1981). The splenocytes from the mice were fused with myeloma P3U1. Hybridoma antibodies were screened by ELISA (enzyme-linked immunosorbent assay). The antibody

which recognized the polypeptide with a molecular weight of 38 KD in the synaptic vesicle fraction was selected as the antibody for synaptophysin by SDS-PAGE and immunoblot methods. This antibody was used for the immunohistochemical localization of the polypeptide in the rat cerebellar cortex. The antibody clearly labeled the large glomeruli in the granular layer and small punctates in the molecular layer. These biochemical and histochemical properties of the antibody resembled those of the monoclonal antibody to synaptophysin characterized by previous investigators (WIEDENMANN and FRANKE, 1985; NAVONE et al., 1986; OBATA, et al., 1987).

Immuno-electron microscopy

Adult rats were fixed by perfusion via the left ventricle with a mixture of 4% paraformaldehyde and 0.1% glutaraldehyde in 0.1 M phosphate buffer (PB) at pH 7.4. The cerebellar cortex was dissected out and cut in sagittal planes into sections 20 μm thick with a microslicer (Dosaka Instruments, Japan). The slices were incubated with the antibody for 4–16 h at 4°C after blocking with 1% bovine serum albumin. The materials were rinsed in phosphate buffered saline (PBS), and then incubated with a second antibody, biotinylated horse antimouse IgG (Vector Lab. Inc. USA) for 2–3 h. The sections were then reacted with ABC reagent (Vector Lab. Inc. USA), rinsed with 0.05 M Tris-HCl buffer (pH 7.6) and incubated with DAB-reagent (0.1% diaminobenzidine tetrahydrochloride in Tris-buffer containing 0.02% H_2O_2) for 30 min at room temperature. After rinsing with PBS, the slices were postfixed with 1% osmium tetroxide in PB and stained en bloc with 1% uranyl acetate in 50 mM acetate buffer, pH 5.2, for 14–16 h at 4°C . Subsequently the specimens were dehydrated with a series of graded ethanol solutions and embedded in Epon 812. Some ultrathin sections were lightly stained with lead citrate while others were not. They were examined with an electron microscope JEM 1200 EX.

Figs. 1–5. Electron micrographs showing the localization of synaptophysin in the molecular layer of the rat cerebellar cortex. Labeled with a monoclonal antibody to synaptophysin, a synaptic vesicle membrane protein with a M.W. of 38 KD. Lightly stained with lead citrate except Figure 2.

Fig. 1. Molecular layer at a low magnification. Various types of nerve endings (*T*) are strongly labeled. $\times 30,000$

Fig. 2. High power electron micrograph showing that the synaptic vesicles (*SV*) and presynaptic membrane are strongly labeled. Intraterminal vacuoles (*V*) occurring on the outskirts of the synaptic vesicle accumulation are labeled. A smooth-surfaced tubular membrane (*arrow*) is weakly labeled in the preterminal area on the top right. The section was not stained with lead citrate. $\times 40,000$

Figs. 1 and 2. Legends on the opposite page.

Figs. 3-5. Legends on the opposite page.

RESULTS

Subsynaptic localization of synaptophysin

To identify the subsynaptic localization of synaptophysin, we immunocytochemically examined the cerebellar cortex with an electron microscope. Reaction products with the antibody were found in every kind of nerve terminal in the cortex: mossy nerve endings and Golgi Type II terminals in the granular layer, and climbing fiber terminals, parallel fiber terminals and other kinds of small nerve terminals in the molecular layer (Fig. 1). However, no labeling was found in the axon or in the postsynaptic dendrites.

High power electron micrographs more clearly delineated the subcellular localization of synaptophysin (Figs. 2-5). The reaction products were concentrated on the membrane of synaptic vesicles and on the presynaptic membrane (Figs. 1-3, 5). In addition, intraterminal vacuoles and coated vesicles, near the synaptic vesicle clusters, were labeled with the antibody (Figs. 2, 4, 5). On the other hand, as shown in Figures 4 and 5, the antibody marked the vacuoles and a coated pit which were distributed apart from the synaptic vesicle clusters, presenting a labeling pattern unlikely to result from the diffusion of the immunocytochemical precipitates from the synaptic vesicles. No labeling was found on the axolemma except the terminal area and postsynaptic membrane (Figs. 1, 2).

The smooth-surfaced tubular membranes near or among synaptic vesicles were intensely labeled in the nerve terminal (Figs. 3, 5; large arrows). A long tubule, similar in shape to the axonal SER and a little apart from synaptic vesicle clusters, was weakly labeled in the preterminal area (Fig. 4). In contrast, the short tubular structures lying just below the axolemma were not stained in the preterminal and nerve terminal areas (small arrow, Fig. 5).

Mitochondria in the nerve terminal were often labeled. However, the reaction product was often precipitated on their surface apposing the synaptic vesicle clusters. Mitochondria distributed in the axon, perikaryon and dendrite were not labeled (Figs. 1-5). From this staining pattern it is doubtful that the labeling of mitochondria is related to the epitope of synaptophysin present in this organella.

DISCUSSION

We prepared a monoclonal antibody to a component protein of the synaptic vesicle membrane, synaptophysin, with a molecular weight of 38 KD. The subcellular localization of this protein was immunocytochemically examined by electron microscopy in the rat cerebellar cortex. The morphology of the smooth-surfaced tubular membranes was scrutinized with the antibody with special reference to the axonal smooth endoplasmic reticulum and synaptic vesicles.

Synaptophysin has been selectively localized on the synaptic vesicle membrane (JAHN et al., 1985; WIEDENMANN and FRANKE, 1985) and simultaneously on the presynaptic membrane which was more intensely labeled with the antibody following stimulation (VON WEDEL et al., 1981; BUCKLEY et al., 1983; ROBITAILLE and TREMBLEY, 1987; VALTORTA et al., 1988). The present study has confirmed these earlier findings and supported the concept that the synaptic vesicle membrane is fused with and then incorporated into the presynaptic membrane by exocytosis (HEUSER and REESE, 1973; CECCARELLI et al., 1973; HEUSER et al., 1979; LENZ and CHESTER, 1982).

Coated vesicles were labeled with the antibody confirming previous reports (PFEFFER and KELLY, 1985; WIEDENMANN et al., 1985; TIXIER-VIDAL et al., 1988), suggesting that they transiently participate in the vesicular traffic involving synaptic vesicles. The antibody conjugated with colloidal gold, however, failed to label the coated vesicles in the nerve terminals (WIEDENMANN and FRANKE, 1985; NAVONE et al., 1986) or in the PC-12 cells. The absence of anti-synaptophysin labeling on coated membranes in these experiments may be due to limited accessibility for gold particles (JANETZKO et al., 1989).

Synaptophysin was present on the intraterminal vacuoles apparently of macropinocytotic origin (KADOTA and KADOTA, 1982). The protein was also found on plasmalemmal invaginations (WIEDENMANN and FRANKE, 1985; NAVONE et al., 1986). These findings strongly support our previous suggestion that the macropinocytotic vacuoles — but not coated vesicles — in the nerve terminal work as a primary device to remove rapidly the excess of terminal

Fig. 3. A short tubule (*arrow*) labeled in a small nerve terminal. $\times 50,000$

Fig. 4. A long tubule (*arrow*) located at the left end in a preterminal area is partially labeled. A short tubule is weakly labeled (*double arrow*). A coated pit invaginating from the axolemma is labeled in a neighboring process (*arrowhead*). $\times 50,000$

Fig. 5. A short tubule lying in a cluster of synaptic vesicles is strongly labeled (*large arrow*) in a large nerve terminal. A C-shaped vacuole (*V*) on the top left is also labeled. A short tubule located below the axolemma (*small arrow*) is not labeled. $\times 48,000$

surface membrane containing the synaptic vesicle-membrane proteins (KADOTA and KADOTA, 1982). A recent report on synaptophysin expression on the endosomes in the PC-12 cells supports this suggestion (CLIFT-O'GRADY et al., 1990).

The antibody to synaptophysin also labeled the smooth-surfaced tubular membranes, short in length, which were present in the relatively interior parts of the nerve terminal and preterminal areas, but not those localized near the axolemma. These short and tubular smooth-surfaced membranes located in the preterminal area as well as in the nerve terminal have been suggested ultrastructurally and histochemically to originate from the axonal SER (PALAY, 1958; DROZ et al., 1975; REINICKE and WALTHER, 1978; KADOTA and KADOTA, 1985). Moreover, the axonal SER has been shown to share histochemical properties with synaptic vesicles (DROZ et al., 1975; REINICKE and WALTHER, 1978; HOLTZMAN and MERCURIO, 1980; KADOTA and KADOTA, 1985). DROZ et al. (1975) have suggested that the axonal SER composes a continuous three-dimensional network passing through the preterminal area and ends in the nerve terminal to supply synaptic vesicles. The present authors have reported that the thin tubular network of the axonal SER increasingly enters the preterminal area and the nerve ending during transmitter release, where vesicular structures, indistinguishable in size and appearance from the synaptic vesicles, are budded off from the short and tubular smooth-surfaced membranes of the SER (KADOTA and KADOTA, 1985). These short tubular membranes of the SER are morphologically consistent with the smooth-surfaced tubular membranes shown in the present study, which are positively stained with the antibody to synaptophysin and occupy interior portions of the nerve terminal and the preterminal areas. Similar observations, however, were not available from the short tubules lying directly below the axolemma. Taken together, the short tubular membranes in the preterminal area and the axon ending would be immunocytochemically divided into two subclasses; one of these, positively labeled with the antibody, is related to synaptic vesicles and involved in the presynaptic membrane movements, and the other occurring below the axolemma is not. Alternatively, the latter might be too immature to be labeled with the antibody.

Some investigators have examined the intracellular localization of synaptophysin in immature neurons, and demonstrated that this protein was present on the innermost cisterns of the Golgi apparatus, and also on the numerous small vesicles near this organelle (NAVONE et al., 1986; TIXIER-VIDAL et al., 1988). They supposed that synaptophysin was released

from the Golgi apparatus in a vesicular form after glycosylation and then transported to the nerve terminal. The workers also observed that the short tubular membranes containing synaptophysin localized near the small vesicles. However, these groups did not determine a transporting pathway of synaptophysin in the axon. A recent report showed the immunocytochemical localization of a synaptic vesicle membrane protein, termed "SV2", on the vesiculo-tubular structures in the axon and the nerve terminal (JANETZKO et al., 1989). This work has claimed that the vesiculo-tubular structures compose a membrane compartment of synaptic vesicles different from the axonal reticulum described by the preceding investigators (DROZ et al., 1975; RAMBOURG and DROZ, 1980; KADOTA and KADOTA, 1985). In short, the arguments concerning the precursor membrane structures for synaptic vesicles are still controversial. At present, it remains unclear whether or not tubular membranes in the preterminal and terminal areas shown in the present report are relevant to the vesiculo-tubular structures (JANETZKO et al., 1989).

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