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Synaptic vesicles in electromotoneurones. I. Axonal transport, site of transmitter uptake and processing of a core proteoglycan during maturation

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We were able by using an *in vivo* pulse-label technique to trace part of the life cycle of a secretory organelle, the acetylcholine-storing synaptic vesicle from electromotoneurones of *Torpedo marmorata*. This technique uses [^{35}S]sulphate incorporation into the cell bodies of the electromotoneurones which results in radioactive labelling of a synaptic vesicle heparansulphate proteoglycan — a major core component. Vesicles are anterogradely transported in the axons at a fast rate as 'empty' organelles (VP_0 population). In the nerve terminal, maturation of the granule to a population (VP_1) fully charged with acetylcholine and ATP occurs. Finally after a longer time interval a change to a third population (VP_2) is observed. This population is reduced in diameter as compared to VP_0 and VP_1 suggesting, in agreement with earlier reports, that it has undergone exo-endocytosis. The changes from VP_0 to VP_1 and VP_2 are accompanied by a degradation of the core proteoglycan as measured by gel filtration of the ^{35}S-labelled compound. The results show that vesicles are axonally transported as preformed organelles, exist in the neurone at least in three different populations and that the nerve terminal is the major site of transmitter uptake.

Key words: synaptic vesicles/electromotoneurones/proteoglycan

Introduction

Synaptic vesicles are the characteristic small neurotransmitter-storing organelles, abundant in nerve terminals, which play a central role in neurotransmission. Evidence has been accumulating that they are directly involved in transmitter release via exocytosis (see Torri-Tarelli *et al.*, 1985 for further references). Interest in synaptic vesicles has greatly increased since it was found that they are associated with neurone-specific (Huttner *et al.*, 1983; Jahn *et al.*, 1985; Wiedenmann and Franke, 1985; Walker *et al.*, 1986) or transmitter-specific antigens (Wedel *et al.*, 1981; Walker *et al.*, 1982), thereby providing valuable markers for neurocytochemistry and for the study of vesicle function (Jones *et al.*, 1982; Buckley *et al.*, 1983).

In contrast to many other secretory systems, the site of release of secretory products in neurones is physically separated from the cell body and its protein- and membrane-synthesizing activity by the long and thin axon. This raises a question as to the nature of the mechanism of the axonal transport of synaptic vesicles and their site of packaging with neurotransmitter, a problem which so far has not been solved.

Although it has been shown that small membrane-bound organelles are transported at a fast rate anterogradely in axons (Tsukita and Ishikawa, 1980), until now the lack of specific and stable vesicle markers has so far impeded the unequivocal identification of these organelles as synaptic vesicles. This limitation applies equally to studies using extruded squid axoplasm and video-enhanced stereomicroscopy (Allen *et al.*, 1982; Brady *et al.*, 1982) where particles in the size range of synaptic vesicles can be observed being transported along microtubules at a fast rate (Schnapp *et al.*, 1985). In motor axons, material likely to represent cholinergic vesicles accumulated rapidly on both sides of a ligature, suggesting their fast axonal transport (Dahlström *et al.*, 1985). Besides axonal transport of vesicles, it has been suggested that acetylcholine-storing vesicles are formed locally in the nerve terminal either by 'budding off' from axoplasmic reticulum (Droz *et al.*, 1975) or by endocytotic retrieval from presynaptic plasma membrane (Zimmermann, 1979).

The only well-characterized synaptic vesicle preparations available at present are the acetylcholine-containing vesicles from electromotoneurones of electric rays (Carlson *et al.*, 1978; Tashiro and Stadler, 1978). These vesicles store high concentrations of acetylcholine and ATP in an aqueous phase at an acidic pH ~5.5 (Stadler and Füldner, 1980; Füldner and Stadler, 1982). An ATP-dependent proton pump (Harlos *et al.*, 1984), a nucleotide carrier (Lee and Witzemann, 1983; Stadler and Fenwick, 1983) and a heparansulphate proteoglycan in the core (Stadler and Dowe, 1982; Carlson and Kelly, 1983) have been identified (see Stadler *et al.*, 1985 for overview). The vesicle preparation isolated from electric organ extracts on shallow sucrose gradients is heterogeneous. A population (VP_1) fully charged with acetylcholine and ATP and another population (VP_2) at the denser side of the gradient as compared to VP_1 have been described (Tashiro and Stadler, 1978). This population consists probably of VP_2-type vesicles that have undergone exo-endocytotic cycles and is abundant in perfused and electrically stimulated electric organs (Zimmermann and Whittaker, 1977).

We have studied the axonal transport and the site at which vesicles are loaded with transmitter in the electromotoneurones. The heparansulphate proteoglycan was used as a vesicle marker. This component can be labelled *in vivo* by [^{35}S]sulphate injection into the electric lobe, the region of the brain where the cell bodies of the electromotoneurones are located (Stadler and Dowe, 1982). Using this technique and a new vesicle proteoglycan-specific antiserum we have now studied the axonal transport of electromotoneurones and their processing in the nerve terminal.

Results

Axonal transport

Prior to injection of [^{35}S]sulphate into the electric lobe, the axons innervating one of two electric organs were cut. The distribution of radioactivity in the electromotor axons at various times after injecting [^{35}S]sulphate into the electric lobes was then determined. Axons were dissected out, cut into segments, homogenized and submitted to SDS gel electrophoresis followed by autoradiography. The amount of radioactivity was then calculated by scanning the autoradiographs. This method ensures that only [^{35}S]sulphate bound to macromolecules is counted. Figure 1a shows the distribution of radioactivity in the lobe and

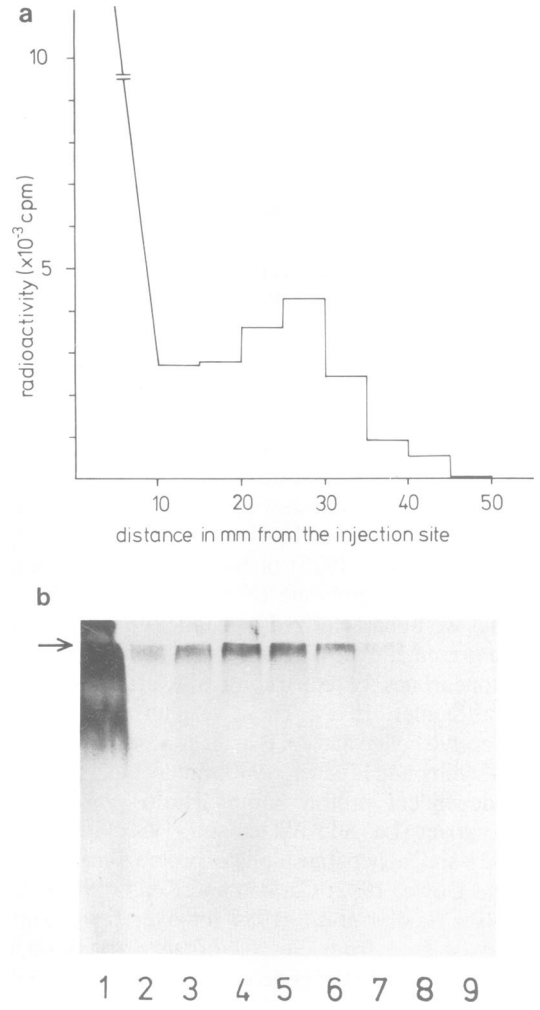

Fig. 1. Fast axonal transport of synaptic vesicle proteoglycan in *Torpedo* electromotoneurones. **(a)** Distribution of radioactivity in nerve II is shown 12 h after injection of 2 mCi [^{35}S]sulphate into the electric lobe. The radioactivity in the lobe and in the axon segments was calculated from scanning SDS gel electrophoresis/fluorography as shown in (b). A peak of radioactivity at ~45 mm axon length is observed; a typical experiment is shown. **(b)** SDS gel electrophoresis/autoradiography of lobe homogenate **(lane 1)** and axon segments **(lanes 2–9)** from the experiment described above. The distribution of radioactivity in the axon segment is characteristic for the vesicular proteoglycan; no other ^{35}S-labelled macromolecules are detectable. A 3% stacking gel and a 11% running gel were used.

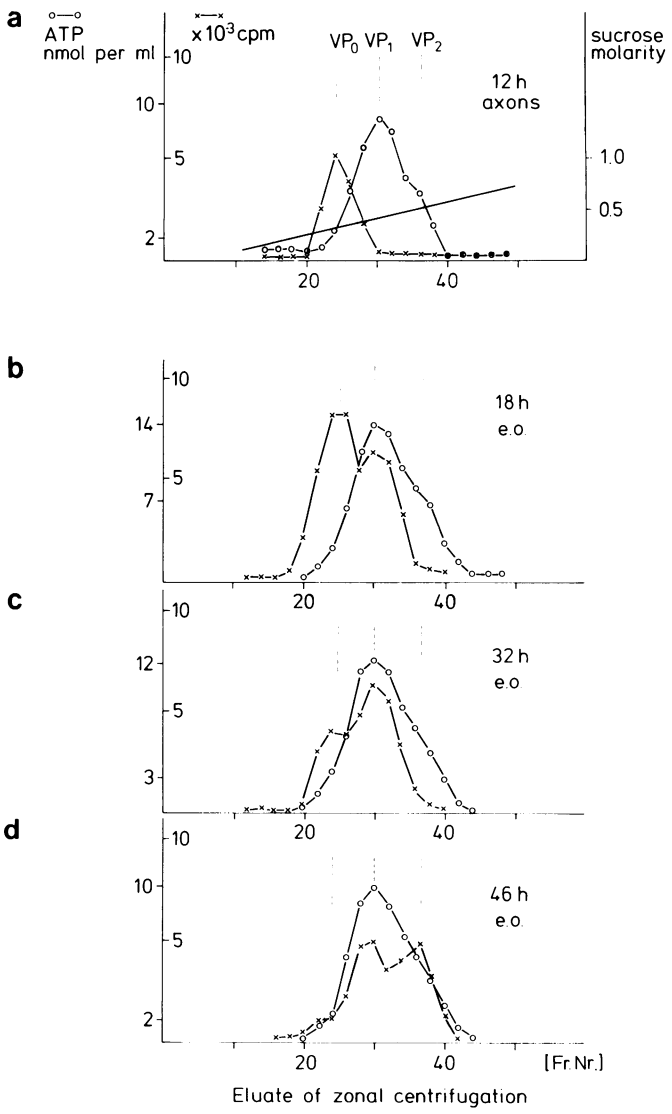

Fig. 2. Isolation of ^{35}S-labelled synaptic vesicles from axons and electric organs by sucrose density gradient centrifugation. Equally sized fish were injected with [^{35}S]sulphate into the electric lobe and after different time intervals vesicles were isolated from axons or nerve terminals. Figures a, b, c and d each obtained from a single fish represent typical experiments. The radioactivity in the fractions was obtained by centrifugation of the eluate of the density gradient followed by counting part of the pellet (densitometric evaluation of the pellets after SDS gel electrophoresis/fluorography showed a similar distribution). The positions of the three vesicle populations VP_0, VP_1, VP_2 in the gradient are indicated by the vertical dotted lines. **(a)** Axons, ^{35}S-labelled, as described in Figure 1 were mixed with (unlabelled) electric organ from another fish. The position of electric organ vesicles was detected by using the classical marker ATP. A major peak (VP_1) and a shoulder (VP_2) are detected. VP_1 is the fully charged vesicle population, VP_2 a denser one with less ATP. The radioactivity from the labelled axons elutes in a major peak (VP_0 position) close to VP_1 but at a lower density. The radioactivity represents the ^{35}S-labelled vesicular proteoglycan suggesting that it is incorporated within a vesicle population lighter than VP_1. **(b, c, d)** Isolation of ^{35}S-labelled vesicles from electric organs. The axonally transported radioactivity was allowed to reach the electric organ and vesicles were subsequently isolated from the organ after 18, 32 and 46 h. Each diagram represents a single experiment. Equally sized fishes with comparable axon lengths were used. Positions of VP_1- and VP_2-type vesicles were detected by their acetylcholine and ATP content. Note that in the VP_0 position no acetylcholine and ATP peak is detected indicating that this population is empty. The experiments show that vesicles mature during their life-cycle from VP_0 to VP_1 and are finally found in a third population (VP_2).

in the axon segments 12 h after injection. It can be seen that a peak of radioactivity distal from the site of injection is present. This peak was not seen in segments from the side where the axons were cut prior to injection and therefore represents an axonal transport phenomenon. From similar experiments at different time intervals (results not shown) it was estimated that the peak of radioactivity migrated anterogradely with a speed of 110 ± 40 mm/day (seawater temperature 17°C). This velocity and the wave-like distribution of radioactivity are characteristic for fast axonal transport systems (Grafstein and Forman, 1980). A more detailed description of fast axonal transport in *Torpedo* electromotoneurones will be presented elsewhere (Tytell *et al.*, in preparation).

Figure 1b shows autoradiographs from the experiment presented in Figure 1a. Whereas in the lobe—the site of injec-

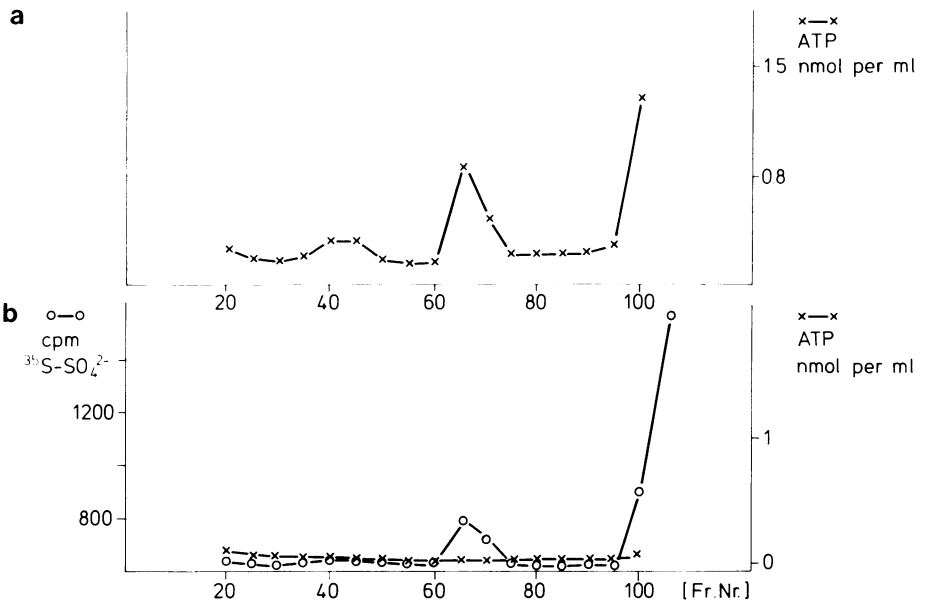

Fig. 3. Sephacryl S 1000 chromatography of axonally transported ^{35}S-labelled material from density gradient centrifugation. (**a**) The column was calibrated first with electric organ vesicles which were detected by their ATP content. (**b**) The radioactively labelled axon material elutes in the same position, indicating that the proteoglycan is within an organelle of similar size to electric organ synaptic vesicles. The peak did not contain any detectable amount of acetylcholine nor ATP suggesting that these organelles are 'empty'.

tion where the perikarya are found—several broad bands are observed, in the axon segments the radioactivity is in the stacking gel and on top of the running gel. As shown previously, this distribution is characteristic for the synaptic vesicle proteoglycan (Stadler and Dowe, 1982). Analysis of axon segments presented here indicates that the only major sulphated macromolecule rapidly transported is the vesicle core proteoglycan. Although relatively high amounts of [^{35}S]sulphate were injected into the lobe, the label efficiency was rather low. This is due to difficult injection conditions resulting in an unavoidable large leakage of radioactivity out of the lobe.

We next investigated whether the proteoglycan is already integrated within synaptic vesicles or is axonally transported in any other form. Synaptic vesicles were isolated from axons by applying the well-established vesicle isolation procedure, utilizing the electric organ (Tashiro and Stadler, 1978), to the axonal material. In this standard procedure, final purification of vesicles as extracted from electric organs is carried out on a shallow sucrose gradient in a zonal rotor. Vesicles are then detected by their acetylcholine and ATP content as a major peak indicating the fully charged population (VP_1), and a second population (VP_2) indicated by a 'shoulder' to the denser side of the peak (see Figure 2).

Axons were collected from ^{35}S-labelled motor neurones at time intervals where the peak of radioactivity was in transit between brain and electric organ as shown in Figure 1. These ^{35}S-labelled axons were then mixed with unlabelled electric organ as 'internal standard' and vesicles were extracted and separated by density gradient centrifugation from the combined homogenates. A peak of radioactivity indicating the presence of vesicle proteoglycan was obtained that eluted close to electric organ vesicles but at a slightly lower density (Figure 2a), suggesting that the proteoglycan is present in the form of vesicles but in a population with a different density.

Further characterization of the radioactively labelled material from the gradient was carried out by size fractionation on a column of Sephacryl S 1000 which separates large membrane fragments and soluble proteins from synaptic vesicles (Stadler and Tsukita, 1984). The Sephacryl S 1000 column was equilibrated with a potassium glutamate/glycine buffer isoosmolar to the vesicle extraction buffer and then calibrated with unlabelled vesicles from electric organ. The radioactively labelled axon material eluted in the same position as electric organ vesicles (Figure 3) indicating that the proteoglycan is present in an organelle-associated form and not as free proteoglycan or bound to larger membrane fragments. These results strongly suggest that the proteoglycan is present in the form of vesicles, nevertheless ACh and ATP present in the fractions either from the gradient or from the column eluate were so in concentrations (per mg protein) that were at least a factor of 30–50 lower. The proteoglycan-associated axonal organelles are therefore practically 'empty' and contain only small amounts of transmitter and ATP as compared to the mature granule.

Incorporation of vesicles into the nerve terminal population

In the next series of experiments the wave of radioactivity was allowed to reach the nerve terminal area in the electric organ. Fish of roughly equal size were used and ^{35}S-labelled vesicles were then isolated after 18, 32 and 46 h (Figure 2b–d). At the shortest time-interval studied, the distribution of radioactivity in the gradient showed a major peak (VP_0) at exactly the same position from which the 'axon-vesicles' were recovered (compare Figure 2a) and a smaller peak coinciding with the major ATP/ACh (VP_1) peak of vesicles. At 32 h after injection the majority of the radioactivity had shifted to a position coincident with VP_1; 46 h after injection radioactivity in the VP_0 position is absent and, in addition to a peak in VP_1, a third peak is found to the denser side (Figure 2d). The position of this peak in the gradient is similar to a previously described population (Tashiro and Stadler, 1978) that is probably identical with VP_2-type vesicles observed in perfused and electrically stimulated electric organs (Zimmermann and Whittaker, 1977) and therefore this population is henceforth called 'VP_2'.

After longer time intervals (3–4 days) more label is found

in the VP_2 fraction and less in the VP_1, but otherwise no significant changes to the 46-h situation were observed (results not shown) suggesting that VP_2 vesicles might have a considerably longer life-time than those in VP_0 and VP_1 fractions. A major finding is that the labelled material isolated from axons (Figure 2a) and the vesicles isolated from electric organs (containing the nerve terminals) after 18- to 32-h intervals elute in exactly the same position. Since the label is subsequently found in the positions of VP_1- and VP_2-type vesicles in the gradient, it is now clear that VP_0 represents a vesicle population and our previous assumption that the axonally transported material is in 'empty' vesicles is supported. These findings are confirmed as well by electron microscopy of VP_0-, VP_1- and VP_2-containing fractions (see below).

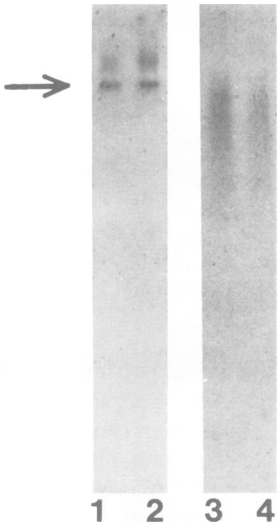

Fig. 4. Analysis of the ^{35}S-labelled proteoglycan by SDS gel electrophoresis/fluorography. **Lane 1**, VP_0-type vesicles; **lane 2**, VP_0 +VP_1-type (from the 18-h experiment); **lane 3**, VP_1 + VP_2; **lane 4**, VP_2-type vesicles (32-h experiment). The low degree of proteoglycan in the stacking gel in lanes 1 and 2 suggests that degradation of the proteoglycan occurs when VP_0 matures to VP_1. A 5% stacking gel and 11% running gel have been used. The arrow marks the top of the running gel.

Analysis of labelled vesicles by SDS gel electrophoresis/autoradiography (see Figure 4) suggests that conversion of VP_0 to VP_1- and VP_2-type vesicles might be accompanied by degradation of the proteoglycan. As compared to VP_0, very little material in VP_1 or VP_2 fractions can be detected in the stacking gel, which probably represents the high mol. wt form of the proteoglycan (Stadler and Dowe, 1982) if a 5% stacking gel is used as is the case here. Three per cent stacking gels, as used with axon segments (Figure 1b), seem to allow the high mol. wt form to enter the running gel.

A further characterization of proteoglycan size was obtained by gel filtration in the presence of 4 M guanidinium hydrochloride, 0.2% CHAPS on Sephacryl S 1000. A vesicle pellet obtained by pooling VP_0, VP_1 and VP_2 fractions from zonal centrifugation from an electric organ 40 h after ^{35}S-labelling was solubilized in sample buffer and chromatographed. Essentially a two-peak (I,II) distribution of radioactivity in the eluate was found (Figure 5); however, the second, larger, peak shows a shoulder and might be composed of two peaks. Analysis of immunoreactivity in the eluate using the proteoglycan antiserum and a quantitative spot assay showed a major reactivity in peak I and a minor one in peak II. The results indicate that the combined vesicle populations contain at least two types of proteoglycan molecules, a larger one present in peak I and a smaller one present in peak II which probably can be assigned to vesicle populations VP_1 and/or VP_2 with regard to the time-course of ^{35}S-labelling which is expected after 40 h to be present mainly in this type of vesicle.

Taken together, the results suggest that the axonally transported proteoglycan contained in the VP_0-type organelle is degraded in the nerve terminal during maturation of the vesicle.

Analysis of vesicle size and protein composition

Morphological examination of vesicle pellets obtained from VP_0, VP_1 and VP_2 peaks showed that all fractions are of high purity. VP_0 and VP_1 seem to be equally sized whereas VP_2 has a somewhat smaller diameter (Figure 6). This agrees well with

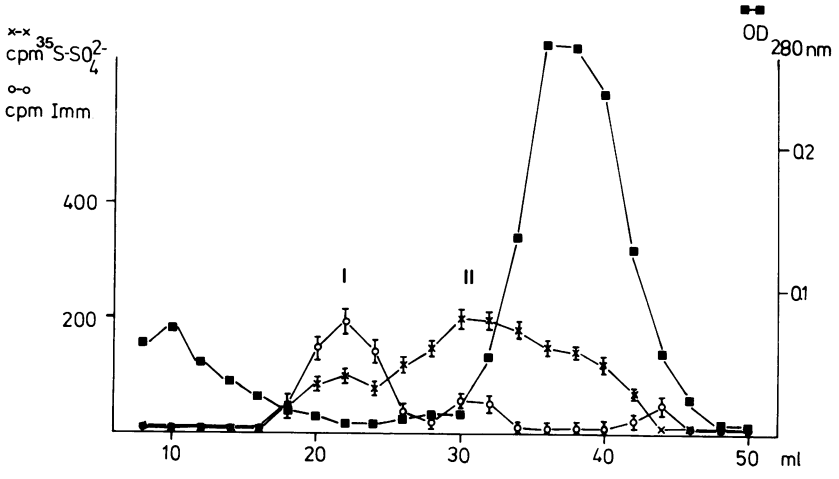

Fig. 5. Size fractionation of vesicular proteoglycan in the presence of detergent. Vesicles were isolated from electric organ 40 h after [^{35}S]sulphate injection where label is in transit between VP_1 and VP_2. The total vesicle fraction containing VP_0-, VP_1- and VP_2-type vesicles was isolated, pelleted and solubilized in 4 M guanidinium hydrochloride, 0.2% CHAPS, 50 mM Hepes pH 7.4 and eluted on a Sephacryl S 1000 column in the same buffer. The ordinate shows radioactivity (cpm)/200-μl aliquot and proteoglycan (cpm Imm) using a proteoglycan-specific antiserum and a quantitative immunassay. The radioactivity representing at this stage of vesicle maturation VP_1, VP_2 -type vesicles elutes essentially in two peaks, the label being present mainly in the broad peak II. By contrast, in the quantitative immunassay (o) representing the proteglycan content of the combined VP_0-, VP_1- and VP_2-type vesicles, the majority is found in peak I. The results indicate that the proteoglycan exists in the vesicle populations at least in two forms, suggesting that VP_1, VP_2 vesicles contain a smaller form than VP_0.

earlier findings of Zimmermann and Whittaker (1977) who reported that after repetitive electrical stimulation of the perfused electric organ a VP_2 population with a 10–20% smaller diameter became abundant in the nerve terminals, indicating that the population described by them indeed corresponds to the VP_2 population described by us. In addition we now show that this population already exists to a significant amount in the electrically unstimulated 'resting' organ.

The protein compositions in the VP_0, VP_1 and VP_2 peak fractions were analysed by SDS gel electrophoresis and staining with Coomassie Brilliant Blue. The patterns were found to be similar to those published previously (Tashiro and Stadler, 1978; Stadler and Tashiro, 1979). A comparison showed that differences seemed to exist only a yet-uncharacterized band in the 40-kd region, suggesting that the overall protein composition does not change during passage from VP_0 to VP_1 and VP_2 stage. Differences in minor bands are, however, not excluded (results not shown).

Discussion

We show here for the first time that a integral component of synaptic vesicles is transported at a fast rate in electromotor axons. This component is found—by a specific [^{35}S]sulphate label technique and by its migration properties upon gel electrophoresis—to be identical to a previously well-described heparansulphate proteoglycan in the core of the vesicles. It is incorporated within axons in an organelle that upon size fractionation is identical to nerve terminal vesicles. This organelle is slightly lower in density than the previously described nerve terminal populations VP_1 and VP_2, probably because it is 'empty'. Since a [^{35}S]sulphate label is only transiently found in the organelle but subsequently in VP_1 and VP_2, we conclude that it represents an immature vesicle population (VP_0). VP_0 vesicles leave the cell body as preformed but 'empty' particles and migrate with the fast axonal transport system to the nerve terminal. The possibility that vesicles are created by 'budding off' from smooth axoplasmic reticulum in the nerve terminal area (Droz, 1975) can be excluded in this system.

Our results do not support earlier preliminary evidence that acetylcholine might be axonally transported in organelle-bound form (see Dahlström *et al.*, 1985 for discussion). These results have, however, been obtained using 'ligature' techniques which might create artificial nerve terminals allowing empty vesicles to accumulate and take up transmitter. Obviously a difference exists here as compared to noradenaline-storing vesicles from sympathetic nerves which seem to be transported as transmitter-containing organelles (compare Lagercrantz, 1976). On the other

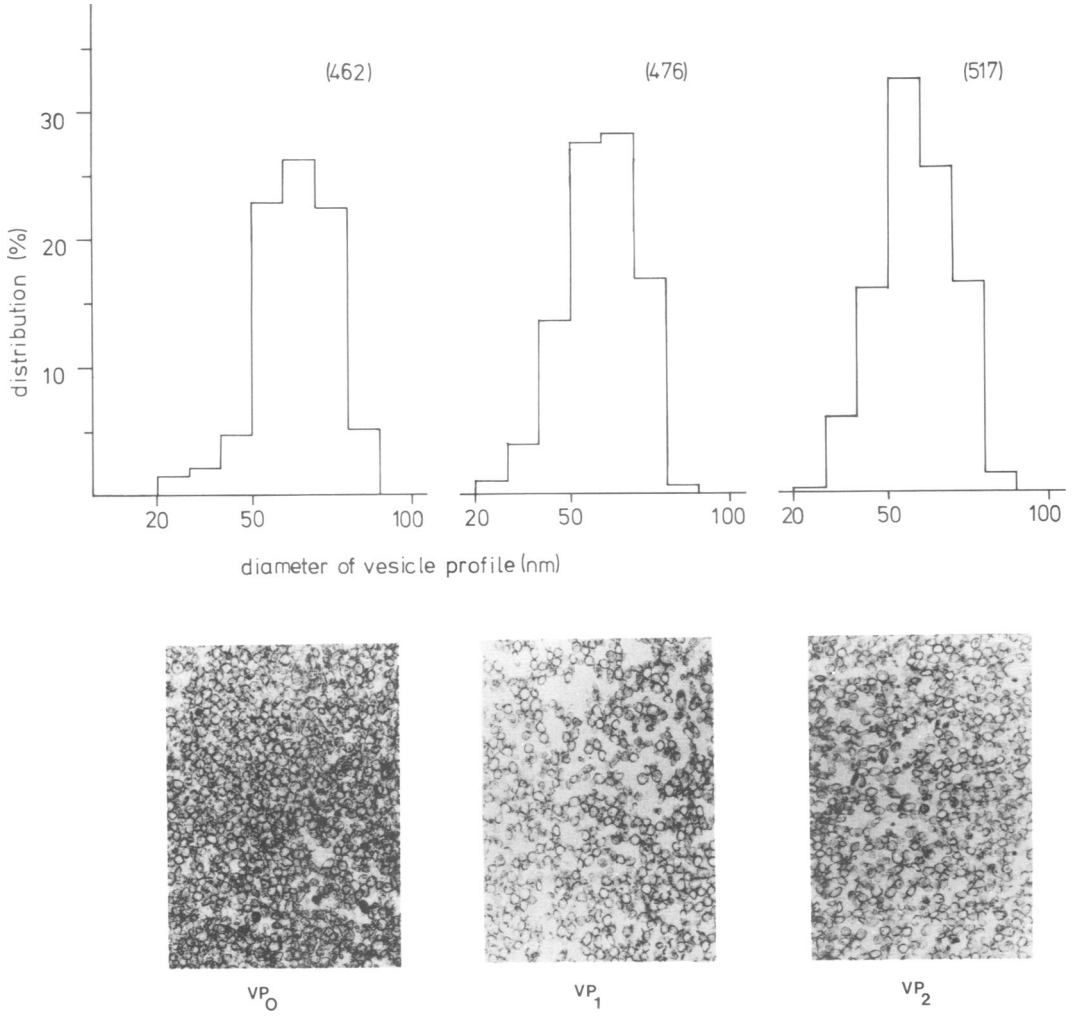

Fig. 6. Electron microscopy of VP_0, VP_1 and VP_2 peak fractions from sucrose density gradient fractions and analysis of vesicle diameter distribution. The block diagrams are normalized frequency distributions of vesicle profile diameters measured at 144 000 magnification uncorrected for fixation, embedding and instrumentation errors. The number of vesicles measured is inserted in brackets. No significant difference in diameter was found between VP_0 and VP_1, whereas VP_2 is smaller.

hand, an 'empty' population of the catecholamine-storing chromaffin granules from the adrenal gland has been described recently (Maret and Fauchère, 1986), suggesting that the presence of an empty population (implying a time-lag between biosynthesis and filling of granules) might occur in other secretory systems as well.

In the nerve terminal, the empty VP_0 population matures into the fully charged VP_1-type which subsequently undergoes a further change to a denser population. The three states are characterized by different densities resulting from differences in contents and size.

VP_2 seems to contain a lower mol. wt form of the proteoglycan: this could be derived by proteolytic cleavage of the larger form present in VP_0 and VP_1. Further evidence in favour of this view is given by Stadler and Kiene (1987). Since the protein composition of VP_2 was otherwise not found to be essentially different from VP_0 and VP_1, the mechanism of retrieval of fused vesicle membranes from the presynaptic plasma membrane appears to keep the identity of the vesicle membrane. Mixing of the two membranes after fusion should be expressed in a different protein composition of VP_2 as compared to VP_0 and VP_1 since the plasma membrane has a different protein composition (Stadler and Tashiro, 1979).

Materials and methods

Animals

Electric rays, *Torpedo marmorata*, were obtained from the Institut de Biologie Marine, Arcachon, France. In all experiments the fish were previously anaesthetized with 0.05% tricaine methanesulphonate in seawater.

Radiolabelling of synaptic vesicles with [^{35}S]sulphate

Sodium [^{35}S]sulphate (25–40 Ci/mg of sulphur) was purchased from Amersham (Braunschweig, FRG) concentrated by lyophilization and disssolved in 0.4 M NaCl. Approximately 2 mCi in 5 μl were injected into the paired electric lobes of *Torpedo*. As a control the nerves from one side of the lobes were cut prior to injection. In all experiments equally sized fishes (25–30 cm length) were used.

The electric nerves and electric organs were dissected at various time intervals between 12 and 46 h after the fish had received the injections. Each experiment at a given time interval represents one fish. For transport experiments with axon segments, nerve II (Fritsch, 1890) was cut into 5-mm pieces, frozen in liquid nitrogen and crushed to a powder in a steel mortar. Then the segments were homogenized in SDS-sample buffer and analyzed by SDS gel electrophoresis/fluorography (see below).

Isolation of synaptic vesicles

Synaptic vesicles were isolated as described by Tashiro and Stadler (1978). Briefly, vesicles were extracted from the crushed frozen tissue, concentrated on a sucrose density step gradient and transferred to a shallow sucrose gradient in a zonal rotor. The fractions of the eluate from the zonal gradients were sedimented (overnight at 36 000 r.p.m. in a Beckman AH 45 rotor). The pellets were dissolved in SDS-sample buffer and an aliquot was taken for counting. The rest was electrophoresed and radioactivity was determined by autoradiography (see below) and compared with the counts obtained directly from aliquots. The recovery of radioactivity of the entire gradient was between 60 and 80%. ATP was detected using a luciferin-luciferase assay (Dowdall *et al.*, 1974). Acetylcholine was detected using an h.p.l.c. separation followed by hydrolysis and oxidation on immobilized acetylcholinesterase and choline oxidase followed by electrochemical detection of the H_2O_2 formed (Stadler and Nesselhut, 1986) in a Biometra (Göttingen, FRG) electrochemical detector.

For isolation of vesicles from axons, three fishes were injected with [^{35}S]sulphate and all axons were cut out (~11 g) and crushed and extracted with 100 g electric organ from another (unlabelled) fish.

Gel electrophoresis and fluorography

^{35}S-labelled samples were dissolved in SDS-sample buffer (2% SDS, 0.1 M dithioerythritol, 0.1 M Tris–HCl pH 6.8 in 10% glycerol) without heating. SDS–PAGE was carried out using either a 3 or a 5% stacking and a 11% separation gel with 0.188 M Tris–glycine running buffer pH 8.7 adapted from Laemmli (1970).

Fluorography was performed using Enlightening (NEN) according to the manufacturer's procedure. Gels were dried and then exposed to Kodak X-Omat R-Film at −80°C. Densitometry was carried out using a Shimadzu Scanner and the areas under the peaks were quantitated using a planimeter. In all experiments stacking and running gels were scanned since a large part of the ^{35}S-labelled material was often present in the stacking gel.

Sephacryl chromatography of vesicles

The ^{35}S-labelled vesicle fractions from zonal centrifugation were pelleted, gently homogenized and chromatographed at 4°C on a column (89 × 1.5 cm) of Sepharcryl S 1000 (Pharmacia) in 0.1 M K-glutamate, 0.6 M glycine, 0.1 mM EGTA, 10 mM Tris–HCl pH 7.4. Fractions of 2.5 ml were collected. The column was calibrated with unlabelled vesicles from electric organ.

Sephacryl chromatography of vesicle extracts

Vesicle-containing fractions from zonal centrifugation were pooled, vesicles pelleted and the pellets suspended in 0.5 ml sample buffer (4 M guanidinium hydrocholoride, 2.0% CHAPS, 0.36 M β-mercaptoethanol, 50 mM Hepes pH 7.4) and chromatographed on a column of Sephacryl S 1000 (43 × 1 cm) in 4 M guanidium hydrochloride, 0.2% CHAPS, 50 mM Hepes pH 7.4. Radioactivity in the fractions was detected by liquid scintillation counting of aliquots. Immunoreactivity was detected using a spot assay (Stadler and Kiene, 1987) and an antiproteoglycan antiserum. Fractions were first desalted on a Sephadex G 25 column equilibrated with 0.2% NH_4HCO_3 and then concentrated by lyophilization.

Electron microscopy

Vesicle-containing fractions from zonal gradient centrifugation were pelleted, fixed and further processed as described by Zimmermann and Whittaker (1974). The diameter distribution was obtained from the electron micrographs as described in the same reference. About 400 vesicle profiles per fraction were analysed.

Acknowledgements

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