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Synaptic vesicles in electromotoneurones. II. Heterogeneity of populations is expressed in uptake properties; exocytosis and insertion of a core proteoglycan into the extracellular matrix

Herbert Stadler and Marie-Luise Kiene

Abteilung Neurochemie, Max-Planck-Institut für biophysikalische Chemie, D-3400 Göttingen, Postfach 2841, FRG

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The three populations of synaptic vesicles in electromotor nerve terminals were analysed quantitatively. Empty vesicles (VP_0), fully charged vesicles (VP_1) and charged but smaller VP_2-type vesicles are present in approximately equal amounts in the nerve terminal. The populations show differences in the kinetics of *in vitro* uptake of acetylcholine, ATP and Ca^{2+}. VP_0 and VP_2 accumulate acetylcholine and ATP but no Ca^{2+}, whereas VP_1 shows negligible acetylcholine and ATP but high Ca^{2+} uptake. Thus the expression of uptake properties of this secretory organelle depend on the stage it has reached in its life cycle and might constitute a signal for processing. VP_2 was found to contain much less core proteoglycan than VP_0 and VP_1 indicating that part of it has been lost by exocytosis. In synaptic extracellular matrix containing fractions an antigen is detectable that cross-reacts with an antiserum against the vesicle proteoglycan. This material elutes upon gel filtration in a position similar to a smaller form of proteoglycan found in vesicles. We conclude that the electromotor nerve terminal releases a proteoglycan by the regulated secretory pathway that is deposited in the extracellular matrix. It might have a function in keeping pre- and postsynaptic structures in alignment constituting a transsynaptic signal. Based on the findings described, a model of the vesicles' life-cycle is discussed, whereby the VP_2 population is the major source of quantal release of acetylcholine.

Key words: synaptic vesicles/electromotoneurones/proteoglycan

Introduction

Secretory organelles are often isolated as a single fraction by density gradient centrifugation or other purification steps and are subsequently treated as a single population although they may be heterogeneous with regard to their composition depending on their functional state (Zimmermann and Denston, 1977) and the stage they may have reached within their life cycle. In the case of the acetylcholine-storing synaptic vesicles from *Torpedo* electromotoneurones, the physical separation of the site of secretory activity and the site of biosynthesis of the granules and, in addition, the relatively slow time-course of axonal transport and nerve terminal maturation as compared to mammalian secretory systems, has allowed us to demonstrate that the well-known resting and reserve populations of vesicles are preceded by and derived from empty, immature vesicles supplied by axonal transport from the cell body (Kiene and Stadler, 1987). There are thus at least three populations of vesicles in the nerve terminal. We have now analysed these populations in more detail using vesicle markers independent of the classical markers acetylcholine and ATP whose concentration depends on the state the vesicles have reached in their life-cycle. The results of the analysis further establish the heterogeneous nature of the total vesicle population.

The isolated cholinergic vesicle from electromotoneurones has been shown to take up acetylcholine (Giompres and Luqmani, 1980; Anderson *et al.*, 1982), ATP (Luqmani, 1981) and Ca^{2+} (Israel *et al.*, 1980; Michaelson *et al.*, 1980) *in vitro*. These experiments were done with the total vesicle population and separation into subpopulations had not been carried out. We have assayed the subpopulations for *in vitro* uptake of acetylcholine, ATP and Ca^{2+} to see whether they differ in these characteristics. Furthermore, we have analysed the populations for their proteoglycan content to see whether this major core component is kept or lost during the individual stages of the vesicles' life-cycle. We have also analysed the electric organ extracellular matrix as a possible target structure for secreted proteoglycan. These results, and our previous ones (Kiene and Stadler, 1987), have led to a revised model of the vesicle dynamics in the nerve terminal.

Results

Quantitative estimation of vesicle populations

We have reinvestigated synaptic vesicles from *Torpedo* electric organ isolated as described by Tashiro and Stadler (1978) by using new vesicle markers differing from the classical markers acetylcholine and ATP used earlier. One of these is a recently described oligomycin- and ouabain-insensitive Mg-ATPase which is probably part of a vesicular proton pump (Harlos *et al.*, 1984). Another, a 34-kd protein, is the vesicle protein V11. This was detected by an antiserum that we have obtained against the highly

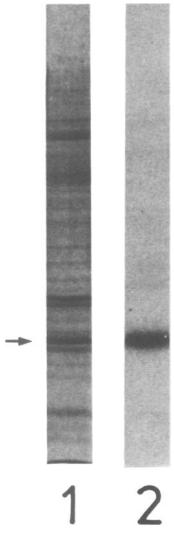

Fig. 1. Immune blotting of a vesicle preparation after SDS gel electrophoresis using antiserum against vesicle protein V11. **Lane 1**, Coomassie Brilliant Blue staining. **Lane 2**, immunoreactivity after transfer to nitrocellulose; autoradiography after incubation with ^{125}I-labelled second antibody. A 5% stacking and 11% running gel was used. Reactivity is seen with a single band at 34 kd corresponding to vesicle protein V11 (arrow), no staining with pre-immune serum was found (results not shown).

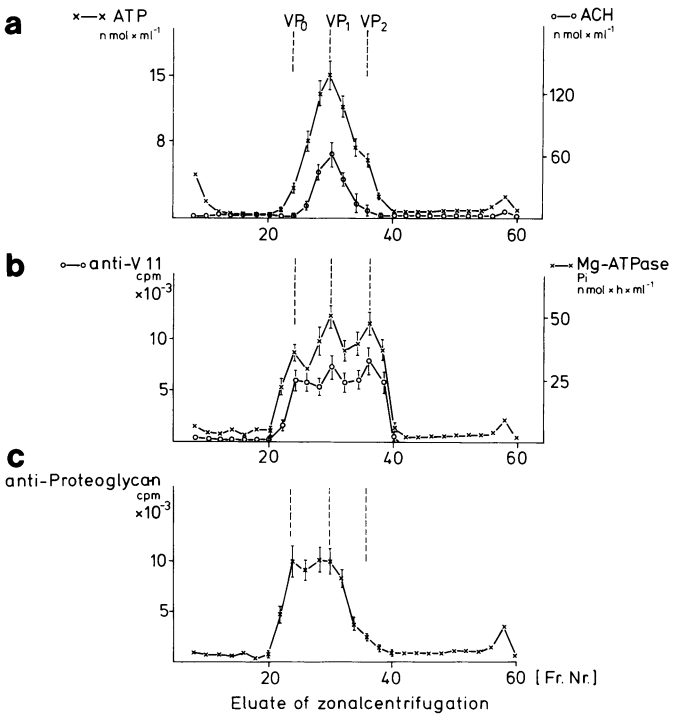

Fig. 2. Isolation of synaptic vesicles on a sucrose density gradient. Analysis of the fractions using markers for the vesicle membrane, a core proteoglycan and the classical markers acetylcholine and ATP. The results shown are averages of four different experiments (±SE). (**a**) Distribution of acetylcholine and ATP shows the typical major peak (VP_1 population) with a shoulder indicating a second population (VP_2). (**b**) Distribution of Mg-ATPase activity and vesicle membrane protein V11 obtained with a quantitative 'dot' immune assay. Beside VP_1 and VP_2 a third population is seen that is not detected with acetylcholine and ATP indicating the empty VP_0 population. Distribution of Mg-ATPase activity is practically identical to V11 and marks all three populations. (**c**) Distribution of the core proteoglycan obtained with a quantitative 'dot' immune assay using antiproteoglycan antiserum. The distribution in VP_0 and VP_1 is similar to the membrane markers V11 and the ATPase but strongly reduced in VP_2 indicating partial loss of the proteoglycan.

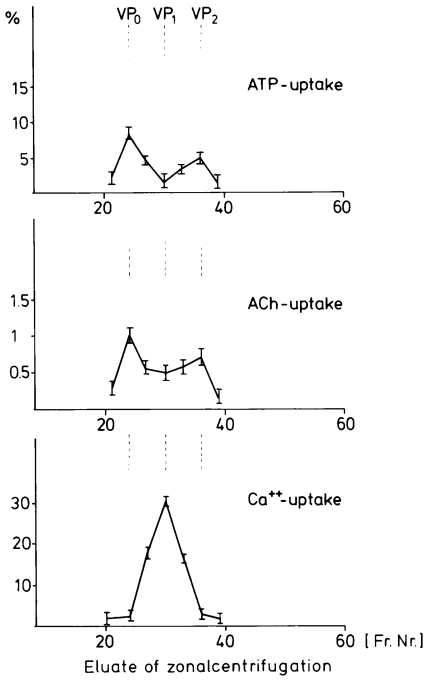

Fig. 3. *In vitro* uptake properties of vesicle populations VP_0, VP_1 and VP_2 towards radioactively labelled acetylcholine, ATP and Ca^{2+}. Uptake is expressed as radioactivity incorporated into vesicles as a percentage of total radioactivity in the assay. Values of each type of uptake experiment are expressed as averages (±SE) of three assays from three different vesicle isolation procedures by zonal centrifugation. Ordinate: fraction number from zonal centrifugation. The results show essentially that acetylcholine and ATP are taken up by VP_0 and VP_2 but not by VP_1, whereas Ca^{2+} is only taken up by VP_1.

purified protein. This protein is an integral vesicle membrane component and has been identified as an ATP carrier (Lee and Witzemann, 1983; Stadler and Fenwick, 1983). Figure 1 shows immune blotting of a vesicle preparation using the anti-V11 antiserum. A response (lane 2) is seen only with the V11 protein (arrow) indicating monospecificity. The third marker is the vesicular core constituent, heparansulphate proteoglycan. We have purified the proteoglycan by gel filtration of vesicle extracts in the presence of sodium dodecylsulphate and raised an antiserum against it. Immune blotting shown in Figure 4 suggests monospecificity of the antiserum, since the distribution of immune reactivity is similar to the one obtained by radioactive labelling of the proteoglycan (Stadler and Dowe, 1982). For both the anti-V11 and the anti-proteoglycan antiserum, estimation of antigen concentration in subcellular fractions was carried out using a 'spot assay' on nitrocellulose paper.

Analysis of vesicle preparations separated by density gradient centrifugation using the two antisera and Mg-ATPase activity is shown in Figure 2. V11 and the Mg-ATPase activity coincide and show a three-peak distribution with similar positions of peaks in the gradient as described in the [^{35}S]sulphate pulse-label experiments in Kiene and Stadler (1987). Although the populations are not fully separated and overlap to a certain extent, it can be estimated that the three populations are usually of roughly equal size, although considerable differences in population sizes existed in some fishes, especially with a tendency to low VP_0 (10–20%) and high VP_2 (40–50%).

Whereas the two membrane markers give a consistent three-peak distribution, the core marker, the proteoglycan clearly shows a different distribution. The VP_2 population is strongly reduced in proteoglycan content as compared to V11 and ATPase distribution (~80% ± 10% SE) indicating partial but not total loss of the core component in agreement with the qualitative findings in the [^{35}S]sulphate experiments which showed that VP_2 still contains radioactively labelled proteoglycan (Kiene and Stadler, 1987).

Furthermore, it is clear from Figure 2 that the classical vesicle markers acetylcholine and ATP do not coincide with the membrane markers. The VP_0 population obviously does not contain them, fully confirming previous results about the existence of an 'empty' axonally transported vesicle population that accumulates in the nerve terminal and is subsequently converted to the VP_1 population. Estimation of the acetylcholine and ATP content in VP_2 as compared to VP_1 suggests that they are lower by a factor of 5–10.

In vitro uptake studies

We have also tested whether the heterogeneity of the three vesicle populations is expressed at the level of acetylcholine, ATP and Ca^{2+} uptake. Acetylcholine uptake (Anderson *et al.*, 1982) showed a maximum in VP_0 and VP_2 and was negligible in VP_1 (Figure 3). Essentially the same result was obtained for ATP uptake (Luqmani, 1981); a minimum uptake was obtained with VP_1 and maxima in VP_0 and VP_2. Ca^{2+}, another storage product of these vesicles (Israel *et al.*, 1980) gave the opposite results

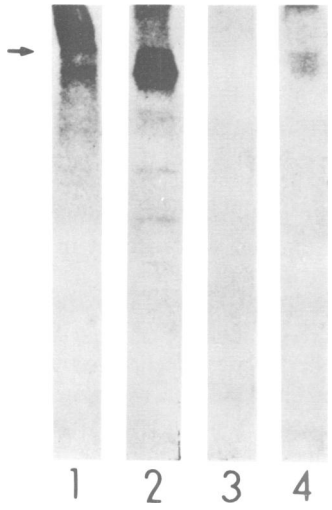

Fig. 4. Presence of synaptic vesicle proteoglycan antigen in fractions containing extracellular matrix from *Torpedo* electric organ demonstrated by immune blotting of electric organ fractions after SDS gel electrophoresis using antivesicle proteoglycan antiserum. **Lane 1**, synaptic vesicles (VP_0 + VP_1 + VP_2) show a smear in the stacking gel and on top of the running gel characteristic for the vesicle proteoglycan. **Lane 2**, extracellular matrix enriched fraction. The distribution of immunoreactivity is closely similar to vesicles but with more material in the running gel and some weak bands at lower mol. wt suggesting proteolytic cleavage of the proteoglycan. **Lane 3**, pellet of an extracellular matrix fraction after extraction with 1 M $MgCl_2$; no immunoreactivity is detectable. **Lane 4**, supernatant of extracellular matrix fraction after extraction with 1 M $MgCl_2$, immunoreactivity is similar to lane 2, indicating that the antigen can be removed at high ionic strength from the matrix.

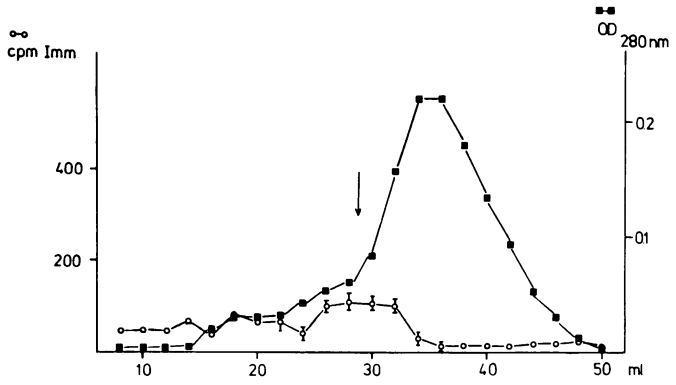

Fig. 5. Chromatography of electric organ extracellular matrix preparations on Sephacryl S 1000. An extracellular matrix preparation was isolated from the electric organ and chromatographed. The peak of immunoreactivity (cpm Imm) using antivesicle proteoglycan coincides with the small mol. wt form of proteoglycan found in vesicles the elution position of which (Kiene and Stadler, 1987) is indicated by an arrow.

in uptake studies. Negligible amounts were taken up in VP_0 and VP_2 and maximal uptake rates coincided with the VP_1 peak using the uptake procedure of Israel *et al.* (1980). These results cannot be explained in terms of varying amounts of vesicles present in the individual fractions: the stable vesicle markers V11 and ATPase showed that the three populations are of comparable magnitude.

Presence of proteoglycan in the extracellular matrix

The fact that VP_2-type vesicles contain much less proteoglycan than VP_0 and VP_1 suggests loss of part of the proteoglycan by exocytosis from VP_1 vesicles. We have therefore tested whether the proteoglycan can be identified in the extracellular matrix of the electric organ, a possible target structure. Preparation of an extracellular matrix fraction from electric organs (Godfrey *et al.*, 1984) involved repeated rigorous extraction by salt solutions of various concentrations including prolonged treatment with 3% Triton X-100, conditions known to solubilize synaptic vesicle proteoglycan (Stadler and Dowe, 1982). Nevertheless, the final fraction clearly cross-reacted upon immunoblotting with the antiproteoglycan antiserum (Figure 4).

Using the quantitative 'spot assay' it became clear that >60% of the vesicle proteoglycan antigen in the original organ homogenate is recovered in the matrix (but only 4–5% of the total protein), suggesting that in the organ of an adult fish a major portion of the vesicle antigen has been deposited in the extracellular matrix. Interestingly, the vesicle antigen was readily extracted from the matrix in the presence of 1 M $MgCl_2$ (Figure 4, lane 3) together with the high salt soluble or asymmetric forms of acetylcholinesterase (Witzemann, 1980) which are known to be associated with the basal lamina components.

At this stage it was essential to test the specificity of the antiproteoglycan antiserum to rule out unspecific cross-reactivity with non-vesicular heparansulphate proteoglycans, since these are common constituents of extracellular matrices (see Hassell *et al.*, 1986 for recent review). The antigen recognized by this antiserum showed a distribution confined to neuronal elements when this was investigated by immunofluorescence cytochemical staining of cryostat sections of electric organ (results not shown) and one identical to that of the vesicular proteoglycan when it was visualized by immunoblotting of electropherograms after SDS gel electrophoresis of vesicles (Figure 4). The antiserum did not react upon immunoblotting with *Torpedo* liver or kidney homogenates, again indicating neurone specificity; furthermore, no reaction was obtained with purified heparansulphate proteoglycan from Engelberth–Holm–Swarm sarcoma (Fujiwara *et al.*, 1984), suggesting specificity of the antiserum for the protein part of the proteoglycan (results not shown).

Further evidence in favour of the release of vesicular proteoglycan and its insertion into the electric organ extracellular matrix came from chromatography of $MgCl_2$ extracts on Sephacryl S 1000 in the presence of guanidinum hydrochloride and CHAPS as already applied to vesicles (Kiene and Stadler, 1987). The vesicle proteoglycan elutes essentially in two mol. wt forms. Figure 5 shows that the matrix proteoglycan immunoreactivity is present in a position co-eluting with the smaller mol. wt form of vesicle proteoglycan suggesting that this form is identical with the vesicular one. Probably it represents a proteolytic cleavage product of the originally intact membrane-bound vesicle proteoglycan.

Discussion

In full agreement with previous results obtained by *in vivo* pulse labelling of vesicles (Kiene and Stadler, 1987) subcellular fractionation of electric organ vesicles on a shallow density gradient shows that using two vesicle membrane markers three peaks are detected representing three vesicle populations. The populations differ in their acetylcholine and ATP content, in the amount of core proteoglycan present as well as in uptake properties. The difference in uptake properties towards acetylcholine and ATP may be an important regulatory step in the vesicle's life-cycle, the molecular basis of which remains to be elucidated.

In the case of Ca^{2+} uptake, calmodulin might be involved. A calmodulin-stimulated Ca^{2+} uptake system has been described

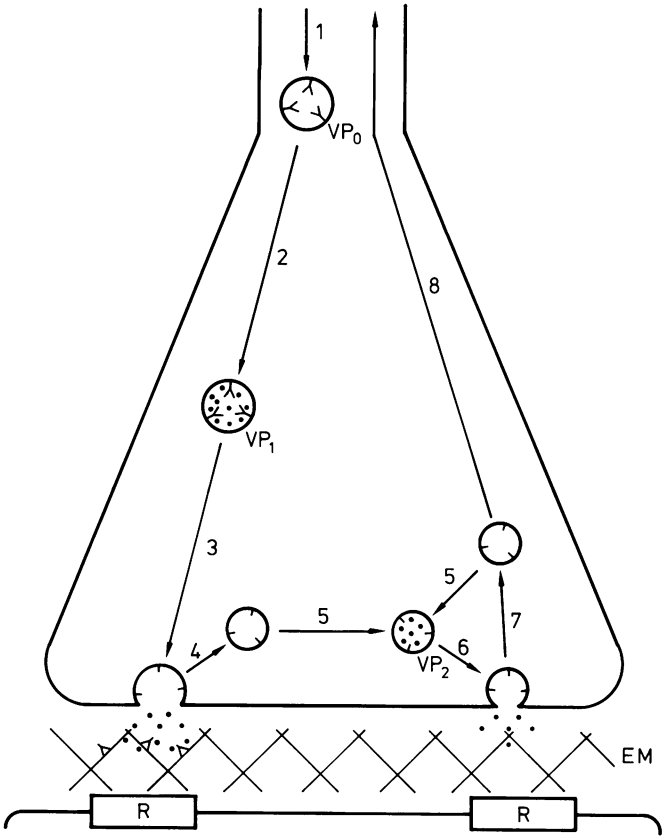

Fig. 6. A tentative model for the life-cycle of synaptic vesicles in the electromotor nerve terminal. The essential features of the model are based on the findings presented in this paper and the preceding one of Kiene and Stadler (1987) and include evidence accumulating in the last decade in favour of the vesicle hypothesis which suggests that acetylcholine release from neuromuscular junctions and electromotor nerve terminals is caused by exocytosis of vesicles (compare Zimmermann, 1979 and Torri-Tarelli *et al.*, 1985, for recent results). (**1**) 'Empty' vesicles (VP_0) arrive by fast axonal transport in the nerve terminal. (**2**) Accumulation of acetylcholine and ATP occurs and vesicles mature to the fully charged VP_1-type population. (**3**) In the vicinity of the presynaptic plasma membrane VP_1 may take up inflowing Ca^{2+} from depolarization events. Possibly Ca^{2+} is an internal signal to activate a protease that splits the core proteoglycan bringing the organelle into an exocytosis-competent status. Under the influence of Ca^{2+} and other factors VP_1 undergoes exocytosis, releases its secretory products—acetylcholine and ATP—together with a major part of the core proteoglycan. This core proteoglycan binds to the extracellular synaptic matrix and may play a role in synapse formation and maintenance. (**4, 5**) Endocytotsis of VP_1 leads to a VP_2 population smaller in diameter than VP_1 and recharged with acetylcholine and ATP. (**6, 7**) VP_2 undergoes further exo-endocytotic cycles and may constitute the major population actively involved in quantal transmitter release. (**8**) VP_2 might eventually be removed from the terminal by retrograde axonal transport and is degraded in the cell body. Y , represents proteoglycan: Y , proteoglycan portion released by exocytosis; VP_2, vesicle with residual membrane anchored proteoglycan; EM, extracellular synaptic matrix; R, acetylcholine receptor; ·, acetylcholine, ATP and Ca^{2+}.

(Rephaeli and Parsons, 1983) as well as calmodulin-binding proteins of the vesicle membrane (Hooper and Kelly, 1984; Walker *et al.*, 1984). Ca^{2+} uptake into VP_1 could constitute a signal for degradation of the proteoglycan by an internal protease to make VP_1 'exocytosis competent'.

The uptake studies fully confirm earlier reports on the presence of these uptake systems in vesicles, but they show in addition that they are a property only of a given population of vesicles, an aspect that should be taken into account in studying uptake properties in other secretory granules.

We show that VP_2 has lost part of its core proteoglycan. Since no other explanation than loss by exocytosis is plausible, VP_2 represents a population that has undergone exo-endocytosis. The large size of the VP_2 population and its relatively long half-life suggests that it is the major source of quantal acetylcholine release in this synapse. VP_2 with its 5–10 times smaller acetylcholine content as compared to VP_1 ($\sim 10^5$ molecules/vesicle; Ohsawa *et al.*, 1979) is in the range of 7000 acetylcholine molecules/quantum which have been observed recently in the *Torpedo* electric organ (Dunant and Muller, 1986).

The finding that VP_2 is labelled in its proteogylcan by the *in vivo* [^{35}S]sulphate technique (Kiene and Stadler, 1987) is not contradictory to the fact described here that VP_2 has lost most of its proteoglycan, since the label experiments can be interpreted only qualitatively and the label may be present in a membrane-bound domain of the proteoglycan, rather than being lost by exocytosis. A similar observation has been made in rat ovarian granulosa cells where a heparansulphate proteoglycan is exposed to the cell surface, partially lost and the remaining membrane bound part reinternalized (Yanagishita and Hascall, 1984). We have shown earlier that the proteoglycan is exposed to the synaptic cleft in the electric organ after electrical stimulation of the electromotoneurones (Jones *et al.*, 1982).

In addition to loss of proteoglycan from VP_2, we show that the electric organ extracellular matrix contains an antigen cross-reacting with antivesicle proteoglycan antiserum and similar in size to a lower mol. wt form of proteoglycan found in vesicles. Recently a glycoprotein of probable nerve terminal origin has been identified in electric organ extracellular matrix preparations. Although this protein shares an epitope with a monoclonal antibody directed against the synaptic vesicle proteoglycan it seems to be larger and differs in its subcellular distribution within the electric organ and in other physical parameters from the vesicle proteoglycan (Caroni *et al.*, 1985; Carlson *et al.*, 1986).

If the heparansulphate proteoglycan described by us is related to heparansulphate proteoglycans of basal laminae of cholinergic synapses reported to play a role in acetylcholine receptor clustering (Anderson and Fambrough, 1983) or acetylcholinesterase aggregation (Fallon *et al.*, 1985), a model of synapse formation and maintenance could be envisaged whereby the nerve terminal releases a proteoglycan by the regulated secretory pathway that acts as a trans-synaptic signal.

Altogether the pulse label and the uptake studies in conjunction with proteoglycan processing and release provide a first framework which allows us to reconstruct part of the life-cycle of the secretory organelle (Figure 6). It shows that synaptic vesicles in this system fall into the category of classical secretory organelles, like the chromaffin granules which upon stimulation release small mol. wt products together with core proteins. Part of the results have been presented previously (Stadler and Kiene, 1986).

Materials and methods

Subcellular fractionation

Isolation of synaptic vesicles from *Torpedo* electric organ was carried out on a shallow sucrose gradient in a zonal rotor as described by Tashiro and Stadler (1978). Distribution of markers or uptake properties through the eluate are expressed as averages (± SE) of several runs using different electric organ tissue. All runs are normalized to the fraction with maxium acetylcholine and ATP content which was reproducibly found at 0.42 M sucrose. Extracellular matrix was isolated from the electric organ according to Godfrey *et al.* (1984). The final preparation was suspended in 2.5 ml 1 M $MgCl_2$ overnight at 4°C then centrifuged in a Sorvall SS 34 rotor for 30 min at 12 000 r.p.m. An aliquot (300 μl) was mixed with 100 μl 4 M guanidinium hydrochloride, 2% CHAPS, 0.36 M β-mercaptoethanol, 50 mM Hepes pH 7.4 and applied to a Sephacryl S 1000

column (43 × 1 cm). Chromatography and determination of immunoreactivity were carred out as described by Kiene and Stadler (1987).

Antisera

Antiserum was raised in guinea-pig against vesicle protein V11 purified by two-dimensional gel electrophoresis as described by Stadler and Fenwick (1983).

An antiserum against the vesicle proteoglycan was obtained as follows: synaptic vesicle pellets containing VP_0-, VP_1- and VP_2-type vesicles were dissolved in SDS-sample buffer, centrifuged at 10 000 *g* for 10 min; the supernatant was applied to a column (44 × 1 cm) of Ultrogel AcA 34 (LKB) and eluted at room temperature with 50 mM NaCl, 0.2% SDS, 20 mM Tris pH 7.4. Fractions of 0.9 ml were collected. The column was calibrated with Dextran blue 2000, BSA and Bromophenol blue. The fraction in the void volume contains highly purified proteoglycan (Stadler and Dowe, 1982) which was used for raising an antiserum. A rabbit was injected at 14-day intervals four times with 100–200 μg of this material. For the first injection the proteoglycan was mixed with Freud's complete adjuvant, for the second, third and fourth, incomplete adjuvant was used. Serum was prepared two weeks after the rabbit had received the last injection.

In the pre-immune serum no immunoreactivity against the vesicle proteoglycan was detected using the tests described below. Immune blotting was carried out according to Newcombe *et al.* (1982) except that ^{125}I-labelled antirabbit IgG (Amersham-Buchler, FRG) from goat was used as second antibody.

Dot assay

Vesicles were pelleted from the eluate of zonal centrifugation and dissolved in 100 μl sample buffer (Kiene and Stadler, 1987). Aliquots (20 μl) were heated in a boiling water bath for 30 s and then cooled on ice and made 10% in trichloroacetic acid and kept on ice for 30 min. After centrifugation at 20 000 *g* for 10 min the pellets were dissolved in 10 μl 0.5 M sodium phosphate buffer pH 7.3 containing 0.2% SDS. Two microlitres of the solution were spotted on nitrocellulose paper (Biorad, FRG). After drying, the paper was suspended in 'dot buffer' (0.05 M Tris–HCl pH 7.5, 0.15 M NaCl, 5 mM EDTA, 0.25% gelatin, 0.05% Nonidet NP 40, 0.02% NaN_3) with the addition of 1% bovine serum albumin and 0.6% defatted milk powder for 30 min with shaking. The dot buffer was then replaced by dot buffer containing 0.1% SDS without albumin and milk powder but Nonidet concentration was raised to 0.5% and incubation of the paper was carried out for 1 h with eight buffer changes after which incubation was carried out with ordinary dot buffer for 30 min including three buffer changes. Then 2% antiserum was made in dot buffer and incubation carried out for 1 h to promote antibody binding. Washing with dot buffer containing 0.2% SDS followed for 30 min with two buffer changes, after which the procedure was repeated with dot buffer without SDS. Then second ^{125}I-labelled antibody (goat antirabbit IgG, Amersham, FRG, for the proteoglycan antiserum) was added. ^{125}I-labelling of the rabbit anti guinea-pig IgG (Dakopatts, Denmark) was carried out using Bolton–Hunter reagent (NEN, FRG). Incubation was carried out for 1 h using $1-2 \times 10^6$ c.p.m. per incubation, subsequently four wash steps for 10 min followed with dot buffer containing SDS, or further wash steps until the wash did not contain radioactivity. From the dried paper the dots were cut out and counted in a gamma counter. For every estimation two dots were made and the results expressed as the average. The proteoglycan and the V11 antisera gave a linear response against purified vesicles over a wide range of concentrations within which the assay was used.

Fractions from Sephacryl S 1000 chromatography were prepared for the spot assay as described by Kiene and Stadler (1987).

Gel electrophoresis was carried out as described by Kiene and Stadler (1987).

In vitro uptake studies

The eluate of the zonal rotor from vesicle isolations was analysed as pools of two 10-ml fractions. From the combined fractions, vesicles were pelleted by high-speed centrifugation (100 000 g_{av} overnight) and the pellets were suspended in the appropriate incubation buffers. In all kinds of uptake studies, three different experiments were undertaken and the averages (± SE) of the three experiments are shown.

Acetylcholine uptake was carried out according to Anderson and Parsons (1982) with a 30-min incubation for maximal uptake and a control experiment in the absence of ATP and carbonate. Ca^{2+} uptake was carried out according to Israel *et al.* (1980) for 4 min to obtain maximal uptake; the control experiment in the absence of ATP did not, however, show a signficant difference. ATP uptake was carried out according to Luqmani (1981) for 40 min to allow maximal uptake; the control was the blocking of uptake by atractyloside. In all cases the control experiments gave background levels in agreement with the published procedure. The only difference in the uptake experiments as compared to the published procedures was that uptake was stopped by gel filtration on Sephadex G 25 columns (9 × 1 cm) eluting at 4°C with incubation buffers not containing the substances to be taken up. Effective separation of free and vesicle-bound (void volume) radioactively labelled uptake substances was obtained within 2–3 min. Uptake was expressed as radioactivity in the void volume as a percentage of total radioactivity in the eluate of the Sephadex column.

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