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Synaptic vesicles contain an ATP-dependent proton pump and show 'knob-like' protrusions on their surface

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Synaptic vesicles from guinea pig brain were highly purified by chromatography on Sephacryl S 1000. They were associated with a Mg-ATPase which could be solubilized with dichloromethane from vesicle membranes, and which elutes upon gel filtration with a mol. wt. ~300 kd. Vesicles accumulate [^{14}C]methylamine in the presence of external ATP, indicating an ATP-dependent proton pump. Electron microscopy using a quick freeze, deep etch, rotary shadowing technique showed characteristic 'knob-like' protrusions on the surface of the vesicle. We suggest that these protrusions represent part of a proton ATPase which may be necessary for packaging of neurotransmitter into synaptic vesicles.

Key words: synaptic/vesicles/proton pump/ATPase

Introduction

Synaptic vesicles play a central role in neurotransmission as the transmitter storing and releasing compartment of the nerve terminal. Although much information has been obtained recently about the acetylcholine containing synaptic vesicles from the electric organ of *Torpedo marmorata* (Zechel and Stadler, 1982; Stadler and Dowe, 1982: Stadler and Fenwick, 1983; Lee and Witzemann, 1983; Walker *et al.*, 1984), much less is known about the constituents of synaptic vesicles from mammalian brain.

The cholinergic synaptic vesicle from *Torpedo* stores acetylcholine at an acidic pH (Stadler and Füldner, 1981; Füldner and Stadler, 1982) suggesting that a proton pump creates an electrochemical gradient across the vesicle membrane. A variety of other intracellular membrane-bound organelles, like secretory granules (for review, see Poisner and Trifaro, 1982), lysosomes (Schneider, 1981) and coated vesicles (Forgac *et al.*, 1983; Stone *et al.*, 1983) are also associated with ATP-driven proton pumps. We have now investigated whether synaptic vesicles of mammalian brain might contain a similar pump.

First we re-examined whether synaptic vesicles are associated with a Mg-ATPase as has been suggested previously (Hosie, 1965; Tsudzuki, 1979). Then we performed a functional test for the presence of a proton pump, and carried out a morphological examination to determine whether the characteristic structural protrusions, correlated with proton ATPases, are detectable. Parts of the results have been published in abstract form (Harlos and Stadler, 1983; Stadler and Lee, 1983).

Results

As seen in Figure 1, synaptic vesicles isolated according to the procedure of Whittaker *et al.* (1964) are enriched in a Mg-ATPase insensitive to ouabain. It is clear that 5'-nucleotidase activity, a marker for lysosomal and plasma membranes (Burnside and Schneider, 1982), and oligomycin-sensitive ATPase activity (a marker for mitochondrial membranes) are abundant in the regions of high sucrose density. However, relatively small residual activities of these enzymes are still present in the vesicle-containing fractions. We have purified these fractions further by using chromatography on Sephacryl S 1000. A typical run is shown in Figure 2. Vesicles elute as a single homogeneous peak, whereas the residual contaminating material, as indicated by the distribution of 5'-nucleotidase and oligomycin-sensitive ATPase, elutes in front of the vesicles. Acetylcholinesterase, a marker for plasma membranes in this tissue, shows a similar distribution. The acetylcholine containing fractions again coincide with a Mg-ATPase activity, now practically insensitive to oligomycin (inhibition <3%). The specific activity (per mg protein) of the ATPase in the vesicle fraction is enriched by a factor of 1.8 as compared with about a 1.6-fold enrichment in transmitter content (see Table I). The almost parallel enrichment of ATPase activity and transmitter content, and the absence of contaminating membrane fragments in the vesicle fraction, strongly suggest that a Mg-ATPase is associated with the vesicles. Thus chromatography on Sephacryl S 1000 is a simple and effective step for removing membraneous contaminants from brain synaptic vesicles. Gel electrophoresis of material in the first peak and in the vesicle peak (Figure 3) shows that although several co-migrating bands are detectable, the overall patterns are quite distinct, suggesting that the vesicles have a characteristic protein composition.

We have further characterized the ATPase using a procedure reported to be specific for the purification of mitochondrial F_1-ATPase (Beechey *et al.*, 1975). This procedure exploits the fact that reagents like dichloromethane split the F_1 part from the F_1F_0 proton ATPase complex, and has recently been successfully applied to chromaffin granules where an F_1-like ATPase' has been isolated (Apps and Schatz, 1979). Treatment of synaptic vesicle pellets with dichloromethane, released into the water phase an active ATPase with a mol. wt. ~300 000, as determined by gel filtration (see Figure 4). Recovery of ATPase activity after this treatment was around 41% (see Table I). Re-extraction of the material released a further 20% activity, suggesting incomplete release by a single-step extraction. Since contamination with oligomycin-sensitive Mg-ATPase in the parent vesicle fraction from chromatography is <3%, we can exclude the possibility that the solubilized ATPase is derived from mitochondrial contamination.

The results suggest that the ATPase present in vesicles resembles the mitochondrial enzyme in mol. wt. and solubilization properties, but differs in oligomycin sensitivity. A oubain- and oligomycin-insensitive Mg-ATPase might now be regarded as an 'enzyme marker' for brain synaptic vesicles.

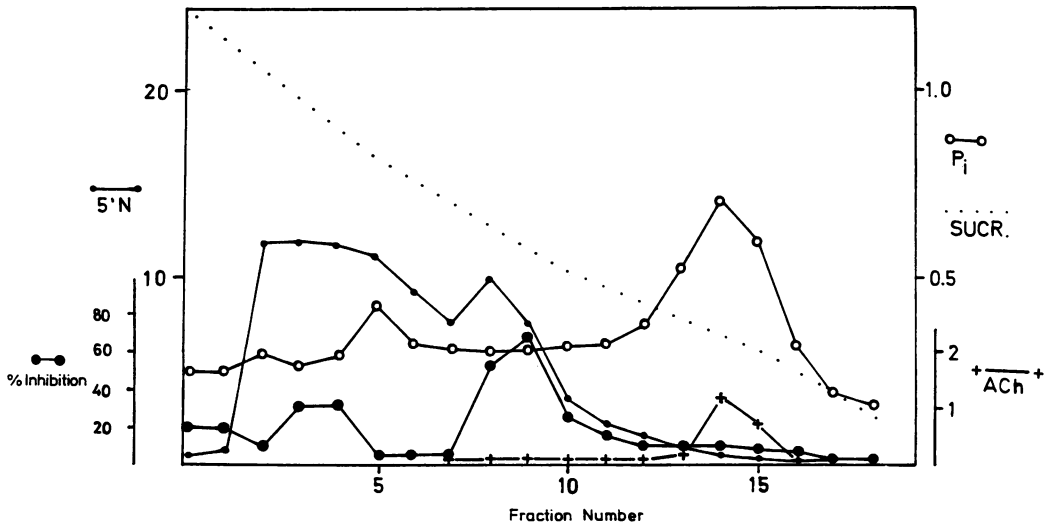

Fig. 1. Isolation of synaptic vesicles by sucrose density gradient centrifugation. Synaptic vesicles as monitored by their acetylcholine content coincide with a major Mg-ATPase peak. The Mg-ATPase was determined in the presence of ouabain and oligomycin. In addition inhibition of the ATPase with oligomycin was determined. It shows that the vesicle fraction contains ~12% oligomycin sensitive Mg-ATPase and trace amounts of 5'-nucleotidase indicating contamination of the vesicle fractions with mitochondrial and lysosomal membranes. These contaminants are removed by Sephacryl S 1000 chromatography (see Figure 2). P_i: Mg-ATPase activity in the presence of ouabain and oligomycin (μmol P_i/ml/h); % inhibition: inhibition of Mg-ATPase activity in the presence of ouabain (= 100% activity) by oligomycin; 5'N: 5'-nucleotidase activity (μmol/min/fraction); ACh: acetylcholine (nmol/ml); Sucr. : sucrose molarity; fraction volume 2 ml.

We examined proton pump activity in vesicles using the technique of [^{14}C]methylamine distribution in a manner similar to that applied to clathrin-coated vesicles (Forgac *et al.*, 1983). Vesicle pellets from Sephacryl chromatography were resuspended and incubated with [^{14}C]methylamine. Incorporation into vesicles was determined by passing the suspension over a column of Sephadex G25, and counting the radioactivity in the void volume. Although gel filtration is a relatively slow method to stop incubation, we assume that the potential error (loss of incorporated radioactivity during gel filtration) is not affected by the presence of ATP and that therefore the values determined are correct. Vesicles were incubated in [^{14}C]methylamine until no further uptake of radioactivity was detectable (zero time value, Figure 5). This occurred after <2 h. Upon addition of ATP to the incubation medium, ~4-fold accumulation of [^{14}C]methylamine into vesicles was observed after 10 min, indicating acidification of the vesicle interior (see Figure 5). Experiments were carried out at 4°C, since at room temperature uptake to saturation level occurred within 2 min. This effect was not found when the proton ionophore nigericin was added in addition to ATP. This result is not compatible with a Donnan equilibrium produced by an acidic buffering capacity of the vesicle core, which should be unaffected by a proton ionophore. Furthermore, N,N'-dicyclohexylcarbodiimide (DCCD), a well-known inhibitor of proton ATPases, blocked ATP-dependent uptake substantially. The results are therefore consistent with the presence of an ATP-dependent proton pump. Oligomycin and ouabain had no effect on ATP-dependent methylamine incorporation, underlining again the absence of mitochondrial and plasma membranes in the fraction. The effects of the various agents on ATP-dependent acidification are listed in Table II.

Although we may exclude submitochondrial particles and 'resealed' lysosomal membrane fragments because no 5'-nucleotidase was detectable in the vesicle fraction, the possibility still remained of contaminating coated vesicles. However, coated vesicles isolated from guinea pig brain elute

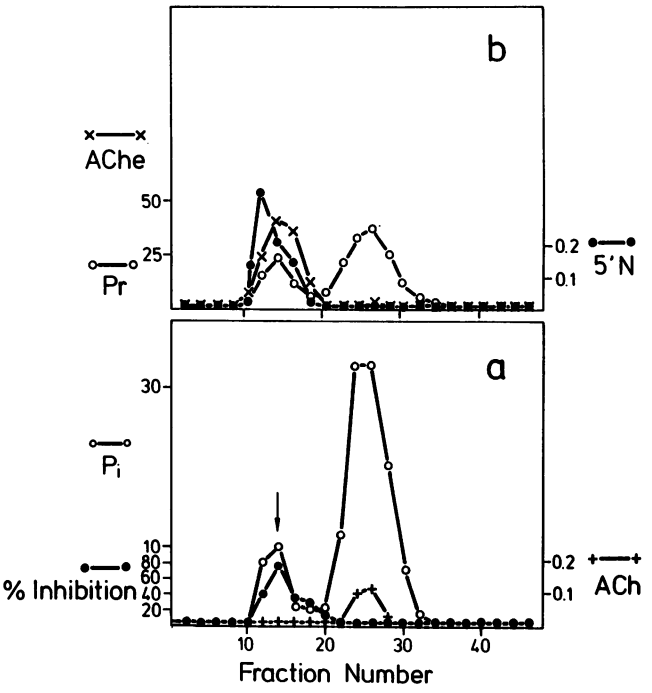

Fig. 2. Chromatography of synaptic vesicles on Sephacryl S 1000. (**a**) Distribution of Mg-ATPase (in the presence of ouabain and oligomycin) and acetylcholine. The vesicle marker acetycholine coincides with an Mg-ATPase insensitive to oligomycin (<3%). Residual amounts of oligomycin-sensitive ATPase from the starting material (compare Figure 1) elute in front of vesicles. P_i: Mg-ATPase activity in the presence of ouabain and oligomycin (nmol/ml/h). % Inhibitition: inhibition of Mg-ATPase activity in the presence of ouabain (= 100% activity) by oligomycin; ACh: acetylcholine (nmol/ml). Fraction volume 2 ml. (**b**) Distribution of protein, 5'-nucleotidase and acetylcholinesterase, a marker for plasma membranes. The enzymes are found exclusively in front of the vesicle peak indicating that vesicles are free of these contaminants. Coated vesicles isolated from guinea pig brain eluted as well in front of the vesicles (arrow). AChE: acetylcholinesterase (nmol/ml/min); Pr: protein (μg/fraction) 5' N: 5'-nucleotidase activity (μmol/min/fraction).

Table I. Activities of the Mg^{2+}-ATPase during purification

Fraction	Mg-ATPase	% Inhibition by oligomycin	Acetylcholine/ mg protein
V_B	25 ± 4	12 ± 3	5 ± 1
V_{Seph}	45 ± 7	< 3%	8 ± 2
CH_2Cl_2 solubilized	620 ± 71	–	–

Results are ± S.D. of three experiments and are given in nmol P_i/mg protein/min determined at 30°C in the presence of ouabain.
V_B: Vesicles from sucrose density gradient centrifugation (peak fractions)
V_{Seph}: Vesicles from Sephacryl chromatography (peak fractions)
CH_2Cl_2 solubilized: ATPase activity released from V_{Seph} after CH_2Cl_2 treatment, recovery is 41 ± 13%.

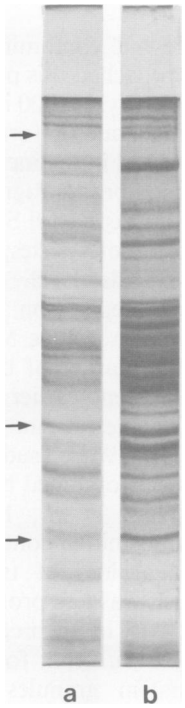

Fig. 3. SDS-gel electrophoresis of fractions from Sephacryl chromatography. Material from fractions 12–16 (**a**) and fractions 22–28 (**b**) (vesicle peak) were combined and electrophoresed in 11% polyacrylamide gels. Vesicles show a distinct protein composition with major bands enriched or absent in fractions 12–16 eluting in front of vesicles. Arrows point to molecular mass markers 200 kd (myosin), 42 kd (actin) and 29 kd (carbonic hydrase) from top to bottom.

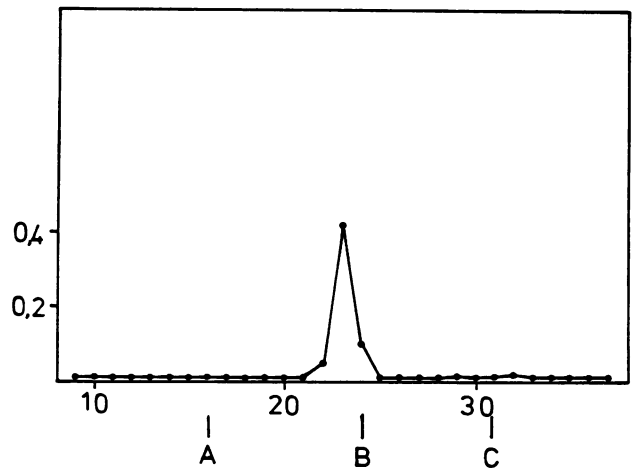

Fig. 4. Gel filtration of dichloromethane-solubilized Mg-ATPase on AcA 34. A single peak of Mg-ATPase activity elutes with a mol. wt. ~300 kd. Mol. wt. standards were ferritin (A), catalase (B) and aldolase (C). Protein was too low to detect. Fraction volume 2 ml.

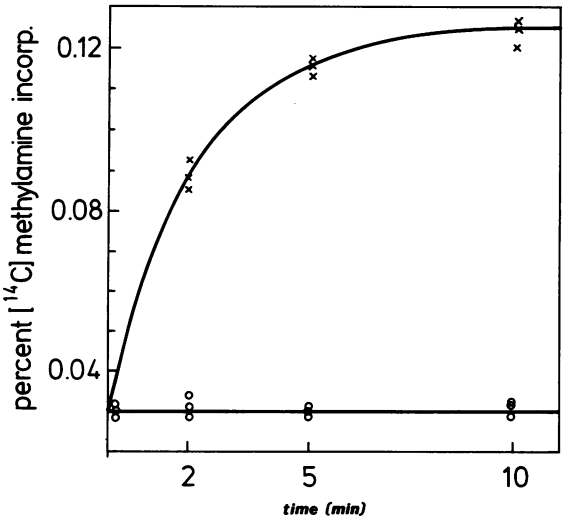

Fig. 5. Uptake of methylamine into isolated synaptic vesicles. Vesicles were first incubated for 2 h in [^{14}C]methylamine until equilibration occurred (zero time values). Then ATP and $MgCl_2$ (x-x) or $MgCl_2$ (O-O) alone were added and accumulation of the radioactivity into vesicles determined. Upon addition of ATP 4-fold accumulation after 10 min incubation time was observed. The ordinates are the percent of total radioactivity incorporated into vesicles.

upon Sephacryl chromatography in front of vesicles (arrow, Figure 2). Furthermore, the vesicle fraction is free of coated vesicles by morphological criteria and clathrin is not detectable by gel electrophoresis (results not shown). The possibility of a contamination with smooth vesicles derived from coated vesicles by 'stripping' off the coat in sucrose (Nandi *et al.*, 1982) is not easily discarded, since relatively high concentrations of sucrose are used during density gradient centrifugation of synaptic vesicles. We have therefore purified coated vesicles from equal amounts of guinea pig brain as used for synaptic vesicle isolation, removed the coat and carried out [^{14}C]methylamine uptake experiments similar to the ones described with these smooth vesicles. The fraction indeed showed ATP-dependent methylamine uptake. However, on a protein basis the amount observed could account for only 10–20% of the synaptic vesicle uptake. In addition the procedure used to isolate coated vesicles is designed to give optimal yield, whereas under the conditions used for synaptic vesicle isolation, yield of 'smooth vesicles' contaminating the final synaptic vesicle fraction might in fact be negligible. We suggest therefore that the proton pump activity observed in the synaptic vesicle fraction is not due to contaminating 'stripped off' coated vesicles.

The F_1-ATPase of mitochondria (e.g., that of submitochondrial particles) has been visualized by morphological studies (Fernandez-Moran *et al.*, 1964) and it has been suggested that characteristic protrusions on the surface of chromaffin granules (Schmidt *et al.*, 1982) might be the F_1-like ATPase described biochemically (Apps and Schatz, 1979).

We have examined deep-etched replicas of isolated and chemically fixed vesicles by electron microscopy. 'Knob-like'

Table II. Effect of various agents on ATP-dependent [^{14}C]methylamine uptake of synaptic vesicles

Condition	Ratio of trapping ± ATP
Standard	4.2 ± 0.1
Ouabain	4.1 ± 0.1
Nigericin	0.9 ± 0.2
Oligomycin	4.2 ± 0.2
DCCD	1.4 ± 0.2

Synaptic vesicles were equilibrated with [^{14}C]methylamine and uptake in the presence or absence of ATP and the additions indicated was measured (see Materials and methods). Values are expressed as the ratio of [^{14}C]methylamine trapped in the presence of ATP and absence of ATP after 10 min incubation time and represent ± S.D. of two experiments

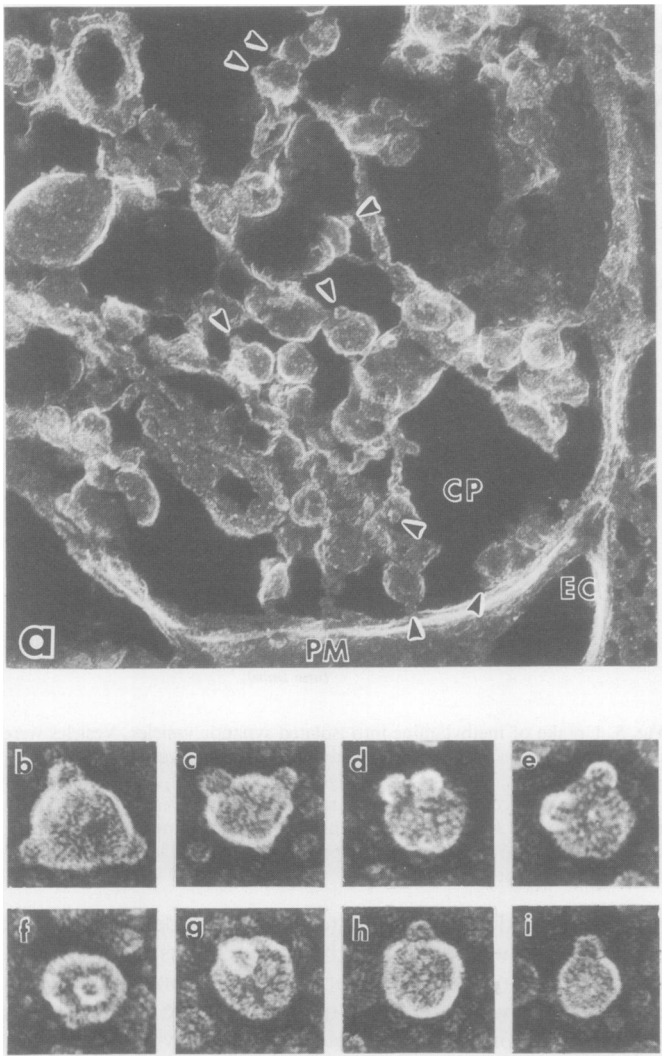

Fig. 6. **(a)** Electron micrograph of rapid-freeze deep-etched replica of unfixed synaptosome. The cytoplasm (CP) of the synaptosomes contained synaptic vesicle and interconnecting filamentous structures. The 'knob-like' protrusions (arrowheads) were observed on the surface of synaptic vesicles. PM: plasma membrane of the synaptosome; EC: extracellular space; x 120 000. **(b – i)** Electron micrographs of freeze-etch replicas of the chemically fixed synaptic vesicles. Most synaptic vesicles show one **(f – i)** or two **(d – e)** knob-like protrusions of ~15 nm in diameter on their surface. Occasionally, three **(b,c)** or four (not shown) extrusions are observed on one vesicle. Magnification x 165 000.

extrusions on the surface of vesicles (Figure 6b – i) can be clearly seen. They measure ~15 nm in diameter, although this is probably an overestimate due to platinum deposition. Most synaptic vesicles showed one or two protrusions but occasionally three or four were seen. The results are in agreement with earlier negative staining studies of Kadota and Kadota (1973) and Kanaseki (1979) which suggested that particles were associated with the vesicle surface. The presence of protrusions on the vesicle surface is further supported by examining synaptosomal cytoplasm in replicas of deep-etched material after rapid freezing without chemical fixation (Figure 6a). As with isolated and chemically fixed vesicles, protrusions can be recognized on the surface of the vesicles present in the cytoplasm, thereby excluding fixation artifacts.

Discussion

It was essential to rule out contaminations of our vesicle preparation, and we approached this problem by introducing chromatography on Sephacryl S 1000 in the purification procedure. The relatively small, and uniformly sized (40 – 50 nm), synaptic vesicles are retained in the included volume, and are separated from larger membrane fragments. The simple and rapid purification step on Sephacryl S 1000 should facilitate the further characterization of vesicles, needed to understand their function at the molecular level.

This highly purified preparation is associated with a ouabain- and oligomycin-insensitive Mg-ATPase which can serve as a useful enzyme marker of brain synaptic vesicles. The results confirm and extend earlier reports, obtained with less-pure preparations, suggesting an association of vesicles with Mg-ATPase (Hosie, 1965; Tsudsuki, 1979). The fact that in vesicle preparations obtained by chromatography on glass bead columns (Nagy *et al.*, 1976) the increases in ATPase activity and transmitter content were not parallel, probably reflects gradual loss of transmitter during the preparation; a loss which was less pronounced with our procedure. The ATPase activity in the preparation (2.9 μmol/mg protein/h) is comparable with that found in other secretory organelles like chromaffin granules (6.3) or cholinergic synaptic vesicles from *Torpedo* electric organ (6.0; compare Breer *et al.*, 1977).

We have demonstrated that synpatic vesicles from guinea pig brain show three essential features of protein ATPases: a Mg-ATPase that can be split off from the membrane, a mechanism of ATP-dependent acidification of the core and characteristic protrusions on the surface of the particle. These findings taken together strongly suggest that brain synaptic vesicles are equipped with a proton ATPase. It is probable that the 'knob-like' protrusions are the morphological equivalents of the ATP hydrolysing enzyme driving the proton pump. In any case the protrusions are characteristic features of the vesicles which might be helpful to identify them in future morphological studies concerning their axonal transport and recycling in the nerve terminal. The proton pump probably generates an electrochemical gradient across the vesicle membrane necessary for packaging of neurotransmitter. It is also conceivable that acidification is necessary for processing of neuropeptide precursors probably stored in vesicles together with a classical transmitter.

Materials and methods

Subcellular fractionation

All procedures including centrifugation were carried out at 4°C.

Synaptic vesicles were isolated from three guinea pig brain cortices as described by Whittaker *et al.* (1964), except that 10 mM Hepes pH 7.4 and 0.1 mM EGTA were included in all the solutions. The crude synaptosomal pellet was osmotically shocked by addition of 10 mM Tris-HCl buffer pH 7.4, 0.1 mM EGTA and centrifuged at 12 000 *g* for 30 min; the supernatant was subjected to sucrose density gradient centrifugation in a Beckman SW 28 rotor and the synaptic vesicle-containing fraction was directly chromatographed on a column (50 x 1.2 cm) of Sephacryl S 1000 (Pharmacia) in 60 mM KCl, 10 mM NaCl, 160 mM glycine, 0.1 mM EGTA, 10 mM Hepes pH 7.4. To avoid adsorption effects the column was first conditioned by passing the pellet fraction of the synaptosomal lysate suspended in elution buffer (20 mg protein/ml) through it. Recoveries for ATPase activities and ACh were between 80 and 95%.

Coated vesicles were isolated from three guinea pig brain cortices with the method described by Pearse (1982). Removal of the coats ('stripping') was carried out by incubating coated vesicles in 10 mM Tris-HCl pH 8.5 (E. Ungewickell, personal communication) and 'smooth vesicles' were collected by centrifugation (100 000 *g* for 3 h). Coated vesicles were detected in the eluate of Sephacryl chromatography by using absorption at 280 nm.

Purification of the vesicle ATPase

Vesicles obtained from Sephacryl chromatography (peak fractions, Figure 2) were centrifuged (100 000 *g* for 3 h), the pellet lysed in 10 mM Hepes pH 7.0 recentrifuged and treated with dichloromethane as described by Apps and Schatz (1979) for chromaffin granules. The water phase was chromatographed on a AcA 34 (LKB) column (95 x 1 cm) in buffers as described (Apps and Schatz, 1979) and the fractions assayed for ATPase activity. The column was calibrated with ferritin, catalase and aldolase. Fraction volumes were 2 ml.

Methylamine uptake

Vesicles isolated by chromatography on Sephacryl S 1000 (peak fractions, Figure 1), were pelleted by centrifugation (100 000 *g* for 3 h) and resuspended (1 mg protein/ml) in 60 mM KCl, 10 mM NaCl, 160 mM glycine, 10 mM Hepes pH 7.4, 0.1 mM EGTA. Vesicle suspensions were then incubated at 4°C with 10 μCi of [^{14}C]methylamine/ml (sp. act. 56 mCi/mmol, concentration 0.22 mmol). After 2 h, equilibration occurred as determined by checking methylamine incorporation at various times as described below. Then samples were made either in 1 mM ATP (sodium salt) and 2 mM $MgCl_2$, or in 2 mM $MgCl_2$ alone and the incubation at 4°C was stopped after various times by gel filtration on a 9 x 1 cm Sephadex G25 column and the amount of trapped [^{14}C]methylamine in the vesicle-containing void volume was determined. Vesicles eluted within 3 min from the column. Uptake of [^{14}C]methylamine was also determined in the presence of oligomycin (5 μg/ml) and nigericin (4 μM), ouabain (0.2 mM) and DCCD (0.15 mM).

Electron microscopy

Synaptosomes were isolated from guinea pig brain according to the method of Whittaker *et al.* (1964). The isolated synaptosomes were dialysed against phosphate-buffered saline (PBS) (145 mM NaCl, 2 mM KCl, 0.5 mM NaH_2PO_4, 10 mM Hepes pH 7.0) for 3 h at 4°C, and then centrifuged at 15 000 *g* for 40 min. The pellet was rapidly frozen, fractured, and deeply etched followed by rotary shadowing with platinum and carbon according to the method used by Tsukita *et al.* (1982, 1983). Isolated synaptic vesicles, from sucrose gradient centrifugation, were attached to polylysine-coated glass by placing a drop of synaptic vesicle suspension on the glass for 2–3 min at 4°C. Non-attached vesicles were discarded by washing the glass with PBS. Then, the synaptic vesicles were fixed with 2.5% glutaraldehyde in sodium cacodylate buffer (pH 7.4) for 30 min at 20°C and washed with PBS. After soaking in 50% methanol, the vesicles still on the glass were frozen by immersing in a slush of liquid nitrogen and freeze-dried *in vacuo* at 2 x 10^{-7} mm Hg followed by rotary shadowing with platinum and carbon. The replicas were floated off the glass in hydrofluoric acid and treated with sodium hypochlorite. After being washed with distilled water, the replicas were picked up on Formvar-filmed grids, and examined on a Hitachi 11-DS electron microscope.

Assays and gel electrophoresis

ATPase (at 30°C in the presence of 0.2 mM ouabain with 5 μg oligomycin/ml included or not), 5'-nucleotidase, acetylcholinesterase, protein assays and gel electrophoresis were done as described previously (Stadler and Tashiro, 1979). Acetylcholine was determined according to Whittaker and Barker (1972).

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References

Apps,D.K. and Schatz,G. (1979) *Eur. J. Biochem.*, **100**, 411.
Beechey,R.B., Hubbard,S.A., Linnett,P.E., Mitchell,A.B. and Munn,E.A. (1975) *Biochem. J.*, **148**, 53.
Breer,H., Morris,S.J. and Whittaker,V.P. (1977) *Eur. J. Biochem.*, **80**, 313.
Burnside,J. and Schneider,D.L. (1982) *Biochem. J.*, **204**, 525-534.
Fernandez-Moran,H., Oda,H.T., Blair,P.V. and Green,D.J. (1964) *J. Cell Biol.*, **22**, 63.
Forgac,M., Cantley,L., Wiedenmann,B., Altstiel,L. and Branton,D. (1983) *Proc. Natl. Acad. Sci. USA*, **80**, 1300.
Füldner,H.H. and Stadler,H. (1982) *Eur. J. Biochem.*, **121**, 519-524.
Harlos,P. and Stadler,H. (1983) *Hoppe-Seyler's Z. Physiol. Chem.*, **364**, 1139.
Hosie,R.J. (1965) *Biochem. J.*, **96**, 404.
Kadota,K. and Kadota,T. (1973) *J. Cell Biol.*, **58**, 135.
Kanaseki,T. (1979) in Otsuka,M. and Hall,Z.W. (eds.), *Neurobiology of Chemical Transmission*, J. Wiley Inc., p. 13.
Lee,D.A. and Witzemann,V. (1983) *Biochemistry (Wash.)*, **22**, 6123.
Nagy,A., Baker,R.R., Morris,S.J. and Whittaker,V.P. (1976) *Brain Res.* **109**, 285.
Nandi,P.K., Israel,G., Van Jaarsvield,P.P., Lippolt,R.E. and Edelhoch,H. (1982) *Proc. Natl. Acad. Sci. USA*, **79**, 5881.
Pearse,B.M.F. (1982) *Proc. Natl. Acad. Sci. USA*, **79**, 451.
Poisner,A.M. and Trifaro,J.M., eds. (1982) *The Secretory Granule*, published by Elsevier Biomedical Press, Amsterdam.
Schmidt,W., Winkler,H. and Plattner,H. (1982) *Eur. J. Cell Biol.*, **27**, 96.
Schneider,D.L. (1981) *J. Biol. Chem.*, **256**, 3858.
Stadler,H. and Tashiro,T. (1979) *Eur. J. Biochem.*, **101**, 171.
Stadler,H. and Füldner,H.H. (1981) *Biomed. Res.*, **2**, 673-676.
Stadler,H. and Dowe,G.H.C. (1982) *EMBO J.*, **1**, 1381.
Stadler,H. and Fenwick,E.M. (1983) *Eur. J. Biochem.*, **136**, 377.
Stadler,H. and Lee,D.A. (1983) *J. Neurochem.*, **41**, S35D.
Stone,D.K., Xie,X. and Racker,E. (1983) *J. Biol. Chem.*, **258**, 4059.
Tsudzuki,T. (1979) *J. Biochem.*, **85**, 567.
Tsukita,S., Usukura,J., Tsukita,S. and Ishikawa,H. (1982) *Neuroscience*, **7**, 2135.
Tsukita,S., Tsukita,S., Usukura,J. and Ishikawa,H. (1983) *J. Cell Biol.*, **96**, 1983.
Walker,J.H., Stadler,H. and Witzemann,V. (1984) *J. Neurochem.*, **42**, 314.
Whittaker,V.P., Michaelson,J.A. and Kirkland,R.J.A. (1964) *Biochem. J.*, **90**, 293.
Whittaker,V.P. and Barker,L.A. (1972) in Fried,R. (ed.), *Methods of Neurochemistry*, Vol. **2**, Marcel Dekker, NY, p. 1.
Zechel,K. and Stadler,H. (1982) *J. Neurochem.*, **39**, 788.

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