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ATP-Dependent Calcium Uptake by Cholinergic Synaptic Vesicles Isolated from *Torpedo* Electric Organ

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Summary. Cholinergic synaptic vesicles were purified from *Torpedo* electric organ to near morphological homogeneity. They were isolated in a K^+ environment. A method is described for the preparation of concentrated synaptic vesicles that allows uptake studies by conventional techniques. An ATP-Mg-dependent calcium uptake associated with synaptic vesicles is characterized. The uptake system transports calcium against a high concentration gradient. The maximum accumulation rate is obtained for the calcium, Mg^{++} and ATP concentrations likely to be found in the nerve terminal cytoplasm. It is suggested that synaptic vesicles are implicated in the removal of the calcium entering the nerve terminal during synaptic activity.

It is considered that intracellular calcium triggers the release of transmitter [23, 29]. An efficient buffering system must be present in the nerve terminal to clear the calcium entering in the course of synaptic activity. Such a role is usually attributed to the nerve-ending mitochondria. A direct demonstration of calcium accumulated into mitochondria of stimulated nerve terminals was indeed shown by Parducz and Joo [32]. In addition to the mitochondrial calcium uptake, there are vesicular structures within presynaptic nerve terminals which can accumulate calcium in the presence of ATP and Mg^{++} [3, 24, 34]. The structural localization of the nonmitochondrial Ca^{++} sequestration mechanism is still a matter of discussion. Blitz et al. [5] demonstrated an ATP-dependent calcium transport system in coated vesicles prepared from brain, resembling the sarcoplasmic reticulum ATPase. Several observations suggest that cholinergic synaptic vesicles are implicated in intracellular calcium regulation:

– Vesicular ACh is high in low calcium [1] and decreases after calcium addition [21, 28].

– Synaptic vesicles may be formed from the intraterminal smooth reticulum [12, 15], which is known to accumulate calcium [17].

– When fixed in the presence of calcium, synaptic vesicles exhibited a dense granule, generally considered to be a calcium precipitate [6, 14, 33, 42]. Finally, a Ca^{++} dependent ATPase is present in a purified fraction of synaptic vesicles [7].

It appeared to us useful to find out if cholinergic synaptic vesicles were able to take up calcium by an ATP-dependent mechanism. For this purpose, we have developed a method to prepare large amounts of concentrated synaptic vesicles from *Torpedo* electric organ. In the past, this cholinergic tissue led to the isolation pure synaptic vesicles [19, 20], but this procedure gave a rather diluted fraction in spite of its high ACh content. This difficulty was not overcome by the scaled-up method using zonal rotors developed by Whittaker et al. [41]. We present here a technique leading to a concentrated fraction of synaptic vesicles purified to near morphological homogeneity. The fraction is able to take up calcium by an ATP-Mg-dependent mechanism.

Materials and Methods

I. Preparation of a Concentrated Fraction of Cholinergic Synaptic Vesicles

Torpedo marmorata came from the Marine Station of Arcachon (France) and were kept in sea water for 1 or 2 weeks. The fishes were chilled on ice for 10 to 15 min. Slices of electric organ were grossly chopped. The mince was gently stirred (magnetic stirrer) in a physiological solution devoid of calcium. It consisted of (in mM: 280 NaCl, 3 KCl, 1.8 $MgCl_2$, 1.2 sodium phosphate buffer

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Table 1. Distribution of ATP-dependent calcium uptake

Fractions	ACh (nmol/g)	ATP (nmol/g)	Proteins (μg/g)	Acetylase (pmol × h^{-1} × g^{-1})	Calcium uptake		Calcium	
					Low calcium (pmol × min^{-1} × g^{-1})	High calcium (pmol × min^{-1} × g^{-1})	Total (nmol/g)	Bound (nmol/g)
1	0.1± 0.1	0.2±0.1	1145±135	697±73	0.5± 0.2	57±27	228±34	28±9
2	18.7± 3.2	3.3±0.7	1106±115	393±43	13.9± 6.3	135±50	164±34	24±6
3	74.7±12.8	19.9±4.3	191± 18	65±11	46.6±17.1	261±71	52±22	16±3
4	8.9± 1.3	4.0±0.7	87± 12	34± 8	26.9±13.3	107±63	29± 7	19±5
5	2.6± 0.8	1.0±0.2	59± 13	24± 7	17.7± 8.3	12±12	28±10	25±9
6	2.8± 0.5	0.9±0.1	248± 36	29± 8	30.7±11.2	92±80	29± 9	13±3
n	(10)	(10)	(10)	(7)	(5)	(3)	(8)	(3)
Recovery	(107%)	(85%)	(101%)	(95%)	ND	ND	(80%)	ND

The distribution of ATP-dependent Ca uptake was measured at low (5–7 μM) and high (50 μM) calcium. The peak of uptake is found, like the peak of ACh and ATP, in the vesicular fraction 3; choline acetylase follows the distribution of proteins: it is mainly soluble. The calcium content of the fractions shows that some soluble calcium is found in top fraction; dialysis permits us to estimate bound calcium. *n* = number of experiments. ND = recovery not determined. Amounts are expressed per g of initial tissue. SRA of ^{45}Ca was not corrected for total Ca content; uptake at low Ca would be × 2 in fraction 3 and × 1.2 in fraction 4.

(pH 6.8), 5.5 glucose, 300 urea, and 100 sucrose. When equilibrated with O_2, the addition of 4 to 5 mM $NaHCO_3$ permits us to adjust the pH at 7.0 to 7.2. In general, 20 to 30 g of minced tissue were washed in 200 to 300 ml of Ca^{++}-free solution which was renewed several times. The total washing procedure lasted 150 to 170 min at room temperature. It was essential to have a good washing in order to remove soluble proteins and calcium; this increases the amount of bound (vesicular) ACh. The physiological solution was then replaced by a "KCl medium" (350 mM KCl, 100 mM sucrose, 10 mM Tris buffer at pH 7.1). The homogenization (20 to 25% wt/vol) and all further steps were carried out at 5 °C. We used a glass-Teflon homgenizer (Potter-Elvehjem) turning at 1000 rpm (6 plus 3 strokes of the pestle separated by 1 to 2 min cooling of the homogenate). The homogenate was spun at 6500 rpm for 20 min in a SS34 rotor of a Sorvall RC2B centrifuge (5100 × g). The pellet was resuspended in 40 to 50 ml (final volume) of the KCl medium. It was frozen and powdered in liquid nitrogen in a porcelain mortar.

The fine powder can be stored for one or two days in plastic vials immersed in liquid nitrogen. The frozen powder was allowed to warm up in a Potter flask until it returned to its initial volume (40 to 50 ml). It now had the consistency of a melting sorbet. This sorbet was homogenized (8 strokes of the pestle); the pestle has to pass smoothly through the sorbet in order to minimize ACh losses. This homogenate was spun at 11,500 rpm (16,000 × *g*) for 30 min in the SS34 rotor. The supernatant was collected and recentrifuged 15 min at the same speed. This supernatant (S_2) was layered on discontinuous gradients (6 to 7 ml of S_2 per SW27 Beckman tube). The gradient consisted of 4 layers (7 ml each) of sucrose-KCl-mixtures buffered with 10 mM Tris (pH 7.1). The top layer consited of: 250 mM sucrose, 300 mM KCl; the second layer: 380 mM sucrose, 250 mM KCl; the third layer: 450 mM sucrose, 200 mM KCl; and the bottom layer: 550 mM sucrose, 150 mM KCl. The gradient was spun at 27,000 rpm for 150 min in the SW 27 rotor of a Beckman L5–65 centrifuge (95,400 × *g* av.). After centrifugation, readily visible bands were found at the first and second interfaces and a pellet was also present. The fractions were numbered from the top to the bottom: the supernatant is fraction 1; the upper band is fraction 2. At the interface 250–380 mM sucrose and penetrating slightly in the 380 mM sucrose layer, we find the second band (fraction 3), which is the synaptic vesicle fraction. Fractions 4 and 5 (interfaces 380–450 mM sucrose and 450–550 mM sucrose) are hardly visible. The pellet (fraction 6) was resuspended in part of the 550 mM sucrose layer. For the determinations of bound calcium, and in calcium uptake measurements of Fig. 8, it was necessary to dialyze the fractions. Five ml were dialyzed in cellulose bags (Nojax 16, Touzart & Matignon, France) against 500 ml of 400 mM KCl, 10 mM Tris buffer (pH 7.1). The bags were stirred for at least 150 min at 4 °C.

II. Biochemical Estimations

ACh was extracted by treating the fraction with 5% trichloracetic acid (TCA). The TCA was washed out with ether (saturated with H_2O) until the pH reached 4. The ACh was assayed using the leech dorsal muscle (method of Szerb [39] as in Israël et al. [20].

ATP was measured in the TCA-ether washed sample by the luciferine-luciferase method [37]. Aliquots of 50 to 100 μl of samples were diluted in 3 ml of 20 mM K phosphate buffer (pH 7.4) containing 4 mM $MgSO_4$. The luminescent reaction was triggered by adding 20 μl of firefly lantern extract (one vial FLE 50 Sigma was dissolved in 5 ml water). After rapid mixing, the luminescent reaction was read at its plateau phase in a dark chamber. A photomultiplier tube Hamamatsu R 374 was used.

Calcium was measured according to Johnson and Shimomura [22] using the luminescent reaction of aequorin with calcium. Aliquots (20 to 500 μl) of fractions were diluted in 4 ml of 200 mM Tris buffer (pH 7.2). The luminescent reaction was triggered by injecting aequorin into the tube through a rubber stopper. Aequorin (A 8513 type I) was purchased from Sigma and one vial was dissolved in 1 ml H_2O. A fivefold dilution in 200 mM Tris-buffer (pH 7.2) was made before the calcium assay, and 20 μl of diluted aequorin were sufficient for each determination. The luminescent reaction gave a flash which was recorded on an oscilloscope. The flash was integrated automatically for a 30-sec period as follows. The amplified signal of the photomultiplier modulated the pulse frequency delivered by a FG 501 Tektronix unit set at 100 Hz. As the amplitude of the flash increased, the pulse frequency increased. The total number of pulses (in 30 sec) was counted with a Tektronix DC 503. Each sample was determined using a standard curve (calcium concentrations ranging from 0.5 to 7.5 nmol calcium/ml). Total calcium was extracted in TCA before dialysis, bound calcium was estimated after dialysis or gel filtration through Sephadex G_{50}.

Choline acetyltransferase (E.C.2.3.1.6.) was measured by the acetylation of choline by labeled acetylCoA as described by Fonnum [13].

Proteins were determined according to Lowry et al. [26] and

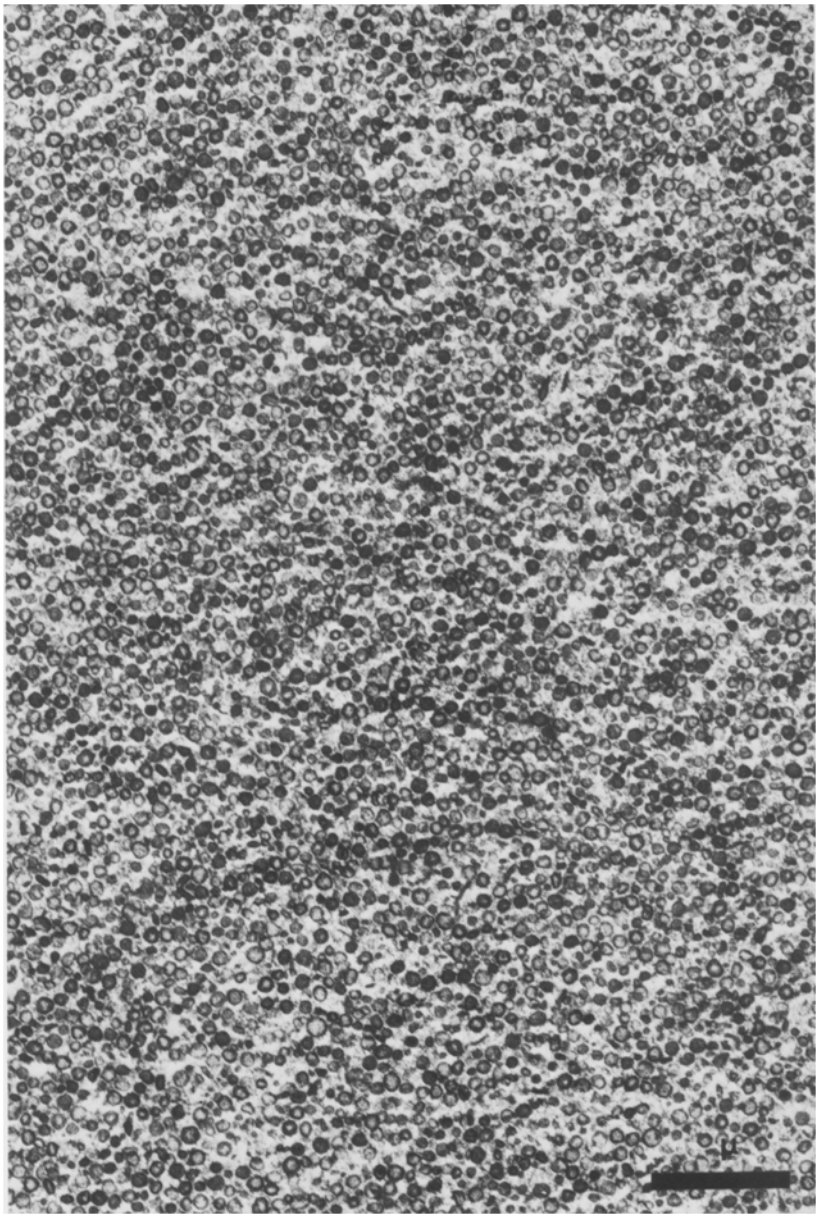

Fig. 1. This large field (bar = 1 μm) shows the morphological homogeneity of the synaptic vesicle fraction (fraction 3). Permanganate fixation method was used

by the Amido Schwarz technique developed by Schaffner and Weissmann [35].

The ATP-dependent calcium uptake was in general determined after Millipore filtration of the fractions. All solutions were made in 400 mM KCl, 10 mM Tris buffer (pH 7.1). The stock solutions of KCl and Tris were usually passed through a column of Chelex 100 (Biorad) to get rid of all traces of calcium. The optimum calcium uptake was obtained for 1 mM ATP, 2 mM $MgCl_2$ and 50 μM $CaCl_2$. The final concentration of ^{45}Calcium chloride (CES 3, The Radiochemical Centre, Ltd., Amersham, England) was 1.1 μCi/ml. The substrates were brought in 550 μl and the reaction was started by addition of 300 μl of fraction. Blanks were obtained by incubating each fraction without ATP. The incubation lasted 4 min unless otherwise stated. At the end of incubation, the whole volume (850 μl) was filtered through a 0.1 μm filter (VCWP 02500 Millipore SA, 67120 Molsheim, France) mounted in a Swinnex capsula (Swinnex 25, SX 02500, Millipore SA) under gentle suction. The filter was washed with 2 or 3 ml of 2 mM EGTA in 400 mM KCl, 10 mM Tris (pH 7.1). The filter surface (opposite the biological material) was blotted. The filter was then counted in a scintillation mixture.

After the whole filtration procedure we recovered 76% ± 3% (4 experiments) of the vesicular ATP and 65% ± 14% (3 experiments) of the vesicular ACh on the Millipore filter.

We also measured the ATP-dependent Ca^{++} uptake by 2 other methods: gel filtration and centrifugation. After incubation, with or without ATP, 0.5 ml of synaptic vesicles were passed through a 5-ml Sephadex (G50 coarse) column equilibrated with 400 mM KCl, 10 mM Tris buffer (pH 7.1). Synaptic vesicles were recovered in the void volume, and radioactivity was determined in 20 μl aliquots.

In the centrifugation method, we used a Beckman Airfuge rotor. The substrates (final concentrations: 1.6 mM ATP, 10 mM $MgCl_2$ and 7 μM $CaCl_2$) were introduced in 35 μl and the incubation was started by addition of 20 μl of concentrated synaptic vesicles (derived from 0.4 g of tissue). The reaction was stopped

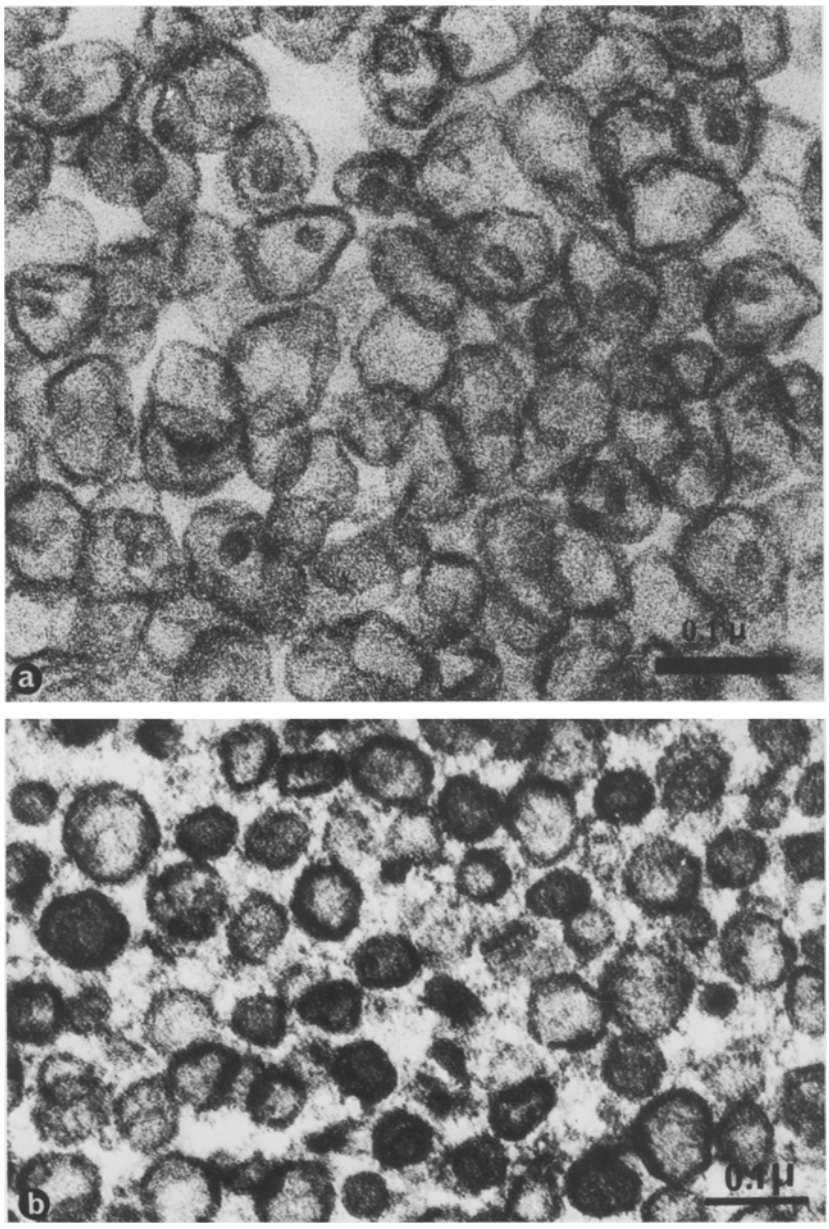

Fig. 2. High magnification of synaptic vesicles (fraction 3). (*a*): Vesicles were fixed in glutaraldehyde-osmium in the presence of 100 μM $CaCl_2$. Dense spots are seen in many vesicles. Bar = 0.1 μm. (*b*): Vesicles were fixed in permanganate. Bar = 0.1 μm

by addition of 120 μl of 2 mM EGTA in 350 mM KCl, 10 mM Tris buffer (pH 7.1). A pellet obtained at $160{,}000 \times g \times 30$ min, was washed with 100 μl of the EGTA solution and counted. Blanks were obtained in the absence of ATP.

Uptake data are given per g of initial tissue; they can be expressed per mg protein by using the estimation of proteins also given per g initial tissue.

Morphological Methods

Permanganate fixation: 7 ml of fraction 3 were fixed in 25 ml of a vol/vol mixture of 2% $KMnO_4$ and veronal acetate buffer (pH 7.4 to 7.7). This buffer contained 56 mM sodium veronal and 34 mM sodium acetate and its pH was adjusted with 0.1 N HCl. Isoosmolarity to *Torpedo* plasma was obtained by adding 250 mM KCl to all solutions. After 30 min at 0 °C, the precipitate was spun at $27{,}000 \times g$ for 30 min. The pellet was dehydrated in acetone, embedded in Spurr's medium [36], and sections were stained with lead citrate.

Glutaraldehyde fixation: 5 ml of fraction 3 were fixed with 40 ml of 2% glutaraldehyde in 350 mM Na cacodylate buffer (pH 7.4). After 45 min, the pellet was obtained by centrifugation ($48{,}000 \times g \times 60$ min). It was postfixed in 2% OsO_4 (in cacodylate buffer), dehydrated in ethanol, and embedded in Spurr's medium. Sections were stained with uranyl acetate and lead citrate. To get a calcium precipitate in synaptic vesicles, it is sufficient to add 100 μM $CaCl_2$ to all solutions.

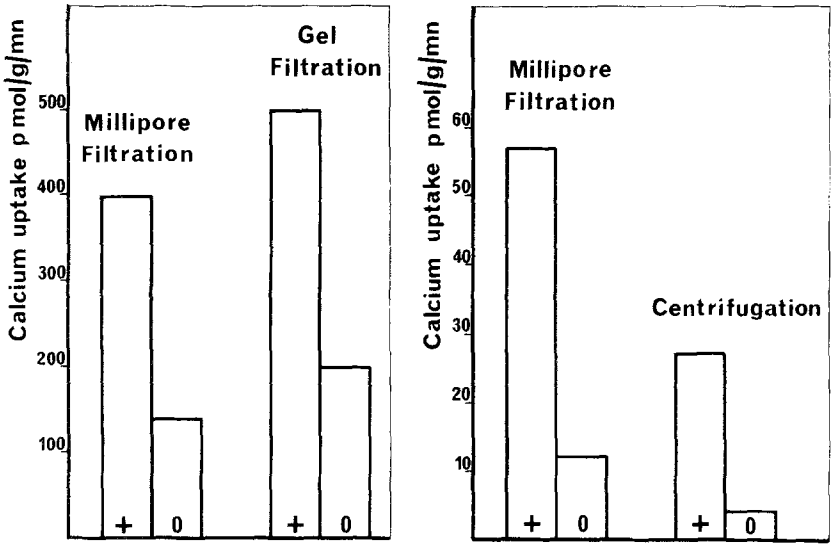

Fig. 3. Demonstration of the ATP-dependent calcium uptake by 3 different techniques. The calcium uptake in the presence (+) or in absence (o) of ATP is compared. *Left:* Millipore filtration was used to separate synaptic vesicles from the incubation medium. The uptake was performed at 50 μM calcium for 6 min. Gel filtration (Sephadex G50) was performed after 20 min incubation in 90 μM calcium. *Right:* Calcium uptake was studied at low (7 μM) calcium concentration. The incubation times were 10 and 3 min for the Millipore filtration and the centrifugation method, respectively. Data given per g initial tissue

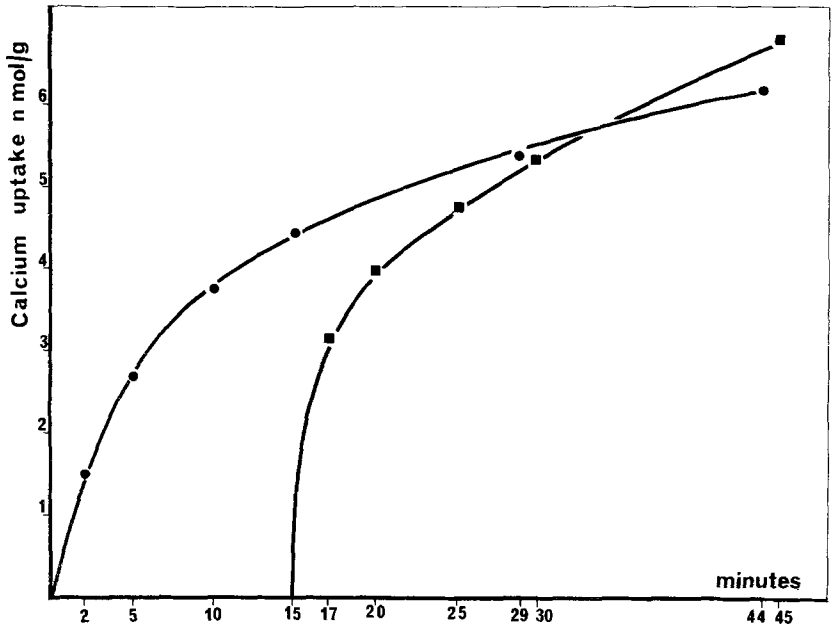

Fig. 4. Time course of ATP-dependent calcium uptake into synaptic vesicles. The incorporation of ^{45}Ca was started by adding the radioactivity either immediately or after 15 min of incubation in the presence of Ca (32 μM), ATP (0.8 mM) and Mg (2.5 mM). The initial rate was not maintained throughout the incubation. The accumulation of nonradioactive calcium did not reduce the initial rate. After 10 min, the accumulation of calcium continues at a slower rate. Data given per g initial tissue

Results

Characterization of the Synaptic Vesicles

The *isolation procedure* described here has 3 main characteristics. First, the electric tissue is washed in a calcium-free solution in order to remove extracellular calcium. This increases the bound vesicular ACh pool [1]. Second, we have fractionated the tissue in KCl sucrose media in order to keep the synaptic vesicle in a K^+ environment close to that of the intracellular medium. Third, the synaptic vesicles are extracted from a low speed pellet and therefore recovered in a volume smaller than in previous techniques which isolate the synaptic vesicles from dilute supernatants. The frozen pellet is powdered and can be stored in liquid nitrogen for several nights. The synaptic vesicles from about 30 g of electric organ can be purified in a single run. It is possible to recover up to 1 mg ACh in about 30 ml of fraction 3.

Table 1 shows that the ACh peak (fraction 3) is found at the top of the 380 mM sucrose layer rendered isoosmotic by KCl addition. Therfore the density of synaptic vesicles is similar to what was reported in the original procedure [20] where they were isolated from the supernatant in sucrose-NaCl media. The

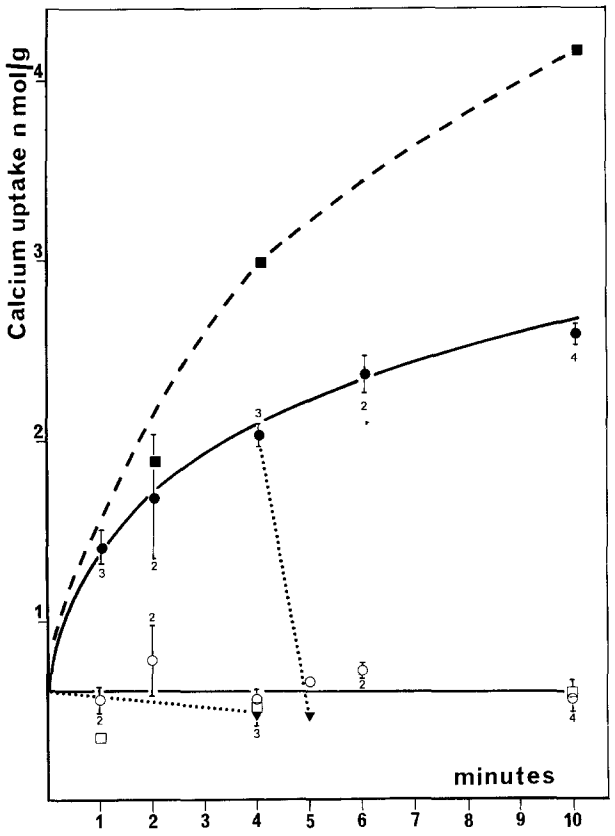

Fig. 5. Effect of oxalate and calcium ionophore (A23187) on calcium uptake. The ^{45}calcium uptake is shown in the presence (●, ■, ▼) or absence (○, □) of ATP. The time course of calcium uptake was determined in the standard conditions (*see* methods). Points (●) are mean ± SEM of the number of determinations indicated. When the same experiment is performed after preincubating the synaptic vesicles in 5 mM ammonium oxalate, for 2 hr at 0 °C, the ATP-dependent calcium uptake is increased (■). The blanks are not modified (□). When calcium ionophore (A23187) was added at 4.7 μM final concentration, at zero time or after 4 min, the calcium uptake was abolished or returned to background within 1 min (▼). Data given per g initial tissue

distribution of ATP follows that of ACh. The ratio ACh/ATP is 4.3 ± 0.3 (15 experiments), close to the value previously reported by Dowdall et al. [10]. The distribution of calcium in the fractions is shown in Table 1. Total calcium is estimated before dialysis of the different fractions, whereas bound calcium is the amount remaining after dialysis of the fractions. Comparison of these two distributions shows that the supernatant fractions contain some soluble calcium. The amount of calcium bound to the synaptic vesicles represents 16 nmol calcium per g initial tissue. The ratio ACh/Ca^{++} is of 4.5 ± 0.9 (4 experiments), the ratio ATP/Ca^{++} being close to 1. The peak of choline acetyltransferase is found in the supernatant (fraction 1 and part of fraction 2). Some of it (10% of supernatant) diffuses in the synaptic vesicle fraction (fraction 3). It can be completely removed from synaptic vesicles by gel filtration [27].

The distribution of proteins (Table 1) is similar to that of choline acetyltransferase, except for the pellet (fraction 6). This suggests that most of the proteins of fraction 3 are soluble proteins diffusing from upper layers. This leads to an underestimate of specific activity of ACh in fraction 3 (799 ± 242 nmol ACh/mg protein (15 experiments, Lowry method). We have found that improving the washing of the minced tissue, at the initial stage of the fractionation procedure, markedly increased ACh specific activity: 1948 ± 617 nmol, ACh/mg protein – Lowry method (4 experiments) or 1778 ± 247 nmol, ACh/mg protein – Amido Schwartz method (4 experiments). If the synaptic vesicles are refractionated, the ACh specific activity can still be increased as in other works [40, 8]. We have removed most of the soluble proteins by flotation on a continuous gradient (*see* below), the ACh specific activity reaching a value of 28,000 nmol of ACh/mg protein (Amido Schwartz method).

The morphological homogeneity of synaptic vesicle fraction is demonstrated in the large field presented in Fig. 1. The diameter of these vesicles shown at higher magnification in Fig. 2 is identical to that of synaptic vesicles *in situ* (about 80 nm). The permanganate fixation technique was used (Figs. 1 and 2*b*) because it traps in a gel all particles in suspension, the precipitate can then be spun at a relatively low speed of centrifugation [19]. No contamination by other particles could be detected at low or high magnification. Moreover, a high speed pellet of synaptic vesicles fixed in suspension with glutaraldehyde is shown (Fig. 2*a*). When calcium (100 μM) is present in the glutaraldehyde-osmium fixative (Fig. 2*a*), a dense spot appears within the isolated synaptic vesicles as it has been reported previously on the fraction or *in situ* [6, 14, 33]. No evident increase was noticed in the presence of ATP, oxalate, or both. These dense spots were not observed after permanganate fixation in the presence of calcium.

Uptake of Calcium Associated with the Synaptic Vesicle Fraction

The ATP-dependent uptake of calcium (Table 1) has been measured at low (5 μM) calcium concentration. The synaptic vesicle fraction exhibits a peak of activity. Some free calcium present in the supernatant (fractions 1 and 2), dilutes the radioactive calcium, leading one to underestimate calcium uptake in fractions 1 and 2. This was checked after dialysis of the

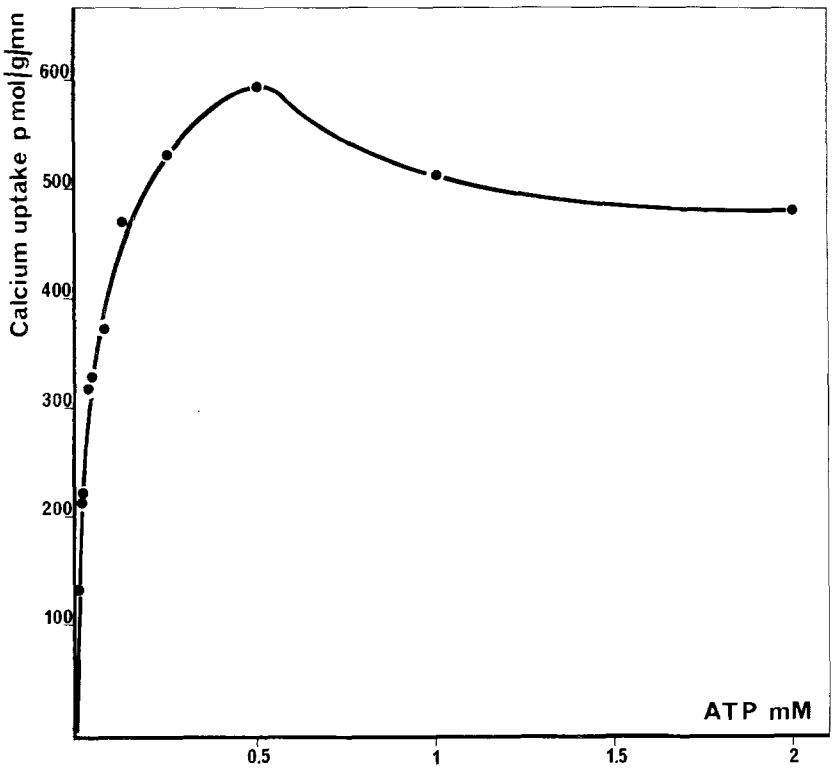

Fig. 6. ATP dependence of calcium uptake. The ATP-dependent calcium uptake was measured for ATP concentrations ranging from 0 to 2 mM. The Ca^{++} and Mg^{++} concentrations were kept constant at, respectively, 50 μM and 2 mM. The maximum uptake was obtained for 0.5 mM ATP. Data given per g initial tissue

fractions. The distribution of ATP-dependent calcium uptake was not modified except for fraction 1, where a fivefold increase was observed. When measured at high calcium concentration (50 μM) and after dialysis of the fractions, the distribution of calcium uptake was not very different from that obtained in low calcium. A tenfold increase in calcium concentration leads to a fivefold increase in calcium uptake in fraction 3. The distribution of ATP-dependent calcium uptake in the gradient shows a single peak coinciding with the peak of ACh and ATP in fraction 3. The ATP-dependent calcium uptake is maximum in the synaptic vesicle fraction in absolute amount and in specific activity (1.48 nmol/min per mg protein). This excludes the possibility that the ATP-dependent calcium uptake in fraction 3 originates from contaminants from neighboring fractions. On refractionation by flotation on a continuous sucrose gradient (Fig. 9*b*), the specific activity of ACh in fractions 11 and 12 is increased 15-fold. The ATP-dependent calcium uptake in the same fractions 11 and 12 is increased 24-fold, reaching a value of 40 nmol/min per mg protein. This shows that the ACh associated with synaptic vesicles and the ATP-dependent calcium uptake co-purify.

The diagram (Fig. 3) demonstrates calcium uptake by the synaptic vesicle fraction using different techniques. On the left, it was measured at high calcium concentration by Millipore filtration and after Sephadex G 50 filtration (*see* Methods). On the right, the calcium uptake was measured at low calcium concentration by the Millipore technique and by a centrifugation technique. In each of the different methods used, the presence of ATP results in a marked increase in the uptake of calcium.

The time course of the ATP-dependent calcium uptake by synaptic vesicles is shown in Figs. 4 and 5. The initial rate, measured at 1 min, is 1 nmol calcium/min per g of original tissue for a calcium concentration of 50 μM. The rate then gradually decreases to a slower but constant rate of about 100 pmol/min per g. This is not due to an exhaustion of ^{45}calcium since less than 5% of the calcium in the solution is taken up by the synaptic vesicles.

In order to decide if this decrease corresponds to a reduction of calcium entry in the synaptic vesicles or to a leakage of labeled calcium from the vesicles, we have in experiment Fig. 4 compared the entry of calcium at the initial time and after the synaptic vesicles have incorporated unlabeled calcium for 15 min. The initial rate was found higher after the preloading. The second curve catches up with the first one. This favors the existence of a leakage which does not balance the entry. This gives a two phase accumulation of calcium in the vesicle.

For lower calcium concentration (5–6 μM), calcium uptake was linear for at least 10 min. When the synaptic vesicles (Fig. 5) are preloaded with ammonium oxalate (5 mM), the ATP-dependent calcium

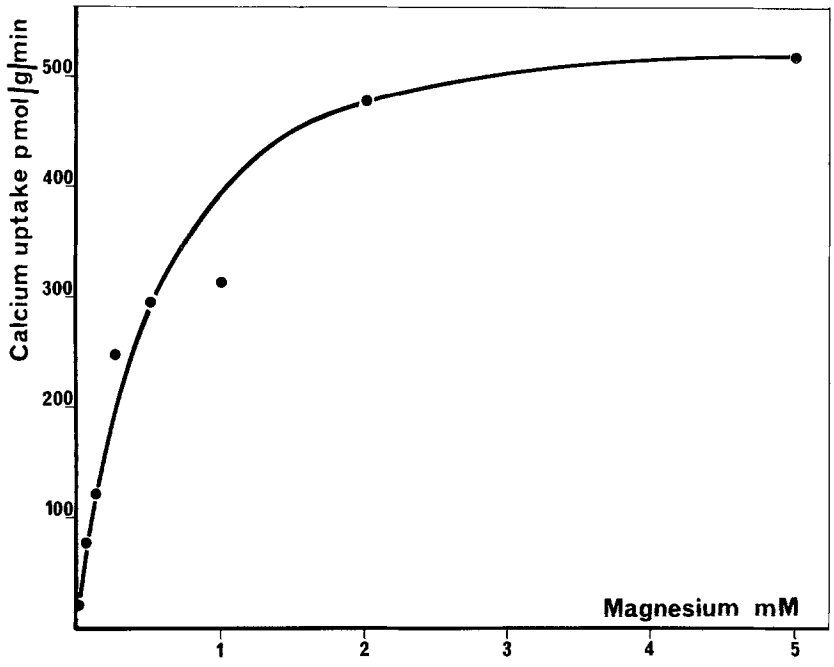

Fig. 7. Magnesium dependence of calcium uptake. The ATP-dependent calcium uptake was measured for a range of Mg^{++} concentrations from 0 to 5 mM. Ca^{++} and ATP were constant at, respectively, 50 μM and 1 mM. The ATP-dependent calcium uptake is strictly Mg^{++} dependent and maximum above 2 mM Mg^{++} Data given per g initial tissue

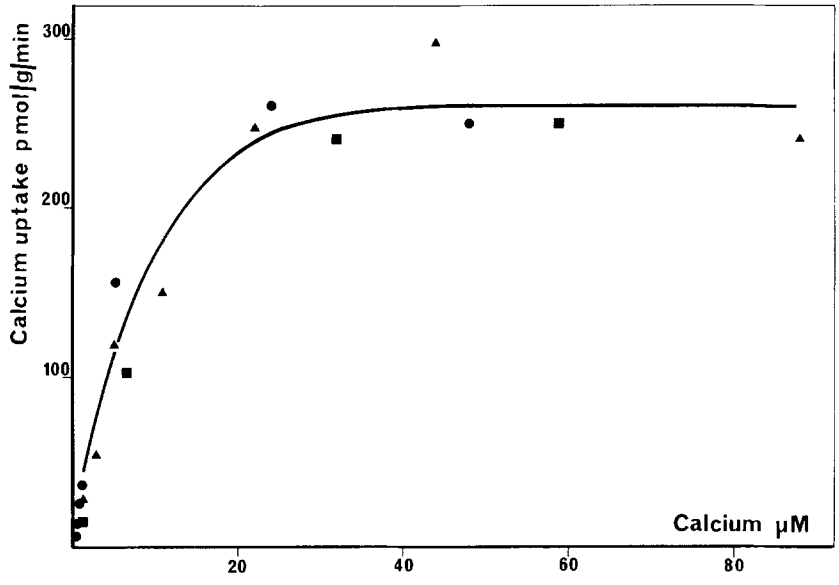

Fig. 8. ATP-dependent calcium uptake as a function of calcium concentration. The concentration of calcium ranged from 90×10^{-9} to 90×10^{-6} M. The uptake shows a clear saturation for calcium concentrations above 20 μM. Three independent experiments are plotted. A K_M value of about 5 μM can be determined. Data given per g initial tissue

uptake is greatly increased. More calcium can be accumulated (160%) within the synaptic vesicles in the presence of oxalate. On the contrary, in the presence of the calcium ionophore A23187, 4.7×10^{-6}M (gift of Dr. Brocklehurst, Eli Lilly, Windlesham, Surrey, England), the ATP-dependent calcium uptake is completely abolished. If this calcium ionophore is added to synaptic vesicles after 4 min of calcium incorporation, the incorporated calcium is lost within 1 min, whereas vesicular ACh and ATP are not modified. The effect of ATP concentration on calcium uptake is shown Fig. 6. The uptake increases as ATP is raised from 0 to 0.5 mM. A slight reduction occurs for higher ATP concentrations. The K_M value is about 30 μM. The effect of Mg^{++} concentration on the ATP-dependent calcium uptake is represented in Fig. 7. In the absence of Mg^{++}, there is no ATP-dependent calcium uptake. This uptake is maximum at about 2 mM $MgCl_2$. The K_M values is about 300 μM (316 ± 116 μM, 3 independent experiments).

In Fig. 8, the rate of ATP-dependent calcium uptake was measured for calcium concentrations ranging from 0 to 90 μM. The curve shows a saturation of the uptake process for calcium concentrations of

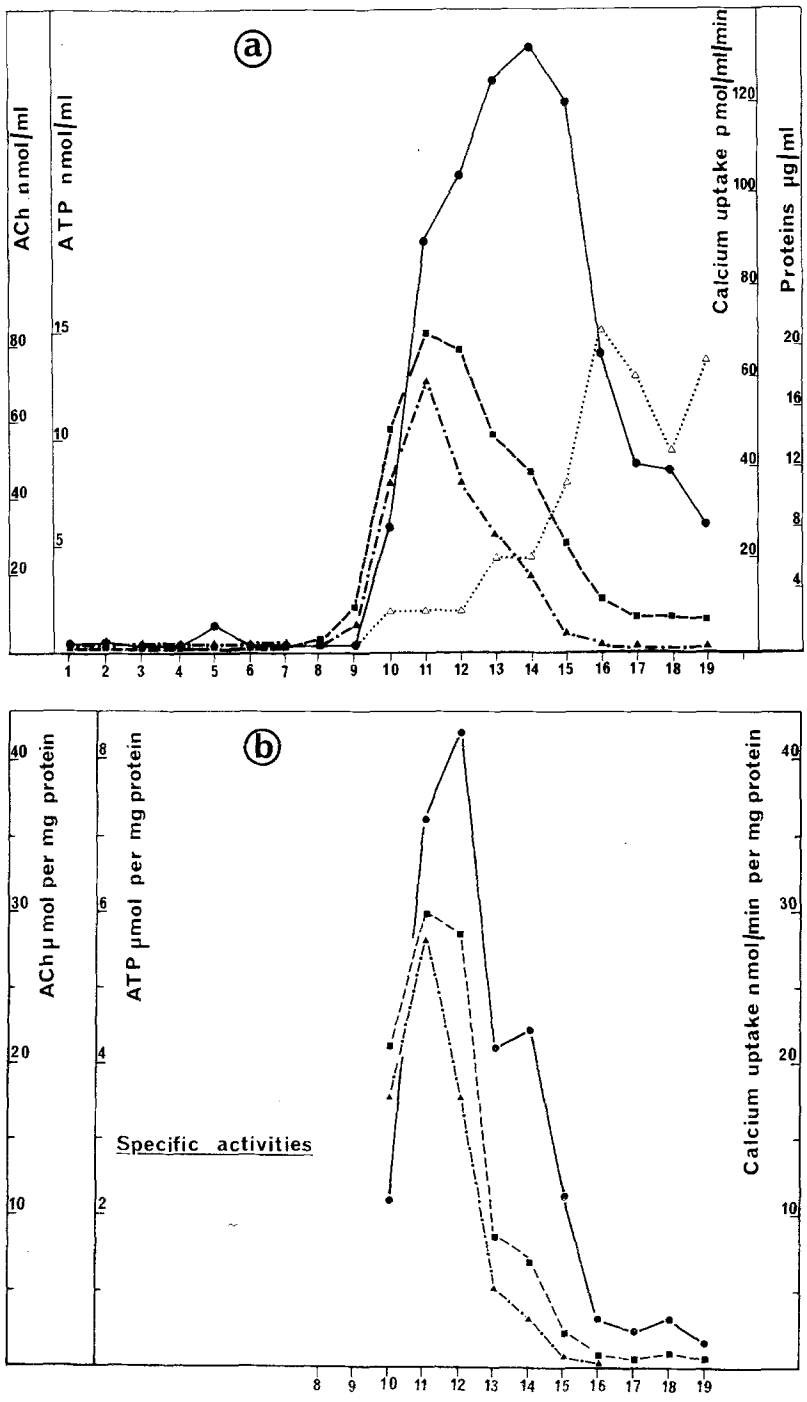

Fig. 9. Refractionation of synaptic vesicles by flotation. Synaptic vesicles (7 ml of fraction 3) are brought to 0.7 M sucrose by adding 3 ml of 2 M sucrose. This step was performed gradually, synaptic vesicles being kept at 0 °C. On top of 8 ml of these synaptic vesicles, a linear continuous gradient of 16 ml expands from 0.5 to 0.25 M sucrose. This gradient is made by mixing a dense 0.55 M sucrose solution in 0.15 M KCl with a 0.25 M sucrose solution in 0.3 M KCl. The pH was adjusted to 7.1 with 10 mM Tris-buffer. An overlayer of 10 ml of 0.15 M sucrose/0.35 M KCl is deposited. After 150 min centrifugation (27,000 rpm in a SW 27 Beckmann rotor) aliquots of 1.2 ml are collected from the top to the bottom of the tube and numbered from 2 to 18. The top and bottom fractions (1 and 19) are, respectively, 9.6 ml and 2.8 ml. ACh (▲), ATP (■), proteins (△), and the ATP-dependent calcium uptake (●) were determined in each fraction. (*a*): Plot of absolute amounts (per ml fraction). (*b*): Plot of specific activities (amounts per mg protein)

20 µM or above. The kinetic appears to be of the Michaelis-Menten type and a K_M value of 5 µM is determined. This value does not take into account a correction for non-ionized calcium. It would have given an about 1.5 times lower value as calculated from Kendrick et al. [25].

Refractionation of Synaptic Vesicles on a Continuous Gradient

In the experiment Fig. 9, isolated synaptic vesicles (fraction 3) were equilibrated in 0.7 M sucrose (by adding a small volume of 2 M sucrose) and refraction-

ated by flotation in a continuous density gradient expanded from 0.5 to 0.25 M sucrose. KCl was added to keep constant the osmolarity. Figure 9*a* gives raw data per ml fraction, while Fig. 9*b* compares the fractions as activities per mg protein. In fractions 11 and 12 at a density close to 0.42 M sucrose, the specific activity peaks for ACh, ATP and calcium uptake are recovered and coincide (Fig. 9*b*). When results are expressed per ml of fraction (Fig. 9*a*), tubes 11 and 12 correspond to the front shoulder of the ATP-dependent calcium uptake which, per ml, is maximum in tubes 14 and 15 at a density of about 0.5 M sucrose. We must notice that fraction 14 and 15, which have a lower specific activity for calcium uptake, correspond to small shoulders of ACh and ATP. In sum, the copurification of ATP-dependent calcium uptake of ACh and ATP (in fractions 11 and 12) does not contradict the observation that there are fractions of slightly heavier density (14 and 15) which take up calcium, but have a low ACh and ATP content. The significance of this will be discussed later.

Discussion

The fractionation procedures presented here enabled us to prepare pure synaptic vesicles from the cholinergic *Torpedo* electric organ. The morphological aspect of the fraction shows that the synaptic vesicles are not contaminated by larger membrane fragments. The ACh specific activity of the synaptic vesicle fraction is high. It could still be increased to very high values by removing the soluble proteins. The present procedure isolates synaptic vesicles in KCl solution, their intracellular environment. The extraction of synaptic vesicles from pellets, rather than from dilute supernatants, enabled us to prepare large amounts of concentrated synaptic vesicles. The overall yield of the isolation procedure was estimated by comparing the ACh content of synaptic vesicles with the homogenate. On the average (10 experiments) we recovered $12.3 \pm 1.0\%$ of the synaptic vesicles of 25 to 30 g of electric organ. This enabled us to study the uptake of calcium by synaptic vesicles with conventional techniques.

The experiments described show that we are dealing with an uptake process, transferring calcium within closed vesicles. Indeed the calcium remained in the vesicles after gel filtration, centrifugation, or Millipore separation. Washing the vesicles with EGTA did not remove the intravesicular calcium. Furthermore, as in the case of sarcoplasmic reticulum [16], the uptake was enhanced after preincubating the vesicles with oxalate, which is known to precipitate the calcium as it accumulated within the vesicles. Moreover, we noticed that the calcium ionophore A23187 (4.7×10^{-6} M) removed the calcium accumulated by the vesicles in less than 1 min.

The uptake of calcium reported here was fully dependent on the presence of ATP and magnesium. It was saturated for concentrations of calcium above 20 μM. This ATP-dependent calcium uptake might be linked to the calcium-magnesium stimulated ATPase described by Breer et al. [7] in highly purified synaptic vesicles. Since ATP-dependent calcium transport systems are present in all sorts of biological membranes, it was important to be sure that the calcium uptake measured was really a property of the synaptic vesicle membrane. The validity of the results presented, i.e., the ATP-dependent calcium uptake by ACh-containing synaptic vesicles, depends on how far we can trust the purity of the fraction. In the absence of known specific markers of the synaptic vesicles membrane, two criteria only can be used. The first one is a morphological analysis, and the second is evaluation of the specific activities of the two indentified substances present in synaptic vesicles (ACh and ATP).

The morphological criterion was tested by two different methods. One was the permanganate technique which trapped in a gel all the particles present, even in dilute suspensions. The particles trapped in the permanganate precipitate can be spun down at low centrifugal speed. Increasing the speed does not increase the precipitation of the particles. As shown in a previous work [19], synaptic vesicles which had not been sedimented at $100{,}000 \times g \times 60$ min in 0.32 M sucrose from a dilute supernatant containing ACh, were precipitated in the permanganate fixative at less than $10{,}000 \times g \times 20$ min.

The large field presented in Fig. 1 excludes membrane contaminants, unless they have the same size and shape as synaptic vesicles, which can be identified at higher magnification in Fig. 2*a* and *b*. In the second morphological method – i.e., the glutaraldehyde fixation – synaptic vesicles were diluted to lower the density in a calcium-containing fixative and spun at high speed ($48{,}000 \times g \times 60$ min). A dense spot can be visualized within the isolated synaptic vesicles. This is similar to what happens with synaptic vesicles in the nerve terminals *in situ* [6, 14, 33]. The calcium nature of these spots were strongly suggested by their disappearance after EGTA treatment [33]. Even if artificial, this method reveals a property of synaptic vesicles which might be related to the existence of some binding sites for calcium.

The biochemical criterion used to evaluate purity was to measure the specific activity of ACh and ATP in the process of purification. The discontinuous sucrose gradient shows a single peak of ACh and ATP

in fraction 3, in absolute amount and in specific activity. ATP-dependent calcium uptake shows the same single peak in fraction 3 in absolute amount and in specific activity. This excludes the possibility that the calcium uptake is due to contaminants from neighboring fractions. Refractionation of fraction 3 by flotation through a shallow continuous sucrose gradient was performed to eliminate soluble components and eventual contaminants which had not been detected by the morphological analysis. The results clearly show that the specific activity peak for calcium uptake coincides with the specific activity peak for ACh and ATP. This co-purification of the calcium uptake and of ACh after refractionation of synaptic vesicles strongly supports the idea that synaptic vesicles are able to take up calcium. We now have to discuss the observation that upon refractionation of synaptic vesicles we find fractions slightly heavier than the ACh peak which also exhibit a substantial calcium uptake. The morphological analysis alone certainly cannot exclude the possibility that they contain microsomal contaminants which have now been removed and separated from the real synaptic vesicles characterized by the specific activity peak for ACh, ATP, and calcium uptake. But one should notice that these heavier fractions still contain some ACh and ATP as indicated by the shoulders when the plots are expressed as amounts per ml. This, together with the high purity of fraction 3, would indicate that we have separated a population of vesicles with much less ACh and ATP but able to take up calcium. It is difficult to know if this heterogeneity reflects a differential depletion of part of the synaptic vesicles in the course of a long fractionation procedure or if it has a physiological significance. The same question was raised in other works. After measuring the specific radioactivity of ACh in isolated synaptic vesicles submitted to gel filtration, Marchbanks and Israël [27] concluded that synaptic vesicles were heterogeneous. The same conclusion was drawn when Dowdall et al. [10,11] studied the ACh/ATP ratio of subfractions of isolated synaptic vesicles. More recently, Zimmermann et al. [38, 43] have attempted to separate the main synaptic vesicular fraction from a smaller more dense population.

Evidently more work is necessary to test the significance of the observed heterogeneity. Presently we have only to consider that synaptic vesicles identified by their high ACh and ATP content have the highest specific activity for calcium uptake. This will now be discussed in relation to other works. First, within the synaptosomes there are vesicular structures other than mitochondria which possess an ATP-dependent calcium uptake system [3]. We were able to confirm these experiments on lysed *Torpedo* synaptosomes (*unpublished results*) in which mitochondria are very infrequent [31]. Furthermore, Rahamimoff and Abramovitz [34] have isolated from rat brain synaptosomes a vesicular membrane fraction able to concentrate calcium.

Second, membrane recycling during synaptic activity suggests that synaptic vesicles can be formed by endocytosis of the presynaptic membrane either directly [9, 43] or through cisternae and coated vesicles [18]. The presynaptic membrane contains a calcium extrusion mechanism (*see* review by Baker [2]) and highly purified coated vesicles transport calcium [5]. It has also been suggested that synaptic vesicles can be derived from smooth endplasmic reticulum [12], which is known to accumulate calcium [17]. All of these works suggest that the synaptic vesicles have kept the calcium transport system during their formation. The present work strongly supports this idea. It is likely that this ATP-dependent calcium uptake by synaptic vesicles operates within the nerve terminal since the ATP concentration in the cytoplasm is in the mM range [30] and magnesium is expected to be found as an intracellular cation. The cytoplamic calcium concentration is normally low (of the order of 10^{-7} M), but during synaptic activity there is an increase of intracellular calcium which triggers ACh release [23, 29]. Efficient buffering systems must exist to clear this calcium and switch off ACh release. Synaptic vesicles, especially those close to the presynaptic membrane, might participate in the calcium removal from the cytoplasm. The synaptic vesicles can accumulate up to 6.5 nmol. calcium per g tissue (Fig. 4). Since we isolated 12% of the synaptic vesicles and since synaptic vesicles occupy 1% of the total tissue volume (*unpublished* morphometric data), we can estimate that calcium concentration within synaptic vesicles would be at least 5.4 mM. If a similar calculation is done, assuming that all the bound calcium of fraction 3 is in the synaptic vesicles, the intravesicular calcium concentration would be 14 mM. This demonstrates that synaptic vesicles are able to transport calcium against a high concentration gradient.

It is important to find methods that can tell us if the synaptic vesicles have different ACh, ATP, and calcium contents during their life span and how calcium is cleared out from the nerve terminal.

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