Biogenesis of Synaptic Vesicles In Vitro

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Abstract. Synaptic vesicles are synthesized at a rapid rate in nerve terminals to compensate for their rapid loss during neurotransmitter release. Their biogenesis involves endocytosis of synaptic vesicle membrane proteins from the plasma membrane and requires two steps, the segregation of synaptic vesicle membrane proteins from other cellular proteins, and the packaging of those unique proteins into vesicles of the correct size. By labeling an epitope-tagged variant of a synaptic vesicle protein, VAMP (synaptobrevin), at the cell surface of the neuroendocrine cell line PC12, synaptic vesicle biogenesis could be followed with considerable precision, quantitatively and kinetically. Epitope-tagged VAMP was recovered in synaptic vesicles within a few minutes of leaving the cell surface. More efficient targeting was obtained by using the VAMP mutant, del 61-70. Synaptic vesicles did not form at 15°C although endocytosis still occurred. Synaptic vesicles could be generated in vitro from a homogenate of cells labeled at 15°C. The newly formed vesicles are identical to those formed in vivo in their sedimentation characteristics, the presence of the synaptic vesicle protein synaptophysin, and the absence of detectable transferrin receptor. Brain, but not fibroblast cytosol, allows vesicles of the correct size to form. Vesicle formation is time and temperature-dependent, requires ATP, is calcium independent, and is inhibited by GTP-γS. Thus, two key steps in synaptic vesicle biogenesis have been reconstituted in vitro, allowing direct analysis of the proteins involved.

THE neuroendocrine pheochromocytoma cell line PC12 has proven useful for the study of synaptic vesicle biogenesis. Much of what we know about synaptic vesicle biogenesis has been gleaned from studying either primary cultures of dissociated hippocampal cells or PC12 cells, which behave in many ways like immature neurons. PC12 cells synthesize vesicles which contain the known synaptic vesicle proteins, have the physical properties of rat brain synaptic vesicles (Clift-O'Grady et al., 1990) and exclude markers of the endocytotic (Linstedt and Kelly, 1991; Cameron et al., 1991) and transcytotic (Bonzelius et al., 1994) pathways. The sorting characteristics found in PC12 cells are similar to those found in primary neurons (Mundigl et al., 1993).

How and where the synaptic vesicle membrane proteins are sequestered away from these other markers is not yet clear (Mundigl and De Camilli, 1994). In one hypothesis, the kiss-and-run model, synaptic vesicle membranes do not fuse with presynaptic plasma membrane, but instead release their neurotransmitter contents via a small channel. In a second, synaptic vesicles arise directly from the plasma membrane either via a clathrin-coated pit or from a large invagination. A third hypothesis postulates that synaptic vesicle biogenesis occurs in two stages, initially by endocytosis from the cell surface to endosomes and subsequently by budding from the endosome.

One way to understand how synaptic vesicle proteins are sorted is to identify a sorting domain. The first example of this strategy is a recent analysis of the synaptic vesicle protein VAMP, also called synaptobrevin. A sorting domain has been identified in VAMP that regulates synaptic vesicle targeting in PC12 cells (Grote et al., 1995), mutations in which can either inhibit or enhance targeting. Targeting was assayed via an epitope tag attached to the lumenal carboxy terminus of the VAMP molecule. Radioactive antibodies to this epitope were internalized by transfected PC12 cells and delivered to synaptic vesicles.

To discover molecules involved in recognizing synaptic vesicle membrane proteins and sorting them to vesicles of the correct size and composition, we have developed a procedure that reconstitutes synaptic vesicle biogenesis in vitro from homogenates of PC12 cells. The assay became possible due to some observations made in vivo. The first is the ability to measure de novo synaptic vesicle biogenesis using VAMP with an epitope tag on its lumenal domain (Grote et al., 1995). Secondly, the fraction of surface labeled VAMP recovered in synaptic vesicles can be increased about ten fold by using a deletion mutant of VAMP (VAMP del 61-70) that is more efficiently targeted to synaptic vesicles. Finally, when PC12 cells transfected with VAMP-TAg del 61-70, are labeled at 15°C, antibody

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is taken up by the PC12 cells but is not targeted to synaptic vesicles.

Materials and Methods

Materials

[^{125}I] and ECL reagents were obtained from Amersham Corp. (Arlington Heights, IL) and Iodogen from Pierce (Rockford, IL). ATP, GTPγS, creatine phosphate, creatine kinase, BSA, and Sephadex G-25 were purchased from Boehringer Mannheim Corp. (Indianapolis, IN). All other reagent grade chemicals were from Sigma Chemical Co. (St. Louis, MO) or Fisher Chemical (Fairlawn, NJ). Cell culture media and reagents were obtained from the University of California Cell Culture Facility (San Francisco, CA), with the exception of Geneticin (G418) which was purchased from GIBCO BRL (Gaithersburg, MD). Female Sprague-Dawley rats were from Bantin and Kingman (Fremont, CA).

Antibodies

Mouse ascites fluid containing monoclonal antibodies against the cytoplasmic domain of the human TfR (H68.4) was generated by BAbCo (Richmond, CA) from cells kindly provided by Ian S. Trowbridge (Salk Institute, La Jolla, CA). Monoclonal antibodies directed against the cytoplasmic domain of synaptophysin (SY38) were purchased from Boehringer Mannheim Corp. HRP-conjugated goat anti-mouse IgG, and purified mouse γ-globulin came from Cappel Research Products (Durham, NC). KT3 monoclonal antibody against the T antigen epitope tag was prepared and iodinated as described (Grote et al., 1995).

Cytosol Preparation

Brains from nine rats were washed and homogenized in ~2 vol bud buffer, 38 mM potassium aspartate (DL), 38 mM potassium glutamate (L), 38 mM potassium gluconate (D), 20 mM potassium MOPS, pH 7.2, 5 mM reduced glutathione, 5 mM sodium carbonate, 2.5 mM magnesium sulfate (Grimes et al., 1992) with 10 passes in a Teflon/glass homogenizer. The homogenate was centrifuged at 10,000 *g* for 15 min and the supernatant centrifuged at 200,000 g_{av} for 1 h. Aliquots were frozen and stored at −80°C until use.

Cell Culture

The pheochromocytoma cell line PC12, stably transfected with rat VAMP-TAg (Grote et al., 1995), was grown in DME H-21 media supplemented with 10% horse serum, 5% fetal calf serum, 100 U/ml penicillin and 100 mg/ml streptomycin, and with 0.25 mg/ml Geneticin (G418; GIBCO BRL) in 10% CO_2 at 37°C. They were treated 18 h before the experiment with 6 mM sodium butyrate to induce VAMP-TAg expression as described (Bonzelius et al., 1995).

In Vivo Labeling and Subcellular Fractionation

Confluent 15 cm-dishes of cells were rinsed once in labeling buffer (PBS with 3% BSA, 0.3 mM $CaCl_2$, 0.3 mM $MgCl_2$, and 1 mg/ml glucose), then incubated in the same buffer with [^{125}I]-KT3 monoclonal antibodies against the T antigen epitope tag (Grote et al., 1995) in the conditions described for each experiment. Control experiments have shown that the presence of the monoclonal antibody does not affect endocytosis (Grote, E., and R. B. Kelly, submitted). To chase the cells, dishes were labeled in the same manner, rinsed with cold labeling buffer and then chased in the absence of labeled antibody for 30–40 min at 37°C in culture medium. Following each condition, cells were washed at 4°C with labeling buffer, scraped from dishes and, after pelleting at 1,000 *g*, resuspended in 300–400 μl per 15 cm dish in buffer A (150 mM NaCl, 10 mM Hepes, pH 7.4, 1 mM EGTA, 1 mM $MgCl_2$). Homogenization was performed by eight passes through a ball-bearing homogenizer (cell cracker EMBL) with a clearance of 12 μm. To analyze vesicles made in vivo, a post-nuclear supernatant (S1; 1,000 *g* for 5 min) was centrifuged at 27,000 *g* for 35 min to generate a supernatant (S2), and a resuspended pellet (P2). To identify synaptic vesicles, 250 μl (3.5 mg/ml) of the S2 was loaded on a 5–25% glycerol gradient prepared in bud buffer over a 50% sucrose cushion, then centrifuged at 218,000 *g* in a SW55 rotor (Beckman Instruments, Palo Alto, CA) for 75 min at 4°C. Fractions (15–16) were collected from the bottom of the gradient and counted in a gamma counter.

In Vitro Budding Assay

Confluent 15 cm dishes of PC12 cells transfected with VAMP-TAg del 61-70 were rinsed once and labeled with [^{125}I]-KT3 antibody as described. Cells were washed at 4°C with labeling buffer, scraped from dishes, washed extensively by pelleting in bud buffer. Cells were homogenized as described before in 1,200–1,500 μl of bud buffer containing protease inhibitors and the labeled homogenate was divided into 300–400-μl aliquots. Although 150 μM $CaCl_2$ was sometimes included in the assay, it was not necessary. Aliquots were incubated at 0° or 37°C for 30 min, in a final volume of 650–750 μl with or without 3 mg/ml cytosol and with an ATP-regenerating system (1 mM ATP, 8 mM creatine phosphate, 5 μg/ml creatine kinase). Budding reactions were stopped in ice water and high speed supernatants (S2) analyzed on glycerol velocity gradients as described before. In contrast to the fractionation of gradients after in vivo labeling, it was necessary to collect 16–17 fractions of the in vitro gradients to better separate the vesicle peak from the soluble label released upon warming.

Immunoadsorption

M-450 Dynabeads, coated with sheep anti-mouse IgG (Dynal; Great Neck, NY), were incubated with SY38, H68.4 ascites or a control of mouse γ-globulin in PBS with 1% BSA for 2 h at room temperature or 16 h at 4°C on a rotator, and then rinsed in PBS, 1% BSA buffer two times for 5 min, once for 20 min with the addition of 1 mg/ml mouse γ-globulin, and twice for 5 min in buffer. Equal amounts of a pool of synaptic vesicle peak fractions (8 and 9) or the P2 of the bud reactions were incubated with beads for 1–2 h at room temperature with 0.5% BSA included. Beads were rinsed three to five times in buffer and counted.

Western Blot Analysis

For Western blot analysis, beads, or gradient fractions were boiled in Laemmli sample buffer and resolved on 10% SDS-PAGE gels, and then transferred to Immobilon-P (Millipore Corp., Bedford, MA) and processed as described (Bonzelius et al., 1994).

Results

Targeting of VAMP-TAg del 61-70 to Synaptic Vesicles In Vivo

Labeled antibodies ([^{125}I-KT3), bound at 0°C to epitope-tagged VAMP on the surface of stably transfected PC12 cells, are rapidly endocytosed and recovered in synaptic vesicles when the intact cells are warmed to 37°C (Grote et al., 1995). The total fraction of counts recovered in synaptic vesicles is small in wild type, but can be increased about five to tenfold by using a deletion mutant of VAMP (VAMP del 61-70). To determine the steady-state distribution of the epitope-tagged VAMP, cells were labeled for 20 min at 37°C, and then chased for 40 min in the absence of labeled antibody. When high speed supernatants from the homogenates were analyzed by velocity sedimentation, the recovery of radioactivity in fractions 8–10, the position of the synaptic vesicles in PC12 (Clift-O'Grady et al., 1990), was significantly higher in the mutant than the wild type transfected cells (Fig. 1). Velocity sedimentation has been extensively used as a way of identifying synaptic vesicles by taking advantage of their unique sedimentation rate (Clift-O'Grady et al., 1990). Unbound antibody was detected at the top of the gradient. The small amount of radioactivity collected in the sucrose pad at the bottom of the gradient (fraction 2), is mainly endosomal material. It may result from endosomes fragmented during homogenization since the amount of radioactivity on the pad varies considerably between experiments. The recovery of radioactivity in the synaptic vesicle peak is 3.2% for wild type and 16% for the del 61-70 mutant, making clear that under

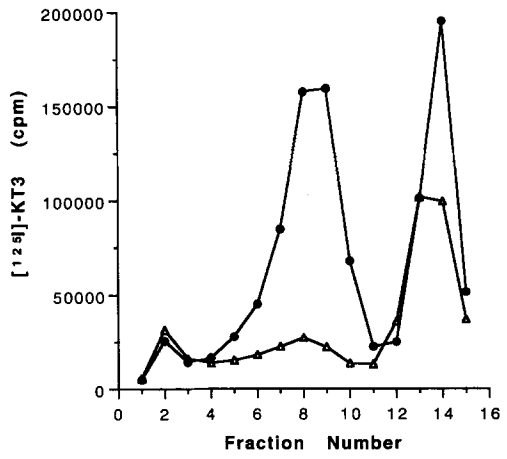

Figure 1. More efficient targeting of VAMP-TAg to synaptic vesicles with VAMP-TAg mutant del 61-70. PC12 cells transfected with wild type VAMP-TAg (-△-) or del 61-70 VAMP-TAg (-●-) were incubated with antibodies ([^{125}I]-KT3) to the epitope tag for 20 min and chased for 40 min at 37°C. After velocity sedimentation of a high-speed supernatant (from which the plasma membrane and most endosomes have been removed), PC12 synaptic vesicles are recovered in fractions 7–10 as described (Clift-O'Grady et al., 1991). Labeled VAMP-TAg recovered in the synaptic vesicle peak is five times higher in the del 61-70 mutant.

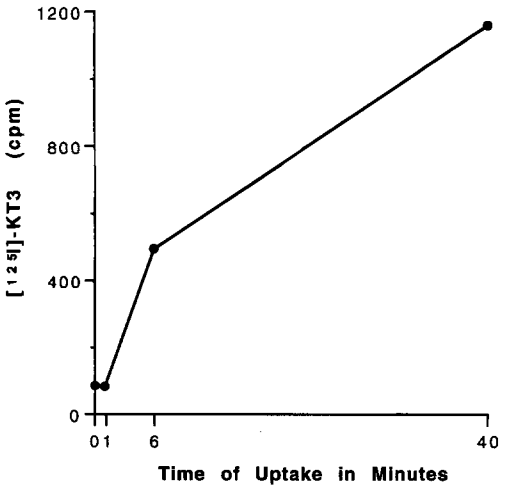

Figure 2. In vivo kinetics of VAMP-TAg recycling. The curve represents the amount of [^{125}I]-KT3 recovered in the synaptic vesicle peak (pool of fractions 8–9) after different lengths of chase at 37°C of VAMP-TAg del 61-70 PC12 cells labeled with [^{125}I]-KT3 at 0°C for 40 min.

close to steady-state conditions, a significant amount of the epitope-tagged VAMP, labeled at the cell surface recycles into synaptic vesicles. Whatever the explanation of the high efficiency of targeting of the VAMP mutant into synaptic vesicles, the mutant facilitates greatly our ability to study synaptic vesicle biogenesis both in vivo and in vitro.

In Vivo Kinetics of VAMP-TAg Recycling

If synaptic vesicles arise from endosomes, a lag may be expected before the epitope-tagged VAMP, labeled at the cell surface, appears in synaptic vesicles. To look for such a lag, transfected PC12 cells were labeled with [^{125}I]-KT3 at 0°C, the label removed and the cells warmed to 37°C. At time intervals high speed supernatants were analyzed for antibody in synaptic vesicles by velocity sedimentation on glycerol gradients. The kinetics of incorporation into synaptic vesicles were indistinguishable for the mutant (Fig. 2) and wild type epitope-tagged VAMP. Endocytosis of the wild type and VAMP del 61-70 in PC12 cells occurs with a half-time of 5 min (Grote et al., 1995). We could find no evidence for a lag of greater than one minute before label began to appear in synaptic vesicles. The half-time of synaptic vesicle formation was about 10 min. Thus, if endosomes are intermediates in the synaptic vesicle pathway, the absence of a detectable lag implies that their proteins must be packaged into vesicles soon after arrival in the endosomes. To confirm that synaptic vesicles form rapidly in PC12 cells, a series of experiments was performed using the del 61-70 variant labeled for three minutes at 37°C, with a 0°C labeling condition subtracted as background. Even in this brief time, 1.44% ± 0.08% (n = 5) of the total label could be recovered in synaptic vesicles which is about 15% of the maximum value reached after long incubations. We conclude that synaptic vesicles form very rapidly after the VAMP molecules leave the cell surface.

Temperature Effect on VAMP-TAg Recycling to the Synaptic Vesicles

Some steps in membrane trafficking appear to be more temperature-sensitive than others. Synaptic vesicle formation appears to be one of those temperature-sensitive steps. When PC12 cells transfected with VAMP-TAg del 61-70 were labeled for 40 min with [^{125}I]-KT3 at 15°C, endocytosis occurred since 25% of the radioactivity became resistant to an acid strip of the cell surface (data not shown). The plasma membrane and also an endosomal compartment become labeled (data not shown), but no label is recovered in synaptic vesicles (Fig. 3, *open circles*). Endogenous synaptic vesicles were present in fractions 8 and 9 as confirmed by an immunoblot of gradient fractions from the 15°C labeling condition with anti-synaptophysin (data not shown). A subsequent chase of these cells at 37°C in vivo demonstrated efficient delivery, 10.7% ± 0.5 (n = 2) of the total label, to synaptic vesicles (Fig. 3, *filled circles*).

In Vitro Budding of Synaptic Vesicles

Synaptic vesicles could arise directly from the plasma membrane or from an endosomal intermediate. Because de novo biogenesis of PC12 synaptic vesicles via the plasma membrane was readily detectable in vivo, we attempted to reconstitute vesicle formation from labeled plasma membrane in vitro. The cell surface of transfected PC12 VAMP-TAg del 61-70 cells was labeled with [^{125}I]-KT3 at 0°C. After washing away the unbound antibody, the cells were homogenized and the homogenate incubated at 37°C in the presence of rat brain cytosol and an ATP-regenerating system (Fig. 4). To detect synaptic vesicle-sized organelles, the budding reaction mix was centrifuged after the reaction to pellet large membranes, and the

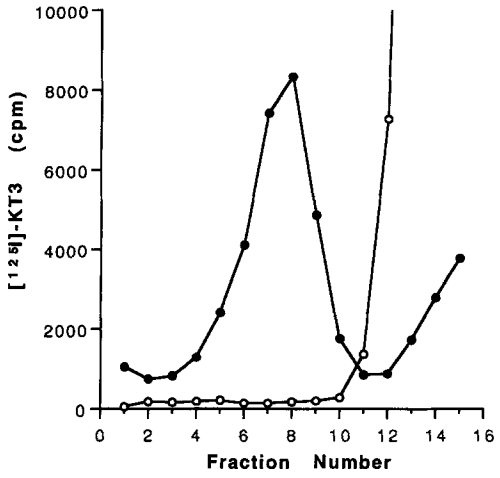

Figure 3. Absence of labeled synaptic vesicles after antibody uptake at 15°C. When PC12 cells, transfected with VAMP del 61-70, were incubated with [^{125}I]-KT3 at 15°C for 40 min, no labeled VAMP-TAg was recovered in the synaptic vesicle peak (-○-). When the cells were washed free of antibody and warmed to 37°C (-●-), a significant amount of [^{125}I]-KT3 co-sedimented with the synaptic vesicles, as expected from previous work (Grote et al., 1995).

high speed supernatant was analyzed by velocity sedimentation. No labeled synaptic vesicles were present, either before (Fig. 4, *open circles*) or after (*filled circles*) an in vitro 30-min incubation of the cell homogenate at 37°C. Although incubation at 37°C did increase somewhat the recovery of [^{125}I]-KT3 antibodies associated with rapidly sedimenting vesicles there was no obvious peak of radioactivity in the synaptic vesicle region (fractions 8–10) previously identified using antibodies to synaptophysin, synaptotagmin, and SV2 (Clift-O'Grady et al., 1990). As a control the same labeled cells were warmed to 37°C in vivo and a clear peak of synaptic vesicles was seen (Fig. 4, *open squares*).

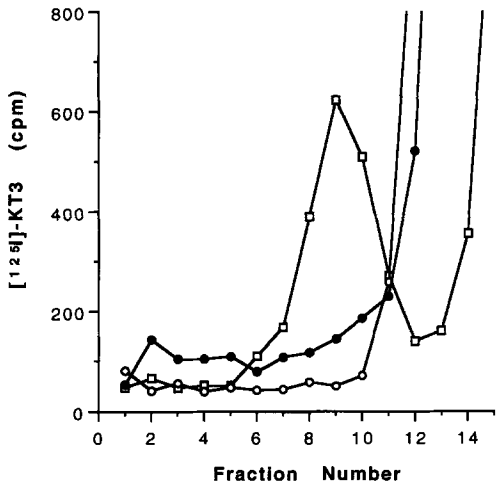

Figure 4. In vitro budding from a cell homogenate labeled at 0°C. Synaptic vesicles cannot be generated in vitro from a homogenate of VAMP-TAg del 61-70 transfected PC12 cells labeled with [^{125}I]-KT3 at 0°C for 30 min. Labeled homogenate was incubated in the presence of rat brain cytosol at 0°C (-○-) or 37°C (-●-) and the high speed supernatants were analyzed on a velocity gradient. However, when cells labeled in the same manner at 0°C were chased for 30 min at 37°C in vivo (-□-), recycling of [^{125}I]-KT3 into the synaptic vesicle peak was observed.

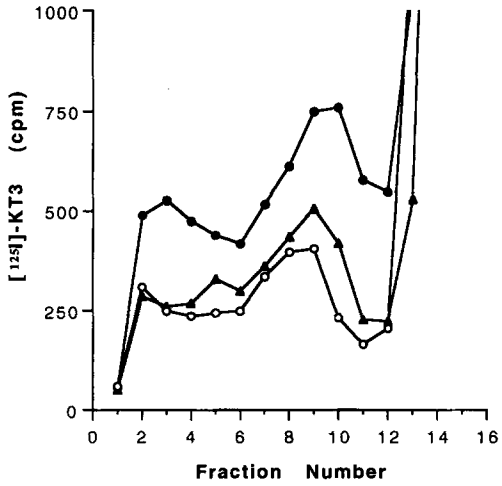

Figure 5. In vitro budding from a cell homogenate labeled 3 min at 37°C. Conditions were the same as in Fig. 4, except that cells were warmed to 37°C for 3 min, in the presence of [^{125}I]-KT3 prior to homogenization. The data from incubations with brain cytosol at 0°C (-○-) and 37°C (-●-) are given. Also included are the results of incubating at 37°C in the absence of added cytosol (-▲-).

Since we could not observe SV formation directly from plasma membrane, we allowed antibody uptake for 3 min at 37°C prior to homogenization. In this way we hoped to label potential donor compartments that might generate synaptic vesicles in vitro. In the absence of incubation in vitro at 37°C (Fig. 5, *open circles*) label was recovered in the synaptic vesicle region (fractions 8–10) and in the faster sedimenting membranes that accumulated on the pad. An increase in the amount of label was seen when rat brain cytosol was present (Fig. 5, *filled circles*) but not in its absence (Fig. 5, *filled triangles*). There was also an apparent increase in the labeled membranes that sedimented faster than synaptic vesicles, but recovery of label on the pad is variable. It therefore appeared that a fraction with the characteristics of synaptic vesicles could be generated in vitro by incubation in the presence of cytosol, but there was a considerable background due to labeled synaptic vesicles made in vivo and present before the in vitro incubation began.

The signal to noise ratio of the in vitro reaction was markedly improved by the use of homogenates of transfected cells labeled at 15°C, which contain no pre-existing labeled synaptic vesicles (Fig. 6). When such homogenates were incubated at 37°C in the presence of rat brain cytosol, a peak of [^{125}I]-KT3 was detected on velocity gradients that co-migrated with PC12 synaptic vesicles (Fig. 6, *filled circles*). Vesicle formation does not occur at 0°C (Fig. 6, *open circles*) or in the absence of brain cytosol (Fig. 6, *filled triangles*) or with CHO cytosol (Fig. 6, *filled squares*). In addition to the synaptic vesicle peak, a heterogeneously sized population of labeled vesicles was often generated in vitro, and sedimented faster than synaptic vesicles. Hetero-

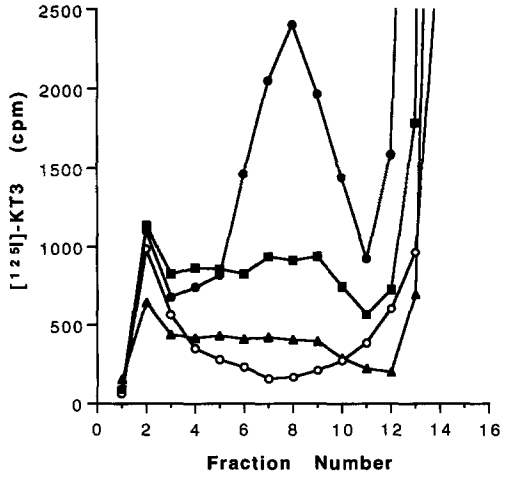

Figure 6. Cytosol and temperature-dependent in vitro budding of synaptic vesicles from 15°C-labeled cell homogenate. Vesicle production in vitro was assayed from a homogenate made from VAMP-TAg del 61-70 PC12 cells incubated at 15°C for 40 min. Labeled synaptic vesicles do not form in homogenates incubated at 0°C (-○-). In the presence of rat brain cytosol, with ATP and at 37°C, the formation of synaptic vesicles from the 15°C-labeled cell homogenate can be detected (-●-). Omission of cytosol (-▲-) or replacement with CHO cytosol (-■-) did not generate a vesicle peak.

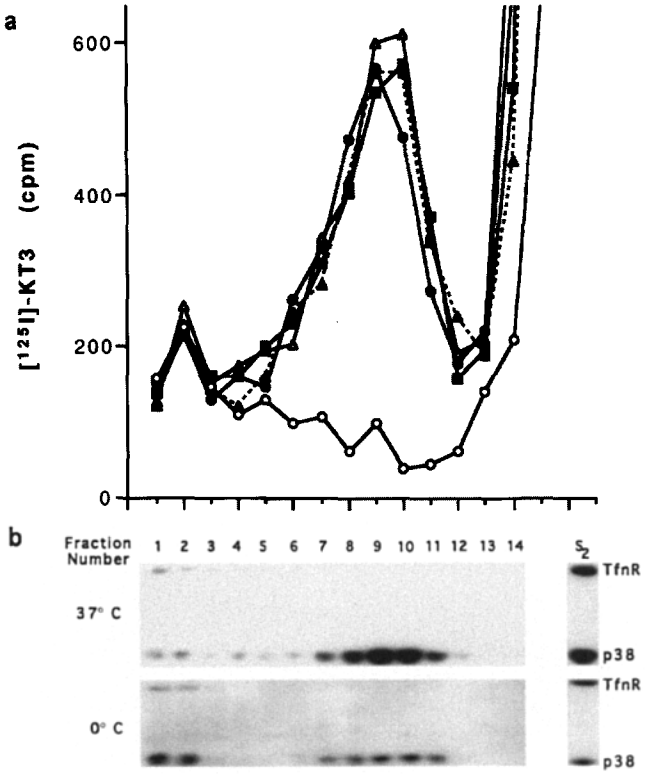

Figure 7. Calcium dependence of the in vitro budding reaction. (*a*) In vitro vesicle production in the presence or absence of calcium or EGTA was measured. Homogenates were prepared as described for Fig. 6. Budding reactions were performed with no additions (-▲-), with 150 μM $CaCl_2$ (-●-), with 2 mM EGTA (-■-), or with 150 μM $CaCl_2$ and 2 mM EGTA (-△-). The 0°C control bud reaction (-○-) contained neither calcium nor EGTA and confirms that labeled synaptic vesicles do not form at 0°C. (*b*) Synaptophysin but not transferrin is detected in synaptic vesicle peak fractions of an in vitro budding reaction. Fractions 1 through 14 of the bud reactions with no additions, at 0°C and at 37°C (Fig. 7 *a*) were pelleted in a TL-120.1 rotor (Beckman Instruments) after diluting 1:3 in bud buffer. After SDS-PAGE, the synaptophysin (p38) and transferrin receptor (*TfnR*) were measured by Western blotting. Film exposure times are the same for both parent fractions (S_2), while the exposure of the 0°C gradient immunoblot is 10-fold more than that of the 37°C gradient. The immunoblot of the 37°C bud reaction with calcium and no EGTA (-●-) confirms that the synaptic vesicles generated in vitro cosediment with the peak of radioactivity in fractions 8, 9, and 10. The transferrin receptor is mostly present in faster sedimenting fractions. The 0°C gel was exposed for a longer time than the 37°C gel to make the signals comparable. Immunoblot of equal amounts of the S_2 for both gradients is shown at right. An increase in the total amount of TfnR and p38 is seen in the 37°C fraction over the 0°C control.

geneously sized vesicles were also formed from extracts of CHO cells, transfected with the same epitope-tagged VAMP construct and incubated the same way, but no peak corresponding to synaptic vesicles was detected (data not shown). Since the recovery of heterogeneously sized vesicles was much less predictable than that of synaptic vesicles. We have not explored further their nature or biogenesis.

Characterization of the Budding Reaction

Because of the conflicting data on the calcium dependence of endocytosis (see Discussion), we examined whether the presence or absence of calcium affected the budding reaction. No effect was seen, even at the relatively high concentration of 150 μM calcium (Fig. 7 *a*).

Western blotting of fractions through the gradient (Fig. 7 *b*) confirmed that the labeled vesicles co-migrated with the endogenous vesicles, recognized by anti-synaptophysin antibodies. The transferrin receptor, an endosomal marker, was mostly present in faster sedimenting vesicles. We have noted an increased recovery of synaptophysin and transferrin receptor in the S2 fractions from in vitro budding reactions. The increase in synaptophysin is in the synaptic vesicle peak (note the difference in exposure time of the 0° and 37°C samples). We cannot tell whether this represents de novo vesicle generation in large amounts or the release of pre-formed vesicles from binding sites on rapidly sedimenting structures.

We next explored the nucleotide requirements for budding. Vesicle formation was enhanced when an exogenous ATP-generating system was present (Fig. 8 *a*). GTP-γS has been reported to prevent the generation of endocytotic vesicles (Carter et al., 1993) and synaptic vesicles (Takei et al., 1995). Its presence in the reconstituted system inhibited the generation of the synaptic vesicle-sized organelles (Fig. 8 *a*).

Efficient reconstitution of synaptic vesicle budding in vitro required the use of rat brain cytosol. Although there was some PC12 cytosol in the reaction mix, without added rat brain cytosol, no vesicles were seen (Fig. 6, *filled trian-*

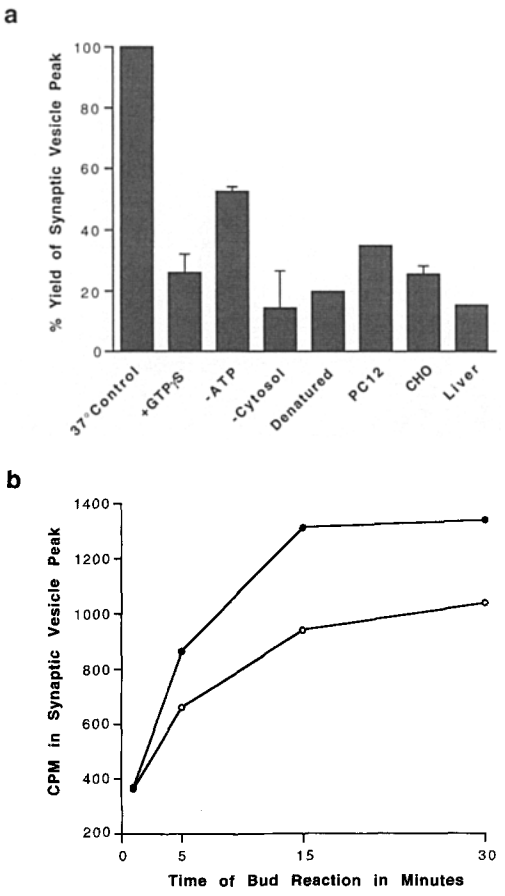

Figure 8. Characterization of the in vitro budding reaction. (*a*) Nucleotide and cytosol dependence of the budding reaction are summarized. To normalize the data, yield of vesicles (radioactivity in fractions 8–10 of the glycerol velocity gradients) is expressed as a percentage of the yield obtained using the complete budding reaction (rat brain cytosol and ATP at 37°C). The 0°C control is subtracted as background (15.8% ± 2.7 of complete reaction, $n = 7$). Error bars represent the range of the results for two independent experiments. The cytosol concentration in all experiments was 3 mg/ml protein and rat brain cytosol was denatured by boiling. The concentration of GTPγS in the budding reaction was 200 μM. (*b*) Kinetics of the in vitro budding reaction. The amount of [^{125}I]-KT3 recovered in the synaptic vesicle peak (pool of fractions 8–9) after a complete budding reaction as described in Fig. 6 was measured as a function of time (–●–). To confirm that the increase of the signal is not due to the release of free [^{125}I]-KT3, aliquots of the same fractions were immunoadsorbed with 4 μg M-450 Dynabeads (sheep anti-mouse IgG) coated with monoclonal antibodies to synaptophysin (SY-38) (–○–). Most of the radioactivity present in the vesicle fractions bound to the immunoadsorbent.

gles; Fig. 8 *a*). Incubation of the labeled membranes with equivalent amounts of cytosol from CHO fibroblasts allowed less efficient generation of only the heterogeneous population of vesicles that did not have the same size distribution as synaptic vesicles (Fig. 6, *filled squares*, Fig. 8 *a*). Thus, both cytosols supported the production of heterogeneously-sized vesicles, but only rat brain cytosol supported the formation of vesicles with the characteristics of synaptic vesicles. Presumably, brain cytosol is rich in factors required for synaptic vesicle formation since cytosol from CHO cells or rat liver gave a signal indistinguishable from a denatured cytosol control and supplementing the reaction with cytosol from PC12 cells at the same protein concentration gave only a modest enhancement (Fig. 8 *a*).

The production of synaptic vesicles had a half-time of about 5 min. To demonstrate that the radioactivity in the peak fractions is associated with synaptic vesicles, an immunoadsorption of aliquots of the peak fractions was performed. Most of the radioactivity (74%) was bound to the anti-synaptophysin coated immunoadsorbent (Fig. 8 *b*, *filled circles*). Part of the unbound radioactivity could be free antibody contaminating our fractions.

The rat brain cytosol titration (Fig. 9) showed that the quantity of vesicles generated depended on the amount of rat brain cytosol added. Generation of antibody labeled synaptic vesicles in vitro from membranes labeled at 15°C and incubated in vitro at 37°C with brain cytosol and ATP was highly reproducible. The fraction of radioactivity recovered in synaptic vesicles generated in vitro was 2.9% ± 0.23 ($n = 7$) of the total label in the homogenate, an efficiency about 20–30% of that observed in vivo. The label was associated with synaptic vesicles since it could be immunoadsorbed with antibodies to synaptophysin (Fig. 8 *b*, *open circles*).

15°C Donor Compartment

The organelle that generates the vesicles was not lost by removing 90% of the plasma membrane by differential centrifugation. The homogenate was subject to a low speed centrifugation at 1,000 *g* for 5 min prior to the reaction (S1). Incubation of S1 at 37°C in the presence of rat brain cytosol resulted in the generation of synaptic vesicles with only slightly reduced efficiency compared to homogenates. In repeat experiments of this type the average recovery of budding activity from the S1 was 63.5% ± 9.6 ($n = 4$). Budding from the S1 fraction consistently pro-

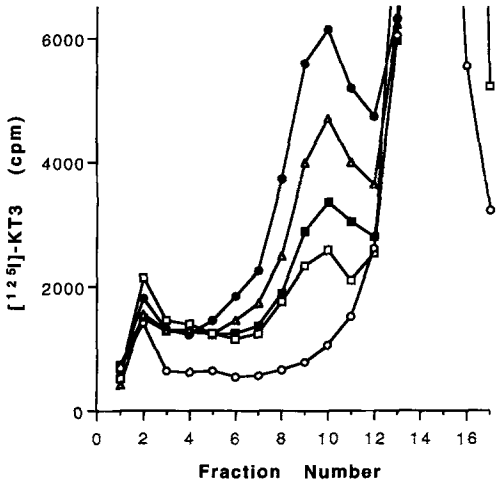

Figure 9. Rat brain cytosol concentration effect on synaptic vesicle formation in vitro. Conditions were standard except that different concentrations of rat brain cytosol were used: 0.5 mg/ml (–□–), 1.0 mg/ml (–■–), 2.0 mg/ml (–▲–), 4.0 mg/ml (–●–). The 0°C incubation control was done with a rat brain cytosol concentration of 2.0 mg/ml (–○–).

duced fewer of the more rapidly sedimenting endosomal vesicles than did budding from homogenates (data not shown).

Sorting Efficiency of the Budding

The rat brain cytosol gave rise to a homogeneously sized population of labeled vesicles that sedimented at the rate expected of synaptic vesicles. Generation of synaptic vesicles requires sorting from other membrane proteins as well as regulation of vesicle size. Sorting is known to occur in vivo because synaptic vesicles are immunoadsorbed by antibodies to a synaptic vesicle protein, synaptophysin, but not by antibodies to the transferrin receptor, which has been shown to be excluded from synaptic vesicles (Linstedt and Kelly, 1991; Cameron et al., 1991). Synaptic vesicles labeled in vivo with [^{125}I]-KT3 antibodies were also selectively adsorbed by anti-synaptophysin antibodies, but not by antibodies to the transferrin receptor (data not shown). Using vesicles generated in vitro, the maximum amount of radioactivity adsorbed to anti-synaptophysin coated beads was considerably larger than that obtained with anti-transferrin receptor coated beads (Fig. 10). The antibody coated beads were equally effective when immunoadsorbing larger membranes from a P2 fraction. Western blot analysis confirmed that the anti-transferrin receptor coated beads were ineffective in binding material from the synaptic vesicle peak, but could recognize larger transferrin-receptor containing membranes as efficiently as anti-synaptophysin coated beads (Fig. 10).

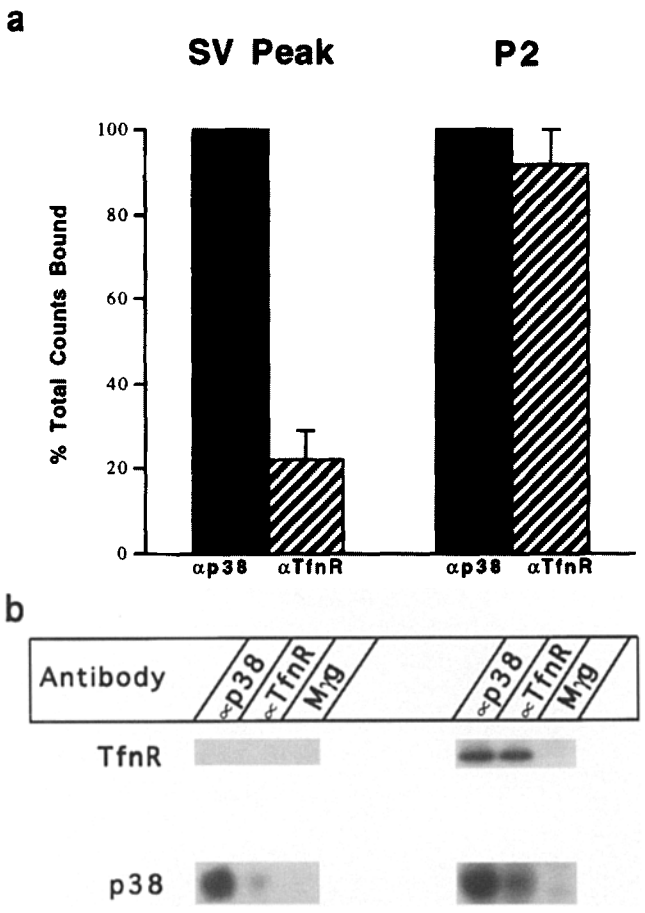

Figure 10. Sorting occurs in the budding reaction. (*a*) Synaptic vesicles formed during the in vitro budding reaction and isolated by velocity sedimentation contain synaptophysin, but not transferrin receptor. Up to 70% of the radioactivity associated with synaptic vesicles could be immunoadsorbed by antibodies to synaptophysin. The radioactivity bound to beads coated with the transferrin receptor antibody (*striped columns*) is expressed as a percent of the total radioactivity bound to beads coated with anti-synaptophysin (*filled columns*). Radioactivity in the P2 fraction containing labeled plasma membranes and endosomes, was adsorbed with equal effectiveness by both coated beads. (*b*) Confirmation of bead specificity by Western blotting. Membranes bound to beads coated with anti-synaptophysin (α38) and anti-transferrin receptor (αTfnR) and mouse gamma globulin (Mγg) as a control, were eluted from beads by boiling in sample buffer. After SDS-PAGE, the amounts of synaptophysin (p38) and transferrin receptor (TfnR) were measured by Western blotting. As expected, there was no TfnR in the SV peak fractions. The beads were equally effective in adsorbing TfnR containing vesicles from the P2 fraction, compared with the negative control of non-specific mouse antibodies.

Discussion

Membrane traffic within the nerve terminal is highly efficient. When synaptic vesicles are labeled, for example, with a fluorescent dye they can be shown to fuse with the plasma membrane with a half-time of seconds to minutes, depending on the frequencies (Betz and Bewick, 1992; Ryan and Smith, 1995). Newly synthesized vesicles are capable of exocytosis as early as 10 s after membrane proteins leave the plasma membrane (Ryan and Smith, 1995). To compensate for this exuberant exocytotic rate, nerve terminals must have a highly efficient biosynthetic apparatus capable of replenishing synaptic vesicles at a rapid rate. Although PC12 cells are at best analogs of immature neuronal cells, they too are capable of making synaptic vesicles from the plasma membrane at a very rapid rate (Fig. 2).

It appears as if both steps in synaptic vesicle biogenesis, namely membrane protein sorting and the creation of vesicles of the correct size, has been recreated in extracts of PC12 cells. Besides being quick and quantitative, the assay using labeled KT3 antibodies allowed us to examine synaptic vesicle generation in cases where no pre-existing labeled synaptic vesicles existed. The vesicles were generated during a 37°C incubation with kinetics not much different from that in vivo but with only about 30% of the in vivo efficiency. We believe that the assay is monitoring accurately the production of synaptic vesicles and not other labeled organelles because of the characteristic sedimentation velocity of the vesicles produced, because of their absence in transfected non-neuronal cells, because they can be immunoadsorbed with antibodies to a synaptic vesicle protein, synaptophysin, and finally because their production only occurs in the presence of cytoplasm from neuronal cells. Their formation is time, cytosol, ATP and temperature-dependent. The sensitivity of the budding to GTP-γS is similar to what is seen in the generation of endocytotic vesicles from the plasma membrane (Carter et al., 1993; Takei et al., 1995). In contrast, GTP hydrolysis is

not required for budding of vesicles from the ER and the Golgi complex (Schwaninger et al., 1992; Rothman and Orci, 1992).

The calcium dependence of endocytosis has been examined in earlier work by using permeabilized cells, by measuring surface capacitance decrease and by dye uptake. Little consistency has been found. In some in vitro assays (Lin et al., 1991) but not in others (Lamaze et al., 1993), calcium is required for endocytosis related events. In patch-clamped endocrine cells (Neher and Zucker, 1993; Thomas et al., 1994; Heinemann et al., 1994), calcium stimulates endocytosis, whereas in patch-clamped retinal bipolar neurons, calcium inhibits endocytosis (von Gersdorff and Matthews, 1994). In permeabilized chromaffin cells (von Grafenstein and Knight, 1993) there was no requirement for calcium or nucleotides to be present during endocytosis. Using a dye-uptake assay, calcium was not required during vesicle recycling at Drosophila neuromuscular junctions (Ramaswami et al., 1994). These assays all measure budding from the plasma membrane and ours may not. In our experiments we could detect no changes in the rate of vesicle production in vitro, even at calcium levels as high as 150 μM.

Vesicle biogenesis in PC12 cells has been proposed to occur in two steps, transport of vesicle proteins from the cell surface to endosomes and subsequently, biogenesis from the endosome (Regnier-Vigouroux et al., 1991; Mundigl and De Camilli, 1994; Südhof, 1995). Two arguments have been raised to support the proposition that synaptic vesicle biogenesis involves an endosomal intermediate. One is that some but not all synaptic vesicle membrane proteins are targeted to endosomes when transfected into non-neuronal cells (Linstedt and Kelly, 1991; Cameron et al., 1991; Feany et al., 1993). The weight of this argument has been diminished by the finding by De Camilli and his collaborators that the targeting is to housekeeping endosomes at the cell body and not necessarily to axonal endosomes (Mundigl et al., 1993). The second argument in favor of endosomes as an intermediate comes from the work of Huttner and his colleagues (Bauerfeind et al., 1993) who showed a significant lag before a bulk phase label added to the outside of PC12 cells appeared in synaptic vesicles. We observe little evidence for such a lag using our assay system.

The absence of a lag could be explained if synaptic vesicles arise from the plasma membrane directly, or if the generation of synaptic vesicles from the endosomal intermediate is very fast. Our data suggest that the plasma membrane itself is not a direct precursor of synaptic vesicles in PC12 cells. We can find no budding of vesicles from labeled plasma membranes directly. In order to see vesicle biogenesis we must allow the label to go into a 15°C compartment. We know that, at 15°C, endocytosis into an intracellular compartment does occur. Thus synaptic vesicle biogenesis in vitro correlates with the ability to direct the label into an endosomal compartment. We also find that the centrifugation of the plasma membranes from the reaction mix causes only a small reduction in the budding efficiency in our reactions. Although these data would be consistent with generation of synaptic vesicles from a more slowly sedimenting endosomal compartment that fills at 15°C other interpretations are possible. For example, the precursor of synaptic vesicles could be a special region of plasma membrane, such as a dynamin-coated invagination (Takei et al., 1995) that forms at 15°C but not at 0°C and that we cannot generate in vitro.

Now that synaptic vesicle biogenesis can be observed in vitro, it should be possible to identify components in the rat brain cytosol that are necessary to sort membrane proteins into synaptic vesicles and to determine vesicle size. Furthermore, it will be important to identify the nature of the 15°C compartment from which the vesicles arise and the synaptic vesicle membrane proteins recognized by the sorting machinery.

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