Endocrine Secretory Granules and Neuronal Synaptic Vesicles Have Three Integral Membrane Proteins in Common

A. W. Lowe,*‡ L. Madeddu,* and R. B. Kelly*

*Department of Biochemistry and Biophysics and ‡Department of Medicine, University of California, San Francisco, California 94143-0448

Abstract. In response to an external stimulus, neuronal cells release neurotransmitters from small synaptic vesicles and endocrine cells release secretory proteins from large dense core granules. Despite these differences, endocrine cells express three proteins known to be components of synaptic vesicle membranes. To determine if all three proteins, p38, p65, and SV2, are present in endocrine dense core granule membranes, monoclonal antibodies bound to beads were used to immunoisolate organelles containing the synaptic vesicle antigens. [^{3}H]norepinephrine was used to label both chromaffin granules purified from the bovine adrenal medulla and rat pheochromocytoma (PC12) cells. Up to 80% of the vesicular [^{3}H]norepinephrine was immunoisolated from both labeled purified bovine chromaffin granules and PC12 postnuclear supernatants. In PC12 cells transfected with DNA encoding human growth hormone, the hormone was packaged and released with norepinephrine. 90% of the sedimentable hormone was also immunoisolated by antibodies to all three proteins. Stimulated secretion of PC12 cells via depolarization with 50 mM KCl decreased the amount of [^{3}H]norepinephrine or human growth hormone immunoisolated. Electron microscopy of the immunoisolated fractions revealed large (>100 nm diameter) dense core vesicles adherent to the beads. Thus, large dense core vesicles containing secretory proteins possess all three of the known synaptic vesicle membrane proteins.

Among the cell types that have regulated secretion, neuronal and endocrine cells appear to be the most closely related (Anderson and Axel, 1986; LeRoith et al., 1982). The two cell types, however, differ in how their secretory vesicles are generated. Endocrine cells secrete soluble proteins that are packaged into maturing secretory granules in the Golgi region. After exocytosis, the granule membrane components return to the Golgi region and are reutilized in the synthesis of new secretory granules (Patzak and Winkler, 1986). In contrast, synaptic vesicles in neuronal cells recycle their membranes locally in the nerve terminal; formation of new synaptic vesicles does not involve return to the Golgi complex (Ceccarelli et al., 1973; Heuser and Reese, 1973). Although synaptic vesicle membranes can recycle from nerve terminal plasma membrane, newly synthesized vesicle proteins must be transported from the Golgi to the nerve terminal. Neurons have dense core vesicles, analogous to secretory granules in the regulated path of secretion in endocrine cells (Gumbiner and Kelly, 1982; Kelly, 1985). Synaptic vesicle components could be transported to the plasma membrane in dense core vesicles, or by a constitutive pathway. Alternatively, a third pathway may exist for the exclusive generation and transport of synaptic vesicle proteins to the nerve terminals.

The presence in all vertebrate synaptic vesicles of three different integral membrane proteins facilitates the study of synaptic vesicle biogenesis. The three proteins have been identified by monoclonal antibodies and are referred to as p38 or synaptophysin (Jahn et al., 1985; Wiedenmann and Franke, 1985), p65 (Matthew et al., 1981), and SV2 (Buckley and Kelly, 1985). The latter is a highly glycosylated protein of 95–100 kD. Since all three proteins are readily detectable in endocrine cells they can be used to study the relationship between endocrine and neuronal cells.

A crucial question is whether or not endocrine dense core secretory granules possess synaptic vesicle membrane proteins. The data are conflicting. There is evidence suggesting that the SV2 and p65 antigen are present in secretory protein containing granules of the rat brain and the pheochromocytoma cell line, PC12 (Floor and Leeman, 1985; Matthew et al., 1981; Schweitzer and Kelly, 1985; Buckley and Kelly, 1985). In contrast, the p38 protein was not detectable in chromaffin granules by biochemical measurement (Wiedenmann and Franke, 1985). Furthermore, careful immunoelectron microscopy showed that the p38 protein was absent on dense core granules but localized to small electron-lucent vesicles in endocrine and neuroendocrine cells (Navone et al., 1986).

Dr. Lowe and Dr. Madeddu contributed equally to this paper. The order of the first two authors is arbitrary.

Dr. Madeddu's present address is Department of Pharmacology, University of Milan, Via Vanvitelli 32, 20129 Milan, Italy.

We have reexamined the presence of the three synaptic vesicle antigens in secretory granules using a sensitive immunoprecipitation technique. With this approach, we have demonstrated that all three of the synaptic vesicle membrane proteins are present in large dense core vesicles that contain secretory proteins.

Materials and Methods

Materials

Polyacrylamide rabbit anti-mouse Immunobeads were obtained from Bio-Rad Laboratories, Richmond, CA; Dynabeads M-450, magnetic polystyrene beads coated with goat anti-mouse IgG, were purchased from Dynal Inc., Fort Lee, NY; Geneticin (G418) from Gibco, Grand Island, NY; l-[7,8^{3}H]norepinephrine, 39 mCi/mM ([^{3}H]NE) from Amersham Corp., Arlington Heights, IL. Rabbit polyclonal antimurine kappa light chain antibodies were obtained from Miles Scientific, Naperville, IL.

All remaining chemicals, including reserpine, pargyline, and carbamylcholine (carbachol), were obtained from Sigma Chemical Co., St. Louis, MO.

Bovine Adrenal Chromaffin Granule Isolation

Purified chromaffin granules were prepared according to Bartlett and Smith (1974). 20–40 bovine adrenal glands (Hohener's Meat Co., San Leandro, CA) from freshly slaughtered animals were used for each isolation. In this procedure, the large granule fraction was centrifuged through an isosmotic solution of 0.27 M sucrose, 19.5% wt/vol Ficoll in D_2O at 80,790 *g* for 1 h at 4°C. The pellet of highly purified chromaffin granules at the bottom was resuspended with 0.32 M sucrose, 10 mM Hepes, pH 7.4. For the uptake experiments the granules were used immediately. Electron microscopy was performed on granules that had been stored at −70°C.

Cell Culture

PC12 cells, a clonal line of rat pheochromocytoma originally described by Greene and Tischler (1976), were obtained from stocks at the University of California (San Francisco) tissue culture facility. The cells were grown in 10% CO_2 at 37°C in DME H-21 containing 10% FCS, 5% horse serum, penicillin (100 U/ml), and streptomycin (100 U/ml). The transfected clones, PC12pMT:hGH and PC12MPC-11K6, were grown in the presence of the antibiotic Geneticin (G418) at 0.25 mg/ml to maintain a selection pressure for plasmid retention.

Cell Transfection

PC12 cells were transfected to yield two clones, PC12pMT:hGH and PC12-MPC-11K6, essentially as described previously (Schweitzer and Kelly, 1985). Transfections (by Dr. E. Schweitzer) resulting in the PC12pMT:hGH clone used two plasmids, pSV2 neo, which confers antibiotic resistance, and a second plasmid containing a metallothionein promoter followed by the genomic human growth hormone DNA (construction #111, Palmiter et al., 1983).

The PC12MPC-11K6 clone, PC12 cells that secrete the kappa light chain immunoglobulin, were kindly provided by Dr. L. Matsuuchi. To produce this clone, PC12 cells were cotransfected with pSV2 neo as described above and with an expression vector containing a genomic clone for the murine MPC11 kappa light chain and the Rouse sarcoma virus long terminal repeat promoter/enhancer combination.

Subcellular Fractionation

To isolate secretory granules, homogenates of PC12pMT:hGH and PC12-MPC-11K6 cells were fractionated on Ficoll equilibrium density gradients as described by Wagner (1985). Cells were grown to confluence in 10-cm tissue culture plates. The cells were incubated at 37°C for 1 h with 10 μCi [^{3}H]NE per dish in medium containing 0.1 mg/ml pargyline. At the end of the incubation, [^{3}H]NE labeled cells were removed from the culture plate with ice-cold PBS and centrifuged at 200 *g* for 5 min at 4°C. The pellet was resuspended with 1.5 ml of 0.32 M sucrose, 10 mM Hepes, pH 7.4, and homogenized with five passes in a custom-made homogenizer (European Molecular Biology Laboratory, Heidelberg, Federal Republic of Germany) with a clearance of 0.012 mm. The homogenate was centrifuged at 10,000 *g* for 5 min at 4°C. 1 ml of the resulting postnuclear supernatant was loaded onto a 1–40% linear Ficoll gradient made up with the 0.32 M sucrose/10 mM Hepes, pH 7.4, buffer. The gradient was centrifuged at 35,000 *g* for 16 h at 4°C. 1-ml fractions were collected from the bottom of the tube and assayed for protein, ATP, [^{3}H]NE, and human growth hormone (hGH).

Cell Stimulation

Cells were stimulated to secrete in two ways. To examine the kinetics of release, PC12pMT:hGH cells were grown to confluence in 10-cm dishes and loaded with [^{3}H]NE as described previously. After the incubation period, the plates were washed six times with 4 ml of fresh medium without serum. The cells were then incubated with medium containing either 5 mM carbachol or 50 mM KCl. The medium was changed and collected at 2-min intervals. At the end of the stimulation period, the cells were triturated off with medium and freeze-thawed in liquid nitrogen three times followed by sonication for 1 min in a bath sonicator (Sonicor Instrument Corp., Copiague, NY). Both the medium and the cellular fraction were cleared of debris by centrifugation at 1,900 *g* for 15 min at 4°C. To all of the samples, iodoacetamide acid and phenylmethylsulfonylfluoride (PMSF) were added (0.3 mg/ml, final concentration). The samples were then assayed for hGH and [^{3}H]NE. To demonstrate that stimulation reduced the recovery of hGH and [^{3}H]NE in secretory granules, PC12 or PC12pMT:hGH cells were stimulated in a different way. [^{3}H]NE-loaded cells were washed once with PBS, removed from the culture plate, and centrifuged at 200 *g* for 5 min at room temperature. The cell pellets were resuspended in a modified Krebs-Ringer solution containing (in mM/liter): NaCl, 125; KCl, 6; $MgSO_4$ and K_2HPO_4, 1.2; $CaCl_2$, 2; Hepes, 25 (pH 7.4); glucose, 6. The cell suspensions were then split into equal aliquots, one of which served as a control, while the others received either 50 mM KCl or 5 mM carbachol. The incubation was carried out at 37°C for 10 min. At the end of the stimulation period, to measure the amounts of [^{3}H]NE remaining in the cells, aliquots of the cell suspensions were centrifuged at 12,000 *g* for 30 s. Supernatants were discarded and the pellets rinsed with 0.5 ml of ice-cold Krebs-Ringer medium and recentrifuged as above. Final pellets were resuspended and assayed for hGH or [^{3}H]NE. The percent radioactivity released was determined by comparison with unstimulated control samples.

[^{3}H]NE Uptake by Isolated Granules

Bovine chromaffin granules were purified as described above. 100 μl of granule suspension (4.8 mg/ml protein) were incubated at 37°C for 10 min with 2 μCi [^{3}H]NE in 0.32 M sucrose, 10 mM Hepes, pH 7.4, in the presence of 5 mM ATP and 0.1 mg/ml pargyline (final volume 1 ml).

For PC12 experiments, aliquots of the postnuclear supernatant (prepared as described under Subcellular Fractionation; final protein concentration: 0.2 mg/ml) were incubated at 37°C for 10 min with [^{3}H]NE (2 μCi/ml) in buffer containing 0.32 M sucrose, 10 mM Hepes, 5 mM ATP, pH 7.4, with or without 2 μM reserpine.

Since the stock reserpine solution was in dimethylsulfoxide, corresponding amounts of dimethylsulfoxide that never exceeded 0.1% were added to the samples not receiving the drug.

At the end of the incubation with [^{3}H]NE, the samples were immediately diluted with ice-cold immunoprecipitation buffer and mixed with the antibody-coated Immunobeads for immunoprecipitation as described in the following section.

Preparation of Monoclonal Antibody Immunobeads and Immunoprecipitation of Organelles

The following murine monoclonal antibodies were used: (*a*) SY38 (Wiedenmann et al., 1985) purchased from Boehringer Mannheim GmbH, Mannheim, FRG; (*b*) M48 (referred to as serum 48 by Matthew et al. [1981]), graciously provided by Dr. L. F. Reichardt; and (*c*) anti-SV2 (Buckley and Kelly, 1985). Anti-SV4 (Caroni et al., 1985), a monoclonal antibody to an extracellular matrix glycoprotein of the elasmobranch electric organ that does not cross-react with mammalian tissue, was used as a control. 1-ml aliquots of 10 mg/ml rabbit anti-mouse Immunobeads were mixed with 3 ml of each antibody (antibody concentration; 10 μg/ml) and incubated with rotation at 4°C overnight. The Immunobeads were washed three times by centrifugation at 12,000 *g* and resuspended to the original volume (10 mg/ml) with immunoprecipitation buffer (PBS containing 10 mM EGTA and 1%

1. *Abbreviations used in this paper*: hGH, human growth hormone; [^{3}H]NE, l-[7,8^{3}H]norepinephrine; Sac, fixed *Staphylococcus aureus*.

BSA, pH 7.4). 100-μl aliquots of monoclonal antibody Immunobeads were then incubated at 4°C for 1 h with 100 μl of either the PC12 postnuclear supernatant or chromaffin granules in immunoprecipitation buffer (final volume, 0.5 ml). At the end of the incubation, the samples were centrifuged at 12,000 *g* for 5 min at 4°C. The pelleted beads were then resuspended, spun 5 min through a 1-ml pad of 30% sucrose in immunoprecipitation buffer, and washed once by resuspension and centrifugation with the same buffer. The final pellets were resuspended in 0.2 ml of PBS containing PMSF and iodoacetamide (0.3 mg/ml, final concentrations). Samples were prepared for hGH assay by freeze-thawing in liquid nitrogen three times. The Immunobeads were then pelleted by centrifugation (12,000 *g* for 5 min at 4°C) and aliquots of the resulting supernatants were assayed for hGH and [^{3}H]NE. In each experiment, sedimentable [^{3}H]NE and hGH were determined by centrifuging (178,000 *g*, 1 h) control samples in which Immunobeads were substituted with corresponding amounts of immunoprecipitation buffer. The pellets were then assayed for [^{3}H]NE or hGH.

Kappa Light Chain Analysis

Cells secreting the kappa light chain immunoglobulin were analyzed on equilibrium density gradients as follows. One confluent 10-cm dish of PC12MPC-11K6 cells was incubated for 30 min with methionine- and cysteine-depleted DME H-21 medium. The medium was then exchanged for 4 ml of the depleted DME H-21 medium, which included 10% FCS and 1 mCi of a mixture of [^{35}S]methionine and [^{35}S]cysteine (Tran^{35}S-label; ICN, Irvine, CA). After 5 h, the labeled cells were treated as described under Subcellular Fractionation.

Fractions from the equilibrium density gradient were analyzed for kappa light chain by immunoprecipitation, SDS-PAGE, and fluorography. An aliquot of 450 μl from each fraction of the gradient was combined with 10 ml of detergent buffer containing 1% NP-40, 0.4% deoxycholate, 66 mM EDTA, and 10 mM Tris-HCl, pH 7.4 (NDET). SDS was added to 0.3% wt/vol. The samples were then preincubated with fixed *Staphylococcus aureus* (Sac) for 30 min. The Sac was pelleted and the antikappa light chain antibody was added to the supernatants. After an overnight incubation at 4°C, the immune complexes were recovered with Sac. The Sac was resuspended and pelleted through a 30% sucrose wt/vol in NDET plus 0.3% SDS pad. The pellets were then washed again twice with NDET plus SDS buffer. The immune complexes were eluted from the Sac by boiling in sample buffer containing β-mercaptoethanol. The immunoprecipitates were analyzed by SDS-PAGE (Laemmli, 1970). The gels were impregnated with 1 M sodium salicylate for 30 min, dried, and fluorographed at −70°C using preflashed Kodak X-OMAT film. The kappa light chain was quantitated by scanning the gels on a soft laser densitometer (LKB Instruments, Inc., Gaithersburg, MD).

Electron Microscopy

Immunoprecipitation of vesicles from purified chromaffin granule suspensions and PC12 postnuclear supernatants was performed essentially as described above, except that Dynabeads M450 coated with goat anti-mouse IgG were used instead of the polyacrylamide rabbit anti-mouse Immunobeads used in the biochemical experiments. The magnetic beads were easier to fix and embed than the polyacrylamide beads. Monoclonal antibody-coated magnetic beads were prepared by mixing 50 μl of 30 mg/ml Dynabeads M450 with 0.7-ml aliquots of each antibody (antibody concentration, 10 μg/ml). The suspensions were incubated at 4°C overnight followed by four washes with ice-cold immunoprecipitation buffer.

600 μg of beads were added to 0.4-ml samples containing either purified chromaffin granules (240 μg protein) or PC12 postnuclear supernatant (50 μg protein) in immunoprecipitation buffer and incubated with rotation at 4°C for 1 h. The beads were then washed four times with ice-cold immunoprecipitation buffer and fixed in 3% glutaraldehyde, 4% sucrose, 0.1 M phosphate buffer, pH 7.4, overnight. Postfixation was performed in 1% OsO_4 and 1.5% ferricyanide for 1.5 h. The samples were dehydrated in a graded series of ethanol followed by embedding in a 50–50 mixture of Poly/Bed 812 embedding media and Araldite epoxy resin 6005 (Polysciences, Inc., Warrington, PA). The sections were cut on a Reichardt Ultracut-E microtome and examined on a JEOL 100B TEM electron microscope.

hGH Assays

Human growth hormone was determined with a radioimmunoassay kit by utilizing a murine monoclonal antibody (Allegro TM hGH Immunoassay System; Nichols Institute, San Juan Capistrano, CA).

Protein Assay

The Amido Schwarz protein assay (Schaffner and Weissman, 1973) was used with BSA as a standard.

ATP Assay

ATP was measured by a fluorometric assay as described by Stanley and Williams (1969). ATP from Sigma Chemical Co. (St. Louis, MO) was used as a standard and fluorescence was measured on a luminometer (LKB-Wallac, Finland).

Results

Immunoprecipitation of Chromaffin Granules

By Western blotting, chromaffin granule preparations contain detectable amounts of the SV2 antigen (Buckley and Kelly, 1985), a synaptic vesicle protein, but not of p38 (Wiedemann and Franke, 1985). Immunoisolation of intact granules has several advantages over Western blotting or protein immunoprecipitation. Since granule contents are measured, the antigen is known to truly be a component of secretory granules, rather than contaminating membranes. A second advantage of assaying contents is that the sensitivity is high since there are many content molecules per membrane protein. Finally, if all the vesicle content is immunoprecipitated, we can conclude that all granules contain at least one copy of the protein in their membranes. To take advantage of this last feature of immunoisolation, all experiments were done in the presence of immunoadsorbant excess.

Chromaffin granules of the adrenal medulla contain catecholamines and ATP, and have a characteristic dense core in electron micrographs. After isolation, they are still capable of catecholamine uptake in the presence of ATP and Mg^{+2} (Kirschner, 1962). We have used this property to label

Table I. Percentage of Sedimentable [^{3}H]NE or hGH Immunoprecipitated

Antigen	Chromaffin granules (percent [^{3}H]NE)	PC12 S1* (percent [^{3}H]NE)	PC12 cells‡ (percent [^{3}H]NE)	PC12pMT:hGH cells‡ (percent hGH)
p38	82.4 ± 5.0	86.5 ± 2.6	80.9 ± 4.1	99.2 ± 5.4
p65	76.6 ± 4.1	53.7 ± 3.8	63.1 ± 0.9	89.3 ± 14.3
SV2	35.6 ± 0.7	64.0 ± 2.8	65.2 ± 6.6	99.2 ± 9.1
Controls	22.8 ± 4.0	9.2 ± 2.9	7.8 ± 3.1	27.8 ± 3.0

Efficiency of immunoprecipitation of [^{3}N]NE and hGH. [^{3}H]NE-labeled purified chromaffin granules, PC12, or PC12pMT:hGH postnuclear supernatants (S1) were incubated with monoclonal antibody-coated Immunobeads as described in Materials and Methods. Samples in which the Immunobeads were substituted with corresponding amounts of immunoprecipitation buffer were used to estimate the sedimentable [^{3}H]NE or hGH content by centrifugation (178,000 *g*, 1 h).
*S1 refers to experiments in which [^{3}H]NE was taken up after cell lysis.
‡ Refers to comparable experiments where [^{3}H]NE was taken up into intact cells. Controls represent immunoprecipitation with the anti-SV4 antibody. (n = 4, ± SD).

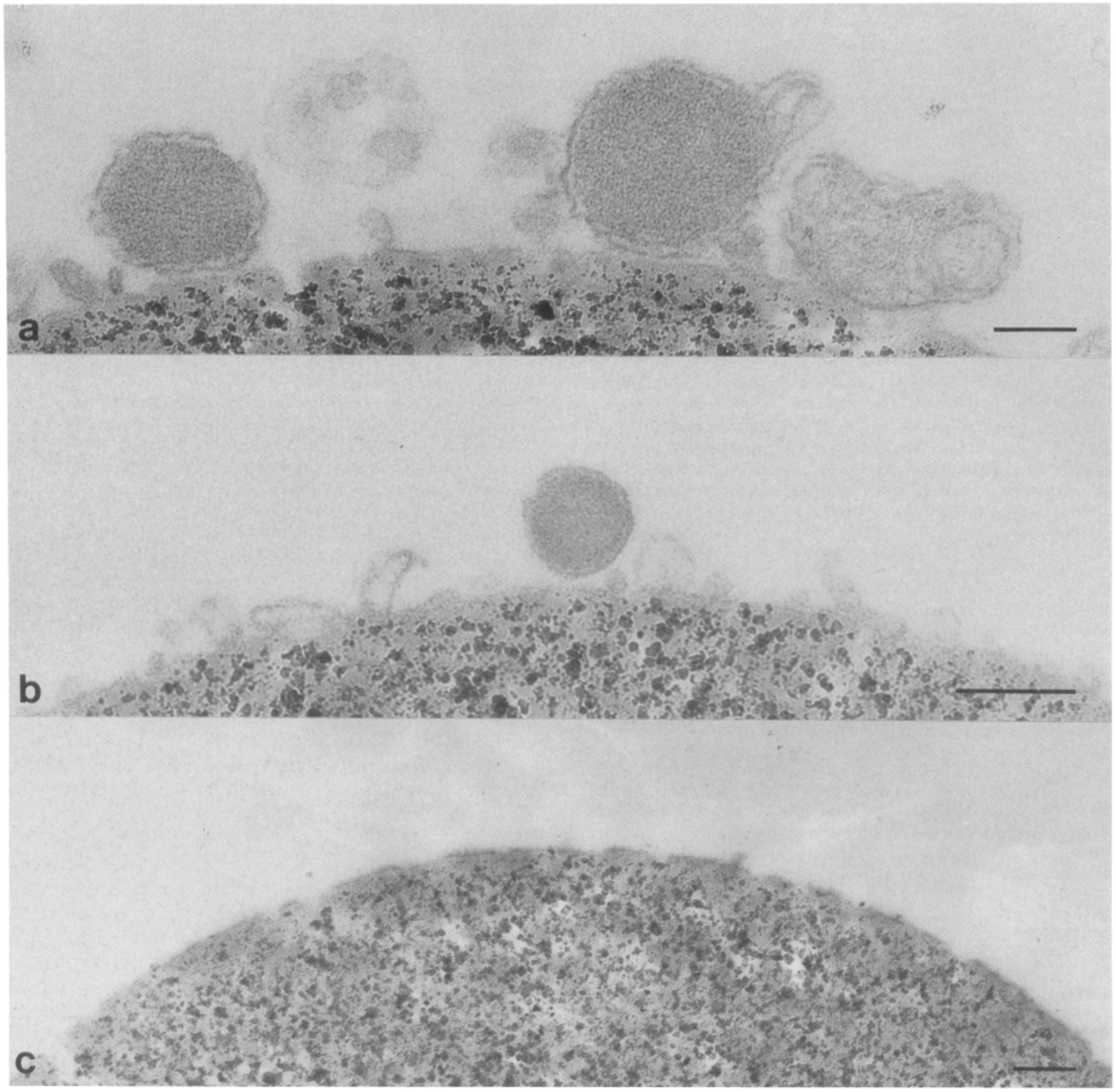

Figure 1. Electron microscopy of immunoprecipitated vesicles. Purified chromaffin granules (*a*) or PC12 postnuclear supernatants (*b*) were incubated with anti-p38 antibody bound to Dynabeads M450 and then processed for electron microscopy as described in Materials and Methods. In both micrographs sectioned beads with attached dense core granules are shown. In PC12 cells (*b*), small clear vesicles (<60 nm) are also immunoprecipitated by the anti-p38 antibody. (*c*) Immunoprecipitation of chromaffin granules with beads prepared with the control antibody. Bars, 100 nm.

chromaffin granules purified from bovine adrenal medullas by the method of Bartlett and Smith (1974). Monoclonal antibodies are available to three highly conserved synaptic vesicle proteins. When bound to a bead, each antibody immunoprecipitated [^{3}H]NE-labeled chromaffin granules. 80% of the total chromaffin granules, measured as sedimentable [^{3}H]NE, were brought down by antibodies to p38 and p65, while antibodies to SV2 brought down 36% (Table I). We can conclude that the majority of the chromaffin granules contained at least one copy of at least two of the synaptic vesicle proteins. We have no explanation of why antibodies to SV2 are less effective in this case; they are equally effective in other situations (see below).

Electron microscopy provided morphologic evidence that the immunoprecipitated granules had dense cores. Electron microscopy of the immunoprecipitated fractions revealed 200–400-nm dense core vesicles characteristic of chromaffin granules (Fig. 1 *a*). Immunoprecipitation with control antibodies resulted in no visible membranous particles bound to the beads (Fig. 1 *c*). Specific binding of dense core vesicles

to the beads was seen for all three of the monoclonal antibodies examined (only micrographs for the antibody to p38 are shown). Membranous structures that lacked dense cores may have been the result of disruption of chromaffin granules during the preparation.

Immunoprecipitation of NE-containing Vesicles from PC12 Cells

Similar experiments have been performed using the PC12 cell line derived from a rat pheochromocytoma. This cell line has retained many of the characteristics of the adrenal chromaffin cell including the ability for synthesis, storage, uptake, and release of catecholamines. It can also be induced to express neuronal-like properties by exposure to nerve growth factor (Greene and Tischler, 1976, 1982; Greene and Rein, 1977; Schubert and Klier, 1977). PC12 cells were incubated with [^{3}H]NE to allow uptake into secretory granules. The cells were then washed, homogenized, and centrifuged to remove the nuclear material. The postnuclear supernatant was then mixed with polyacrylamide beads coated with the different monoclonal antibodies. Specific isolation of [^{3}H]NE radioactivity was seen for all antibodies to synaptic vesicle proteins (Table I). In contrast to the results obtained with chromaffin granule preparations, in PC12 homogenates the anti-SV2 antibody was nearly as effective in immunoprecipitating [^{3}H]NE from PC12 postnuclear supernatants as antibodies to p38 and p65. Earlier studies with antibody to p65 gave similar results (Matthew et al., 1981).

Two criteria were used to verify that the [^{3}H]NE radioactivity was indeed in secretory vesicles; sensitivity of uptake to reserpine and release of contents on stimulation. Reserpine is a selective inhibitor of catecholamine uptake into secretory vesicles (Slotkin et al., 1973). To show inhibition, we measured uptake into vesicles in vitro (Rebois et al., 1980). The postnuclear supernatant from PC12 cells was incubated with [^{3}H]NE in the absence or presence of 2 μM reserpine before immunoprecipitation. The presence of reserpine resulted in a >90% decrease in immunoprecipitated counts when compared with the control (Fig. 2). Similar results were obtained if the [^{3}H]NE was taken up into whole cells in the presence or absence of reserpine before homogenization (data not shown). To demonstrate that the immunoprecipitated [^{3}H]NE was contained in a releasable compartment, cells loaded with [^{3}H]NE were stimulated to secrete with 5 mM carbachol or 50 mM KCl. Carbachol released 20% of stored [^{3}H]NE while 50 mM KCl released 47% (data not shown). When recovery of [^{3}H]NE in immunoprecipitable material was analyzed, stimulation with carbachol resulted in a 15–22% decrement, while 50 mM KCl resulted in a 44–47% decrease for all three antibodies (Fig. 3). Thus, all three antibodies recognize vesicles with a reserpine-sensitive catecholamine uptake. Furthermore, the release of 20–40% of the vesicle content by exocytosis is reflected in a similar loss of immunoprecipitable radioac-

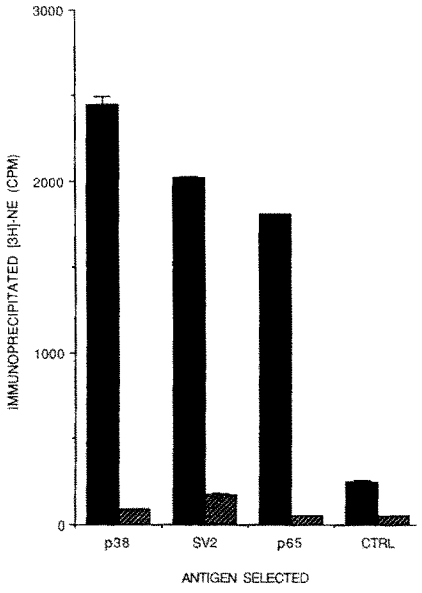

Figure 2. Immunoprecipitation of [^{3}H]NE from PC12 cells. Parallel aliquots of PC12 postnuclear supernatants were incubated with [^{3}H]NE in the presence (▨) or absence (■) of 2 μM reserpine. Immunoprecipitations were then carried out essentially as described in Materials and Methods, except that reserpine (2 μM, final concentration) was included in the immunoprecipitation buffer. Each bar represents the average of duplicate samples in one of two experiments and the error bars represent the range. Control (*CTRL*) represents immunoprecipitation with the anti-SV4 antibody.

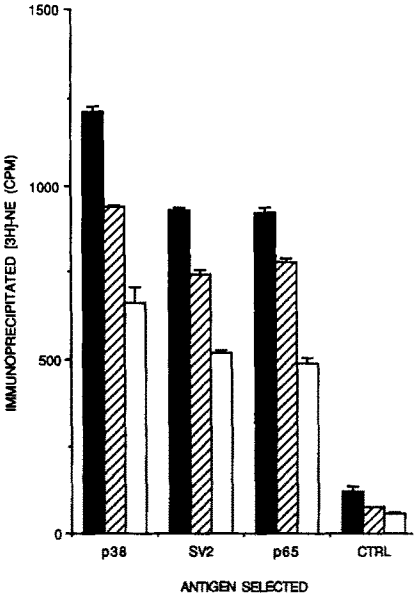

Figure 3. Immunoprecipitation of [^{3}H]NE from PC12 cells after release is stimulated with either carbachol or 50 mM KCl. [^{3}H]NE-loaded cells were pelleted and resuspended in Krebs-Ringer medium (10 × 10^6 cells/ml). The cell suspension was then split into three equal aliquots, two of which received either 5 mM carbachol (▨) or 50 mM KCl (□), while the other served as a control (■). At the end of the stimulation period, each sample was diluted threefold with ice-cold homogenization buffer (0.32 M sucrose, 10 mM Hepes, pH 7.4), homogenized, and centrifuged at 10,000 *g* for 5 min at 4°C. Parallel aliquots of the resulting postnuclear supernatant (volume, 100 μl; protein concentration, 0.5 mg/ml) were then immediately mixed with the monoclonal antibody Immunobeads and immunoprecipitations were carried out as described in Materials and Methods. Each bar represents the average of duplicate samples in one of two experiments and the error bars represent the range. Controls (*CTRL*) represent immunoprecipitation with the anti-SV4 antibody.

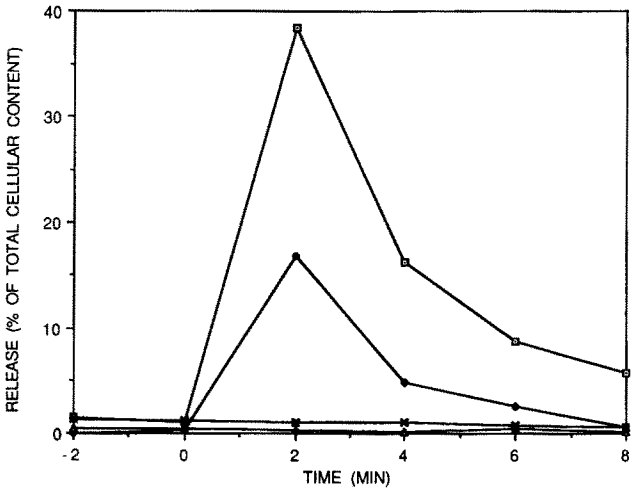

Figure 4. Release of [^{3}H]NE and hGH from PC12pMT:hGH cells. [^{3}H]NE-loaded PC12pMT:hGH cells were washed five times with fresh medium. Beginning at t = 0 min, parallel dishes received medium without ([^{3}H]NE, ■; hGH, △) or with ([^{3}H]NE, ⊡; hGH, x) 50 mM KCl. The media were changed every 2 min and assayed for [^{3}H]NE and hGH. At the end of the stimulation period, the amounts of [^{3}H]NE and hGH remaining in the cells were measured. Secretion is expressed as the percent of total cellular content of [^{3}H]NE (300,000 cpm/dish) and hGH (275 ng/dish).

tivity. We conclude that the antibodies recognize vesicles that take up catecholamine selectively and release it on stimulation.

Immunoprecipitation of Protein-containing Vesicles

Since there are two types of catecholamine-containing vesicles, vesicles that contain and vesicles that lack soluble protein content, it was important to determine whether protein-containing vesicles were immunoprecipitated by antibodies to synaptic vesicle membrane proteins. For these studies, a clone of PC12 was used that had been transfected with DNA encoding hGH. The clone used in this work, PC12pMT:hGH, was similar to the previously described transfected cells (Schweitzer and Kelly, 1985) except that the expression of hGH was under the control of a metallothionein promoter, which resulted in significantly higher expression of hGH than before. To show that the PC12pMT:hGH clone also released stored hGH and norepinephrine in response to stimulation, PC12pMT:hGH cells incubated with [^{3}H]NE were stimulated to secrete by exposure to medium containing 50 mM KCl. The medium was then assayed for [^{3}H]NE and hGH. The stimulated release was virtually complete within 8 min (Fig. 4), demonstrating that both NE and hGH were in secretory vesicles of the regulated type and are released with apparently identical kinetics. The presence of hGH in dense secretory granules was confirmed using an equilibrium density gradient sedimentation procedure described by Wagner (1985). Fractionation of postnuclear supernatants from PC12pMT:hGH cells on 1–40% Ficoll gradients yields a dense secretory granule fraction containing a high specific activity for [^{3}H]NE, ATP, and hGH (Fig. 5 *a*).

The secretory granule peak was well separated from the bulk of protein in the fraction (Fig. 5 *b*). It was also well separated from other intracellular membrane fractions. This was shown using another clone of PC12 cells, PC12MPC-11K6, which had been transfected with DNA encoding the kappa light chain immunoglobulin. This protein, which is excluded from secretory granules, is a marker for the constitutive pathway (Matsuuchi, L., and R. B. Kelly, unpublished observations). The kappa chain would be expected to be localized mostly in endoplasmic reticulum and Golgi vesicles. When the distribution of intracellular kappa chain was examined on a parallel gradient (Fig. 5 *b*), it was found in vesicles of lighter density than hGH-containing vesicles. We conclude that at least some of the intracellular hGH is in dense secretory granules.

To ask if membrane vesicles containing hGH had detectable amounts of synaptic vesicle membrane proteins, PC12-pMT:hGH cells were labeled with [^{3}H]NE and homogenized. Vesicles from the postnuclear supernatant were immunopre-

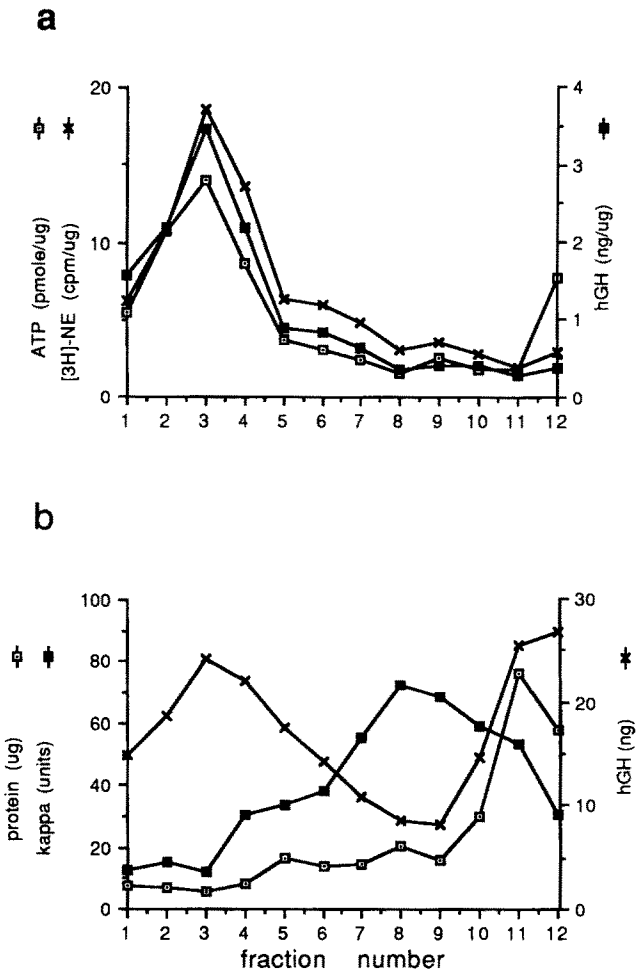

Figure 5. Equilibrium density gradients of extracts of hGH-secreting PC12 (PC12pMT:hGH) and kappa-secreting PC12 (PC12MPC11K6) cells. 1 ml of postnuclear supernatant (0.38 mg) was loaded onto a 1–40% Ficoll gradient and centrifuged for 16 h at 35,000 *g*. 1-ml fractions were collected from the bottom (fraction *1*) and assayed for hGH, ATP, [^{3}H]NE, and protein. (*a*) Values are expressed as the specific activity (absolute amount per total micrograms protein in each fraction). (*b*) Fractionation of PC12MPC-11K6 superimposed on that of PC12pMT:hGH. Values are expressed as absolute amounts. The amount of the immunoglobulin light chain kappa was determined as described in Materials and Methods.

cipitated with the monoclonal antibodies and assayed for hGH. Greater than 95% of the sedimentable hGH could be isolated (Table I). Thus, virtually all of the hGH is associated with vesicles possessing at least one copy of each of the three synaptic vesicle membrane proteins. Immunoprecipitates from homogenates of [^{3}H]NE-labeled PC12pMT:hGH cells contained as large a fraction of the sedimentable radioactivity as untransfected cells (data not shown).

To demonstrate that the population of hGH-containing vesicles attached to the immunoadsorbent included vesicles capable of secretion, homogenates from cells stimulated with 50 mM KCl were compared with unstimulated controls (Fig. 6). Stimulation of exocytosis resulted in a 30% decrease in the hGH that could be immunoprecipitated, comparable to the decrease in stored hGH in whole cells (Fig. 4). Thus, secreted protein must have been contained within secretory granules that possess the synaptic vesicle membrane proteins.

The Immunoadsorbed Vesicles in PC12 Cells Include Dense Core Vesicles

Morphologic correlation was again obtained to demonstrate that the immunoprecipitated vesicle population from PC12 cells included the dense core vesicle. Dense core vesicles of up to 100–200 nm in diameter were found attached to the beads in electron micrographs of the immunoadsorbed fractions (Fig. 1 *b*). Also seen attached to the beads were many smaller nondense core vesicles of 30–60 nm, presumably representing the presence of the vesicle proteins in membranous structures other than secretory granules.

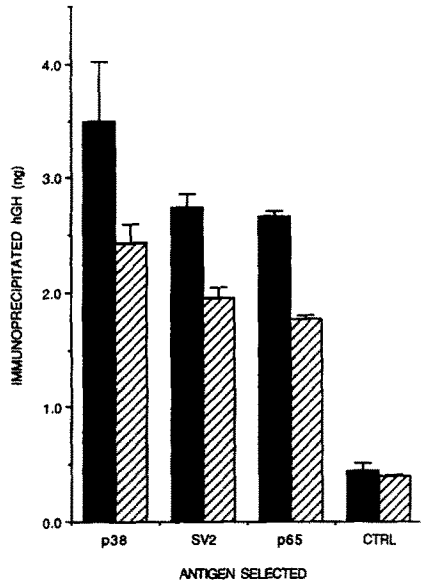

Figure 6. Immunoprecipitation of hGH from PC12pMT:hGH cells after stimulation of release with 50 mM KCl. Cell pellets were resuspended in Krebs-Ringer medium (15 × 16^6 cells/ml), split into two equal aliquots, and incubated at 37°C for 10 min with (▨) or without (■) 50 mM KCl. The experiment was then carried out as described in the legend to Fig. 4. At the end of the immunoprecipitation, aliquots of each sample were assayed for hGH. Each bar represents the average of two experiments and the error bars represent the range. Controls (*CTRL*) represent immunoprecipitation with the anti-SV4 antibody.

Discussion

Immunoisolation of intact vesicles has demonstrated that the synaptic vesicle membrane proteins, p38, p65, and SV2, are all present in the membranes of dense core secretory granules from bovine adrenal medulla and rat pheochromocytoma PC12 cells. Using a biochemical approach, [^{3}H]NE containing-vesicles were isolated from both the purified chromaffin granule preparation and the postnuclear supernatant of PC12 cells. In PC12 cells, the immunoisolated vesicles possessed two of the expected characteristics of the mature secretory vesicle, namely, a catecholamine uptake system that is reserpine sensitive, and the ability to release norepinephrine by exocytosis in response to an external stimulus. Isolation of secretory granules from a clone of PC12 cells that express human growth hormone verified that all three synaptic vesicle proteins were present in granules that contain secretory proteins. When the organelles attached specifically to the immunoadsorbent were examined by electron microscopy, chromaffin granules from the adrenal medulla and dense core vesicles (100–200 nm diameter) from PC12 cells were seen. The failure to observe p38 in dense core secretory granules by immunoelectron microscopy (Navone et al., 1986) can be attributed to the higher sensitivity of the immunoisolation technique. Since almost all the secretory granules could be brought down by antibodies to each of the three proteins, most granules would contain one copy or more of each protein. Thus, despite the differences in the life cycle of secretory granules in endocrine cells and synaptic vesicles in neural cells, both types of secretory vesicle have the same three synaptic vesicle proteins in their membranes.

Although the synaptic vesicle proteins are clearly present in secretory granules, they are not restricted to them. The electron microscopic observations of Navone et al. (1986) demonstrated the presence of large amounts of p38 in membranes of electron-lucent vesicles. Immunoisolated fractions from PC12 cells revealed many membrane structures smaller than secretory granules bound to the immunoabsorbent (Fig. 1 *b*). Immunofluorescent studies with antibodies against SV2 and p38 detect considerable expression in juxtanuclear Golgi regions of endocrine cells and of immature neurons (Buckley and Kelly, 1985; Navone et al., 1986). The Golgi region would be expected to contain newly synthesized p38, and, if the secretory granule membrane is recycled, vesicle antigens returning from the plasma membrane. A recent study by Patzak and Winkler (1986) demonstrated that a granule membrane protein, gpIII, inserted into the plasma membrane during stimulated secretion was recycled via coated and smooth vesicles first to the Golgi region and then into nascent secretory granules.

Both the immunofluorescence and the electron microscopic data suggest that the majority of the vesicle antigens in endocrine cells are not in dense core granules. Combining the results of this study and that of Navone et al. (1986) suggests that the vesicle antigens are much more concentrated in small electron-lucent vesicles than they are in dense core vesicles. Unfortunately the origin of the small electron-lucent vesicles is unknown. If the small vesicles are generated by endocytosis of secretory granule membrane (Fig. 7 *a*), there must be a concentration of vesicle proteins at some point. Evidence for concentration of a membrane protein in membrane domains of Golgi-associated and endocytic vesi-

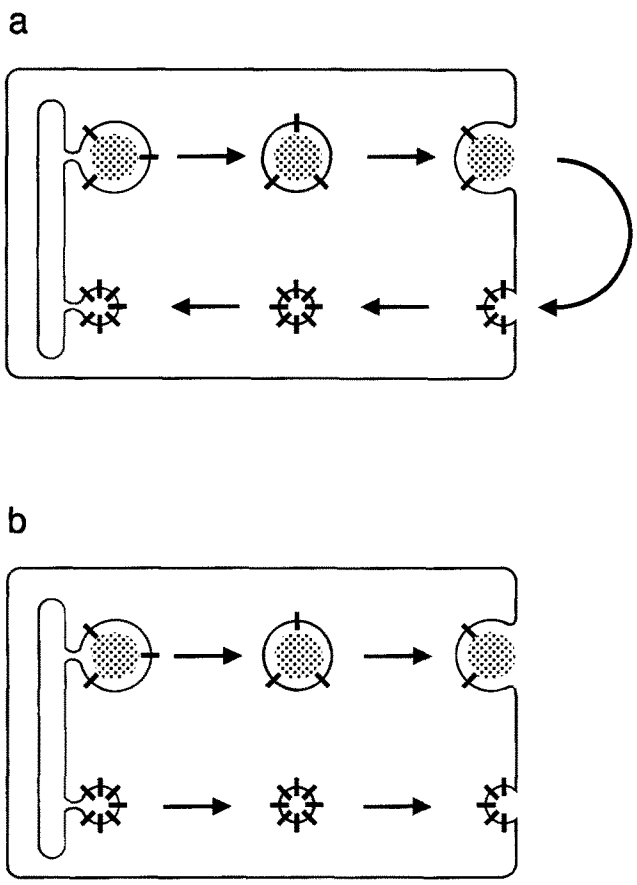

Figure 7. Proposed models for synaptic vesicle biogenesis. (*a*) Recycling model in which synaptic vesicles are generated from dense core granule membranes by exocytosis. (*b*) Model in which synaptic vesicles are generated by a pathway independent of that used for the generation of dense core secretory granules. See Discussion for details.

cles has been obtained in the case of the asialoglycoprotein reeptor (Geuze et al., 1987). Alternatively, the small vesicles could transport newly synthesized vesicle proteins either by a constitutive pathway or by another regulated pathway distinct from that carrying mature vesicles (Navone et al., 1986). In the latter case, sorting, if present, must be inefficient since the data presented here argue for the presence of vesicle proteins in dense core granules (Fig. 7 *b*). If the small vesicles are generated by endocytosis, then the route taken to the plasma membrane, constitutive or regulated, might be of no major physiological significance.

The morphological and immunological similarity between the small electron-lucent vesicles in endocrine cells and synaptic vesicles in neurons suggested to Navone et al. (1986) that endocrine cells have a separate secretory pathway analogous to that used by neurons. An alternative possibility seems to us equally attractive. The small electron-lucent vesicles in endocrine cells could be endocytotic, and their similarity to neuronal synaptic vesicles is because synaptic vesicles can arise by endocytosis of dense core vesicle membranes. The possibility that dense core secretory vesicles give rise to synaptic vesicles has already been suggested for catecholaminergic neurons. Sympathetic nerve terminals contain two populations of catecholamine-containing vesicles, a large population of small vesicles that lack internal protein, and a small population of large vesicles that contain internal soluble proteins. Both vesicles have dopamine β-hydroxylase in their membranes (Neuman et al., 1984). When axonal transport is blocked, however, the majority of the vesicles that accumulate proximal to the block are of the large type. This has led to the frequent suggestion that the dense core vesicles are converted to small vesicles in the nerve terminal (Geffen et al., 1969; Tomlinson et al., 1975; Thureson-Klein, 1983). The number of both large and small catecholamine-containing vesicles is reduced on nerve stimulation, and small electron-lucent vesicles increase in number (Thureson-Klein, 1983), suggesting that conversion may require exocytosis and endocytosis. Since dense core vesicles are also found in many types of nerve terminals other than catecholaminergic ones, it is possible that their membranes are always precursors of synaptic vesicles. A recycling model of this type also suggests a plausible link between endocrine and neuronal cells; recovery of secretory granule membrane by endocytosis could be adapted by neurons so that instead of recycling to the Golgi apparatus, the endocytotic membranes remain at the periphery as synaptic vesicles. In such a scheme, the Golgi region of the immature neuron is rich in synaptic vesicle proteins, and maturation triggers accumulation of vesicle proteins at the nerve terminal in small endocytotic vesicles that are inhibited from returning to the Golgi region.

The authors wish to express their indebtedness to Debbie Crumrine for her assistance with the electron microscopy, to Dr. Daniel Cutler (Imperial College, London), who participated in some of the early experiments, to Dr. Erik Schweitzer (University of Wisconsin, Madison, WI) and Dr. Linda Matsuuchi (University of California at San Francisco) for allowing use of their PC12 clones before publication, to Leslie Spector for her aid with the manuscript, and to the members of the Kelly laboratory for their many constructive suggestions.

This work was funded by grants from the National Institutes of Health (NS09878, DK33937, and NS15927) to Dr. Regis B. Kelly. The immunoelectron microscopy was funded by a Program Project grant (NS16033). Dr. A. W. Lowe acknowledges the support of a Physician-Scientist Award (AM01458-02) and Dr. Luisa Madeddu was supported by a European Molecular Biology Organization fellowship (86-1986).

Received for publication 29 July 1987, and in revised form 8 October 1987.

References

Anderson, D. J., and R. Axel. 1986. A bipotential neuroendocrine precursor whose choice of cell fate is determined by NGF and glucocorticoids. *Cell.* 47:1079–1090.

Bartlett, S. F., and A. D. Smith. 1974. Adrenal chromaffin granules: isolation and disassembly. *Methods Enzymol.* 31:379–389.

Buckley, K., and R. B. Kelly. 1985. Identification of a transmembrane glycoprotein specific for secretory vesicles of neural and endocrine cells. *J. Cell Biol.* 100:1284–1294.

Caroni, P., S. S. Carlson, E. Schweitzer, and R. B. Kelly. 1985. Presynaptic neurones may contribute a unique glycoprotein to the extracellular matrix at the synapse. *Nature (Lond.).* 314:441–443.

Ceccarelli, B., W. P. Hurlbut, and A. Mauro. 1973. Turnover of transmitter and synaptic vesicles at the frog neuromuscular junction. *J. Cell Biol.* 57: 499–524.

Floor, E., and S. E. Leeman. 1985. Evidence that large synaptic vesicles containing substance p and small synaptic vesicles have a surface antigen in common in rat. *Neurosci. Lett.* 60:231–237.

Geffen, L. B., and A. Ostberg. 1969. Distribution of granular vesicles in normal and constricted sympathetic neurones. *J. Physiol. (Lond.).* 204:583–592.

Geuze, H. J., J. W. Slot, and A. L. Schwartz. 1987. Membranes of sorting organelles display lateral heterogeneity in receptor distribution. *J. Cell Biol.* 104:1715–1723.

Greene, L. A., and G. Rein. 1977. Release, storage and uptake of catecholamine by a clonal cell line of nerve growth factor (NGF) responsive pheochromocytoma cells. *Brain Res.* 129:247–263.

Greene, L. A., and A. S. Tischler. 1976. Establishment of a noradrenergic

clonal line of rat adrenal pheochromocytoma cells which respond to nerve growth factor. *Proc. Natl. Acad. Sci. USA.* 73:2424–2429.

Greene, L. A., and A. S. Tischler. 1982. PC12 pheochromocytoma cultures in neurobiological research. *Adv. Cell. Neurobiol.* 3:373–414.

Gumbiner, B., and R. B. Kelly. 1982. Two distinct intracellular pathways transport secretory and membane glycoproteins to the surface of pituitary tumor cells. *Cell.* 28:51–59.

Heuser, J. E., and T. S. Reese. 1973. Evidence for recycling of synaptic vesicle membrane during tramsmitter release at the frog neuromuscular junctions. *J. Cell Biol.* 57:315–344.

Jahn, R., W. Schiebler, C. Ouimet, and P. Greengard. 1985. A 38,000-dalton membrane protein (p38) present in synaptic vesicles. *Proc. Natl. Acad. Sci. USA.* 82:4137–4141.

Kelly, R. B. 1985. Pathways of protein secretion in eukaryotes. *Science (Wash. DC).* 230:25–32.

Kirschner, N. 1962. Uptake of catecholamines by a particulate fraction of the adrenal medulla. *J. Biol. Chem.* 237:2311–2317.

Laemmli, U. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature (Lond.).* 227:680–685.

LeRoith, D., J. Schiloach, and J. Roth. 1982. Is there an earlier phylogenetic precursor that is common to both the nervous and endocrine system? *Peptides (Fayeteville).* 3:211–215.

Matthew, W. D., L. Tsavaler, and L. F. Reichardt. 1981. Identification of a synaptic vesicle-specific membrane protein with a wide distribution in neuronal and neurosecretory tissue. *J. Cell Biol.* 91:257–269.

Navone, F., R. Jahn, G. D. Gioia, H. Stukenbrok, P. Greengard, and P. DeCamilli. 1986. Protein p38: an integral membrane protein specific for small vesicles of neurons and neuroendocrine cells. *J. Cell Biol.* 103:2511–2527.

Neuman, B., C. J. Wiedermann, R. Fischer-Colbrie, M. Schober, G. Sperk, and H. Winkler. 1984. Biochemical and functional properties of large and small dense-core vesicles in sympathetic nerves of rat and ox vas deferens. *Neuroscience.* 13:921–931.

Palmiter, R. D., G. Norstedt, R. E. Gelinas, T. E. Hammer, and R. L. Brinster. 1983. Metallothionein-human GH fusion genes stimulate growth of mice. *Science (Wash. DC).* 222:809–814.

Patzak, A., and H. Winkler. 1986. Exocytic exposure and recycling of membrane antigens of chromaffin granules: ultrastructural evaluation after immunolabeling. *J. Cell Biol.* 102:510–515.

Rebois, R. V., E. E. Reynolds, L. Toll, and B. D. Howard. 1980. Storage of dopamine and acetylcholine in granules of PC12, a clonal pheochromocytoma cell line. *Biochemistry.* 19:1240–1248.

Schaffner, W., and C. Weissmann. 1973. A rapid, sensitive and specific method for the determination of proteins in dilute solutions. *Anal. Biochem.* 56:502–514.

Schubert, D., and F. G. Klier. 1977. Storage and release of acetylcholine by a clonal cell line. *Proc. Natl. Acad. Sci. USA.* 74:5184–5188.

Schweitzer, E. S., and R. B. Kelly. 1985. Selective packaging of human growth hormone into synaptic vesicles in a rate neuronal (PC12) cell line. *J. Cell Biol.* 101:667–676.

Slotkin, T. A. 1973. Hypothetical model of catecholamine uptake into adrenal medullary vesicles. *Life Sci.* 13:675–683.

Stanley, P. E., and S. G. Williams. 1969. Use of the liquid scintillation spectrometer for determining adenosine triphosphate by the luciferase enzyme. *Anal. Biochem.* 29:381–392.

Thureson-Klein, A. 1983. Exocytosis from large and small dense core vesicles in noradrenergic nerve terminals. *Neuroscience.* 10:;245–252.

Tomlinson, D. R. (1975). Two populations of granular vesicles in constricted post-ganglionic sympathetic nerves. *J. Physiol. (Lond.).* 245:727–735.

Wagner, J. A. 1985. Structure of catecholamine secretory vesicles from PC12 cells. *J. Neurochem.* 45:1244–1253.

Wiedenmann, B., K. Lawley, C. Grund, and D. Branton. 1985. Solubilization of proteins from bovine brain coated vesicles by protein perturbants and Triton X100. *J. Cell Biol.* 101:12–18.

Wiedenmann, B., and W. W. Franke. 1985. Identification and localization of synaptophysin, an integral membrane glycoprotein of M_r 38,000 characteristic of presynaptic vesicles. *Cell.* 41:1017–1028.