

Selective Packaging of Human Growth Hormone into Synaptic Vesicles in a Rat Neuronal (PC12) Cell Line

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ABSTRACT We have introduced the gene for human growth hormone (hGH) into PC12 cells, a rat pheochromocytoma-derived cell line with neuronal characteristics, and have isolated stable cell lines that express this protein. hGH is stored within the cells in membrane-bounded vesicles that are indistinguishable from the endogenous catecholaminergic synaptic vesicles. When the transfected cells are stimulated by carbachol or direct depolarization, they release norepinephrine and hGH with parallel kinetics. Treatment of the transfected cells with nerve growth factor results in a twofold increase in the amounts of hGH stored in and secreted from the cells. Not all proteins are packaged into the synaptic vesicles, since the rate of release of laminin, a soluble secreted protein endogenous to PC12 cells, is not stimulated by carbachol. This neuronal cell line therefore possesses at least two distinct pathways for secretion and can selectively package a foreign endocrine hormone into the regulated pathway.

All nucleated cells secrete cellular products into the extracellular medium. This function is highly developed in some cells, notably granulocytes, endocrine, exocrine, and neuronal cells, in which the secretory process is regulated by physiological stimuli. In all these cells, proteins or small molecules are stored within specialized secretory vesicles and are released from the cell by fusion of the vesicle membrane with the plasma membrane. However, some details of this process differ between these cell types. Neurons, for example, package different compounds into vesicles than do endocrine cells, transport these vesicles great distances from the cell body, and release them at specialized synaptic sites. We do not know whether all these cells use similar molecular machinery to carry out these secretory processes, or whether each has developed distinct pathways. If similar machinery is needed by various cells, one would predict that secretory vesicles of different cell types would share common components. However, analysis of the major proteins of adrenal chromaffin granules, zymogen granules from the exocrine pancreas, and serotonin granules from platelets revealed no components that were common to all vesicles (31). In contrast, immunological analysis has identified two antigenic components that are shared between the secretory vesicles of endocrine and neuronal cells (2, 24).

The classic way to show that different cell types carry out a cellular process with similar molecular machinery is to recon-

stitute the process *in vitro* and interchange components from different origins. In this way, for example, the machinery for translocating newly synthesized secretory proteins across rough endoplasmic reticulum membranes was shown to be universal (39). More recently, it has been possible to show similarities in molecular mechanisms by introducing heterologous gene products by DNA transfection. Thus, when African green monkey kidney cells (11), L cells (20), or COS cells (18) are transfected with DNA encoding proinsulin, the cells secrete proinsulin into the medium constitutively, that is, without storage and in the absence of an external signal (11, 20). When similar plasmids are transfected into a mouse pituitary cell line, AtT-20, that stores adrenocorticotrophic hormone (ACTH)¹ in secretory vesicles, the proinsulin is stored in the secretory vesicles and proteolytically cleaved. When the cells are stimulated to release stored ACTH, they also release stored insulin (27). A similar capacity for storage and regulated secretion was observed when GH4 cells were transfected with DNA encoding preproparathyroid hormone (13). These experiments showed that African green monkey

¹ *Abbreviations used in this paper:* ACTH, adrenocorticotrophic hormone; DME, Dulbecco's modified Eagle's medium; G418, geneticin; hGH, human growth hormone; N-det, buffer containing 1% Nonidet P-40, 0.4% deoxycholate, 66 mM EDTA, and 10 mM Tris-HCl, pH 7.4; NE, norepinephrine; NGF, nerve growth factor; SAC, *Staphylococcus aureus* cells.

kidney, COS, and L cells lack the machinery to store and process proinsulin, whereas in pituitary-derived secretory cells, the molecular machinery for packaging endogenous hormones into secretory vesicles can recognize peptides from different cell types and different species. The capacity for proteolytic processing is not limited to specialized secretory cells, since preprosomatostatin and preproparathyroid hormone are processed to mature hormones in COS cells (40) and 3T3 cells (13), respectively.

In this paper, we extend the DNA transfection approach to look for similarities between endocrine and neuronal cells. We have developed procedures for transfecting DNA encoding an endocrine product, human growth hormone (hGH), into a rat pheochromocytoma cell line (PC12) that has neuronal properties. These include the ability to synthesize and package norepinephrine (NE) and acetylcholine into synaptic vesicles, sensitivity to NGF (10), and the ability to send out long neuritic processes (21) and to form synapses with cultured myotubes (35). We have therefore used PC12 cells to examine the capacity of cells with a neuronal phenotype to package and secrete a foreign endocrine hormone. Gumbiner and Kelly (12) first reported the coexistence of constitutive and regulated pathways for protein secretion in AtT-20 cells. It has recently been possible to use DNA transfection procedures to demonstrate that there is also a segregation of secreted proteins into these two pathways (reference 3; and Moore, H. P., and R. B. Kelly, personal communication). By studying the kinetics of secretion release of dopamine β -hydroxylase, McHugh et al. (25) have provided evidence for the existence of a regulated and a constitutive pathway in PC12 cells. They suggest that dopamine β -hydroxylase enters both pathways so that a fraction of dopamine β -hydroxylase is secreted constitutively, while the remainder is stored within the cells and released upon stimulation. In addition to demonstrating that PC12 cells can package and secrete a foreign endocrine hormone, we present additional evidence to support the existence of constitutive and regulated secretory pathways in PC12 cells. We also show that PC12 cells can sort secretory proteins selectively into one of these two pathways with a high efficiency.

MATERIALS AND METHODS

Materials and Solutions

Geneticin (G418) was obtained from Gibco Laboratories (Grand Island, NY). The G418 was titrated for the killing of untransfected PC12 cells, and 0.5 mg/ml of active drug was found to be optimal. The drug was mixed with culture media in 100 \times stock solutions, adjusted to neutral pH, sterile filtered, and stored at -20°C for no longer than 2 mo.

The TE buffer used for DNA transfections contained 10 mM Tris, 1 mM EDTA, pH 8.0. HeBS contained 137 mM NaCl, 5 mM KCl, 5.5 mM glucose, 0.8 mM Na_2HPO_4 , and 21 mM HEPES, pH 7.05. The detergent extraction buffer used for determining the hGH content of cells contained 150 mM NaCl, 1.5 mM MgCl_2 , 0.5% Triton X-100, and 10 mM Tris, pH 7.5. Just before use, protease inhibitors were added in the form of a 100 \times stock solution in 100% ethanol of phenylmethylsulfonyl fluoride and iodoacetamide to final concentrations of 300 $\mu\text{g}/\text{ml}$ each. N-det buffer for immunoprecipitations contained 1% Nonidet P40, 0.4% deoxycholate, 66 mM EDTA, and 10 mM Tris-HCl, pH 7.4. Phosphate-buffered saline (PBS) contained 150 mM NaCl and 20 mM NaH_2PO_4 , pH 7.4.

DNA Vectors

The pRSV-CAT (chloramphenicol acetyltransferase) has been described by Gorman et al. (7); pSV2neo:hGH was a generous gift from Dr. Michael Walker (University of California, San Francisco) and consists of a 2.6-kb genomic hGH

sequence (5), including the 5' flanking sequences, inserted into the EcoRI site of pSV2neo (36) in both orientations. Thus, both the selected marker (neo) and the nonselected marker (hGH) were present on the same plasmid.

Cell Culture

PC12 cells, originally described by Greene and Tischler (9), were obtained from University of California (San Francisco) cell culture stocks and grown in Dulbecco's modified Eagle's medium (DME) H-21 + 10% fetal calf serum + 5% horse serum at 37°C in 9.5% CO_2 (pH 7.4). Cells were grown on untreated tissue culture plastic (Falcon Labware, Oxnard, CA) and passaged by treatment with trypsin and EDTA. Nerve growth factor (NGF) was purified from mouse salivary glands by the method of Mobley et al. (26) and added and assayed for biological activity by monitoring the outgrowth of cell processes by PC12 cells. When used in these experiments, NGF (2–10 ng/ml) was added at 10–50 times the amount needed for maximal effects on morphology.

Transfection of PC12 Cells

PC12 cells were transfected by a modification of the calcium phosphate procedure described by Moore et al. (27). On the day before transfection, 3×10^6 PC12 cells were plated onto poly-D-lysine-coated 10-cm plastic tissue culture dishes and allowed to adhere overnight. The plasmid DNA was added to HeBS-rinsed cells in the form of a calcium phosphate precipitate (8) and allowed to settle onto the cells for 20 min at room temperature. DME H-21 + 5% fetal calf serum was then added, and the cells were placed in an incubator for 4–7 h to permit the uptake of the DNA- CaPO_4 precipitate. The cells were then subjected to osmotic shock by incubation in 25% glycerol in DME H-21 for 1 min and then rapidly washed with DME H-21 (without serum). The medium was then switched to standard culture medium, and the cells were replaced in the incubator. The remainder of the transfection was done essentially as described by Moore et al. (27).

The optimal amount of DNA for transfection was determined using a transient expression assay with an RSV-CAT plasmid (7). Expression of CAT was approximately linear up to 25 μg of DNA/ 3×10^6 cells; above this amount, the level of expression plateaus. For stable transfections, 20 μg of DNA/ 3×10^6 cells was routinely used. This amount generally resulted in the selection of 10–30 clones/10-cm dish. For selection of cells transfected with pSV2neo:hGH, medium containing 0.5 mg/ml G418 was added to the cells on the second day after transfection and fed with the same medium as needed. After ~ 3 wk, individual clones were transferred to wells containing medium + 0.25 mg/ml G418 and grown to sufficient density to screen for hGH secretion. No clones grew in dishes that were treated identically except for the omission of DNA in the CaPO_4 precipitate. When the selected clones reached about half confluency, the culture media were assayed for hGH content. Positive clones were maintained in 0.25 mg/ml G418 thereafter.

Cell Stimulations

The standard stimulation protocol was performed on parallel 10-cm dishes of PC12 cells at about one-half confluence. To quantitate stimulated secretion, cells were incubated for several hours in the presence of 1 μCi [^3H]NE (Amersham Corp., Arlington Heights, IL) and then rinsed three times with fresh medium. The cells were incubated in 4 ml of medium \pm 5 mM carbachol, and the medium was changed at 2-min intervals. At the end of the experiment, the cells were rinsed three times with PBS to remove serum and extracted with 4 ml of detergent extraction buffer. Cells were removed from the dish by scraping, freeze-thawed three times, and centrifuged to remove insoluble cell debris. This extract was then diluted and assayed for hGH.

Subcellular Fractionation

Cells were fractionated on a sucrose equilibrium density gradient essentially as described by Schubert and Klier (34). PC12 cells were grown to near-confluency in 15-cm dishes. Several hours before harvesting, fresh medium containing 5 μCi [^3H]NE was added. Cells were removed by rinsing the dish twice with PBS and then adding PBS containing 5 mM Mg-EGTA at 0°C . Cells were removed by trituration and homogenized in a tight-fitting Dounce homogenizer (Kontes Glass Co., Vineland, NJ) with 25 strokes at 0°C . This entire homogenate was layered onto a linear sucrose gradient (0.6–2.0 M) containing 1 mM Mg-EGTA and 10 mM HEPES, pH 7.4. The gradients were centrifuged at 100,000 g for 3 h at 4°C , and the fractions were collected from the bottom of the tube and assayed for protein, [^3H]NE, hGH, and vesicle antigen. Before aliquots of these fractions were assayed for hGH, they were added to an equal volume of detergent extraction buffer, and freeze-thawed once. Sucrose concentration was determined by refractive index.

Immunoprecipitations

PC12/1B6 VESICLES: [³H]NE-labeled cells were removed from the culture dish with PBS + 10 mM EGTA, and homogenized by 10 passes at 0°C in a custom-made homogenizer (EMBL, Heidelberg, FRG), with a 0.012-mm clearance. 100- μ l aliquots of this homogenate were incubated with 2 μ l of culture medium or culture medium containing monoclonal antibodies directed against synaptic vesicles at 0°C for 2 h. The antibodies used were anti-SV2 (2) and anti-P65 (referred to as serum 48 in reference 24). Fixed *Staphylococcus aureus* cells (SAC) (Pansorbin, Calbiochem-Behring Corp., La Jolla, CA) were pretreated with unlabeled, untransfected PC12 cell membranes to reduce non-specific binding and then incubated with the antibody-treated homogenate for 30 min at 0°C. The suspension was then centrifuged 2 min in a Fisher microfuge (12,000 g) (Fisher Scientific Co., Pittsburgh, PA), and the pellet was resuspended in 1 ml PBS containing 1% bovine serum albumin (BSA) and 5 mM EGTA. This was layered on top of 0.5 ml of 30% sucrose in PBS/BSA/EGTA buffer and centrifuged for 5 min in a microfuge. The resulting pellet was resuspended in detergent extraction buffer including phenylmethylsulfonyl fluoride and iodoacetamide, and was assayed for [³H]NE and hGH.

³⁵S-LABELED PROTEINS: Immunoprecipitations from culture media were done by incubating media samples, pretreated with SAC and prespun for 15 min in a microfuge, with the appropriate dilution of antiserum for 12 h at 4°C. Antibodies used were goat anti-hGH (Antibodies Inc., Davis, CA), rabbit anti-laminin serum kindly provided by Janet Winter (University of California, San Francisco), or the corresponding nonimmune goat or rabbit serum. Pre-washed SAC was added to these samples and incubated at 25°C for 15 min. The SAC was then pelleted by spinning 1 min in a microfuge, resuspended in N-det plus 0.3% SDS, and pelleted through 30% sucrose in N-det plus SDS. The resulting pellet was washed once in N-det plus SDS, and once in water, changing tubes at each step. Antigen was solubilized by boiling the final pellet in SDS PAGE sample buffer and was applied directly to the gel for electrophoresis.

SV2 Immunoreactivity

The amount of SV2 antigen in membranes was determined by quantitative dot blotting of fractions essentially as described by Carlson and Kelly (4). Fractions were solubilized in 2% SDS and applied to nitrocellulose filters in a 96-well manifold (Bio-Rad Laboratories, Richmond, CA). The filters were then blocked with 50% fetal calf serum and incubated with monoclonal anti-SV2 and an ¹²⁵I-goat anti-mouse IgG probe.

hGH Assays

hGH was determined with a murine monoclonal radioimmune assay obtained from Hybritech Inc. (La Jolla, CA). This radioimmune assay showed no detectable cross-reactivity to rat growth hormone (unpublished observations). Samples were prepared for hGH assay either by collecting the culture medium or by extracting the cells with detergent. Standard curves were generated in both the presence and absence of detergents.

Protein Assays

Protein concentrations in sucrose gradients were determined by the method of Bradford (1). Cell extracts, which contained detergent, were assayed with the Amido Schwarz assay (33) using BSA as a standard.

SDS PAGE and Autoradiography

Samples were boiled in SDS and β -mercaptoethanol-containing buffer and separated on polyacrylamide gels according to the method of Laemmli (16), using 12% acrylamide gels for hGH and ³⁵S-labeled media, and 4–12% gradient gels for laminin, using Sigma molecular weight markers (MW-SDS-70L and MW-SDS-200, Sigma Chemical Co., St. Louis, MO). For autoradiography, gels were soaked in 1 M salicylate for 1/2 h, dried, and used to expose Kodak X-ray film at -70°C.

RESULTS

Expression and Storage of hGH in Transfected PC12 Cells

Growth hormone is normally expressed and secreted in somatotroph cells of the anterior pituitary (14). These cells

store growth hormone in dense granules contained in the cytoplasm. To examine how a cell line with a neuronal phenotype would secrete this endocrine hormone, we transfected (rat) PC12 cells with a vector containing both the gene for hGH and the selectable marker for neomycin resistance. Selection with G418-containing medium resulted in the isolation of 31 clones. These clones were then screened for their ability to express and secrete hGH by assaying the culture medium with a monoclonal radioimmune assay that specifically detected human but not rat growth hormone (unpublished observations). Thirteen of the clones (42%) expressed detectable levels of hGH. We chose one of these clones (PC12/1B6) for further characterization.

To determine whether the transfected PC12 cells were secreting authentic hGH, we metabolically labeled PC12/1B6 cells with [³⁵S]methionine and [³⁵S]cysteine. The culture medium was collected and immunoprecipitated with goat anti-hGH antibody. As shown in Fig. 1, one major protein was precipitated that co-migrated with the purified hGH standard (22,000 mol wt). A second, minor band was present with an M_r of 2,000 less than that of the major form; this band probably represents the 20,000 form of hGH that is found in human pituitary at a level of ~1/10 the 22,000 form (19). Neither of these bands was present in precipitations carried out with nonimmune serum (lane 1) or in immunoprecipitations of untransfected PC12 cells (data not shown). A third, faint band represents a nonspecifically precipitated protein of M_r 21,000. On other gels, this band is clearly distinct from the two specifically precipitated bands. Densitometry of autoradiograms from two independent collections and immunoprecipitations gave a ratio of 10.6 ± 0.8 for the 22,000 and 20,000 forms secreted by the transfected PC12 cells. Since we have introduced only one gene for hGH into PC12 cells, the present data constitute direct evidence that both 22,000 and 20,000 forms of hGH can be produced from the same gene. It is clear that PC12 cells possess the proper machinery to express and secrete hGH in a form indistinguishable from that produced by the human pituitary.

In previous experiments in which growth hormone has been introduced into fibroblast (6) or monkey kidney cells (28), no storage of the gene product was observed. In contrast, PC12 cells, which store neurotransmitter and release it on stimulation, have the capacity to store hGH (Table I). If we assume that all of the hGH that is synthesized is eventually secreted, then at a steady-state the rate of synthesis should be equivalent to the rate of basal release of hGH. By dividing the total amount of hGH present in cell extracts by the steady-state rate of secretion, we obtained a storage index, which is a measure of the amount of hGH that is stored intracellularly. Thus, in the experiment shown in Table I, PC12/1B6 cells stored substantial amounts of hGH equivalent to the amount synthesized in 137 min. This index is similar to that obtained for all forms of ACTH secretion by the pituitary cell line AtT-20 (Burgess, T., and H.-P. Moore, personal communication) and is higher than that observed for the proinsulin expressed by L cells, which do not store secretory products intracellularly (storage index = 66 min, calculated from data in reference 27). Since intracellular storage is a property of the regulated secretory pathway (37), this result suggested that PC12/1B6 cells were packaging hGH into some form of regulated secretory vesicles.

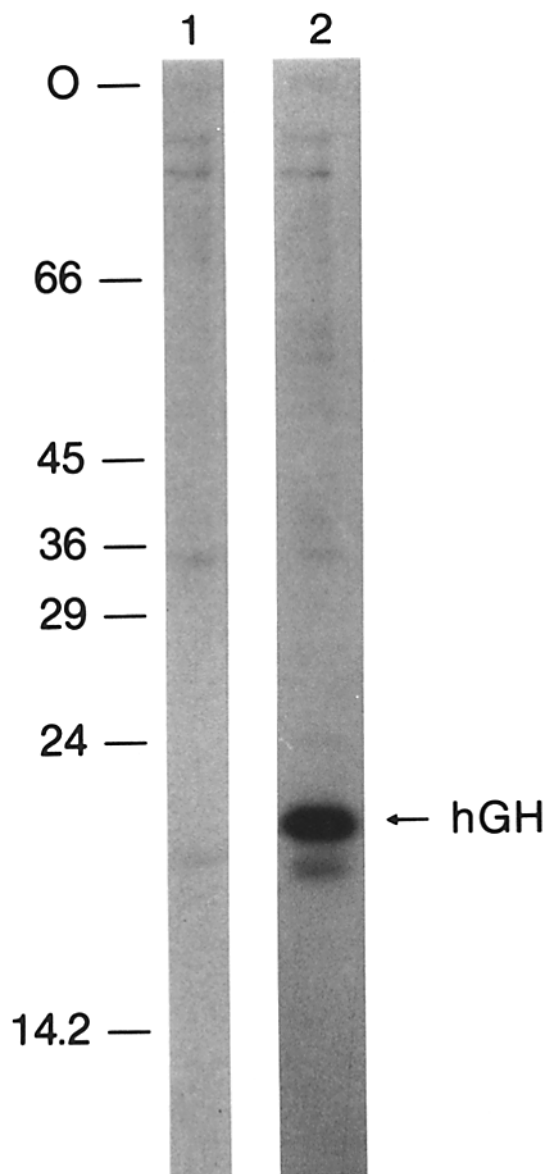


FIGURE 1 Immunoprecipitation of hGH secreted by PC12/1B6 cells. Two 10-cm dishes of PC12/1B6 cells, grown in the absence of NGF, were labeled by incubation in medium containing 1/50 the normal amount of cysteine, 10% dialyzed fetal calf serum, and 0.25 mCi [35 S]cysteine. After 9 h, the medium was removed and precipitated as described in Materials and Methods. After immunoprecipitation, the samples were dissolved in SDS PAGE sample buffer by boiling, and separated on a 12% gel. Purified hGH and molecular weight markers were run in adjacent lanes; arrows indicate the mobility of these standards. After staining the gel with Coomassie Brilliant Blue, the gel was soaked for 30 min in 1 M salicylate, dried, and exposed for autoradiography. Lane 1, precipitation with normal goat serum; lane 2, parallel precipitation with goat anti-hGH.

Release of hGH and NE

If the stored hGH is contained in normal PC12 synaptic vesicles, it should be possible to increase the rate of secretion by treating the cells with physiological secretagogues. Since PC12 cells possess acetylcholine receptors, we used 5 mM carbachol to depolarize the cells. Such treatment produced a 15-fold increase in the rate of release of hGH (Table I). Similar results (data not shown) were obtained by depolarizing the cells with 51 mM KCl.

As judged by morphological criteria (cell flattening and outgrowth of multiple neuritic processes), PC12/1B6 cells were indistinguishable from untransfected control cells in their response to NGF. Table I indicates that NGF also affected the storage and secretion of hGH. Whereas the rate of basal secretion (and presumably synthesis) of hGH was not significantly altered by NGF treatment, the storage capacity of the cells increased more than twofold (storage index = 300). NGF treatment also increased the stimulated release about twofold to more than 25 times the basal rate. Since both the storage and stimulated release of hGH increased in parallel, the additional storage appears to reflect an increased capacity of the NGF-treated cells to package and store hGH in synaptic vesicles. The increase in the efficiency of this storage with NGF treatment suggests that differentiation is accompanied by an increased efficiency of protein sorting into synaptic vesicles. We conclude that at least part of the hGH stored in the cells is in some type of secretory vesicle whose fusion with the plasma membrane can be stimulated by cell depolarization. The properties of this secretion can be contrasted with those observed in the endocrine cell line, AtT-20, in which cAMP causes a stimulation of secretion that is two to five times the basal rate; depolarization of these cells with KCl is even less effective (12, 24).

In contrast to the slow rate of stimulated secretion of hormones by endocrine cells (24), PC12 cells release NE within minutes of stimulation (10). If the pool of vesicles containing hormone differed from the pool that contained neurotransmitter, hGH and NE might be secreted with different kinetics. We found that when NGF-treated PC12/1B6 cells were exposed to 5 mM carbachol, there was a rapid stimulation of NE release that peaked within 2 min (Fig. 2). In spite of the continued presence of secretagogue, the release rate returned to baseline over the next 10 min. Since such a decrease has been observed in the release of transmitter from PC12 cells stimulated with 51 mM KCl (10), this decay of release is most likely due to inactivation of the secretory process rather than repolarization of the cells due to desensitization of the acetylcholine receptor. Both the rise and fall of hGH release paralleled that of [3 H]NE from these same cells (Fig. 2), suggesting that the secretion of both compounds is under similar regulation. In this experiment, the total amount of [3 H]NE released in 10 min was $51.1 \pm 0.2\%$ of the total, whereas $42 \pm 2\%$ of the hGH was released (Fig. 3). The slightly lower fraction of hGH released may reflect its presence in other, nonreleasable compartments in the cell, such as rough endoplasmic reticulum and Golgi. Examination of the amount of hormone recovered in cell extracts demonstrated that hGH is depleted from the cells concurrently with its appearance in the bathing medium. Stimulation for 10 min resulted in a 42% decrease in hGH content of the cells (Fig. 3); this decrease can be completely accounted for by the hGH recovered in the bathing media. We conclude that protein degradation plays no significant role in the turnover of hGH, at least on this time scale. Similar experiments performed on non-NGF-treated cells gave qualitatively similar results. However, as indicated by Table I, the amount of stimulation compared with the basal release was not as great.

Packaging of hGH into Synaptic Vesicles

The most likely explanation for the increased rate of secretion produced by secretagogue is that hGH is packaged into

TABLE I. Storage and Secretion of hGH by PC-12 Cells

| | Total hGH content | Basal release | Storage index | Stimulated release | Stimulated/ basal |
|-------------|-------------------|--------------------------------|---------------|------------------------------|----------------------|
| | ng/mg protein | ng/mg min (% total/min) | min | ng/mg min (% total/min) | |
| No NGF | 5.6 ± 1.6 | 0.041 ± 0.014 (0.61 ± 0.18) | 137 | 0.63 ± 0.02 (7.7 ± 0.4) | 15 |
| NGF-treated | 17.3 ± 2.0 | 0.057 ± 0.005 (0.31 ± 0.01) | 304 | 1.48 ± 0.13 (8.99 ± 0.28) | 26 |

Basal and stimulated secretion of hGH. Cells were grown in 10-cm dishes for 4 d either in the presence or absence of 10 ng/ml NGF, and fed daily. To measure rates of secretion, media (± 5 mM carbachol) were changed at 2-min intervals. The collected media were centrifuged at 200 g for 5 min to remove cell debris, and then assayed for hGH. At the end of 10 min of collection, the hGH remaining in the cells was determined by rinsing the cells three times with PBS to remove protein from the media, and then extracting with detergent extraction buffer. This extract was also centrifuged to remove nuclei and cell debris, and then assayed for hGH and protein. The storage index represents the total hGH content divided by the rate of basal release.

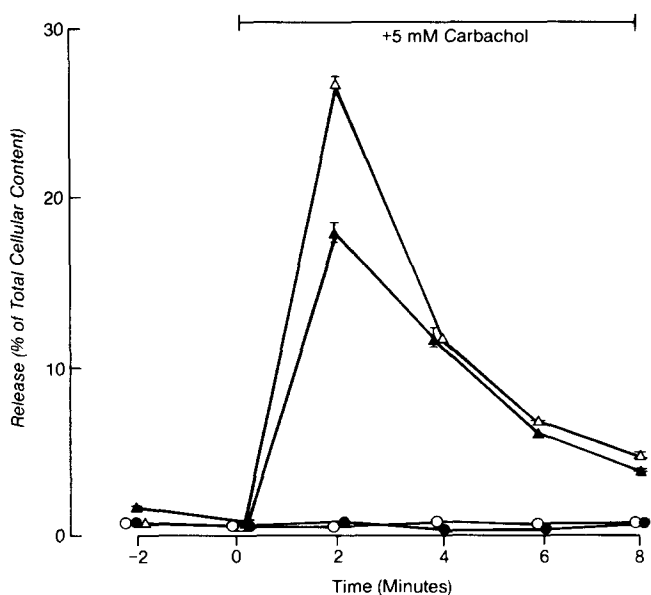


FIGURE 2 Stimulated secretion of $[^3\text{H}]\text{NE}$ and hGH. PC12/1B6 cells were plated into four 10-cm dishes and grown for 4 d in the presence of NGF. The cells were loaded for 2 h with $1 \mu\text{Ci } [^3\text{H}]\text{NE}$ and then washed three times with normal medium. The media were then changed in 2-min intervals; beginning at $t = 0$, one pair of dishes received medium without (O, ●) or with (Δ , \blacktriangle) 5 mM carbachol. The media were collected, centrifuged to remove any cell debris, and assayed for $[^3\text{H}]\text{NE}$ (O, Δ) and hGH (●, \blacktriangle). At the end of the stimulation period, the cells were extracted with detergent, and the amounts of $[^3\text{H}]\text{NE}$ and hGH remaining in the cells were determined. Secretion is expressed as percentages of the total $[^3\text{H}]\text{NE}$ and hGH recovered in the media and extracts.

the endogenous synaptic vesicles in PC12 cells. To test this hypothesis, we have used two separate criteria to determine the intracellular localization of the hGH: immunoprecipitation with vesicle-specific antibodies and co-purification with $[^3\text{H}]\text{NE}$ on subcellular fractionation.

Synaptic vesicles from PC12/1B6 cells were immunoprecipitated from homogenates using monoclonal antibodies known to bind proteins on the cytoplasmic surface of secretory vesicles. The first antibody, BM48, binds to a protein (M_r 65,000) in the vesicle membrane, and has been used to immunoprecipitate catecholaminergic vesicles from PC12 cells (24). The second antibody, anti-SV2, binds to the cytoplasmic portion of a vesicle-specific glycoprotein (2). Both vesicle antigens are found in many species and in a wide variety of endocrine and neuronal tissue. Table II shows that incubation of the PC12 homogenate with these antibodies followed by precipitation with SAC resulted in the specific

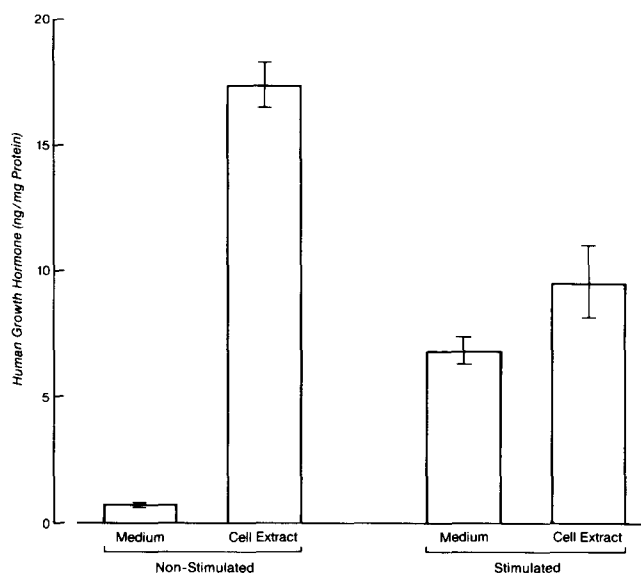


FIGURE 3 Depletion of hGH from cells by stimulation. The total amount of hGH released in the experiment described in Fig. 2 was obtained by summation of hGH recovered in the media during 10 min of incubation ± 5 mM carbachol. Both the amounts of hGH secreted and recovered in the cell extracts are normalized to the amount of cellular protein recovered in the cell extracts. These amounts were 2.1 ± 0.2 and 2.2 ± 0.2 mg/dish for the unstimulated and stimulated cells, respectively.

precipitation of synaptic vesicles, as indicated by the precipitation of $[^3\text{H}]\text{NE}$. Moreover, both these antibodies could specifically immunoprecipitate 40–45% of the total hGH in the PC12 cell membranes; this figure coincides with the amount released by stimulation in the preceding experiment. By immunological criteria, the hGH in PC12 cells is contained in synaptic vesicles.

Synaptic vesicles can also be isolated from PC12 cells by equilibrium density centrifugation. These vesicles have been reported to band at 1.2 M (34) or 1.4 M (29) sucrose. As shown in Fig. 4a, when $[^3\text{H}]\text{NE}$ -loaded PC12/1B6 cells were fractionated on such a gradient, there is a peak of $[^3\text{H}]\text{NE}$ found at 1.4 M sucrose (open triangles). In addition to this peak, there is some $[^3\text{H}]\text{NE}$ found at the top and bottom of the gradient; the former presumably represents $[^3\text{H}]\text{NE}$ released from vesicles lysed during homogenization, and the latter, vesicles trapped in undisturbed cell debris. To confirm the identity of the secretory vesicle peak, the amount of the secretory vesicle antigen, SV2, was assayed across the gradient. The binding of anti-SV2 (open circles) also peaked at 1.4 M sucrose, even though the total amount of protein in this region

is small. Determinations of the distribution of hGH across the gradient (closed triangles) revealed that it co-localized with the [³H]NE and SV2 antigen, supporting the hypothesis that the hGH is contained in catecholaminergic vesicles. It is important to note that the hGH is enriched in the vesicle peak, rather than simply following the protein distribution. The specific activity (ng hGH/mg protein) is highest in fraction 9 (1.4 M sucrose), coincident with the peak of vesicular [³H]NE. We therefore conclude that the hGH is selectively

localized within membrane vesicles that co-purify with PC12 catecholaminergic vesicles.

If these vesicles form the basis for the stimulated secretion illustrated in Fig. 2, it should be possible to deplete this vesicle peak of hGH. Fig. 4b shows a gradient profile from cells that were treated in parallel with those in Fig. 4a, except that these cells were stimulated for 10 min with 5 mM carbachol before homogenization. In this experiment, stimulation resulted in a 25% decrease in the total cellular hGH. A comparison of parts a and b of Fig. 4 reveals that there is a decrease in the amounts of [³H]NE (open triangles) and hGH (closed triangles) recovered in the vesicle peak, although there is no decrease in either the total protein or the amount of vesicle antigen. Summation of the hGH recovered in the corresponding vesicle fractions (1.1–1.5 M sucrose) in gradients 4a and 4b indicate that the hGH recovered in this region of the gradient is decreased by 24% (7.0 ng vs 5.3 ng). There is also a decrease in the [³H]NE and hGH recovered at the top and bottom of the gradient, as would be expected if these represent lysed and trapped vesicles. The above data demonstrate that PC12 cells can package hGH into membranous vesicles that

TABLE II. Immunoprecipitation of PC12/1B6 Vesicles

| Antibody | [³ H]NE cpm (% total) | hGH ng (% total) |
|----------------|--------------------------------------|---------------------|
| Culture medium | 295 ± 9 (28) | 0.16 ± 0.01 (33) |
| Anti-SV2 | 733 ± 44 (70) | 0.37 ± 0.05 (78) |
| Anti-P65 | 822 ± 82 (78) | 0.34 ± 0.30 (73) |

[³H]NE-labeled PC12/1B6 cells were homogenized in PBS + 10 mM EGTA, and the membranes were incubated with culture medium or monoclonal antibody-containing medium. The vesicles were precipitated with SAC as described in Materials and Methods. The total [³H]NE and hGH contained in the PC12/1B6 membranes was determined by centrifuging aliquots at 18,000 g for 15 min and assaying the pellets for [³H]NE and hGH.

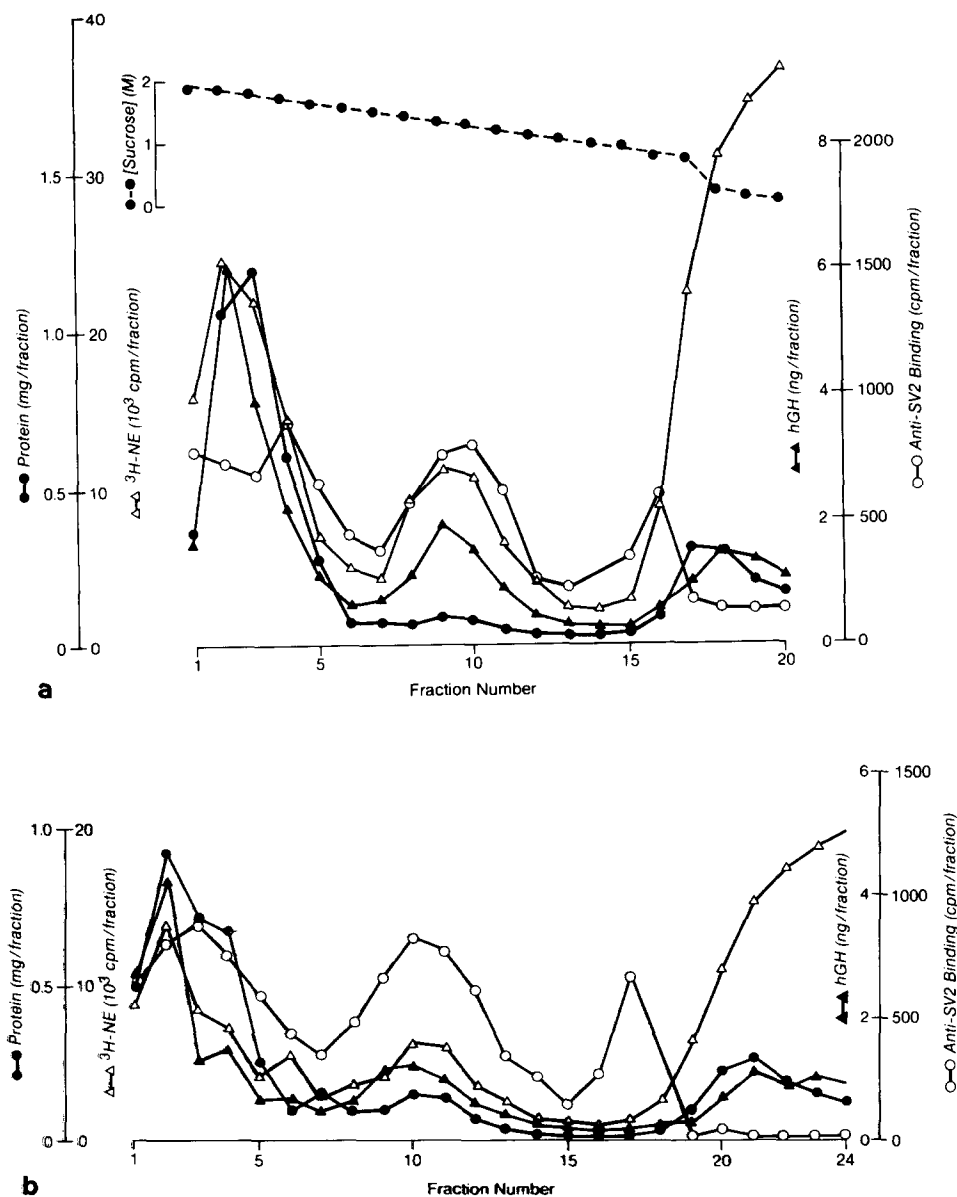


FIGURE 4 Subcellular fractionation of PC12/1B6 cells with or without stimulation. Two parallel 15-cm dishes of cells were loaded with 5 μ Ci [³H]NE each for 12 h. The cells were then washed twice with fresh medium and then incubated for 10 min in medium without (a) or with (b) 5 mM carbachol, washed twice with PBS, and removed from the dish with PBS containing 10 mM EGTA at 0°C. The cells were then homogenized, layered onto sucrose gradients, and centrifuged to equilibrium. Recovery of protein from the two gradients was 5.10 and 5.12 mg. Concentration of sucrose (—●—) was calculated from refractive index. Protein (—●—), [³H]NE (Δ), hGH (\blacktriangle), and SV2 immunoreactivity (\circ) were assayed as described in Materials and Methods.

are indistinguishable by density and antigenic properties from the endogenous catecholaminergic vesicles, and that the cells can release hGH from this vesicle pool upon appropriate stimulation.

Sorting of Secreted Proteins into Two Pathways in PC12 Cells

If PC12 cells package all newly synthesized protein into secretory vesicles, it would not be surprising to find hGH colocalizing with NE. On the other hand, some cells possess two separate pathways for secretion of proteins (12). To determine whether separate secretory pathways exist in PC12 cells, we pulse-labeled PC12/1B6 cells with [³⁵S]methionine and followed the secretion of proteins during four 10-min chase periods. As shown in Fig. 5, there is a gradual chase-out of labeled proteins (*a*, lanes 1, 2, 3, and 4) in the absence of stimulation. However, the addition of carbachol during the third and fourth chase periods (*b*, lanes 3 and 4) resulted in a dramatic stimulation of the secretion of some proteins (compare *a*, lane 3, with *b*, lane 3), for example those of M_r 39,000 and 78,000 (closed arrows). Although we have not identified these stimulated proteins, they are likely to include the chromogranins, which are soluble protein components of chromaffin granules (29, 41). In contrast, other proteins such as those of M_r 32,000 and 81,000 (open arrows) were secreted constitutively, i.e., their secretion was not increased by carbachol. We interpret this result to mean that only a subset of all newly synthesized proteins are packaged into the transmitter-containing synaptic vesicles whose fusion with the plasma membrane is stimulated by carbachol.

A third pattern of secretion is exemplified by the band at M_r 73,000 (star), which is released in substantial amounts in the absence of stimulation, and increases about twofold in the presence of carbachol. This may represent the superposition of two proteins, one secreted constitutively and the other released by stimulation. An alternative explanation is that this band represents a third class of proteins that is not sorted efficiently between the two pathways. McHugh et al. (25) have recently presented evidence that dopamine β -hydroxylase (M_r 73,000; reference 32) is secreted constitutively and that its release is stimulated only about twofold by depolarization.

To provide further evidence for the segregation and separate secretion of PC12 cell proteins, we followed the secretion of laminin, a soluble protein which is known to be secreted by PC12 cells (17), but which we did not expect to be packaged into secretory vesicles. Laminin secretion was quantitated by immunoprecipitation with rabbit anti-laminin serum of the media samples from the same experiment described in Fig. 5. On reducing gels, laminin migrates as a heavy and light chain of M_r 400,000 and 200,000 (38). Bands co-migrating with laminin standards were specifically immunoprecipitated by antiserum (Fig. 6*b*) but not by nonimmune rabbit serum (Fig. 6*a*). Detectable amounts of laminin are secreted in 10 min in the absence of stimulation (Fig. 6*b*, lanes 1 and 2); this secretion is not increased by carbachol (Fig. 6*b*, lane 4). Laminin is therefore an example of a constitutively secreted protein. This lack of stimulation can be directly contrasted with the nonspecifically precipitated protein of M_r 66,000; the secretion of this protein is markedly stimulated by carbachol. We conclude from these experiments that PC12 cells segregate secreted proteins into at least two distinct pathways, distinguishable by their sensitivity to carbachol. The packag-

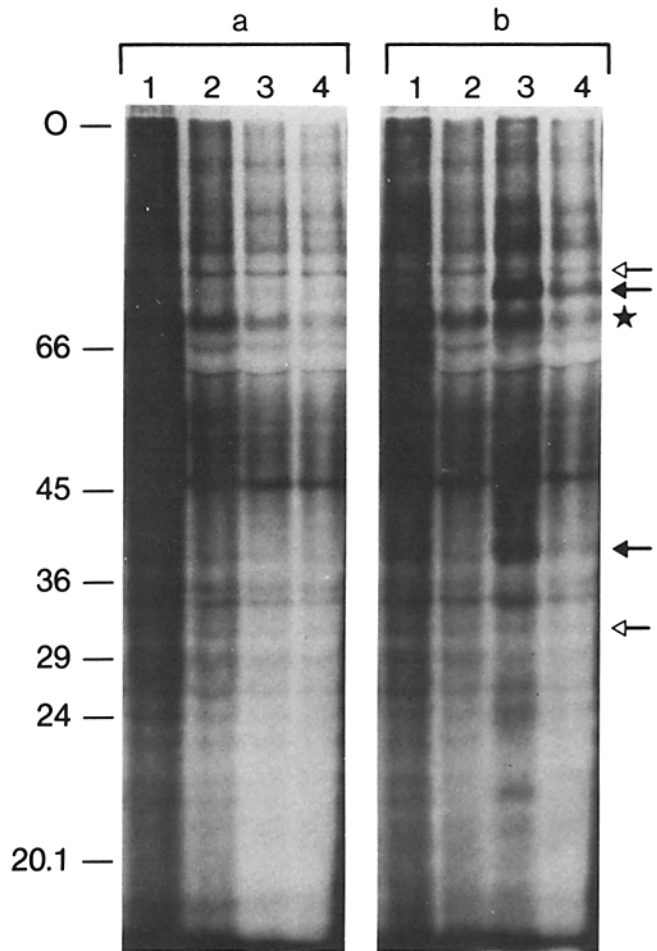


FIGURE 5 ³⁵S-labeled proteins secreted by PC12/1B6 cells. Four 10-cm dishes of PC12/1B6 were labeled for 10 h with 1 mCi [³⁵S]methionine, 0.5 mCi [³⁵S]cysteine, and 2 μ Ci [³H]NE in medium containing 1/100 the normal amounts of methionine and cysteine. At the end of the labeling period, the media were removed and replaced with fresh media at 10-min intervals. One pair of dishes (*a*) was incubated with normal medium, collected at 10 min (1), 20 min (2), 30 min (3), and 40 min (4) after labeling was discontinued. The second pair of dishes (*b*) was incubated in the same way except that the first two incubations (1 and 2) were in normal medium, and the second two (3 and 4) were in medium containing 5 mM carbachol. Duplicate samples were pooled, and 5 μ l of each sample was separated on a 12% SDS polyacrylamide gel and autoradiographed. The position of molecular weight markers (in kilodaltons), visualized by Coomassie Blue stain, are indicated along the left; closed arrows indicate examples of stimulated proteins, open arrows indicate nonstimulated proteins. The star marks a band of M_r 73,000.

ing of hGH into secretory vesicles is therefore not simply a reflection of the bulk flow of secreted proteins, but most involve an active sorting step at some point along the secretory pathway. PC12 cells can selectively package hGH but not laminin into the endogenous, regulated secretory vesicles.

DISCUSSION

We have genetically inserted a foreign peptide hormone into a neuronal cell line and have demonstrated that this altered cell line can secrete normal hGH in a physiologically regulated fashion. The ability of PC12 cells to carry out such secretion implies that they possess the cellular machinery to express the

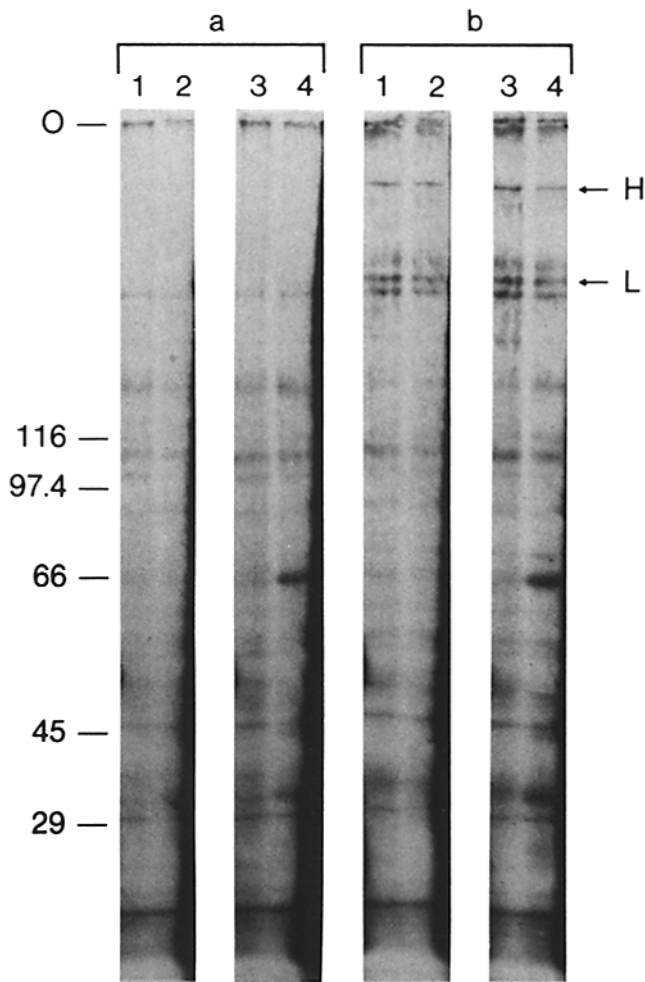


FIGURE 6 Immunoprecipitation of laminin secreted by PC12/1B6 cells. Pooled media from the experiment described in Fig. 6 were incubated with normal rabbit serum (a), or rabbit anti-laminin serum (b), precipitated with SAC and applied to a 4–12% gradient SDS polyacrylamide gel. The gel was stained to visualize laminin and molecular weight standards and processed for autoradiography. Lanes 1 and 2 represent media collected during two successive 10-min incubations in normal medium; lanes 3 and 4 represent the same two periods, the first in normal medium (3) and the second in 5 mM carbachol (4). The location of laminin light chain (L) and heavy chain (H) are indicated along the right; molecular weight standards (in kilodaltons) are indicated along the left.

gene, process the RNA, and package and release the peptide as if it were an endogenous neuropeptide transmitter.

The construct used for these studies contained the genomic hGH coding sequence and 5' flanking region. The expression in PC12 cells of hGH encoded by this plasmid is not surprising since hGH has been expressed in a variety of cells, including fibroblasts and monkey kidney cells (28), as well as AtT-20 cells (27). The expression of hGH in PC12 cells, albeit at low levels, has permitted its use as a marker for the study of specificity in subsequent steps in packaging and secretion of peptides in a neuronal cell line. Recent results indicate that approximately 50-fold higher levels of expression can be obtained by linking the hGH gene to the metallothionein promoter (unpublished observations). With such cell lines, immunocytochemical localization of the hGH in PC12 cells should be feasible.

The transfected PC12 cells secrete two peptides that are

specifically immunoprecipitated by anti-hGH serum. The mobilities of these peptides correspond to those of the 22,000- and 20,000-mol-wt forms of hGH produced by human pituitary cells (19). The smaller form, which is probably generated by alternative RNA splicing (5), has not been seen in monkey kidney cells containing the hGH gene (28). At present we do not know the molecular basis for the formation of the two forms observed in PC12 cells (but see reference 30).

PC12 cells are specialized for the storage and rapid release of transmitters. The experiments presented here demonstrate that these cells can store hGH in secretory vesicles until it is released by an appropriate secretagogue. In the absence of NGF, transfected PC12 cells can store an amount of hGH equivalent to the amount they secrete in 2 h without stimulation. Treatment of the cells with NGF does not necessarily affect the expression of hGH but increases the amount stored by 2.5-fold, indicating that differentiated PC12 cells are more efficient in storing this secretory peptide. The increased storage capacity could be due either to an increase in the number of storage vesicles with NGF, or to an increased amount of peptide packaged into each vesicle. The amount of hGH that is releasable by carbachol appears to reflect the amount of hGH in the synaptic vesicle pool, since NGF treatment produces parallel increases in stimulated release and storage of hGH.

Whereas PC12 cells store hGH with about the same efficiency as ACTH is stored by pituitary AtT-20 cells (Burgess, T., and H.-P. Moore, personal communication), they release large amounts of the stored peptide hormones much more rapidly; carbachol causes a 26-fold stimulation of hGH secretion by NGF-treated PC12 cells, resulting in the release of 40% of the total stored hGH in 10 min. In contrast, stimulation for AtT-20 cells causes a 3–5-fold increase in ACTH release by AtT-20 cells (23), resulting in a 20% depletion in 1 h (Moore, H.-P., personal communication). The high level of regulation and the rapid rate of secretion seen in PC12 cells may be a reflection of their neuronal character.

The observation that the rate of hGH secretion is indistinguishable from that of NE is significant for several reasons. First, it demonstrates that hGH is packaged into endogenous secretory vesicles in PC12 cells that not only share the same physical properties but also possess the same regulatory elements as do the synaptic vesicles. PC12 cells can therefore recognize a peptide hormone normally expressed in a different cell type in a different species and selectively package this hormone into a specific type of secretory vesicle. Despite any differences that exist between vesicles from various sources, the mechanism that permits selective packaging of peptides must be highly conserved. A second conclusion from the rapid kinetics of hGH release is that neurosecretory vesicles can be rapidly secreted on the first round of fusion with the plasma membrane. Neurons can package transmitters such as acetylcholine and NE into preformed vesicles from cytoplasmic pools. Such a mechanism permits a neuron to retrieve, reload, and reuse vesicle membrane at great distances from the cell body. Peptides, on the other hand, must be packaged into vesicles at the Golgi apparatus and are presumably released during the first fusion event with the plasma membranes. In the case of vesicles that originally contain both neurotransmitter and peptides, such a system would create a difference between vesicles that have never fused with plasma membrane, and recycled vesicles, which would be depleted of peptides. Indeed, one explanation for the different frequency

and calcium sensitivity seen between peptide and neurotransmitter release (22; see also reference 15) would be that newly synthesized vesicles fuse slowly with the plasma membrane, whereas recycled vesicles fuse rapidly. The experiments presented here indicate that, at least in PC12 cells, the hGH-containing vesicles can carry out secretion as rapidly as would be expected for vesicles containing neurotransmitter alone.

Third, these experiments demonstrate the feasibility of experimentally producing a cell that will secrete any particular peptide in a rapid and highly regulated manner. If the gene for that specific peptide is introduced or turned on in a neuronal cell, the existing cellular machinery will package and secrete the peptide in a manner characteristic of neuronal secretion.

We have demonstrated by kinetic, immunological, and subcellular fractionation criteria that the peptide hGH is packaged into vesicles indistinguishable from the endogenous NE-containing vesicles. Laminin, an endogenous secretory protein often associated with the extracellular matrix, does not appear to be released from these same vesicles. PC12 cells thus possess at least two separate pathways for protein secretion and can segregate proteins selectively into at least one of the two pathways. The co-existence of two separate pathways, regulated and constitutive, was originally described in the ACTH-secreting endocrine cell line, AtT-20 (12; see also reference 3). PC12 cells provide a second example of the co-existence of these two pathways within a single cell. In addition to the ability to synthesize and package NE, PC12 cells make and package acetylcholine, especially after treatment with NGF (10). Since these two transmitters have been reported to be packaged into separate vesicles (29, 34), there is a possibility the PC12 cells possess more than one regulated pathway for secretion. It will therefore be important to determine whether different soluble proteins are packaged into the two vesicle types. Such a difference would imply the existence of sorting not only between the regulated and constitutive pathways, but between two distinct regulated pathways as well.

Transfection of PC12 cells has been used here to study only one property of neurons, the capacity to package peptide hormones correctly. Now that a neuronal cell line can be successfully transfected, however, the opportunity exists to create new cell lines that express additional experimentally useful properties, to examine the regulation of neuronal gene expression and the modulation of this expression by NGF, and the elucidation of other steps in the process of neurosecretion.

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