# Sorting during Transport to the Surface of PC12 Cells: Divergence of Synaptic Vesicle and Secretory Granule Proteins

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*Abstract.* PC12 cells, a cell line derived from a rat pheochromocytoma, have both regulated and constitutive secretory pathways. Regulated secretion occurs via large dense core granules, which are related to chromaffin granules and are abundant in these cells. In addition, PC12 cells also contain small electron-lucent vesicles, whose numbers increase in response to nerve growth factor and which may be related to cholinergic synaptic vesicles. These could characterize a second regulated secretory pathway. We have investigated the trafficking of protein markers for both these organelles.

We have purified and characterized the large dense core granules from these cells using sequential velocity and equilibrium gradients. We demonstrate the copurification of the major PC12 soluble regulated secretory protein (secretogranin II) with this organelle. As a marker for the synaptic vesicle-like organelles in this system, we have used the integral membrane glycoprotein p38 or synaptophysin. We show that the p38-enriched fraction of PC12 cells comigrates with rat brain synaptic vesicles on an equilibrium gradient. We also demonstrate that p38 purifies away from the dense core granules; <5% of this protein is found in our dense granule fraction. Finally we show that p38 does not pass through the dense granule fraction in pulse-chase experiments. These results rule out the possibility of p38 reaching the small clear vesicles via mature dense granules and imply that these cells may have two independently derived regulated pathways.

THE way in which simple organelles such as neuroendocrine secretory granules are assembled is of considerable interest. One aspect of assembly that has received great attention is how the cell targets the appropriate proteins to the site of organelle assembly. Much progress in refining this question has been made in recent years. This has in part arisen out of work exploiting the advantages of tissue culture systems embodied in the AtT-20 cell line (for reviews see references 3 and 14) to investigate sorting during export. This work has led to the establishment of the "two-pathway" paradigm for granule biogenesis; there are two exocytic routes in cells specialized for secretion—constitutive and regulated (11). The former is rapid, not responsive to secretagogues, and exocytosis occurs from an ill-defined organelle. The regulated pathway is slow, requires external stimulation for release, and proceeds via the secretory granule. Exported proteins are sorted between these two pathways as they pass along the secretory system of the cell.

The two-pathway description has proved to be both influential and useful to the field. However, an aspect of the model that has not yet been biochemically tested is whether or not the membrane proteins of the regulated pathway follow the same pattern of behavior as the soluble proteins. We do not yet know how regulated membrane proteins are partitioned between exocytic routes in cells possessing both constitutive and regulated pathways to the cell surface.

A second area that has received scant biochemical attention is that of cellular systems that possess more than one regulated secretory pathway. For example, neurons can have large dense core granules in addition to their small synaptic vesicles (e. g., 16), and may therefore have two regulated secretory pathways. How proteins are sorted between these different structures remains to be elucidated. To investigate the relationship between more than two exocytic pathways and to look at the sorting of membrane proteins associated with regulated export, we have begun to exploit the PC12 cell system.

The PC12 cell line has been developed from a transplantable rat pheochromocytoma (9). It has many large dense core granules (25), small electron-lucent vesicles (19, 28) which may be related to cholinergic synaptic vesicles, and responds well to secretagogues (8, 10). Like other cells related to the sympathetic nervous system, it has been suggested that PC12 cells may have more than one regulated secretory pathway. In addition to the regulated pathway based on large (up to 350 nm) dense core granules that are related to chomaffin granules, there may be a second regulated pathway based on the small (50–70-nm) clear synaptic-like vesicles. This latter pathway may be partly inducible by treating the cells with nerve growth factor (8, 10, 19).

In this paper we describe the purification of the dense core granules from PC12 cells and demonstrate that a synaptic vesicle membrane protein (p38 or synaptophysin; 2, 13, 20, 22, 33, 34) purifies away from the dense chromaffin-like granules. We also demonstrate that the subcellular fraction of PC12 cells containing p38 has a density similar to that of rat brain synaptic vesicles. Finally, we use pulse-chase protocols combined with subcellular fractionation to show that

p38 does not pass through mature dense core granules on route to its cellular destination. This implies that the p38-containing organelle arises independently of the dense granules, and suggests that a membrane protein that has been shown to be associated with regulated exocytosis (30) can effectively be sorted between different exocytic pathways.

## Materials and Methods

### Cell Culture

PC12 cells obtained from the cell repository, University of California, San Francisco, were grown in DME (Gibco UK, Paisley, Scotland) supplemented with 10% horse serum and 5% FCS in an atmosphere of 10% $CO_2$. They were passaged approximately once a week, and were not used for experiments until at least 48 h after passaging.

Radiolabeling of cells was performed in methionine-free MEM (Gibco UK). Pulse labeling was done with $^{35}$S-labeled amino acids (Tran $^{35}$S-Label; ICN Biomedicals Ltd., High Wycombe, UK) as the only source of methionine. 1 mCi was used for each 10-cm dish, 250 μCi for each 3.5-cm dish. For these experiments, cells were starved of methionine for 15 min before labeling began. Overnight labeling was done in methionine-free medium supplemented with 0.1 vol of normal DME including serum. 1 mCi of Tran $^{35}$S-Label was used for each 10-cm dish of cells. Labeling of cells with $^{3}$H-dopamine (Amersham International, Amersham, UK) was done by adding 5 μCi of tritiated amine to each dish of cells 1–2 h before cell fractionation. Stimulation of regulated secretion was performed by the addition of 5 mM carbamylcholine (Sigma Chemical Co., St. Louis, MO) to the medium.

### Subcellular Fractionation

Cells were rinsed with and then scraped into 1 ml of an ice-cold solution of 0.32 M sucrose, buffered with 10 mM Hepes, pH 7.4. They were then passed nine times through a custom-made ball-bearing homogenizer (European Molecular Biology Labs workshop, Heidelberg, FRG; see reference 1) with a clearance of 0.012 mm. The lysate was centrifuged at 11,000 rpm for 5 min in a microfuge. The postnuclear supernatant was then layered onto a 1–16% (velocity) gradient of Ficoll 400 (Sigma Chemical Co.) dissolved in buffered sucrose. This gradient was centrifuged in a rotor (SW40Ti; Beckman Instruments Inc., Palo Alto, CA) at 30,000 rpm and 4°C for 45 min after which 1-ml fractions were collected. Samples were either analyzed directly or the granule-containing fractions were identified by scintillation counting of samples from cells incubated with tritiated catecholamine, pooled, and layered onto a 16–40% (equilibrium) ficoll gradient. This was centrifuged in a rotor (SW40Ti; Beckman Instruments Inc.) at 25,000 rpm and 4°C for 17 h before 1-ml fractions were collected.

Ficoll 400 varies considerably between batches. We dissolve it in 0.32 M sucrose to give a final concentration of 40% (wt/vol) and then each batch is tested by running velocity gradients loaded with homogenates made from cells labeled with $^{3}$H-dopamine. The concentration of Ficoll to be used is then determined empirically by the dopamine distribution. The concentrations of 1 and 40% are always used but the intermediate concentration can vary from the 16% used in the experiments described in this paper to as low as 10%.

Sucrose gradients (modified from ref. 32) were used to determine the equilibrium density of rat brain synaptic vesicles and p38-containing fractions from PC12 cells. Rat brain synaptic vesicles were prepared as described (12). The P3 fraction from three rat brains was resuspended in 0.32 M sucrose, loaded onto a gradient consisting of 2-ml steps of 1.2, 1.0, 0.8, 0.6, and 0.4 M sucrose in 10 mM Hepes, and centrifuged in a rotor (SW40Ti; Beckman Instruments Inc.) for 17 h at 25,000 rpm and 4°C.

### Immunoidentifications

Cells to be immunoprecipitated were rinsed with ice-cold PBS, and lysed on ice with 1 ml 1% Nonidet P-40, 0.6% deoxycholate, 66 mM EDTA, 10 mM Tris, pH 7.5 (NDET)[1] supplemented with PMSF (1 mM). Nuclei and debris were removed by centrifugation at 14,000 rpm for 5 min in a microfuge (Eppendorf Gerätebaul, Hamburg, FRG). The supernatant was adjusted to 0.3% SDS and fixed *S. aureus* cells (Pansorbin; Calbiochem-Behring Corp., La Jolla, CA, 60 μl of a 10% suspension) added. This suspension was rotated at 4°C for 0.5–1 h before removing the Pansorbin by centrifugation. The supernatant was taken, primary antibody (usually 5 μl of anti-p38 serum or 10 μl antisecretogranin II serum) added, and the sample then incubated at 4°C for between 2 h and overnight. Fresh Pansorbin (60 μl) was added and the sample then incubated at 4°C for a further 0.5–1 h. The pellet was then collected, resuspended in 600 μl NDET/SDS, and layered over a sucrose cushion (30% sucrose in 0.5 × NDET/SDS). The Pansorbin was pelleted (5 min, 14,000 rpm, microfuge), washed once more in NDET/SDS, and then finally resuspended in 50 μl sample buffer for electrophoresis. SDS–PAGE was performed according to the method of Laemmli (15).

Samples taken from gradient fractions were diluted 10-fold in NDET/SDS to a final volume of 1 ml and then treated as outlined above, except that the preincubation with Pansorbin was omitted.

Western blotting of gradient fractions was performed as described (29). 5-μl aliquots of gradient fractions were run on a 10% SDS gel and transferred for 16 h at 100 mA. The blots were incubated with the anti-p38 serum at 1:1,500 and a peroxidase-linked goat antirabbit serum (Jackson Immunoresearch Laboratories Inc., West Grove, PA) at 1:3,000 and developed using the diaminobenzidine reaction. To determine the density of p38-containing fractions from PC12 cells, fractions from near the top of a velocity gradient containing the bulk of p38 following overnight labeling were centrifuged on a sucrose step gradient as described above followed by immunoprecipitation and SDS–PAGE.

### Antibodies

The antibody used to purify p38 (2) was obtained by repeated intradermal immunization of a rabbit with a washed membrane fraction from purified rat brain synaptic vesicles (32). 100 μg of protein was used at each boost. The antibody used to quantify secretogranin II (6, 24) was made by collecting serum-free medium from PC12 cells stimulated to secrete by the application of carbamylcholine. The secreted proteins were concentrated by TCA precipitation and separated by SDS–PAGE, and the appropriate gel fragments excised, pulverized, and injected subcutaneously into a rabbit with two popliteal boosts. The monoclonal antibody SY38 (33) was purchased from Boehringer-Mannheim Biochemicals (E. Sussex, UK).

### Quantitation of Proteins

Quantitation of secretogranin II and p38 was performed by scintillation counting of gel fragments containing the relevant immunoprecipitated band, which had been located using the autoradiograph and then excised.

### Enzyme Assays

ATP assays were performed using a crude firefly extract (FLE-50; Sigma Chemical Co.) according to the manufacturers instructions. Luminescence was monitored in a luminometer (Model 1250; LKB Instruments, Inc., Gaithersburg, MD). β-*N*-acetylglucosaminidase and succinate-INT-reductase were assayed as described (21, 27). Endo β-*N*-acetylglucosaminidase H (Endo H; Boehringer-Mannheim Biochemicals) was used on immunoprecipitates according to the manufacturers instructions. Thus, instead of resuspending immunoprecipitates into sample buffer, the antigen-antibody-Pansorbin complex was washed twice in 10 mM Tris, pH 7.6, and resuspended in citrate buffer (pH 5.5). This mix was incubated for 18 h with 10 μU/μl enzyme, and then the Pansorbin washed twice in 10 mM Tris, pH 7.6, and resuspended in sample buffer for electrophoresis.

## Results

### Purification of Dense Core Granules

We have developed our granule purification from methodology established by Wagner (31; and our unpublished results). PC12 cells are broken with a ball-bearing homogenizer (1), and a postnuclear supernatant is loaded onto a velocity gradient made of Ficoll in sucrose. After centrifugation for 45 min, those fractions from the gradient that contain the granule peak are pooled and loaded onto a second Ficoll gradient for overnight centrifugation to equilibrium (see Materials and Methods for details).

We monitor the presence of the granules on velocity gradi-

1. *Abbreviations used in this paper*: Endo H, Endo β-*N*-acetylglucosaminidase H; NDET, 1% Nonidet P-40, 0.6% deoxycholate, 66 mM EDTA, 10 mM Tris, pH 7.5.

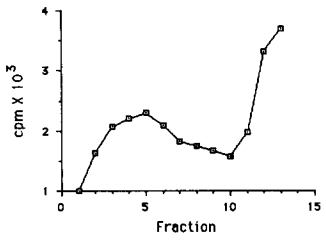


*Figure 1.* Distribution of $^3$H-dopamine on a velocity gradient. A 10-cm dish of PC12 cells was incubated with labeled dopamine for 90 min. The cells were rinsed, removed, and homogenized. A postnuclear supernatant was prepared and then centrifuged on a standard velocity gradient. The gradient was fractionated and 50-μl samples were counted. The gradient fractions are numbered from the bottom.

ents by following the presence of $^3$H-dopamine, with which this organelle can be preloaded (26). The profile of tritiated material shows a peak of granule-associated counts in the lower third of the gradient and a peak of cytoplasmic label (data not shown) at the top of the gradient (Fig. 1). The fractions to be pooled and recentrifuged on the equilibrium gradient are chosen on the basis of this dopamine analysis.

The granule purification can also be monitored by SDS–PAGE of metabolically labeled gradient samples (Fig. 2). The autoradiograph shows a velocity gradient loaded with material taken from cells after 24 h of continuous labeling. This shows that one major band with an apparent molecular mass of ~80–85 kD (identified by an arrowhead in the figure), copurifies with the dopamine, peaking in fractions 3–5 on this velocity gradient. This band probably corresponds to secretogranin II (6, 24), a marker for neuroendocrine-regulated secretion.

Secretogranin II is functionally associated with the regulated secretory pathway in these cells. A gel comparison (Fig. 3) of a purified granule sample with the proteins released from PC12 cells in the presence or absence of secretagogue stimulation indicates that this band is the major regulated secretory protein of these cells.

For some experiments, the purification afforded by the velocity gradient is sufficient. However, further purification of the granule fraction beyond that accomplished by the velocity gradient can be obtained by the use of an equilibrium gradient when required (see Materials and Methods). Fig. 4 is an SDS–PAGE analysis of metabolically labeled samples from an equilibrium gradient. It shows that this procedure further purifies the granule marker secretogranin II (peaking in fractions 5 and 6) away from many of the other proteins present in the pooled granule fraction obtained from the velocity gradient. The profile of the gel track of the granule

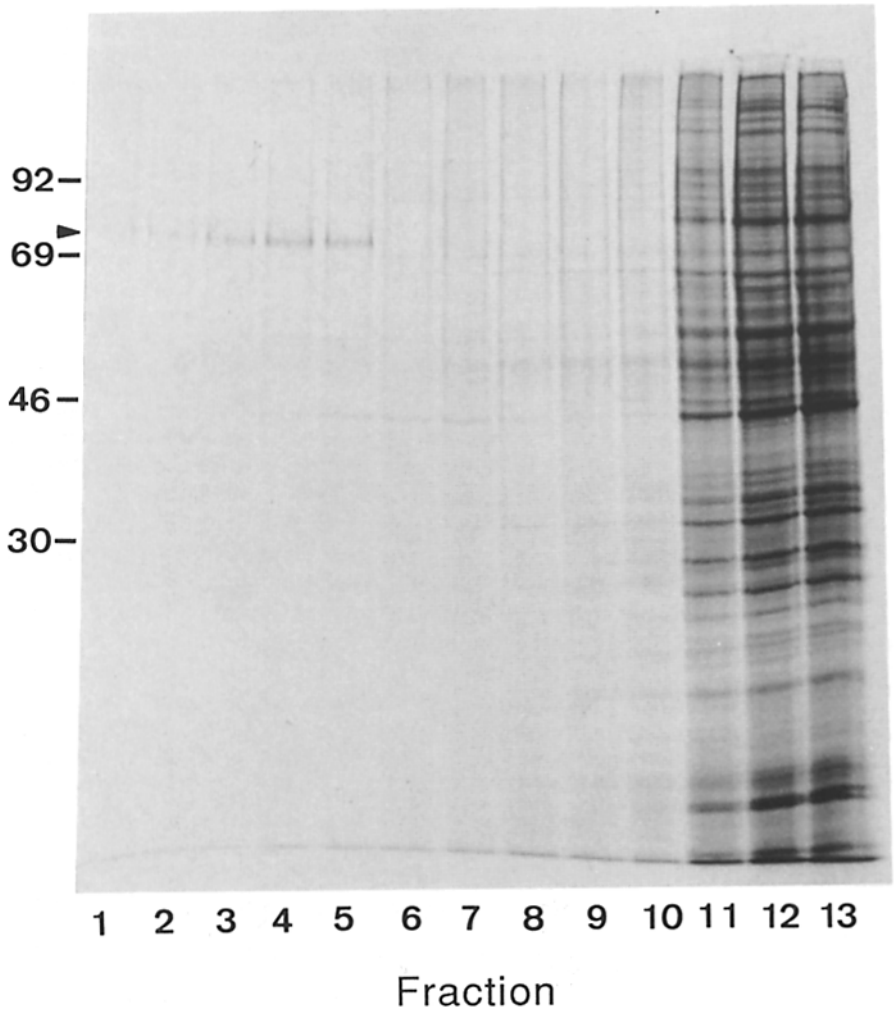


*Figure 2.* Distribution of radiolabeled proteins on a velocity gradient. One 10-cm dish of cells was labeled for 24 h. A postnuclear supernatant was then prepared and centrifuged on a velocity gradient. 10-μl samples of each fraction were then electrophoresed on a 10% polyacrylamide gel. The gradient fractions are numbered from the bottom. The molecular masses of marker proteins ($\times 10^{-3}$) are indicated. The major granule-associated protein secretogranin II is indicated with an arrowhead.

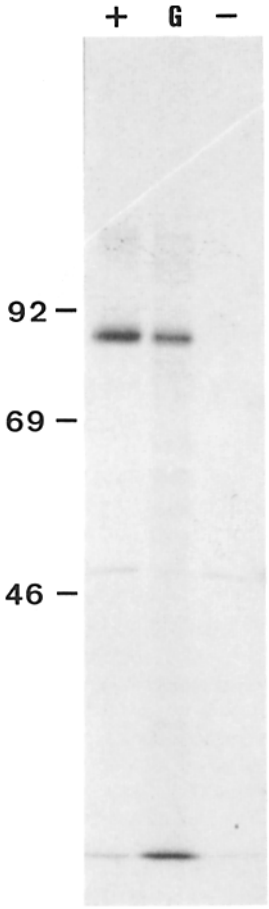


*Figure 3.* Comparison of purified granules with medium taken from stimulated and unstimulated cells. Two 10-cm dishes of PC12 cells were labeled for 4 h followed by a 40-min chase. 4 ml of medium with or without 5 mM carbamylcholine were added for 10 min and then removed. 30 μl samples of the media were electrophoresed on a 10% gel along with a gradient sample highly enriched in granules. The molecular masses of marker proteins ($\times 10^{-3}$) are indicated. + and − refer to media from stimulated and unstimulated cells, respectively. G, a granule-rich velocity gradient fraction.

fraction seen in Fig. 3 and also in Fig. 4 is dominated by the major secretory protein, implying a highly enriched granule fraction.

The kinetics of transport of secretogranin II into a compartment from which release can be stimulated by carbachol have been determined (data not shown). After a 15-min pulse, half-maximal release of secretogranin II after stimulation is reached by a chase of 10 ± 2 min ($n$ = 4). Using a 15-min pulse in this experiment enables comparisons with other experiments described below (See Figs. 7 and 8) to be made. These kinetics suggest that after a 24-h labeling the bulk of secretogranin II will be in the dense granules and that this protein can therefore be used as a marker for this compartment under these conditions. We have therefore quantitated (see Materials and Methods) the distribution of secretogranin II in gradient fractions derived from cells labeled for 24 h. These experiments show that this granule marker is enriched ~100-fold during the two-gradient procedure. 30% of the secretogranin found in the postnuclear supernatant is recovered in the equilibrium gradient granule fractions.

After secretagogue-induced release of proteins from PC12 cells, under conditions where 30% of intracellular dopamine is released, the amount of secretogranin II present on the equilibrium gradient was reduced by approximately one third (data not shown). In addition, luciferase-based assays (not shown for this experiment, but see Fig. 6) of equilibrium gradient fractions also show a peak of ATP that colocalizes with secretogranin II; dense core granules from these cells have been shown to contain significant stores of ATP (31).

We have also determined the degree of contamination of the equilibrium gradient purified granules by other dense organelles: mitochondria and lysosomes. Marker enzyme assays (see Materials and Methods) show that we have removed >96% of the cellular complement of a lysosomal marker (β-*N*-acetylglucosaminidase) and >99% of a mitochondrial marker (succinate-INT reductase).

### *A Synaptic Vesicle Membrane Protein Does Not Copurify with Dense Granules in PC12 Cells*

The neuronal character of PC12 cells has been emphasized by the morphological identification of vesicles similar to synaptic vesicles (19, 28) and the demonstration of the presence of synaptic vesicle membrane proteins in these cells (7, 20). To investigate the relationship between these small clear vesicles and the dense granules, we decided to characterize the intracellular transport of synaptic vesicle membrane proteins with regard to the dense granules.

We have raised a polyclonal serum against highly purified rat brain synaptic vesicle membranes (see Materials and Methods, and reference 2). This serum crossreacts at high dilutions with PC12 cells in immunofluorescence, Western blotting, and immunoprecipitation assays. Western blotting and immunoprecipitation reveal that the serum binds almost exclusively to a protein of ~38 kD. This polyclonal serum effectively competes in a nitrocellulose filter-binding assay with the mAb SY38 (33) which recognizes the protein synaptophysin or p38 which has been extensively documented (2, 13, 20, 22, 33, 34) and is known to be very antigenic. This suggests that most of the reactivity of our serum is directed against this protein. For the rest of this paper the serum will be referred to as an anti-p38 serum.

To investigate the distribution of p38 relative to PC12 dense granules, cells were labeled for 24 h, and fractionated on a velocity gradient; then the fractions were immunoprecipitated with anti-p38 serum. Immunoprecipitates were then subjected to SDS–PAGE and quantified. When the distribution of p38 (Fig. 5) is compared with the distribution of granule markers on a similar gradient (Figs. 1 and 2) it is clear that most p38 purifies away from the dense core granules. The highest concentration of p38 is to be found towards the top of the velocity gradient, implying that it is present in a low-density organelle. However, some of the p38 is found on velocity gradients at the same density as the dense core granules (i.e., fractions 3–5).

To determine whether this colocalization is fortuitous, we have further purified the dense core granules by using an equilibrium gradient as well as a velocity gradient. The distribution of granule markers and p38 were then analyzed (Fig. 6). Most of the small amount of PC12 p38 that is found in the same fraction as dense granules on a velocity gradient purifies away from secretogranin II and ATP on an equilibrium gradient. The p38 is seen peaking in fractions 8–9, whereas the dense core granules on this gradient are found in fractions 4–5. Quantitation shows that <1.5% of the total p38 in a postnuclear supernatant colocalizes with dense core granule markers on an equilibrium gradient (note the small shoulder of p38 in fractions 4 and 5). Because the recovery of secretogranin II is ~30% during the two-gradient granule

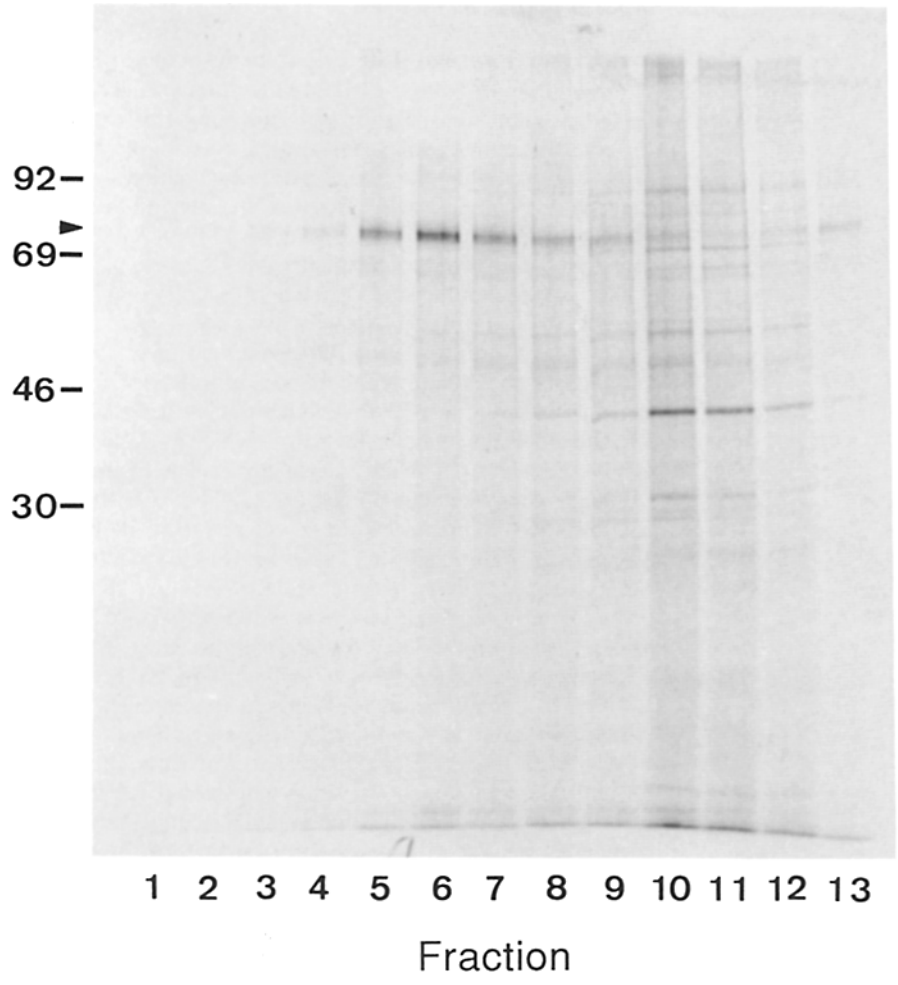


*Figure 4.* Distribution of radiolabeled proteins on an equilibrium gradient. A 10-cm dish of cells was labeled for 24 h, and a postnuclear supernatant was prepared and then centrifuged on a velocity gradient. The fractions corresponding to the granule-associated dopamine peak were pooled and centrifuged on an equilibrium gradient. 20-$\mu$l samples from gradient fractions were electrophoresed on a 10% gel. The gradient fractions are numbered from the bottom. The molecular masses of marker proteins ($\times 10^{-3}$) are indicated. Secretogranin II is indicated with an arrowhead.

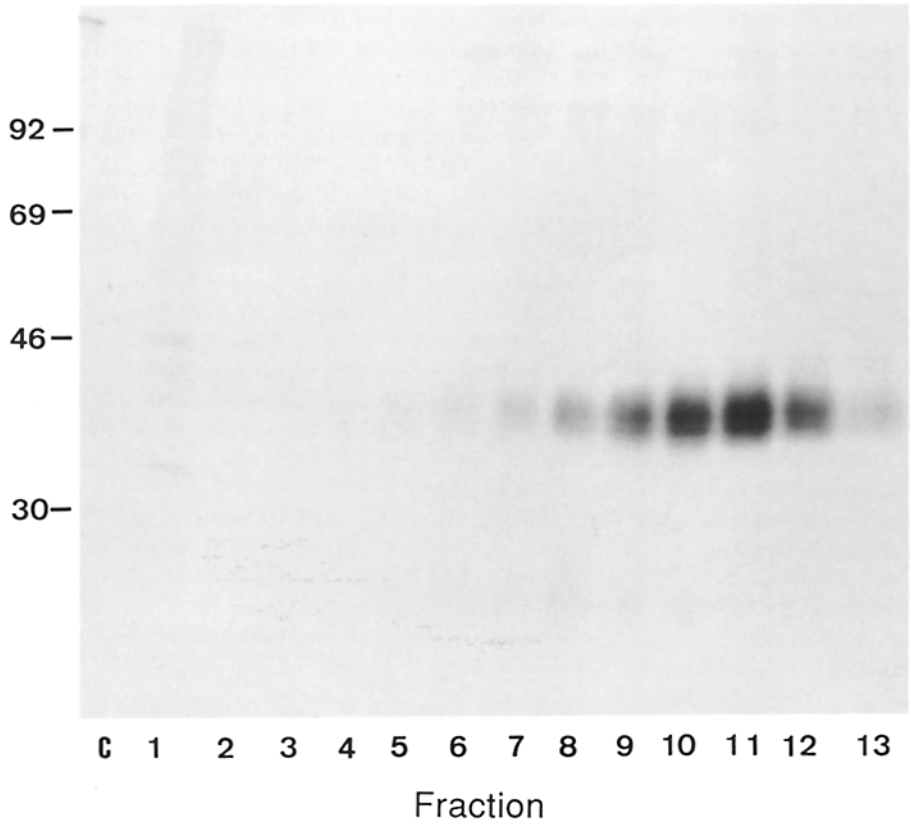


*Figure 5.* Distribution of p38 on a velocity gradient. One 10-cm dish of cells was labeled for 24 h and fractionated on a velocity gradient. Samples from each fraction were immunoprecipitated with anti-p38 serum before electrophoresis on a 10% gel followed by fluorography. The gradient fractions are numbered from the bottom. The molecular masses of marker proteins ($\times 10^{-3}$) are indicated. The track labeled *C* is a control immunoprecipitation of material pooled from fractions 9–11 performed without anti-p38 serum. The additional bands seen in fraction 1 are contaminating insoluble material found at the bottom of velocity gradients.

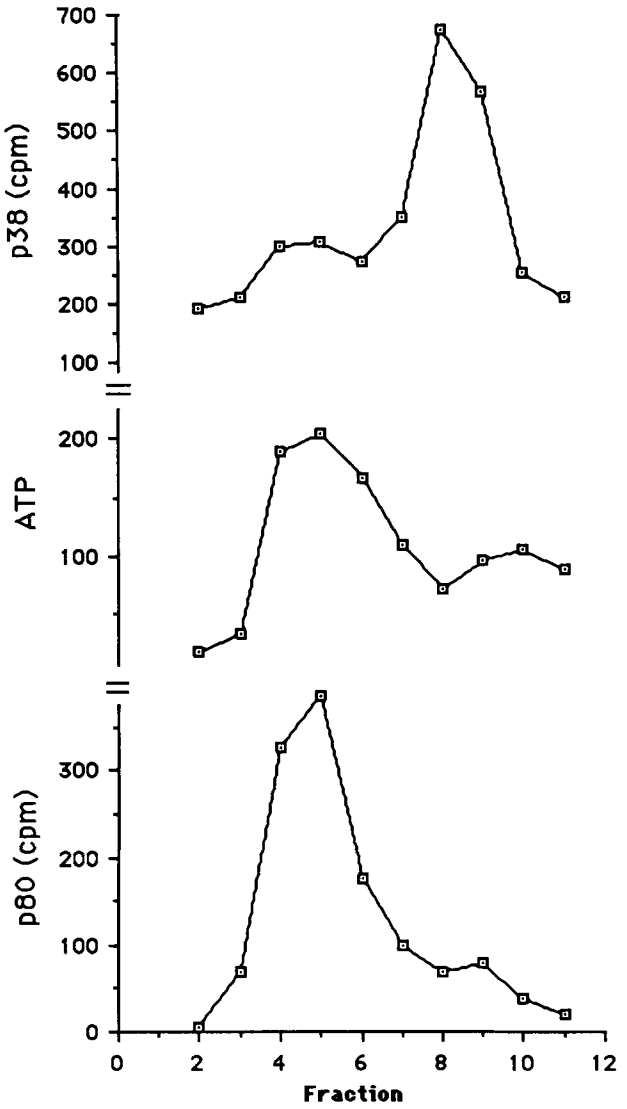


*Figure 6.* Distribution of p38 and dense core granule markers on an equilibrium gradient. Two 10-cm dishes of PC12 cells were labeled overnight and then fractionated on velocity and equilibrium gradients. Samples of the equilibrium gradient were electrophoresed either directly or after immunoprecipitation, and p38 and secretogranin II quantitated. Fractions were also analyzed for the presence of ATP. ATP is measured in arbitrary luminescence units.

isolation, the fraction of cellular p38 found in the dense granule fraction after a 24-h labeling could be at most 5%.

### *P38 Does Not Pass through the Dense Granules on Route to Its Cellular Destination*

It has been suggested that p38 reaches the small clear vesicles by endocytosis after its insertion into the plasma membrane by secretagogue-induced fusion of the large secretory granules (18). Although we have shown that at steady state in unstimulated PC12 cells most p38 is not associated with the dense granules (as might be expected if the above suggestion were true), the possibility still exists that it might be passing through this organelle on its way to the small clear vesicles that are its likely destination (18). This is because even in the absence of stimulation, the level of basal fusion of dense granules with the plasma membrane might still allow for a rapid transport through this compartment. Thus, the small amount of p38 found associated with the granules might simply reflect the rate at which synaptophysin passes through this compartment. We have investigated this possibility by analyzing the movements of p38 on velocity gradients in pulse–chase experiments.

We have determined the kinetics of acquisition of resistance to Endo H of p38. This will give a measure of the time taken to arrive in the medial Golgi compartment, and thus can be used as a rough guide to the likely time of transport to the *trans*-Golgi; if p38 were to be transported via a dense granule this is the compartment from which granule biogenesis would occur (5). Moreover, using the data from this experiment it may be possible to correlate the extensive post-translational modifications found on p38 with its position along the secretory pathway. This would aid in the interpretation of the pulse–chase gradient analysis. Fig. 7 shows that p38 is rapidly modified by the addition of N-linked sugars as expected from the sequence of this protein (2, 17). After a 15-min pulse and a 6-min chase essentially all the protein is found in a single band which is sensitive to digestion but which thereafter is rapidly modified to higher molecular weight forms that are Endo H resistant. The chase time taken for half of a population of newly synthesized p38 to become resistant to digestion is 25–30 min. This information was used in designing a pulse–chase experiment to determine whether p38 passes through the dense granules on its way to the small clear vesicles.

Cells were pulse labeled, chased for various times, and then fractionated on velocity gradients. Gradient samples were immunoprecipitated and analyzed by SDS-PAGE. After a 15-min pulse labeling, p38 runs as a single band that is distributed over much of the gradient (Fig. 8 *a*). This corresponds to the Endo H–sensitive form. After a chase of 30 min (Fig. 8 *b*) two things occur: the protein is seen as several forms of increasing molecular weight (Endo H resistant), and its distribution on the gradient alters. At the same time as it increases in molecular weight, reflecting its passage along the secretory pathway, the protein begins to concentrate at the top of the gradient. Thus, the higher molecular weight Endo H–resistant forms of the protein are to be seen mainly in the low-density fractions, while the earlier sensitive form (the lowest in molecular weight of the forms to be seen) is more broadly distributed. After a chase of 60 min (Fig. 8 *c*) the sensitive form has completely disappeared and the protein has reached a distribution similar to that seen after overnight labeling (Fig. 5). At this time each gradient fraction containing p38 exhibits the same spectrum of the different forms of this protein, as is the case with overnight labeling (not shown). Further chases of up to 180 min (Fig. 8, *d* and *e*) do not change the distribution of p38 from that seen at 60 min (Fig. 8 *c*).

This experiment strongly suggests that a synaptic vesicle membrane protein is not transported via the mature dense core granules in PC12 cells. At none of the chase times do we see any significant concentration of high molecular weight forms of p38 in the portion of the gradient containing the dense granules.

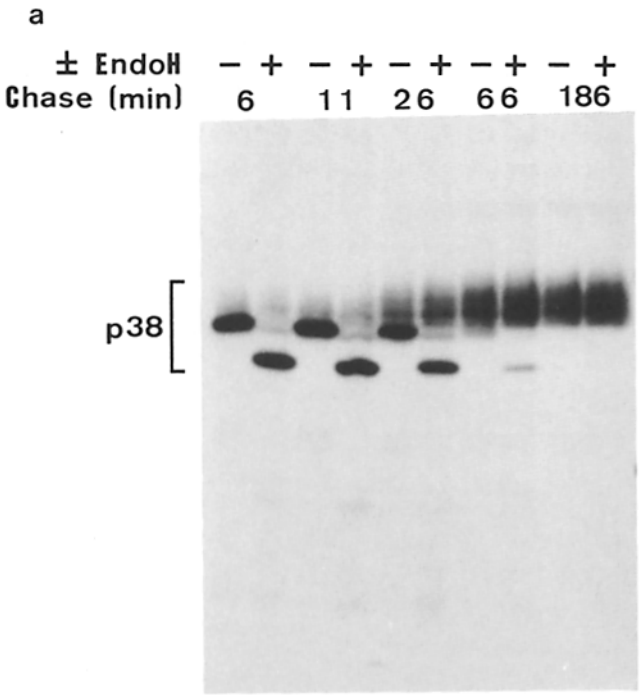


*Figure 7.* Kinetics of acquisition of Endo H resistance of p38. Five 3.5-cm dishes were pulse labeled for 15 min, chased for 6, 11, 26, 66, and 186 min, respectively, and then immunoprecipitated with anti-p38 serum. After resuspension in digestion buffer, the precipitated material was divided in two and 10 μU/μl of Endo H added to one of the portions. Resuspensions were incubated for 18 h at 37°C and then analyzed by SDS-PAGE (see Materials and Methods). (*a*) Fluorogram showing the banding pattern of p38 with and without Endo H with increasing chase time. (*b*) Plot of the percent of p38 that is resistant to Endo H out of total p38 at each chase time.

This interpretation of our data is dependent on p38 at 180 min having been chased along the secretory pathway beyond the *trans*-Golgi. Even though we have shown that the $t_{½}$ for transport to the medial Golgi is 25–30 min and that there is no change in p38 distribution when chase times longer than 60 min are used it is still a formal possibility that the protein has not yet reached its final destination. However, if the p38 were to become Endo H resistant with a $t_{½}$ of 25–30 min but not to have reached the dense granules within 3 h then we would expect to see an accumulation of p38 in the perinuclear area of these cells. Immunofluorescence microscopy, using either our anti-p38 serum or a monoclonal antibody to p38 (33) and comparing results with those obtained with an anti-Golgi antibody (4), shows no accumulation of p38 in this region of the cell (data not shown).

### *P38 Is Found in an Organelle of Similar Density to Synaptic Vesicles*

P38 has been identified in several neuronal and neuroendocrine cell types including PC12 cells. Morphological studies show that the protein is confined to synaptic vesicles and small electron-lucent vesicles that resemble synaptic vesicles (7, 20). This result implies that they are synaptic vesicle–like organelles. We have begun to characterize our p38-containing fraction from PC12 cells to see if it resembles synaptic vesicles by biophysical criteria.

As starting material we have taken those fractions from near the top of velocity gradients that contain the bulk of cellular p38 (i.e., fractions 9–11). Our characterization has concentrated on establishing whether the p38-containing material has a similar density to that of synaptic vesicles. We have therefore centrifuged our fractions to equilibrium on sucrose gradients (see Materials and Methods) designed for the purification of synaptic vesicles (33). A gel analysis of immunoprecipitates from such a gradient is shown in Fig. 9 *a*. The p38 is found by immunoprecipitation to peak in fractions 4–6. When a fraction of rat brain highly enriched in synaptic vesicles (12) is centrifuged on a gradient of this type and fractions are analyzed for the presence of p38 by Western blotting, a similar result is obtained (Fig. 9 *b*). Thus, the rat brain synaptic vesicles have a similar density to our p38-containing fraction of undifferentiated PC12 cells.

## *Discussion*

In this paper, we describe an isolation of the large dense core granules from PC12 cells. The isolation of granules from PC12 cells has been previously described but we have achieved an enrichment of granule-specific markers significantly greater than that reported elsewhere (23, 25, 31) while retaining a high yield. Our results suggest that this highly reproducible preparation should be of use in studies of granule biogenesis in PC12 cells.

We have used the granule purification to address the controversial question of the intracellular location of p38 in PC12 cells. P38 or synaptophysin has been described as a synaptic vesicle marker (13). However, it has also been found in adrenal medulla (33, 34). Navone et al. (20) have shown by morphological techniques that the localization of p38 in these neuroendocrine cells was not on the large dense core granules but on small clear vesicles. Franke et al. (7) have also presented some evidence suggesting that it is present on small clear vesicles in PC12 cells after treatment with nerve growth factor. Similarities between the large vesicles from neurons and the large dense core granules found in chromaffin cells (discussed in 16) as well as these morphological studies suggested that p38 in PC12 cells would not be found on the large dense core granules. It was therefore surprising when

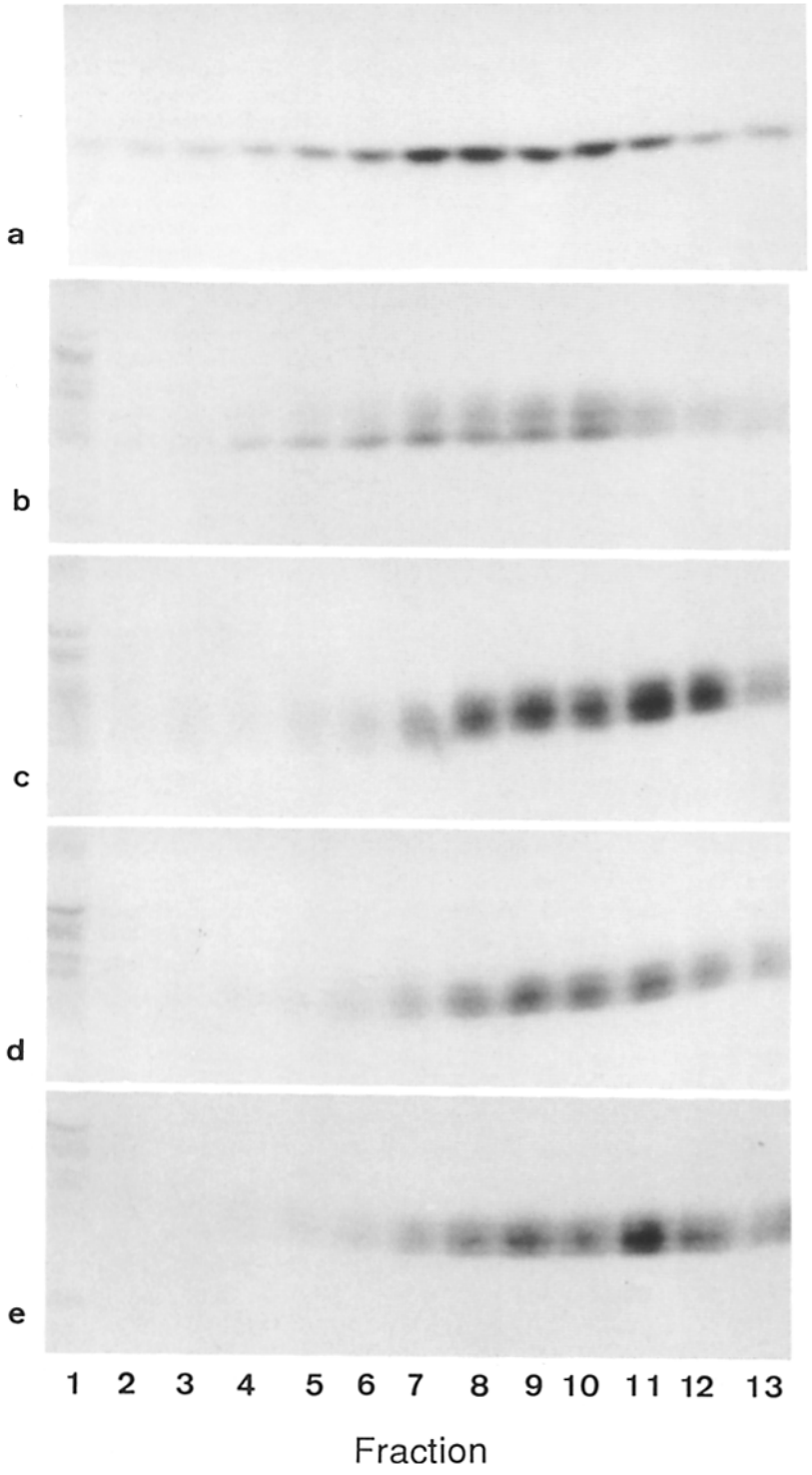


*Figure 8.* Distribution of p38 on velocity gradients in a pulse–chase experiment. Five 10-cm dishes of PC12 cells were labeled for 15 min, and then fractionated directly (*a*) or chased for 30 (*b*), 60 (*c*), 90 (*d*), or 180 min (*e*), and then fractionated on velocity gradients. Samples of each fraction were immunoprecipitated with anti-p38 serum, electrophoresed, and fluorographs prepared. Only the portion of each fluorograph showing p38 is displayed.

Lowe et al. (18) found that an antibody to p38 could be used as an immunoadsorbent with which to isolate large dense granules from PC12 cells. Our results support the morphological analysis of Navone et al. (20) and of Franke et al. (7) in showing that the bulk of p38 purifies away from the mature dense core granules. Moreover, Weidenmann et al. (35) have shown that bovine chromaffin granules contain very little p38. Nevertheless our data can be reconciled with those of Lowe et al. (18) in that we do find a very small amount of p38 copurifying with the dense core granules (note the small shoulder of p38 in fractions 4 and 5, Fig. 6). The different results obtained by these groups probably reflect the different sensitivities of immunoadsorbtion and immunoelectron microscopy.

We interpret the intracellular p38 that is present in these organelles (maximum 5%) as being a measure of the degree of missorting that is occurring between two exocytic pathways. Nevertheless, it is possible that the small amount of

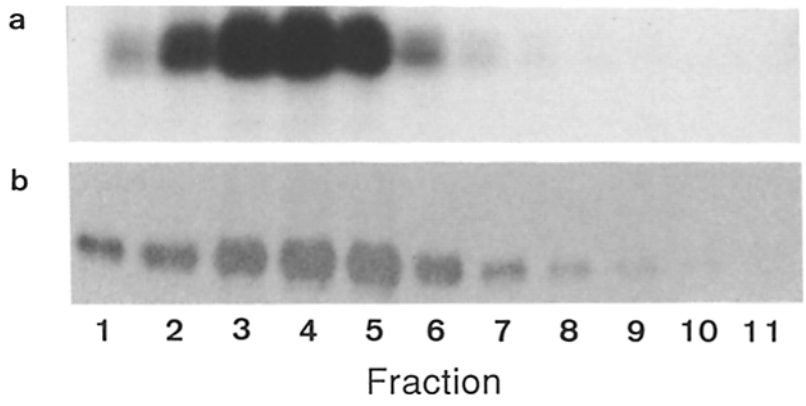


*Figure 9.* Comparison of the density of p38-containing material from PC12 cells and rat brain synaptic vesicles. A 10-cm dish of PC12 cells was labeled for 4 h and then fractionated on a velocity gradient. The fractions from the top of this gradient containing the bulk of p38 (i.e., fractions 9–11) were pooled and loaded onto a sucrose gradient (0.4–1.2 M sucrose). After centrifugation for 17 h at 25,000 rpm, samples of each gradient fraction were immunoprecipitated with anti-p38 serum and electrophoresed on a 10% gel. The portion of a fluorograph of this gel containing the p38 is shown (*a*). Rat brain synaptic vesicles were loaded onto a similar gradient, centrifuged, and samples of each fraction electrophoresed on a 10% gel and then analyzed by Western blotting, using the anti-p38 serum as primary probe. The portion of the blot with the p38 is shown (*b*).

p38 present in the dense core granules represents the functionally competent component of this protein, and that it is the other 95% that is "missorted." Thus, the small minority could be reaching a regulated secretory compartment via the dense granules and cell surface whereas the rest of the p38 enters some nonphysiological compartment. However, immunoelectron microscopic studies (7) suggest that most p38 in PC12 cells is found associated with a population of small vesicles resembling the expected functional compartment; i.e., synaptic vesicles. This suggests that it is unlikely that only 5% of this protein is in a functionally competent compartment. The similarities between the PC12 system and the sympathetic neuron led Lowe et al. (18) to speculate that if the small clear vesicles were closely related to synaptic vesicles and that if p38 was present in the large dense vesicles then these large granules could be the precursors of the small clear vesicles. Thus, p38 could be delivered to the plasma membrane after exocytosis of the dense granules and would then be endocytosed to form the small clear vesicles. This model implies an accumulation of p38 in mature dense granules in the absence of regulated exocytosis. This was contrasted to a model in which there were two regulated secretory organelles, both having an independent biogenesis. If this were the case one would not expect to find significant quantities of p38 in dense granules. We show that after 24-h labeling there is very little p38 present in mature granules. However, this low level of p38 (up to 5% of cellular synaptophysin) could be due to rapid kinetics of transport through this compartment facilitated by a basal level of granule fusion in the absence of external secretagogue. Our pulse–chase experiments were designed to explore this possibility. Our data showing that p38 does not accumulate in granules at any time during a 3-h chase seem to exclude a model where the bulk of p38 is transported to small clear vesicles by any route involving traffic through mature dense granules. However, our data do not allow us to distinguish between several remaining possible models for the biogenesis of the small "synaptic" vesicles. For example, these organelles may be derived directly in the *trans*-Golgi region or by endocytosis not involving granules. Our data simply rule out one of the possible models; that of transport to the plasma membrane via the dense secretory granules followed by endocytosis.

The separation of p38 from the dense core granules provides biochemical evidence for PC12 cells ability to efficiently sort a membrane protein normally associated with regulated secretion between exocytic pathways. Moreover, if the small clear vesicles of PC12 cells are capable of exocytosis after stimulation then our data show that these cells are capable of effectively sorting p38 between two different regulated pathways. This degree of discrimination would be a remarkable result since Kelly (14) and Burgess and Kelly (3) have shown that the neuroendocrine cell line AtT-20 has a much broader sorting specificity.

There have been other reports of separation of the organelles of two regulated pathways in PC12 cells. Schubert and Klier (25) and Roda et al. (23) reported that catecholamine- and acetylcholine-containing particulate fractions could be separated on a sucrose gradient. Unfortunately, in neither case were the proteins of either fraction characterized in any detail.

Although we find p38 at an anomalously high density compared with values reported for synaptic vesicles (0.8 vs. 0.4 M sucrose [e.g., reference 32]) we find that purified brain synaptic vesicles do band at the same density as our p38-containing fraction on sucrose gradients. We therefore assume this to be due to differences in gradient composition since gradient composition can have dramatic effects on density of synaptic vesicles (32).

Our results may have implications for the biogenesis of cholinergic synaptic vesicles. However, for the PC12 system to be a good in vitro model for a neuron, the p38 must be present on a vesicle that is closely related to a synaptic vesicle. Here we demonstrate that the fraction of PC12 cells containing p38 has a similar density to that of brain synaptic vesicles. There is also data from Weidenmann et al. (35) showing that at least some of the p38 in PC12 cells behaves similarly to synaptic vesicles in size exclusion chromatography. However, we do not yet have any functional evidence that p38 is in a regulated exocytic vesicle in these cells. Further characterization of the fraction enriched in p38 may provide an answer to this and other related questions. It might also provide us with the opportunity to investigate sorting of proteins between these exocytic pathways.

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