Overall Lack of Regulated Secretion in a PC12 Variant Cell Clone*  
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A stable clone of PC12 neuroendocrine cells, named 27, known from previous studies to exhibit a defect of regulated secretion (lack of regulated secretory proteins, of synaptophysin, of dense granules and of catecholamine uptake and release; Clementi, E., Racchetti, G., Zacchetti, D., Panzeri, M. C., and Meldolesi, J. (1992) Eur. J. Neurosci. 4, 944–953) was characterized in detail to clarify the nature of its phenotype and the mechanisms of its establishment. The neuroendocrine nature of the PC12-27 phenotype was documented by specific markers: synapsins, neurofilament subunit H, neuronal kinesin, and α-latrotoxin receptor. Moreover, various intracellular membrane systems of PC12-27, including the endoplasmic reticulum and the Golgi complex, appeared similar to control PC12 in both morphology and marker expression. In contrast, all the investigated markers located either in dense granules (dopamine-β-hydroxylase), in synaptic-like microvesicles (the acetylcholine transporter) or in both these regulated secretory organelles (VAMP2/synaptobrevin-2, synaptotagmin) were missing in PC12-27 cells, and the same was true also for the cytosolic and plasmalemma proteins involved in regulated exocytosis (Rab3, SNAP25, syntaxin). Pulse labeling and in vitro translation experiments revealed the defect to consist in a protein synthesis blockade that mRNA studies (reverse transcription-polymerase chain reaction, Northern blotting, and actinomycin D experiments) revealed to take place primarily at the transcriptional level. The secretion defect of PC12-27 cells was modified neither by various types of long term stimulation nor by nerve growth factor treatment. Moreover, when one of the missing regulated secretory proteins, chromogranin B, was expressed by cDNA transfection, it was secreted, however via the constitutive pathway. Our results demonstrate that PC12-27 cells are fully incompetent for both branches of regulated secretion, those of dense granules and synaptic-like microvesicles, possibly because of the impairment of a general expression control system that appears to operate independently of neuroendocrine cell differentiation.
Defect of Regulated Secretion

Defect of Regulated Secretion

EXPERIMENTAL PROCEDURES

Materials and Antibodies—[35S]Sulfate and Trans-35S-label were purchased from Amersham International (Buckinghamshire, UK) and ICN Pharmaceuticals (Irvine, CA), respectively; the expression vector pREP4 from Invitrogen (San Diego, CA); fura-2 from Calbiochem, (San Diego, CA); a-latrotoxin was purified to homogeneity as described (27). The monoclonal and polyclonal antibodies against human CgB (hCgB) were raised as described previously (28). Other antisera and monoclonal antibodies used in this study were kindly provided by the following scientists: anti-mannosidase II by M. G. Farquhar (29), anti-TGN38 (EMBL database accession number X13618, 1988), anti-physin (X06388, 1987). First strand cDNA synthesis was conducted using an oligo(dT) primer according to the standard protocol (46). The RT-PCR products were obtained after 35 cycles of the following conditions: denaturation for 1 min at 94 °C, annealing for 30 s at 61 °C, and extension for 45 s at 72 °C using 1 unit of Taq polymerase/30 μl (Perkin Elmer, Ampli-Taq kit). For Northern blotting, probes were prepared from RT-PCR fragments isolated from polyacrylamide gel by the “crush and soak” method (46), and labeled with [α-32P]dCTP (3000 Ci/mmol; Redivue, Amersham) by random-priming oligolabeling (47). 10 μl of total RNA of each PC12 clone were separated on 1% agarose, 2.2 M formaldehyde denaturing gel and transferred onto nylon membranes (Hybond-N, Amersham). Hybridizations were carried out at 65 °C in a mix containing 125 mM Na2HPO4, 1 mM EDTA, 250 mM NaCl, 7% SDS, 100 μg/ml denatured salmon sperm DNA, and 106 cpm/ml radiolabeled probe. Washes were performed at 65 °C first in 2 × SSC and 0.1% SDS, then twice in 0.1 × SSC and 0.1% SDS for 20 min before film exposure. Northern blot signals were normalized to the glyceraldehyde-3-phosphate dehydrogenase mRNA detected as described (48). The transcription rate of the SgII gene was evaluated by run-on experiments carried out with nuclei from PC12-27 and control cells, processed as described in Ref. 49, using the specific cDNA.

Labeling and Stimulation of the Cells—Control cell clones and subclones were grown in 24-well plates until near confluence. The cells were then labeled overnight with 100 μCi/ml [35S]sulfate as described (50). For long term stimulations, before their labeling with [35S]sulfate, the cells were treated for up to 4 days with either 55 mM KCl, 20 nM TPA, or 1 mM dibutyryl-cAMP.

Hormone and Gene Transfer—Control cell clones and subclones were grown in 24-well plates until near confluence. The cells were then labeled overnight with 100 μCi/ml [35S]sulfate as described (50). For long term stimulations, before their labeling with [35S]sulfate, the cells were treated for up to 4 days with either 55 mM KCl, 20 nM TPA, or 1 mM dibutyryl-cAMP.

Mitochondria were purified to homogeneity as described (27). The monoclonal and polyclonal antibodies against human CgB (hCgB) were raised as described previously (28). Other antisera and monoclonal antibodies used in this study were kindly provided by the following scientists: anti-mannosidase II by M. G. Farquhar (29), anti-TGN38 (EMBL database accession number X13618, 1988), anti-physin (X06388, 1987). First strand cDNA synthesis was conducted using an oligo(dT) primer according to the standard protocol (46). The RT-PCR products were obtained after 35 cycles of the following conditions: denaturation for 1 min at 94 °C, annealing for 30 s at 61 °C, and extension for 45 s at 72 °C using 1 unit of Taq polymerase/30 μl (Perkin Elmer, Ampli-Taq kit). For Northern blotting, probes were prepared from RT-PCR fragments isolated from polyacrylamide gel by the “crush and soak” method (46), and labeled with [α-32P]dCTP (3000 Ci/mmol; Redivue, Amersham) by random-priming oligolabeling (47). 10 μl of total RNA of each PC12 clone were separated on 1% agarose, 2.2 M formaldehyde denaturing gel and transferred onto nylon membranes (Hybond-N, Amersham). Hybridizations were carried out at 65 °C in a mix containing 125 mM Na2HPO4, 1 mM EDTA, 250 mM NaCl, 7% SDS, 100 μg/ml denatured salmon sperm DNA, and 106 cpm/ml radiolabeled probe. Washes were performed at 65 °C first in 2 × SSC and 0.1% SDS, then twice in 0.1 × SSC and 0.1% SDS for 20 min before film exposure. Northern blot signals were normalized to the glyceraldehyde-3-phosphate dehydrogenase mRNA detected as described (48). The transcription rate of the SgII gene was evaluated by run-on experiments carried out with nuclei from PC12-27 and control cells, processed as described in Ref. 49, using the specific cDNA.

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Defect of Regulated Secretion

farads. Cells were then diluted to $1 \times 10^7$/ml in Dulbecco's modified Eagle's medium supplemented with serum and plated in 4 × 10-cm dishes (1 × 10^6 cells/dish). The selection of hygromycin B-resistant cells was started 48 h after the electroporation using 450 μg/ml antibiotic. After 15 days, individual clones were transferred into 24-well plates and grown to sufficient density to screen for hCgB (see below). The stably transfected clones were cultured in the continuous presence of hygromycin B (450 μg/ml).

Immunoprecipitation—Cleared cell lysates and media were diluted in immunoprecipitation buffer (50 mm Tris-HCl, pH 7.4, 500 mm NaCl, 5 mm EDTA, containing either 0.3% or 1% Triton X-100) and incubated overnight at 4 °C with the appropriate concentrations of specific antisera followed by incubation for 1 h with 15 μl (packaged gel) of protein A conjugated to Sepharose CL-4B (Pharmacia Biotech, Uppsala, Sweden). The immunoprecipitates thus obtained, as well as aliquots of the cleared cell lysates and media, were analyzed by SDS-PAGE followed by fluorography, as described previously (21). Fluorograms were quantitated by densitometric scanning using an LKB Ultroscan (Pharmacia).

Values are expressed as the percent of the hCgB or heparin sulfate proteoglycan present in the cleared cell lysates at the end of the 15-min pulse.

Immunofluorescence and Electron Microscopy—PC12 cells were plated onto poly-l-lysine-coated coverslips, grown for 2–4 days, and then analyzed by indirect immunofluorescence (22). For electron microscopy, cell monolayers were washed in phosphate-buffered saline, fixed for 30 min at 4 °C with 4% paraformaldehyde, 0.2% glutaraldehyde in 0.1 m sodium cacodylate buffer (pH 7.3), post-fixed in 2% OsO_4, scraped from the dishes, pelleted, dehydrated, and embedded in Epon as described (52). Thin sections, cut at an Ultra-Cut “E” microtome (Reichert-Jung, Wien, Austria), were examined with a Philips 400 electron microscope. For immunoelectron microscopy the cells, fixed with aldehydes as above, were scraped from the dishes and sedimented. The pellets were included in 10% gelatin, fixed for an additional 60 min, and then sliced into small pieces, which were passed through increasingly concentrated sucrose solutions (up to 2.3 μ) in phosphate-buffered saline, and finally frozen in liquid nitrogen. Ultrathin cryosections were cut at −100 °C using an Ultra-Cut “E” microtome equipped with an PC4 cryochamber, transferred to Formvar-coated nickel grids, and finally immunolabeled as described previously (28), using an antiserum directed against the C terminus of hCgB revealed by an anti-rabbit IgG conjugated to 5-nm colloidal gold particles.

RESULTS

The Clone 27 Cells Exhibit an Overall Defect of Regulated Secretion—In a previous study (26), the 27 clones variant of PC12 cells was shown to lack secretory granules as well as CgB and SgII, the major regulated secretory proteins of PC12 cells (21). To study in more detail the extent of the defect, expression of known protein markers for various organelles and functions in neuroendocrine cells was investigated. Fig. 1 shows Western blots of total homogenates prepared from the defective clone PC12-27 and from the control clone 251, which exhibits the typical PC12 phenotype. Panel A is focused on proteins active in the Golgi area. βCOP, a well characterized member of the protein complex forming the coats of the vesicles budding from the ER and the Golgi cisternae (see Ref. 7, and references therein), was revealed at similar levels in both the 27 and 251 clones. Likewise, Rab6, a small GTP-binding protein thought to be involved in the regulation of constitutive vesicle transport from the TGN (53), and mannosidase II, an integral membrane protein of Golgi stacks, were immunodetected in both clones, although their levels in clone 27 appeared lower than those detected in clone 251 (Fig. 1A). Parallel immunofluorescence experiments, carried out using antibodies against mannosidase II and TGN38, an integral membrane protein of the TGN, suggested a normal organization of the Golgi complex (data not shown). After treatment with brefeldin A, the two markers redistributed similarly in PC12-251 and -27 cells, becoming colocalized with ER and endosomal markers, respectively (data not shown).

Completely different results between clones 27 and 251 emerged when we examined the expression of components specific for one of the secretory organelles (granules or synaptic like microvesicles), common to both these organelles or participating in their exocytosis. As shown in Fig. 1B, dopamine-β-hydroxylase, the noradrenaline synthesizing enzyme located at the luminal surface of the granule membrane, and the vesicular acetylcholine transporter, a trans-membrane protein exclusively localized in the synaptic-like microvesicles (35), were both undetectable in PC12-27 cells but clearly expressed in control cells. Since recent results have demonstrated that in PC12 cells (36, 38) the docking and fusion of both synaptic-like microvesicles and secretory granules involves a number of identified proteins that are present in or interact with the vesicles and the plasma membranes (see Refs. 7–9, and references therein), the expression of VAMP2, synaptotagmin I, SNAP25, syntaxin, and the small GTP-binding protein Rab3 was also examined. Fig. 1B shows that none of these proteins was detected in clone 27, whereas they were all abundant in rat brain and present in considerable amounts in PC12-251 cells.

Further experiments were focused on the expression of non-granular proteins specific for neurons and neurosecretory cells. Blots shown in Fig. 1C demonstrate that tyrosine hydroxylase, the rate-limiting enzyme for catecholamine synthesis, is abundant in 251 and scarce, yet clearly appreciable, in PC12-27 cells. Similarly, synapsin I and IIa, the members of a peripheral membrane protein family known to establish a con-
Ca²⁺-sensitive granules, are devoid of it (56). Among the effects of receptor stimulation of synaptic terminals and found also on typical PC12 cells, were very similar, whereas bovine chromaffin cells remained negative for CgBand SgII (Fig. 3A). At variance with the dephosphorylated form (55), PC12 cells exhibited a lower than in the brain. Of the two neurofilament H subunit isoforms of kinesin (42), whose doublet of 120–130 kDa was not due to the absence of the relevant enzymatic activity.

To further characterize the defect of clone 27, cells were exposed to nerve growth factor (50 ng/ml, for 4 days), the treatment that induces the classical neuron-like differentiation of the cells from which PC12 originate), and chromaffin cells (i.e. the cells from which PC12 originate), grain expression was shown to increase after long term treatment with stimulants such as dibutyryl-cAMP, depolarizing concentrations of KCl and the phorbol ester, TPA (57, 58). Experiments were therefore carried out to investigate whether expression of CgB and SgII could be induced in PC12-27 cells when these treatments were prolonged for up to four days. With KCl (55 mm) and TPA (20 nm), the intracellular levels of [³⁵S]sulfate-labeled CgB and SgII did increase in PC12-251 cells. In contrast, the two secretory proteins remained inappreciable in PC12-27 cells (Fig. 3A). Similar results were obtained when dibutyryl-cAMP (1 mm) was employed as the stimulant.

In separate experiments, the cells of the two clones were exposed to nerve growth factor (50 ng/ml, for 4 days), the treatment that induces the classical neuron-like differentiation of the cells from which PC12 originate), and chromaffin cells (i.e. the cells from which PC12 originate), and synaptophysin mRNAs is due to an acceleration of the molecular mechanisms of the Clone 27 Defect—In order to establish whether in PC12-27 cells expression of the mRNAs coding for the missing proteins was completely absent, as expected for deletions of the corresponding genes, non-quantitative RT-PCR analyses were carried out. In these experiments, we focused on the mRNAs of one secretory protein (SgII) and three membrane proteins that in control cells are located in both granules and vesicles (VAMP2, synaptotagmin I, and synaptophysin). The results obtained exclude gene deletions, inasmuch as in all four cases clearly amplified cDNA signals were recorded (Fig. 4A). The levels of expression of the corresponding mRNAs were established quantitatively by Northern blot analysis. As can be seen in Fig. 4B, some degree of variability (<50%) appeared already between two granule-containing control clones, 251 and another clone, denominated 1 (Ref. 26). With PC12-27 cells, however, the differences with respect to controls were very large. Among the mRNAs analyzed in the defective clone, that of synaptotagmin I was barely appreciable while that of VAMP2 accounted for the 11% of control clone 251. In the case of synaptophysin and SgII, the values approached 10 and 5% of the control, respectively (Fig. 4B).

The latter two mRNAs were further investigated with the aim of clarifying the mechanisms of their reduction and lack of translation in clone 27 cells. Sucrose gradient centrifugation experiments, carried out in parallel in clones 27 and 251, failed to reveal any difference in the nuclear and cytoplasmic distribution of the two mRNAs (not shown). These results exclude the possibility that in clone 27 the lack of translation of the SgII and synaptophysin mRNAs is due to an acceleration of the mRNA transport across the nuclear envelope. Another possi-
Results shown are representative of three experiments. A, PCR and/or Northern blot (N.) analyses of the control 251 and the defective 27 clones. A, experiments were carried out with the PCR primers designed for amplification of SgII, VAMP2, synaptotagmin I (SytI), or synaptophysin (Sph) cDNAs, under the conditions specified under "Experimental Procedures." One μg of total RNA extracted from PC12-251 or -27 cells was used as starting material for each reaction. For each sample two lanes are shown: one obtained in the presence (left) and the other in the absence (control, right) of RT. Results shown are representative of three experiments. B, total RNAs (10 μg) prepared from two control clones, 251 and 1, and from the defective clone 27 were analyzed for Northern blotting using specific probes for SytI, VAMP2, Sph, or SgII mRNAs prepared as described under "Experimental Procedures." The densitometric data, normalized based on glyceraldehyde-3-phosphate dehydrogenase data obtained in the same nylon membranes, are expressed as percent of those obtained for the control clone 251 (broken line). Results shown are representative of five experiments.

Fig. 5. mRNA turnover in PC12-251 and -27 clone cells. Cells were cultured for 6, 12, 18, or 24 h in the presence of actinomycin D (5 μg/ml). Total RNA (10 μg for each sample) was extracted from PC12-251 and -27 cells and analyzed by Northern blot as described in Fig. 5. Values, expressed as percent of those found in each clone at time 0, are averages of three experiments. For relative values see Fig. 4B.

(1) Defect of Regulated Secretion

Expression of Transfected hCgB in Clone 27—To study whether the lack of secretory granules in clone 27 cells is the simple consequence of the absence of regulatable secretory proteins, transfection experiments with the cDNA for hCgB were carried out. Various stable subclones, isolated from the transfected cell population, were labeled with [35S]sulfate. When the proteins of cells and media obtained from three such subclones (referred to as c4, c5, and c7) were analyzed by SDS-PAGE followed by fluorography, a labeled band of 120 kDa, the expected molecular mass of mature hCgB, was detected accompanied in the media by two bands of lower molecular mass (105 and 90 kDa, respectively) (Fig. 6, A and B). These findings suggest a proteolytic processing just before and/or after secretion. Neither the subclones and their parent clone 27 nor the control clone 251 showed detectable levels of the proprotein convertases, PC1/3 and PC2 (data not shown), i.e. of the proteases that in other neuroendocrine cells are known to catalyze limited proteolysis of prohormones (see Ref. 3, and references therein). Thus, other enzymes, e.g. furin or a furin-like enzyme, which are more ubiquitous, might be involved in the proteolytic processing of hCgB in the various subclones. Using an antibody directed against the C terminus of hCgB, the [35S]sulfate-labeled 120-kDa band as well as the smaller bands were immunoprecipitated from the total homogenates and media of the three transfected subclones. In contrast, the preparations obtained from the parental clone 27 remained negative (Fig. 6, C and D). Treatment of these gels with 1 M HCl at 100 °C for 5 min largely removed the [35S]sulfate labeled from the hCgB indicating that [35S]sulfate was mainly bound to tyrosine residues (data not shown), as is the case with control cells. The amount of hCgB expressed by the various transfected subclones was different. Since c7 cells synthesized more hCgB than c4 and c5 (Fig. 6, A and B), this subclone was selected for further experiments.

Fig. 3. Metabolic labeling of PC12 cells. A, right panel. PC12 cells from clones 251 and 27 were labeled overnight with [3H]tyrosine. Equal volumes (15 μl) from the cell lysates (TH, right panels) and the heat-stable fractions (HSS, left panels) were analyzed by SDS-PAGE followed by fluorography. A, left panel. PC12-251 and 27 cultured for 4 days in the conventional medium (−) or in the medium supplemented with high KCl (55 mM, K) or TPA (20 nM, TPA) were labeled overnight with [35S]sulfate. Equal amounts of proteins of cell lysates (3 μg) and equal volumes of the heat-stable fractions (HSS) were analyzed as above. Note the absence of the granins in clone 27, no matter whether incubated without any treatment or after long term treatments with high KCl or TPA. B, PC12 cells from clones 251 and 27 were labeled for 7 min with the Trans[35S]-label ([35S]-Methionine) and proteins from the cell lysates were either analyzed by SDS-PAGE followed by fluorography (1 μg of protein, TH) or subjected to immunoprecipitation (30 μg of protein, IP) using an anti-rat SgII antiserum, before SDS-PAGE. Open arrows, rat CgB; arrowheads, SgII; brackets, heparan sulfate proteoglycans. Results shown are representative of at least two consistent experiments.

Fig. 4. RT-PCR (A) and Northern blot (B) analyses of the control 251 and the defective 27 clones. A, experiments were carried out with the PCR primers designed for amplification of SgII, VAMP2, synaptotagmin I (SytI), or synaptophysin (Sph) cDNAs, under the conditions specified under "Experimental Procedures." One μg of total RNA extracted from PC12-251 or -27 cells was used as starting material for each reaction. For each sample two lanes are shown: one obtained in the presence (left) and the other in the absence (control, right) of RT. Results shown are representative of three experiments. B, total RNAs (10 μg) prepared from two control clones, 251 and 1, and from the defective clone 27 were analyzed for Northern blotting using specific probes for SytI, VAMP2, Sph, or SgII mRNAs prepared as described under "Experimental Procedures." The densitometric data, normalized based on glyceraldehyde-3-phosphate dehydrogenase data obtained in the same nylon membranes, are expressed as percent of those obtained for the control clone 251 (broken line). Results shown are representative of five experiments.

Discussion

A number of techniques and approaches have been used to investigate the mechanism of regulated secretion. These include the use of proteases such as furin or furin-like enzymes, to degrade prohormones in a cell-free system (see Ref. 3, and references therein). These studies have shown that proteolytic processing of prohormones is a common feature of neuroendocrine cells, and is likely to be involved in the maturation of prohormones in vivo. In the present study, we have used a different approach to study the mechanism of regulated secretion in PC12 cells.

Characterization of PC12 Cells

PC12 cells are a well-characterized model system for the study of regulated secretion. They express a number of key components involved in the process of regulated secretion, including the vesicle-associated membrane protein 2 (VAMP2), synaptotagmin I (SytI), and the granin family of proteins. These proteins are involved in the fusion of secretory vesicles with the plasma membrane, and in the recognition of the target membrane. In addition, PC12 cells express the neurotrophin nerve growth factor (NGF), which is known to stimulate regulated secretion in these cells.

Regulated Secretion in PC12 Cells

Regulated secretion in PC12 cells is a complex process that involves the fusion of secretory vesicles with the plasma membrane. This process is regulated by a number of factors, including the presence of neurotransmitters, growth factors, and ion channels. In the present study, we have investigated the mechanism of regulated secretion in PC12 cells using a combination of biochemical and molecular biological approaches.

Conclusion

In conclusion, we have shown that regulated secretion in PC12 cells is a complex process that involves the fusion of secretory vesicles with the plasma membrane. This process is regulated by a number of factors, including the presence of neurotransmitters, growth factors, and ion channels. The results of this study provide new insights into the mechanism of regulated secretion in PC12 cells, and may have implications for the understanding of regulated secretion in other neuroendocrine cells.

References

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further experiments.

**Transfected hCgB Is Secreted Constitutively from Subclone c7**—The results described in the preceding section show that the transfected subclones are able to synthesize, post-translationally modify, and secrete hCgB. Pulse-chase experiments, however, demonstrated that in these subclones secretion of the granin is different from that in the control 251 clone. In the latter, newly synthesized, [35S]sulfate-labeled granins are largely retained intracellularly upon chase because these proteins are efficiently (95%) sorted to secretory granules (12). In contrast, when c7 cells were [35S]sulfate-labeled for 1 h and then chased for 4 h without any stimulation, only 7% of the total labeled hCgB was recovered intracellularly. The majority (70%) was found in the chase medium, accounted for by the mature, 120-kDa form together with the degradation products indicated previously (Fig. 6, E and F). The incomplete recovery observed in these experiments (see also Fig. 7A), suggests that a fraction of hCgB is processed to small peptides, undetectable by SDS-PAGE under the employed conditions.

Further pulse-chase experiments were carried out with stimulation of regulated exocytosis. Cells from subclone c7 and the control clone 251 were labeled for 15 min and then chased for 10, 20, and 60 min (Fig. 7). To stimulate regulated exocytosis, some dishes were exposed to depolarizing concentration of KCl immediately after labeling, i.e., when the majority of hCgB was still intracellular also in clone c7. As shown in Fig. 7, the kinetics of hCgB release from unstimulated c7 cells was similar to that of heparan sulfate proteoglycan released from PC12-251 cells, a well characterized marker of constitutive secretion (12), except that the recovery of labeled hCgB was incomplete after 60 min of exposure (Fig. 7E). In control PC12-251 cells, depolarization induced secretion of endogenous CgB via the regulated pathway (Fig. 7D), whereas in c7 cells hCgB secretion was not stimulated (Fig. 7B). From these data we conclude that in subclone c7 secretion of hCgB occurs constitutively.

**Immunocytochemical Localization of hCgB in Control and Subclone c7 PC12 Cells**—The intracellular localization of hCgB
in c7 cells was investigated at the electron microscopy level. In the control PC12-251 cells (Fig. 8, A and B), a population of dense-core granules was observed throughout the cytoplasm, especially in the proximity of the plasma membrane, whereas in c7 cells no such granules could be seen (Fig. 8, C and D). The Golgi complex of control cells (GC, B) is highly developed and exhibits granule formation activity with protein condensation in TGN cisternae (arrowhead) and a few, probably newly synthesized granules (arrow). In a fraction of c7 cells, aggregated material (D, arrowheads) is also evident within the lumen of the Golgi complex (GC, D). However, discrete granules never appear even in the Golgi complex area. The hCgB nature of the Golgi luminal protein in c7 cells is revealed by ultrathin cryosection immunolabeling using the anti-C terminus hCgB antiserum decorated with 5-nm anti-rabbit IgG-gold particles (E and F, arrowheads). Bars, 500 nm in A and C; 250 nm in B, D, and E; 100 nm in F.

**DISCUSSION**

In neurons and neuroendocrine cells, regulated secretion is more complex than in other cell types, inasmuch as it includes two distinct, parallel pathways, which, however, share at least some of their molecular components: the pathway of dense granules and that of synaptic-like microvesicles (Refs. 1–4 and 6, and references therein). The mechanisms that govern the acquisition of the dually regulated secretion have never been specifically investigated, and appearance of granules and vesicles has often been conceived as a general differentiation event, occurring in parallel to the acquisition of other typical neuroendocrine properties (59–61).

In a previous study (26) in which 16 permanent clones isolated from the PC12 neurosecretory parental cell population were surveyed biochemically and morphologically, one clone (denominated 27) had already been reported to lack dense granules, two secretory proteins, CgB and SgII, as well as synaptophysin, a membrane protein of synaptic-like microvesicles also present in secretory granules (62, 63). In addition, PC12-27 cells were unable to pick up, store, and regulate release catecholamines (26). In this previous investigation, however, the status of clone 27 cells, i.e. whether they are typical PC12 neurosecretory cells just incompetent for regul-
lated secretion or undifferentiated PC12 precursors, was not established, and the effects of long term stimulations (e.g., depolarization with high K\(^+\) and activation of protein kinases C or A) were not evaluated. These treatments are important because, on the one hand, they are known to increase considerably the secretory activity of control PC12 cells (58); on the other hand, they have been recently shown to induce the appearance of granules in two clones of ATI20 (another type of neurosecretory cell) that, under resting conditions, had appeared incompetent for regulated secretion (64).

The present detailed investigation of clone 27 cells has yielded answers to the above questions. The neuroendocrine nature of the phenotype was demonstrated by the expression of well known markers, in addition to synapsins, also the neurofilament H subunit, the neuronal isoform of kinesin and the receptor of α-latrotoxin (42, 54–56). Also present, although in low amount, is tyrosine hydroxylase, the cytosolic enzyme that constitutes the specific rate-limiting step in the biogenesis of the catecholamines that in normal cells are destined to be segregated within granules (65). This list does not exhaust all neuroendocrine markers of PC12-27 cells. In preliminary experiments both surface N-type Ca\(^{2+}\) channels and the novel G protein XL\(\alpha\)s (66) have been detected at levels comparable to those of controls.\(^2,3\) As far as long term stimulations, and at variance with the ATI20 clones, none of the treatments mentioned above was found to induce any reappearance of regulated secretion in PC12-27 cells, and no reappearance occurred after nerve growth factor, the treatment that induces differentiation of PC12 cells toward the neuronal phenotype (23). We conclude therefore that competence for regulated secretion is not a necessary step in the acquisition of the neuroendocrine phenotype but appears to be regulated independently.

Two additional properties of PC12-27 clone cells deserve further discussion. The first is that the molecular defect of the clone appears to concern mainly the two types of regulated secretory organelles, dense granules and synaptic-like microvesicles, as well as the plasmalemma and the cytosolic components that are needed for their exocytic discharge. All these investigated components were in fact undetectable by Western blotting, and regulated secretion was not functional in PC12-27 cells. In contrast, the components of the other investigated structures in the secretory pathway (i.e., ER, Golgi cisternae, and TGN) were all appreciable in PC12-27 cells, although some of them (mannosidase II, Rab6) were clearly reduced with respect to controls. Moreover, the ER-Golgi-TGN transport of proteins and the constitutive limb of secretion appeared functional. The concomitant lack of both granules and vesicles does not seem to be a simple consequence of their considerable molecular overlapping, with expression in their membranes of common proteins such as synaptophysin, synaptotagmin I, and VAMP2 (7–9, 37, 62, 63). In fact, specific membrane components, such as dopamine-β-hydroxylase (granules) and the acetylcholine transporter (vesicles), were also missing in PC12-27 cells. Taken together these results strongly suggest that expression within neuroendocrine cells of the two organelle types occurs under the control of common factor(s). The nature of the latter is unknown. At the moment we can only exclude that the lack of the cargo, constituted by the secretory proteins addressed to granules, plays such a role. In fact, when hCgB was expressed at high levels in PC12-27 cells after cDNA transfection, it did not cause the reappearance of secretory organelles. Although some granin aggregates (i.e., the initial step of granule formation) could be observed in the TGN lumen of the defective cells, release of the transfected protein did ultimately occur not via the regulated but via the constitutive pathway. Such a diversion of a regulated secretory protein, typical of cells without regulated secretion (1), confirms the full incompetence of PC12-27 cells for the latter process. The possible role of other missing proteins in granule-vesicle acquisition remains to be investigated.

The second information we have obtained concerns the mechanisms sustaining the phenotypic defect of PC12-27 cells. Although not quantitative, the positive RT-PCR results, focused on four components, excluded deletion of the genes for granule/vesicle proteins. On the other hand, the quantitative cellular levels of mRNAs, revealed by Northern blot, were found to be decreased to considerable extents (85–95\%) with respect to controls. These results suggest the existence in PC12 cells of a unique transcriptional control system regulating the expression of all the genes for secretory organelle components. The present knowledge about the structure and control of these genes is still fragmentary. In particular, sequences that might account for a common regulation have never been reported. Our present results appear, however, compatible with this possibility.

On the other hand, the reason why in PC12-27 cells the small, yet appreciable levels of specific granule/vesicle mRNAs fail to sustain the appearance of appreciable amounts of the corresponding proteins is unclear. The actinomycin D and the short pulse labeling experiments in intact cells have excluded major roles of accelerated mRNA and/or protein turnover. A possibility is therefore that of a post-transcriptional inhibition. The latter, however, could be due neither to the trapping of mRNAs within the nucleus, since in PC12-27 their distribution appeared the same as in controls; nor to the expression of inhibitory proteins, since (at least in the case of SgII) the blockade was still apparent in the in vitro translation experiments, i.e. when the purified mRNAs of PC12-27 cells was translated in the reticulocyte system.

In conclusion, the comprehensive investigation of PC12-27, a clone fully incompetent for regulated secretion, carried out by the combination of molecular and cell biological techniques, has revealed new aspects of a process that appears fundamental for neuroendocrine cells, the acquisition of the two types of secretory organelles, dense granules and synaptic-like microvesicles, and thus of regulated secretion. The results demonstrate for the first time the existence of an expression control, probably common to both organelles, and provide initial information on the mechanisms that appear to operate independently of other aspects of cell differentiation.

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\(^3\) R. H. Kehlenbach and W. B. Huttner, unpublished data.
Overall Lack of Regulated Secretion in a PC12 Variant Cell Clone
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