Human Neuroblastoma Cells Acquire Regulated Secretory Properties and Different Sensitivity to Ca^{2+} and α-Latrotoxin after Exposure to Differentiating Agents

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Abstract. IMR-32 human neuroblastoma cells are unable to release [^{3}H]dopamine in response to secretagogues. However, they express a normal complement of membrane receptors and ion channels which are efficiently coupled to second messenger production. In the present study we took advantage of the ability of this cell line to differentiate in vitro in the presence of either dibutyrryl-cAMP or 5-bromodeoxyuridine, to analyze any developmentally regulated changes in its secretory properties. Uptake, storage, and release of [^{3}H]dopamine were studied biochemically and by autoradiography. The calcium ionophore ionomycin, phorbol 12-myristate 13-acetate and the presynaptic acting neurotoxin α-latrotoxin were used in both control and differentiated cells as secretagogue agents. The presence of secretory organelles was investigated by electron microscopy; the expression of secretory organelle markers, such as chromogranin/secretogranin proteins (secretory proteins) and synaptophysin (membrane protein), was detected by Western blotting and immunofluorescence. The results obtained indicate that IMR-32 cells acquire regulated secretory properties after in vitro drug-induced differentiation: (*a*) they assemble "de novo" secretory organelles, as revealed by electron microscopy and detection of secretory organelle markers, and (*b*) they are able to store [^{3}H]dopamine and to release the neurotransmitter in response to secretagogue stimuli. Furthermore, secretagogue sensitivity was found to be different, depending on the differentiating agent. In fact, dibutyrryl-cAMP treated cells release [^{3}H]dopamine in response to α-latrotoxin, but not in response to ionomycin, whereas 5-bromodeoxyuridine treated cells release the neurotransmitter in response to both secretagogues. All together these results suggest that IMR-32 cells represent an adequate model for studying the development of the secretory apparatus in cultured human neurons.

THE coupling between receptor activation and transmitter release, also referred to as "stimulus-secretion coupling," is a fundamental cellular process which is still poorly defined at the molecular level especially in neurons (Augustine et al., 1987; Bell, 1988; Burgoyne, 1987; Gomperts, 1986; Kretsinger and Creutz, 1986). The study of this process would be made much easier if it were possible to dissect the different events that occur between the stimulus and the secretory response, i.e., receptor activation, second messenger production, neurotransmitter synthesis and storage, and vesicle fusion. Human neuroblastoma cell lines, which have several characteristics of neuronal cells and where the expression of neuron-specific enzymes, proteins, and organelles can be modulated according to the culture conditions (Gotti et al., 1987; Gupta et al., 1985; Prasad et al., 1973; Reynolds and Perez-Polo, 1981; Ross et al., 1980; Rupniak et al., 1984; Sidell, 1982; Spinelli et al., 1982) could represent a useful cell model to study the expression of these properties. We have previously shown that IMR-32 cells, established in vitro by Tumilowicz et al. (1970), are able to take up the neurotransmitter [^{3}H]dopamine (^{3}H-DA)[1] by a specific, high affinity, uptake system, but are not able to release it in response to classical secretagogue agents (Clementi et al., 1986).

We have also shown that this "defect" might be at the level of some steps of the regulated secretory machinery itself and not at the level of receptor activation or second messenger modulation, since in these cells membrane receptors and ion channels are present and functioning (Clementi et al., 1986; Gotti et al., 1986; Sher et al., 1988*a*), and different secretagogues are able to modulate intracellular free calcium concentration ($[Ca^{2+}]_i$) levels (Sher et al., 1988*b*).

IMR-32 cells also have the interesting property of differentiating in vitro in response to pharmacological agents, to-

1. *Abbreviations used in this paper*: α-LTx, α-latrotoxin: Bt_2cAMP, N^6-D^2-dibutyrryl-cyclic adenosine 3′5′ monophosphate; BrdU, 5′-bromo-2′ deoxyuridine; $[Ca^{2+}]_i$, intracellular-free calcium concentration; Cg/Sg, chromogranin/secretogranin; ^{3}H-DA, ^{3}H-dopamine; KRH, Krebs-Ringer-Hepes medium; TPA, phorbol 12-myristate 13-acetate.

wards a "mature" neuronal phenotype, which can be evaluated not only morphologically, but also biochemically or functionally (Gotti et al., 1987; Gupta et al., 1985; Prasad et al., 1973; Reynolds and Perez-Polo, 1981; Thompson et al., 1982).

In the present study we investigated whether the ability to release ^{3}H-DA could be acquired by IMR-32 cells after exposure to differentiating agents, such as N^6-O^2-dibutyrryl-cyclic adenosine 3′5′ monophosphate (Bt_2cAMP) or 5′-bromo-2′ deoxyuridine (BrdU). We first studied neurotransmitter storage in control and differentiated cells by analyzing their ability to take up and store ^{3}H-DA in secretory organelles that were visualized by electron microscopy and characterized by the presence of chromaffin granule antigens and synaptophysin, an integral membrane protein of synaptic vesicles. Neurotransmitter release was investigated by the use of three secretagogue agents, which activate different pathways: (*a*) ionomycin (Ca^{2+}-dependent release); (*b*) phorbol 12-myristate 13-acetate (TPA) (Ca^{2+}-independent release possibly related to protein kinase C activation plus synergy with Ca^{2+}-dependent release); and (*c*) α-latrotoxin (α-LTx) from black widow spider venom (Ca^{2+}-dependent plus Ca^{2+}-independent release mediated by unknown mechanisms). IMR-32 cells were found to be able to assemble secretory organelles, to store in them ^{3}H-DA, and to release the neurotransmitter in response to secretagogue agents, only after differentiation. Furthermore, interesting differences in stimulus-secretion coupling were detected in cells differentiated with different drugs.

Materials and Methods

Cell Culture

The IMR-32 cell line (obtained from the American Type Culture Collection, Rockville, MD) was grown as previously described (Clementi et al., 1986) in MEM containing Earle's salts, 10% heat-inactivated FCS, 100 IU/ml penicillin, and 100 μg/ml streptomycin. The experiments were performed on cells between passage 70 and 85. Cells were usually plated at a concentration of $5 \times 10^3/cm^2$ and used 12–13 d later when they reached confluency.

To induce differentiation, the cells were treated after the first medium change (2 d after plating), with 1 mM Bt_2cAMP or 2.5 μM BrdU dissolved in culture medium. Drugs were added every 3 d at each medium change and the cells used 12 d later, unless otherwise indicated.

Cells to be used for immunolocalization studies were grown on gelatin-coated round glass coverslips (24 mm in diameter) inserted in 35-mm Petri dishes.

^{3}H-DA Uptake, Storage, and Release

Uptake experiments were performed as described (Clementi et al., 1986) with slight modifications. Cells were gently detached from the Petri dishes, transferred to siliconized tubes, and centrifuged at 150 *g* for 5 min. Pelletted cells were resuspended (3×10^7 cells/ml) in a Krebs-Ringer-Hepes medium (KRH) containing 125 mM NaCl, 5 mM KCl, 1.2 mM KH_2PO_4 and $MgSO_4$, 2 mM $CaCl_2$, 25 mM Hepes/NaOH buffer (pH 7.4), and 6 mM glucose. The medium also contained 0.1 mg/ml each of ascorbate and the monoamine oxidase inhibitor pargyline plus the desired amount of ^{3}H-DA (from 0.1 to 5.0 μM). Sister cells were incubated in the same mixture to which 2 μM desmethylimipramine was added, which is known to inhibit the high affinity amine uptake system in catecholaminergic neurons and in IMR-32 cells (Richards and Sadee, 1986). Uptake experiments were performed for 30 min at 37°C in an oscillating bath (50 rpm).

For release experiments, cells were incubated in the above medium for 60 min at 37°C. After incubation the cells were pelletted by centrifugation for 5 min at 150 *g* and resuspended in KRH containing 2 μM desmethylimipramine and the various secretagogues to be tested (10 μM reserpine, 1 μM ionomycin, 160 nM TPA, 2 nM α-LTx). Since some drugs were dissolved in dimethylsulfoxide, control cells received the same amount of solvent alone. The cells were then returned to 37°C for 8 min unless otherwise indicated, centrifuged, washed twice with ice-cold KRH buffer, and finally the pellets were counted to determine the amount of ^{3}H-DA remaining in the cells. Samples, dissolved in 200 μl of NaOH and then in 10 ml of Atomlight, were counted in a beta-counter (model L57500; Beckman Instruments Inc., Palo Alto, CA) with an efficiency of ~40%.

Visualization of ^{3}H-DA by Autoradiography

The cultures were incubated for 1 h at 36°C with 0.5 μM ^{3}H-DA in Dulbecco's PBS (D-PBS). Incubation was stopped by means of three D-PBS washes. Cells were then fixed for 1 h at room temperature in 1.5% glutaraldehyde in PBS, extensively washed in PBS, air dried, and coated with Ilford emulsion K5 diluted 1:1 with water. They were exposed for 1 wk in the dark before being developed with D19 developer (Eastman Kodak Co., Rochester, NY).

Electron Microscopy

Cells were fixed with 2% glutaraldehyde in PBS for 1 h, postfixed in 1% OsO_4 for 1 h, dehydrated in acetone, and embedded in Epon. Sections were observed using a Philips CM10 electron microscope.

Measurements of $[Ca^{2+}]_i$ with Quin-2

These experiments were performed as described by Sher et al. (1988*b*). Briefly, the cells were mechanically detached from the dishes, centrifuged for 5 min at 150 *g*, and resuspended at a concentration of 10^7 cells/ml in MEM containing 3% FCS and 50 μM quin2 acetoxymethylester (quin2-AM) in dimethylsulfoxide (0.5% of final volume). After 20 min at 37°C, the cells were further diluted with the same medium and left at 37°C for additional 60 min. Before the fluorescence assay, the cells were pelletted in a microfuge and resuspended at a concentration of 10^6 cells/ml in 1.5 ml of KRH.

All the assays were carried out in a spectrofluorimeter (model LS-5; Perkin Elmer Corp., Eden Prairie, MN) (excitation: 339 ± 5 nm; emission: 492 ± 10 nm), using a cuvette positioned in a thermostatically controlled (36 ± 1°C) cell holder equipped with magnetic stirring. Calibration of the fluorescence signal was performed as described by Tsien et al. (1982). Whenever necessary, the data were corrected for changes in cell autofluorescence.

Detection of Secretory Organelle Markers

Nondifferentiated and BrdU-differentiated pelletted cells were homogenized in 0.25 M sucrose plus 0.5 mM PMSF and centrifuged at 600 *g* for 10 min. The presence of secretory proteins, mainly chromogranins/secretogranins (Cgs/Sgs) (Eiden et al., 1987), in the supernatants was detected by Western blotting (Burnette, 1981). Proteins were separated by two-dimensional PAGE, as described by Zanini and Rosa (1981) on a Mini Protean II cell (Bio-Rad Laboratories, Richmond, CA), and were transferred to nitrocellulose membranes at 300 mA for 5 h. An antiserum raised in mice against human pheochromocytoma chromaffin granules, which recognized both CgA and CgB/Sgl (kindly given by Drs. A. Siccardi and M. Pelagi, Department of Biology, University of Milan), was used diluted 1:200. Horseradish peroxidase–conjugated anti–mouse IgGs were used diluted 1:1,000. The immunoreaction was visualized by means of 3,3′-diaminobenzidine.

To detect synaptophysin, proteins were separated by SDS-PAGE as described by Maizel (1971), in 10% polyacrylamide gel slabs. Immunoblotting was performed by using affinity-purified polyclonal anti–synaptophysin antibodies diluted 1:10 (kindly given by Dr. R. Jahn, Max-Planck-Institut fur Psychiatrie, Munchen, FRG), and the immunoreaction was visualized by means of 4-chloro-1-naphtol.

Synaptophysin was quantitated by densitometric analysis of the immunoblots in a laser densitometer (Ultroscan XL; LKB Instruments, Gaithersburg, MD).

Immunolocalization of Secretory Organelle Markers

For immunolocalization studies, a variation of the method described by Geiger and Singer (1979) was used. Control and differentiated IMR-32 cells, grown on gelatin-coated glass coverslips, were washed twice with D-PBS and fixed in the same buffer, containing 1% paraformaldehyde, for 20 min at room temperature. Cells were then washed with D-PBS plus 0.1% BSA

(3 × 5 min) and, at the second wash, a drop of glycine (1 M, pH 8) was added to quench residual aldehyde activity. The cells were permeabilized by a 5-min incubation with 0.1% Triton in D-PBS, followed by three additional washes. Permeabilized cells were incubated for 1 h at room temperature with either the polyclonal anti-chromaffin granule antiserum (diluted 1:25) or the polyclonal anti-synaptophysin antibodies (diluted 1:10), or an anti-DA antiserum (diluted 1:400) and further washed three times for a total of 30 min. The cells were then incubated in the presence of a biotinylated secondary antibody for 30 min (diluted 1:100) and, after three additional washes, incubated in the presence of Avidin Texas red (diluted 1:100) for 15 min. The coverslips were mounted on a drop of Moviol on glass microscope slides and viewed in a Zeiss Axiophot microscope equipped for epifluorescence. Pictures were taken with a Technical Pan 2415 film (Eastman Kodak Co.) developed with D19 developer.

Materials

MEM, FCS, and antibiotics were obtained from Flow Laboratories, Inc. (Ayrshire, Scotland). Plastic Petri dishes were purchased from Corning Glass Works (Corning, NY) and BrdU, Bt2cAMP, gelatin, pargyline, desmethylimipramine, TPA, and 3,3' diaminobenzidine were purchased from Sigma Chemical Co. (St. Louis, MO). ^{3}H-DA (specific activity 5–15 Ci/mmol) was from Amersham as well as the biotinylated anti-mouse IgG antibodies and Avidin Texas red. Atomlight was from New England Nuclear (Boston, MA) and Moviol from Calbiochem-Behring Corp. (San Diego, CA). Rabbit anti-DA antiserum was obtained from Sera-Lab (Crawley Down, UK). Horseradish peroxidase-conjugated goat anti-mouse IgG were from Organon Teknika-Cappel (Malvern, PA). Ionomycin and quin2-acetoxymethylester were obtained from Calbiochem-Behring Corp., and 4-chloro-1-naphtol was purchased from Bio-Rad Laboratories. α-LTx, purified as described (Frontali et al., 1976), was a kind gift of Dr. L. Madeddu and L. Rosenthal from our institute.

All other reagents were of reagent grade and were purchased from E. Merck (Darmstadt, FRG).

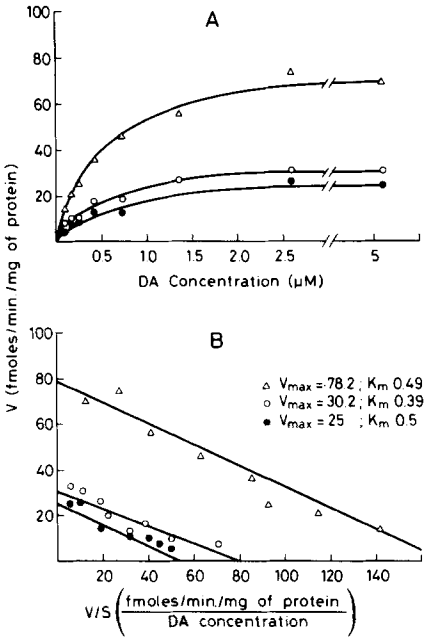


Figure 1. Kinetics of uptake of DA by IMR-32 cells. (*A*) Velocity of initial uptake of DA expressed as a function of total DA concentration in the external medium. Each data point represents the average of triplicate determinations. Final concentration of ^{3}H-DA, 0.1 × 10^{-6} M. (*B*) Hofstee plot (Hofstee, 1959) of data in *A*. Line was fitted by least-squares analysis. ●, control cells; ○, Bt2cAMP-treated cells; △ BrdU-treated cells.

Results

Uptake and Storage of ^{3}H-DA

We have previously shown that neurotransmitter synthesis occurs both in control and differentiated cells (Gotti et al., 1987). We checked if both undifferentiated and differentiated IMR-32 cells were able to take up ^{3}H-DA in a specific, high-affinity and saturable fashion. Both control and differentiated cells take up ^{3}H-DA, but BrdU-differentiated cells take up much more labeled neurotransmitter (V_{max}: 78.2 fmol/min per mg of protein) than Bt_2cAMP-differentiated cells (V_{max}: 30.2 fmol/min per mg of protein) or control cells (Fig. 1 *A*). However, no significant difference was found in the affinity for ^{3}H-DA of the uptake system in the different phenotypes (Fig. 1 *B*). We thus tested if there could be differences between control and differentiated cells in the ability to assemble secretory organelles and store ^{3}H-DA taken up from the medium. To test this hypothesis, storage of dopamine was evaluated autoradiographically, biochemically, and by immunofluorescence. Control and differentiated cells were incubated for 1 h with ^{3}H-DA (0.5 μM), in the absence of pargyline. Under such conditions only ^{3}H-DA stored in secretory granules could be viewed since, unlike cytoplasmic ^{3}H-DA, it is protected from degradation operated by monoamine oxidases. We found that very few control cells were labeled, probably only the spontaneously differentiating cells (Fig. 2 *a*). On the contrary, a greater proportion of cells was labeled in both Bt_2cAMP- and BrdU-differentiated cultures. The intracellular labeling was not uniform, suggesting that ^{3}H-DA was concentrated in discrete subcellular structures. In Bt_2cAMP-treated cells, labeling was concentrated at the periphery of the cell body, in particular in the short neurites (Fig. 2 *b*). In BrdU-treated cells, which exhibit a more differentiated phenotype, the labeling was concentrated along neurites and in varicosities (Fig. 2 *c*). The same type of compartmentalization could also be observed in BrdU-differentiated cells by immunofluorescence using anti-DA antiserum (Fig. 2 *d*), suggesting that both endogenous and exogenous DA was stored in similar structure.

Further indication of the subcellular localization of ^{3}H-DA could be obtained by studying the ability of reserpine (10 μM) to induce depletion of stored ^{3}H-DA by both control and differentiated cells. These experiments were performed in the presence of the monoamine oxidase inhibitor pargyline. Under such conditions, the amount of ^{3}H-DA released ("reserpine-sensitive") is an indirect measurement of the amount of transmitter actually stored in granules (Langhey and Kirshner, 1987). As shown in Table I, in control IMR-32 cells the amount of ^{3}H-DA stored in granules is very low (~7%), whereas it represents a greater proportion in Bt_2cAMP- (~15%) and BrdU- (~25%) differentiated cells.

Electron Microscopy

A striking difference was found between control and differentiated IMR-32 cells at the ultrastructural level. Control cells had none or very few secretory vesicles in the cytoplasm or in the short neurites (Fig. 3 *a*), whereas in both Bt_2cAMP- (Fig. 3 *b*) and 5BrdU- (Fig. 3 *c*) treated cells many secretory organelles were present.

The great majority of these secretory organelles had a "dense core"; they were irregular in shape and highly heterogeneous in dimension. Few "clear" small vesicles, possibly

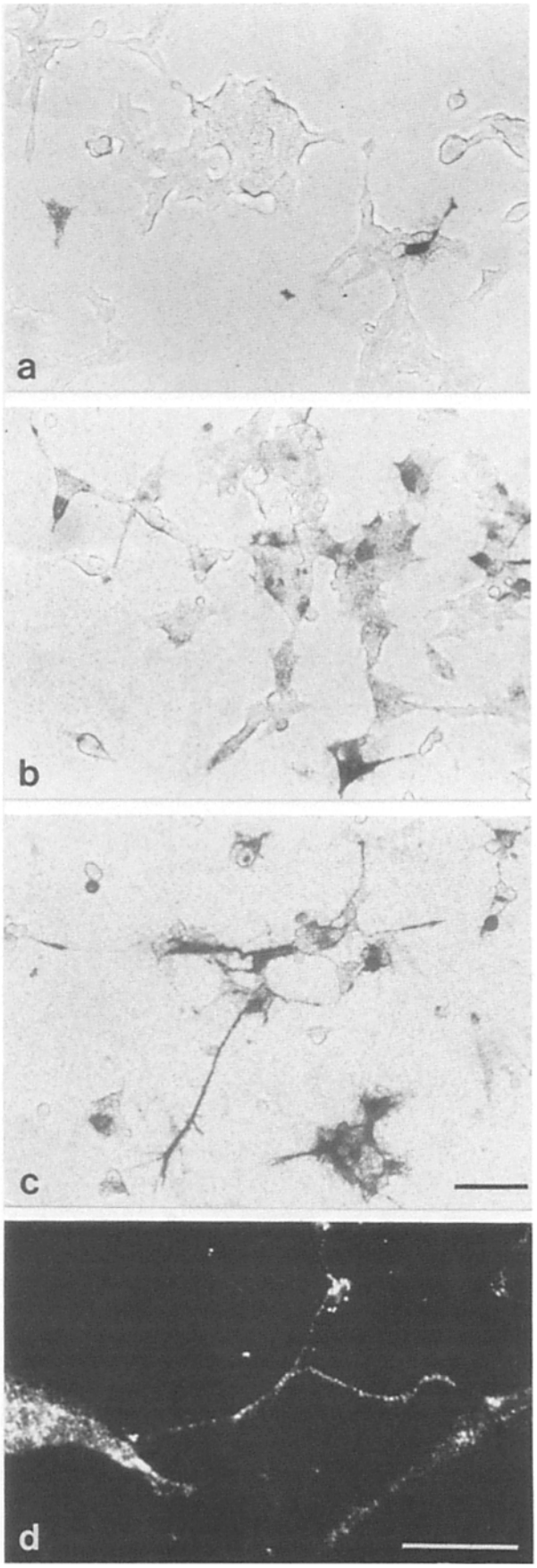


Table I. Effect of Reserpine on Preaccumulated ^{3}H-DA

	Percent increase of release		
	Control	Bt_2cAMP	BrdU
Reserpine (1×10^{-5} M)	7 ± 2	15 ± 2	23 ± 3
DMSO (1%)	2 ± 1	3 ± 1.5	2 ± 1

The cells were incubated for 2.5 h at 37°C with the drug or with the same amount of DMSO alone. Values represent the average ± SEM of three different experiments.

related to synaptic vesicles were found, scattered throughout the cytoplasm and in the neurites.

Detection of Secretory Organelle Proteins

Secretory granule proteins were detected after separation by two-dimensional PAGE from both control and BrdU-differentiated cell homogenates, and immunoblotting with an anti-human chromaffin granule antiserum. As shown in Fig. 4, some proteins were specifically recognized by this antiserum only in differentiated cells; in particular, two acidic components corresponding to CgA and CgB/Sgl were clearly detectable in BrdU-differentiated cells (Fig. 4 *b*) and not in control cells (*a*).

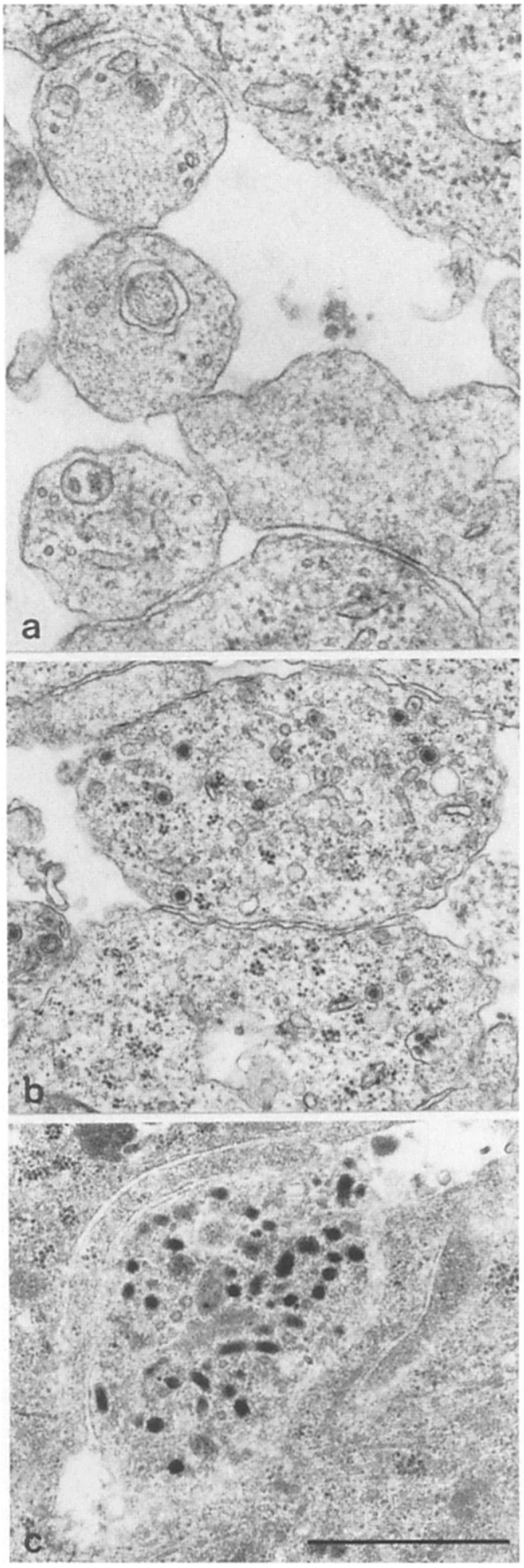
Immunofluorescence experiments with the anti-chromaffin granule antiserum revealed that in control IMR-32 cells only low levels of granule proteins were detectable in a few punctate structures mainly at the periphery of the cell (Fig. 5, *a* and *b*). On the contrary, a very strong dotted signal was found in BrdU-differentiated cells at the level of the Golgi apparatus, near the plasma membrane, along neurites and inside varicosities and "terminals" (Fig. 5, *c–e*). Similar results were obtained in Bt_2cAMP-treated cells (not shown).

Moreover we followed the regulation of expression of a marker of secretory vesicles membranes by using an affinity-purified antibody directed against synaptophysin. This Ca^{2+}-binding transmembrane glycoprotein is probably present on different types of secretory organelles (Navone et al., 1986; Lowe et al., 1988; Obendorf et al., 1988), but it seems to be enriched in small, nonpeptidergic, synaptic vesicles (Navone et al., 1986; Obendorf et al., 1988).

Differentiated IMR-32 cells express more synaptophysin than control cells as revealed by immunoblotting. Densitometer scans of the immunoblots indicate a ~100% increase in the amount of synaptophysin doublet in differentiated cells compared with control cells (Fig. 6 *a*).

In immunofluorescence studies (Fig. 6, *b*, *c*, and *d*), a

Figure 2. Autoradiography of ^{3}H-DA and immunofluorescence with anti-DA antiserum. Control (*a*) Bt_2cAMP- (*b*) and BrdU- (*c*) treated IMR-32 cells were loaded with ^{3}H-DA (0.5 μM, 60 min at 37°C) and processed for autoradiography. Very few cells are labeled in control cultures (*a*), while both types of differentiated cultures accumulate a great amount of neurotransmitter. Note the particular compartmentalization of labeled structures in the short neurites in Bt_2cAMP-treated cells (*b*) and along the long processes of BrdU-treated cells (*c*). In *d* the immunofluorescence pattern obtained with the anti-DA antiserum in BrdU-treated cells is shown. A punctate labeling in the cytoplasm and along neurites is clearly noticeable. Bars, 50 μm.



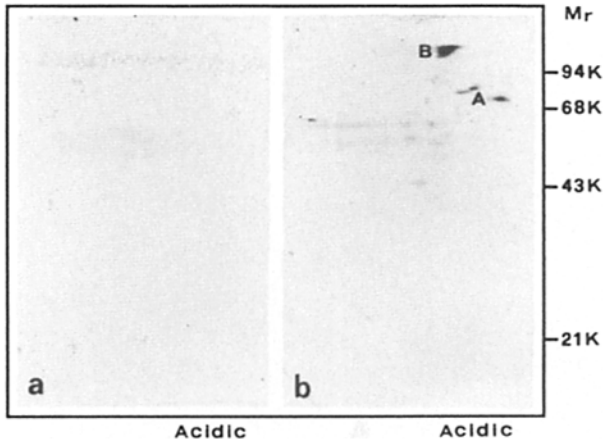


Figure 4. Western blotting with anti-human chromaffin granule antiserum. Proteins from control (*a*) and BrdU-differentiated (*b*) IMR-32 cell homogenates were separated by two-dimensional PAGE. blotted, and immunostained as described in Materials and Methods. Only differentiated IMR-32 cells express a significant amount of the two granule-specific proteins CgA (*A*) and CgB/Sgl (*B*), as well as of other unidentified proteins likely related to chromaffin granules.

punctate labeling was found that was more prominent in differentiated cells, concentrated in the Golgi area, in neurites, and in varicosities. Similar results were obtained in Bt_2cAMP-treated cells (not shown).

^{3}H-DA Release

Spontaneous Release. The rate of spontaneous release of ^{3}H-DA was measured both in control cells and in cells differentiated with either Bt_2cAMP or BrdU. As shown in Fig. 7 during the first 5 min of incubation a significant release (~20%) was detected followed by a slow decline during the next 60 min. The rate of spontaneous release is very similar in the three types of cells.

Secretagogue-induced Release. In Table II the results of secretagogue-induced neurotransmitter release experiments on control and differentiated cells are reported. Regardless of the agent tested (Table II, *Control* column) nondifferentiated IMR-32 cells do not release ^{3}H-DA. On the other hand, BrdU-differentiated cells are able to release ^{3}H-DA either in response to ionomycin or to α-LTx (Table II, *BrdU* column). TPA alone induces only a slight ^{3}H-DA release which is however additive with the ionomycin effect. In contrast, Bt_2cAMP-differentiated IMR-32 cells were not able to release ^{3}H-DA in response to either the calcium ionophore ionomycin, or to TPA (Table II, *Bt_2cAMP* column). Only α-LTx was able to induce a significant release of ^{3}H-DA from such cells, although at a reduced level when compared to BrdU-differentiated cells. Since the level of spontaneous release is equivalent in control and differentiated cells, we can thus exclude the possibility that the different ability to release ^{3}H-DA in response to secretagogues (see results above)

Figure 3. Transmission electron microscopy of control and differentiated IRM-32 cells. Control cells (*a*) are almost devoid of secretory granules. In contrast Bt2cAMP- (*b*) and BrdU- (*c*) treated cells contain many secretory organelles with a typical dense core, which are particularly concentrated in the neurites. Bar, 1 μm.

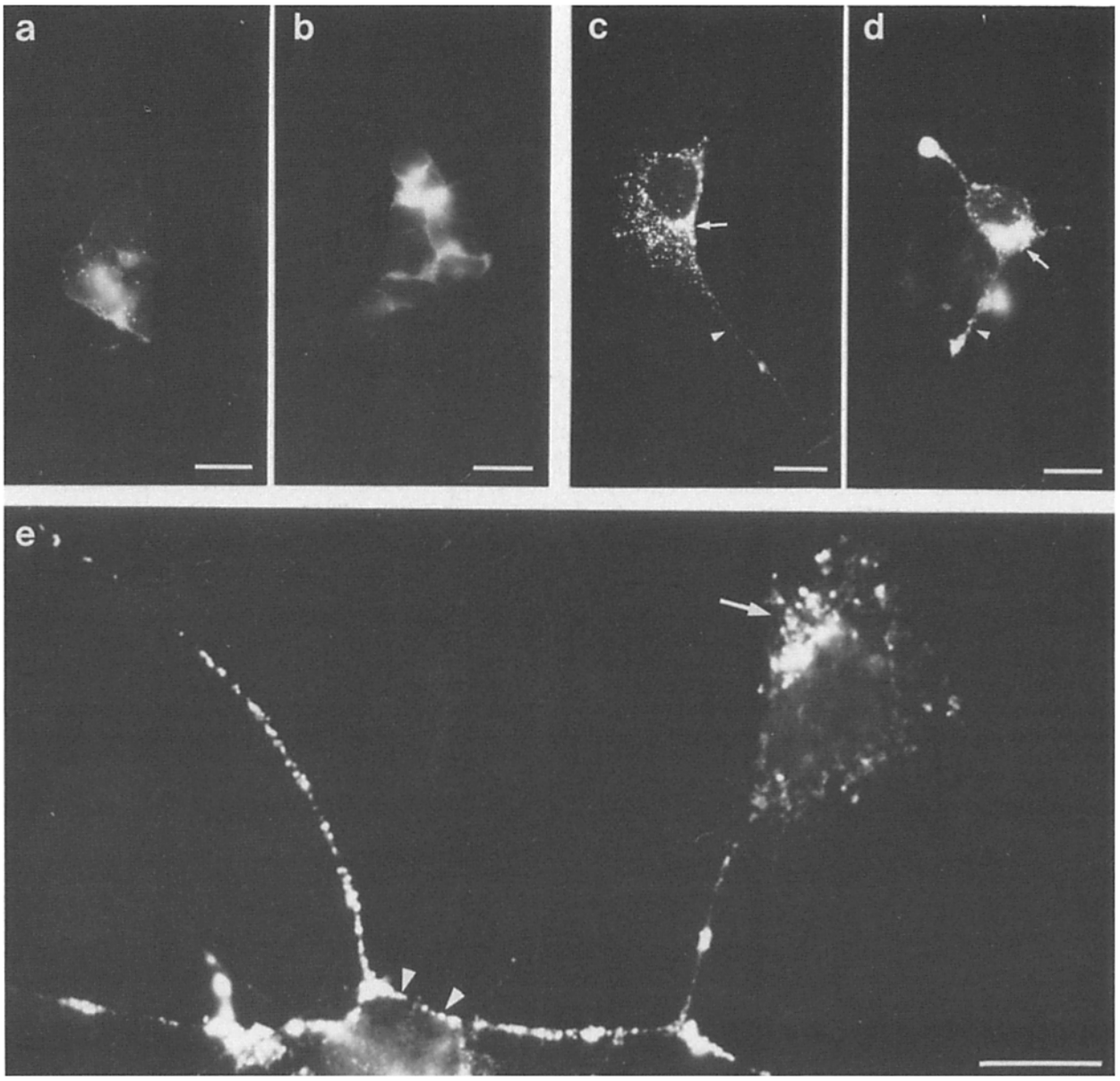


Figure 5. Immunolocalization of Cgs/Sgs in control and differentiated IMR-32 cells. In *a* and *b* two fields of control IMR-32 cultures are shown, with no neurites and very few labeled granules. In *c* and *d* two cells from BrdU-differentiated culture are shown: a typical punctate fluorescence is evident in the Golgi area (*arrows*) and in the neurites (*arrowheads*). (*e*) One field from BrdU-differentiated cells showing the ramification pattern of the heavy-labeled neurites. Note the dotted appearance of the immunostained neurites making contact with a neighbor cell (*arrowhead*). *Arrow*, Golgi area. Bars, 25 μm.

could be due to a modification of the basal rate of release induced by the differentiating drugs. α-LTx in Bt_2cAMP- and BrdU-treated IMR-32 cells, as in other systems (Meldolesi et al., 1986), was able to induce neurotransmitter release even in the absence of extracellular calcium and at levels only slightly lower than those observed in the presence of extracellular calcium (Table III).

Age-dependent Effects of Secretagogues. The difference in response to secretagogues between Bt_2cAMP- and BrdU-differentiated cells might be due to the fact that the two drugs induce different pathways of differentiation or to the fact that there is a lower level of morphological maturation of the Bt_2cAMP-treated cells. In fact, from a morphological point of view, Bt_2cAMP-induced cell differentiation is similar to the early stages of BrdU-induced differentiation. Furthermore cell shape is known to influence dopamine processing at least in PC12 cells (Bethea et al., 1987). We therefore tested the release capacity of cells differentiated for only five d. In Table IV the results of such experiments are reported. "Younger" cells release quantitatively less ^{3}H-DA than "older" cells in response to the various secretagogues. However, a significant difference still exists between Bt_2cAMP- and BrdU-treated cells in terms of the ratio between α-LTx and ionomycin-induced release, indicating that Bt_2cAMP-treated cells do not represent, in this respect, an "intermediate" step in BrdU-induced cell differentiation.

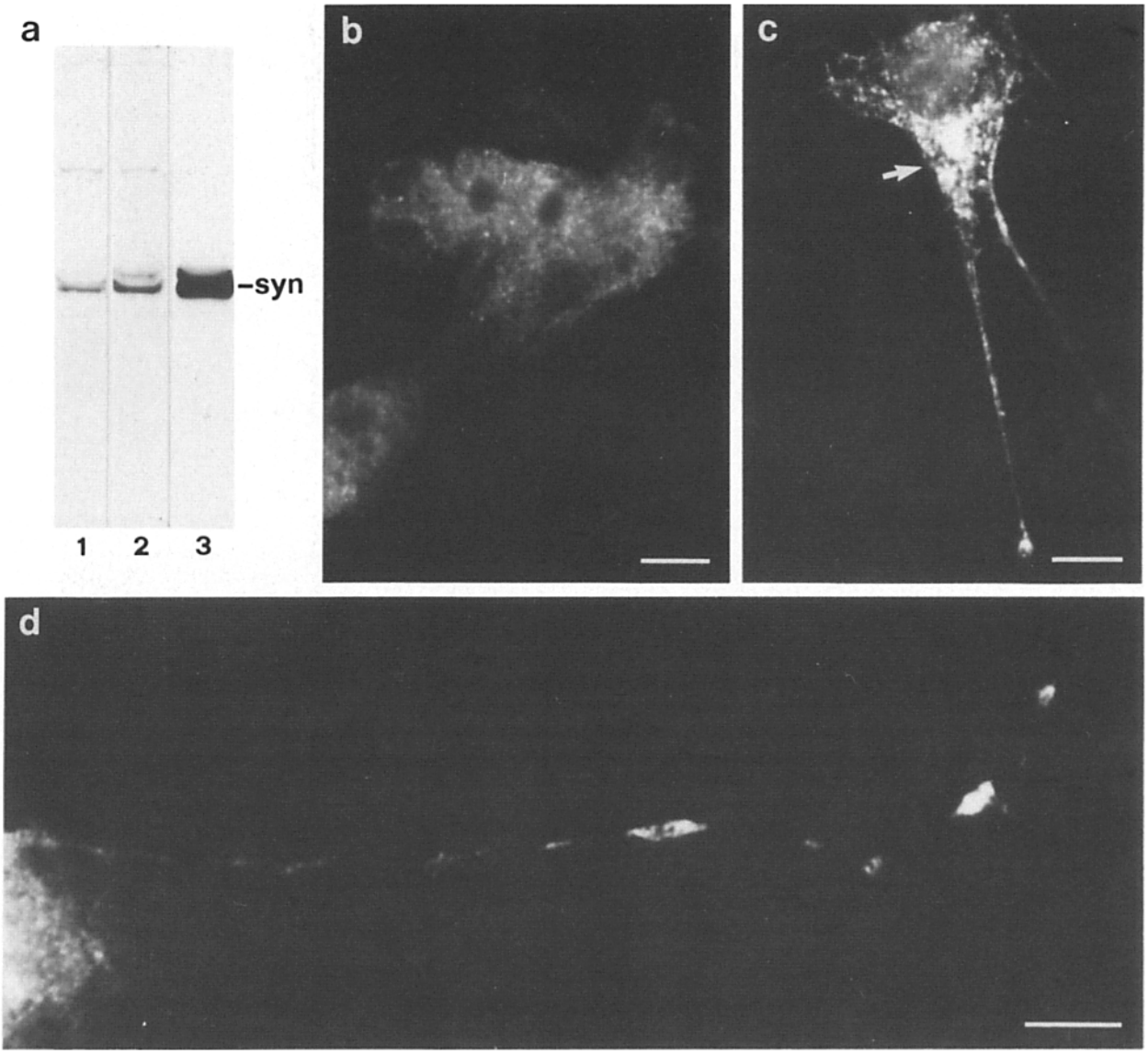


Figure 6. Detection of synaptophysin in control and differentiated IMR-32 cells. (*a*) Western blotting with affinity-purified polyclonal anti-synaptophysin antibodies. Equal amounts of proteins from control (lane *1*) and differentiated (lane *2*) IMR-32 cell homogenates were separated by SDS-PAGE, blotted, and immunostained as described in Materials and Methods. Proteins from bovine posterior pituitary, known to contain synaptophysin (Navone et al., 1988), were loaded in lane *3* as a positive control. A stronger labeling (~100% increase) of the doublet corresponding to the differently glycosylated forms of synaptophysin (*syn*), is evident in differentiated versus control cells. (*b*, *c*, and *d*) Immunofluorescence of synaptophysin in control (*b*) and differentiated (*c* and *d*) IMR-32 cells. It can be seen that particularly in differentiated cells there is dotted labeling scattered in the cytoplasm, in the Golgi area, and in the neurites. Labeled neurite varicosities are shown in *d*. *Arrow*, Golgi area. Bars, 25 μm.

Secretagogue-induced Modification of Intracellular Calcium Levels

Both ionomycin and α-LTx promote an increase in $[Ca^{2+}]_i$ in a calcium-containing medium (Sher et al., 1988*b*). Tracings from experiments performed with the fluorimetric Ca^{2+} probe Quin2 (Fig. 8) support the view that α-LTx–induced ^{3}H-DA release is Ca^{2+} independent. In Fig. 8 *a* a typical tracing obtained when α-LTx is added to Bt_2cAMP-treated cells in a calcium containing KRH medium (see Materials and Methods) is shown: after 30–40 s, $[Ca^{2+}]_i$ increases to very high levels (~1 μM) and decreases only after the addition of 1 mM EGTA. When EGTA is added before the toxin (Fig. 8 *c*), no increase in $[Ca^{2+}]_i$ is caused by α-LTx, indicating that it does not induce calcium redistribution from internal stores. Identical results were obtained in BrdU-treated cells (not shown).

Since in the same experimental conditions (i.e., calcium-free medium plus EGTA), α-LTx is able to induce release from both Bt_2cAMP- and BrdU-treated cells, we can conclude that in both types of cells this release is not caused by calcium influx, nor by calcium redistribution, but is mediated by some other unknown signal(s).

The effects of ionomycin on calcium movements were also compared. In a calcium-containing medium, ionomycin in-

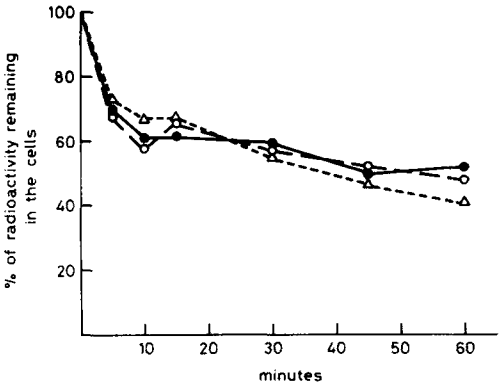


Figure 7. Rate of spontaneous release of ^{3}H-DA from IMR-32 cells. Data are expressed as percent of radioactivity remaining in the cells with respect to their own control. After loading the cells with ^{3}H-DA (0.8 μM, 60 min at 37°C) the cells were washed and left at 37°C for the indicated additional time; then the cells were centrifuged and the pellet radioactivity counted. ●, control cells; ○, Bt_2cAMP-treated cells; △, BrdU-treated cells. Each data point represents the average of triplicate determinations.

duces a great increase in $[Ca^{2+}]_i$ although lower than α-LTx (Fig. 8 *b*). Ionomycin induces a small and transient increase in $[Ca^{2+}]_i$, even in the presence of EGTA (Fig. 8 *d*), probably through the release of calcium from internal stores. The subsequent addition to the medium of an excess of calcium induces a steady increase in $[Ca^{2+}]_i$, most probably due to calcium influx; in the same experimental conditions ionomycin was not able to induce secretion from Bt_2cAMP-treated cells. TPA did not increase $[Ca^{2+}]_i$ either in the presence or in the absence of calcium in the medium (not shown).

Discussion

In the present work we have shown that a human neuroblastoma cell line, which synthesizes dopamine (Gotti et al., 1987) and possesses the dopamine uptake mechanism (Richards and Sadee, 1986), but which is unable to release it in response to secretagogues (Clementi et al., 1986), acquires, during in vitro differentiation, the ability to secrete the neurotransmitter in response to stimulation. Furthermore, we have shown that such cells exhibit alternative pathways of differentiation, which differ, according to the differentiating agent, in the way they regulate secretory events.

Table II. Release of ^{3}H-DA from Control and Differentiated Cells

Drugs	Percent increase of release		
	Control	Bt_2cAMP	BrdU
α-LTx (2×10^{-9} M)	2.0 ± 1	16 ± 2	42 ± 2
Ionomycin (1×10^{-6} M)	4.1 ± 1.5	2 ± 1	27 ± 3
TPA (0.1×10^{-6} M)	3.0 ± 1	1 ± 0.5	6 ± 2
Ionomycin + TPA	6.0 ± 2	3 ± 2	33 ± 6
Ratio αLTx/ionomycin	0.5	8.0	1.5

The cells were loaded with ^{3}H-DA (0.8 μM) and the amount of release determined after 8 min, as described in Materials and Methods. Values are expressed as a percent increase of release over basal ± SEM and represent the average of five to eight different experiments. DMSO alone induced a release of 1–3% in different experiments.

Table III. Release of ^{3}H-DA from Differentiated IMR-32 Cells in the Presence or in the Absence of Ca^{2+} in the Extracellular Medium

Drugs	Percent increase of release	
	Bt_2cAMP	BrdU
α-LTx	13 ± 2	37 ± 4
α-LTx + EGTA	10 ± 2	31 ± 5

The toxin was added for 8 min at 37°C. EGTA concentration was 1 mM. The values represent the average ± SEM of four different experiments.

Storage of ^{3}H-DA

^{3}H-DA autoradiography clearly indicates that Bt_2cAMP- and BrdU-differentiated IMR-32 cells can store ^{3}H-DA, while control cells cannot. We have previously shown that differentiated IMR-32 cells synthesize more neurotransmitters than control cells (Gotti et al., 1987), and exhibit as well a brighter endogenous amine histofluorescence (Denis-Donini, S., and E. Sher, unpublished results). Our present autoradiographic and immunocytochemical results with the anti-DA antiserum, together with the increased sensitivity of differentiated IMR-32 cells to the granule-depleting drug reserpine, suggest that differentiated IMR-32 cells have developed a storage apparatus where they accumulate neurotransmitters, that could be released upon stimulation through exocytosis.

Such results were confirmed by electron microscopy which showed that only differentiated cells contain a large number of secretory organelles, and that the great majority of these structures belongs to the "dense-core" vesicle type.

Moreover, we were able to demonstrate, both by immunochemical and immunocytochemical approaches with an antiserum rasied against human purified chromaffin granules, that secretory granule protein accumulation is greatly increased in differentiated neuroblastoma cells. The most abundant proteins recognized by this antiserum were CgA and

Table IV. Role of Cultures' Age on Stimulated ^{3}H-DA Release

	Percent increase of release			
	Bt_2cAMP		BrdU	
	1 wk	2 wk	1 wk	2 wk
α-LTx (2×10^{-9} M)	13.7	18.7	28.0	41.6
Ionomycin (1×10^{-6} M)	2.6	3.2	18.1	26.8
TPA (0.1×10^{-6} M)	3.5	–	–	–
Ionomycin + TPA	3.0	2.6	21.3	37.0
Ratio α-LTx/ionomycin	5.2	5.8	1.5	1.5

The drugs were present for 5 or 12 d in 1- and 2-wk-old cultures, respectively. Values are expressed as a percentage of release over basal level, as described in legend of Table I, and are representative of at least three different assays.

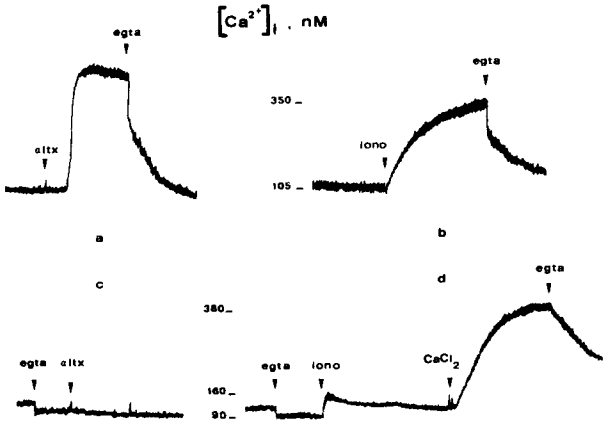


Figure 8. Quin2 measurements of $[Ca^{2+}]_i$ changes in IMR-32 cells exposed to (*a*) α-LTx, 2×10^{-9} M, followed by EGTA 2×10^{-3} M; (*b*) ionomycin, 1×10^{-6} M followed by EGTA 2×10^{-3} M. (*c*) α-LTx, 2×10^{-9} M in calcium-free medium; (*d*) ionomycin in calcium-free medium; values shown represent the $[Ca^{2+}]_i$ (in nM) calibration scale. These particular traces were obtained from Bt_2cAMP-treated cells, but identical results were obtained from control and BrdU-treated cells (not shown).

CgB/Sgl, i.e., the secretory acidic proteins present in chromaffin granules of adrenal medulla (Winkler et al., 1986), human pheochromocytoma (Schober et al., 1987), or PC12 cells (Lee and Huttner, 1983). We have also followed the expression, in control and differentiated cells of a single, specific marker of the membrane of secretory organelles, i.e., synaptophysin whose expression is known to be developmentally regulated (Knaus et al., 1986). Also in this case we have found, by both immunoblotting and immunofluorescence, that differentiated cells express a higher amount of this vesicle protein.

There is still debate on the subtypes of secretory vesicles that carry this antigen (Navone et al., 1986; Lowe et al., 1988; Obendorf et al., 1988), but probably more than one type of vesicle can carry this protein.

Our results therefore show that differentiation of IMR-32 cells considerably affects the expression of storage organelles as indicated by the presence of stored DA, of "dense core" granules, of a protein of the secretory organelle membrane (synaptophysin), and of secretory proteins (chromogranins/secretogranins) known to be stored in secretory granules (Neuman et al., 1984; Somogyi et al., 1984; Rosa et al., 1985; Winkler et al., 1986).

While a similar in vitro induction of storage properties was recently described in a pituitary cell line (Scammel et al., 1986), this is the first time that such a process is described in neuronal cells in culture.

^{3}H-DA Release

As a result of this differentiation-induced storage capacity IMR-32 cells acquire the ability to release ^{3}H-DA in a regulated fashion, i.e., in response to different secretagogue stimuli.

In BrdU-differentiated cells both ionomycin (which induces a great influx and redistribution of calcium into the cells, activating the Ca^{2+}-dependent pathway for secretion [Augustine et al., 1987]), and α-LTx (a presynaptic neurotoxin capable of inducing massive neurotransmitter release from all synapses studied until now [Meldolesi et al., 1986]), induced a significant release of ^{3}H-DA. α-LTx induced ^{3}H-DA release also in a calcium-free medium and without mobilizing calcium from intracellular stores, a behavior similar to that described in PC12 pheochromocytoma cells (Meldolesi et al., 1984). Although TPA alone had little effect in inducing ^{3}H-DA release from the different types of IMR-32 cells, it did, however, have an additive effect to that of the calcium ionophore. This is at variance with the results obtained in PC12 cells, where TPA acts in synergism with Ca^{2+} in inducing ^{3}H-DA release (Pozzan et al., 1984).

Ca^{2+}-dependent and Ca^{2+}-independent ^{3}H-DA Release

A very interesting finding that deserves discussion is the peculiar behavior of Bt_2cAMP-differentiated cells, which reflects important molecular differences from both control and BrdU-differentiated cells. While we found that Bt_2cAMP-differentiated cells (just as BrdU-differentiated cells) contain more catecholamines than control cells (Gotti et al., 1987), and express a higher ability to store ^{3}H-DA in secretory organelles, they do not release ^{3}H-DA in response to the calcium ionophore ionomycin in the presence or absence of TPA, but they do release it in response to α-LTx, even in a calcium-free medium.

This suggests that some step, distal to calcium influx and protein kinase C activation, is not functioning in this particular phenotype and that, therefore, this kind of stimulus-secretion coupling is not efficient. Much evidence is accumulating that exocytosis in several cell systems can proceed in the virtual absence of Ca^{2+} in the medium and without activation of protein kinases (Barrowman et al., 1986; Bittner et al., 1986; Vallar et al., 1987; Neher, 1988). In the same systems GTP analogues can trigger exocytosis by activating a yet unknown GTP-binding protein. Since the experiments were performed by means of considerable manipulation of the cell ionic homeostasis their physiological relevance is still to be clarified.

We here demonstrate, for the first time, that an intact cell exists, where an alternative pathway of secretion, independent from Ca^{2+} and protein kinase C, is physiologically present.

Bt_2cAMP- and BrdU-differentiated IMR-32 cells could represent different temporal steps of the same developmental program or two distinct, although interconnected, lines of differentiation (Gotti et al., 1987). Our results on ^{3}H-DA release in "younger" BrdU-treated cells support the latter hypothesis. "Younger" BrdU-treated cells behave more like "older" BrdU-treated cells than like Bt_2cAMP-treated cells, at least as far as stimulus-secretion coupling is concerned.

In conclusion these human neuronal cells, after drug-induced in vitro differentiation, acquire the ability to release the neurotransmitter ^{3}H-DA in a regulated fashion. This process involves the coordinate expression of secretory granule components and their assembly in mature secretory organelles. These findings suggest that this cellular model could be a very interesting tool for studying the control of transmitter secretion in human neurons and its modulation during neuronal development since it is amenable to manipulation and molecular dissection.

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