

Visualization of the Exocytosis/Endocytosis Secretory Cycle in Cultured Adrenal Chromaffin Cells

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ABSTRACT Cultured bovine adrenal medullary chromaffin cells were stimulated to secrete catecholamines by addition of veratridine or nicotine. The formation of an exocytotic pit exposes a major secretory granule membrane antigen, the enzyme dopamine β -hydroxylase, to the external medium. By including antiserum to this enzyme in the medium, we were able to visualize sites of exocytosis by decoration of bound antibody using a fluorescent second antibody. Internalization of this antibody-antigen complex was then followed in chase experiments: approximately half the surface complex was internalized in 15–30 min. In other experiments, secretion was triggered in the absence of antiserum, and surface enzyme was revealed by binding antibodies at various times after secretion had been halted by an antagonist. Surface patches of antigen remained discrete from the bulk of the plasma membrane for at least 30 min, although a substantial proportion of the antigen was internalized within this time. Cell surface concanavalin A receptors were internalized at a roughly similar rate, suggesting that mechanisms may be similar. After internalization, chromaffin granule membranes fused to larger structures, possibly lysosomes, and were transported over a few hours to the perinuclear region of the cell.

During the process of secretion by exocytosis, secretory granule membranes fuse with the plasma membrane of the cell. Subsequently, membranous material must be retrieved to the interior of the cell to restore the normal dimensions of the plasma membrane. In some cases, for example the parotid gland and mast cells, the cell membrane becomes deeply invaginated, since several rounds of granule fusion occur after the initial round of fusion with the plasma membrane; this seems to outstrip the ability of the cell to recover membrane from its surface (21).

Membrane recovery could occur by any of three processes: (a) Recovery from membrane sites distant from sites of exocytosis, with a concomitant incorporation of the secretory granule membrane into the plasma membrane. (b) Removal of the whole intact granule membrane from its point of fusion with the plasma membrane immediately the granule contents are discharged. (c) Removal of small portions of the granule membrane by vesiculation to form coated pits that are then internalized as coated vesicles.

To investigate the process of membrane retrieval, we used

primary cultures of bovine adrenal medullary cells. These cells are specialized for the secretion of epinephrine and norepinephrine, which are stored in high concentrations in membrane-bound chromaffin granules. During secretion, the interior of the chromaffin granule membrane is exposed to the cell's exterior milieu. One of the most prominent membrane proteins of the chromaffin granule is the enzyme dopamine β -hydroxylase (DBH)¹: this is an intrinsic membrane glycoprotein with its antigenic determinants located on the interior surface of the granule membrane (1, 12). Some DBH is also secreted into the medium.

The secretion of catecholamines and DBH occurs in response to secretagogues such as nicotine and veratridine, which are effective in stimulating the intact adrenal gland. These primary cultures of adrenal cells are now well characterized (14, 29) and have proved useful for investigations of the pharmacology of the secretory process (8, 15, 16). Such

¹ *Abbreviations used in this paper:* Con A, concanavalin A; DBH, dopamine- β -hydroxylase; PBS, phosphate-buffered saline.

studies have been complemented by detailed biochemical investigations of the structure and bioenergetics of the chromaffin granule (30) and by histochemical studies of cell morphology (11, 26). In the present study we used an antiserum to DBH as a marker for the inner surface of granule membranes as they are inserted into the plasma membrane during exocytosis. We show that these membranes are subsequently recovered into the cell but that this is a relatively slow process. A similar approach to the study of membrane recycling has recently been reported which used another chromaffin granule antigen (20).

MATERIALS AND METHODS

Antisera: Antisera to dopamine β -hydroxylase and cytochrome *b₅₆₁* were raised in rabbits and were characterized by immune replicas of gel electrophoretic separations of chromaffin granule proteins (13; and Dr. D. K. Apps, personal communication). The antisera gave only faint background staining with two bovine cell lines, embryonic bovine trachea (fibroblast-like) and kidney epithelial cells. The rabbit antisera to brain spectrin (fodrin) has been described previously (5). Purified goat IgG raised against rabbit IgG and conjugated with fluorescein was purchased from Cappel Laboratories, Inc. (Cochranville, PA).

Cell Cultures: Chromaffin cells were isolated from perfused adult bovine adrenal glands as previously described (8, 14, 29). The basic medium used for cell culture consisted of 50% Dulbecco's modified Eagle's medium and 50% nutrient mixture F12 with 5 mM HEPES and 28.6 mM NaHCO₃ (pH 7.4 at 37°C and 5% CO₂). The cells were suspended at 8×10^5 /ml in this basic medium supplemented with 10% newborn calf-serum, 2×10^{-5} M 5-fluorodeoxyuridine, 10⁵ U/liter penicillin, 40 mg/liter gentamycin. For microscopy the cells were plated on uncoated 12-mm diameter coverslips at an average cell density of 2×10^5 cells per coverslip. After 20 h, the plating medium was replaced with the basic medium supplemented only with 40 mg/liter gentamycin. All experiments were performed within 48 h of this medium change.

Catecholamine Secretion: Cells were plated in multiwell plates (0.4×10^6 cells per well). The serum-free medium was replaced with fresh medium 30 min before initiating secretion. At zero-time, fresh medium containing 100 μ M veratridine, 10 μ M nicotine, or no addition, was added. Aliquots of medium were removed from the wells to 1 M acetic acid, and their catecholamine content, and that of the cells in the well, were determined as described previously (28). Catecholamine released to the medium was expressed as a percentage of that present in the culture.

Immunofluorescence Microscopy: Coverslips were rinsed in phosphate-buffered saline (PBS; 0.13 M NaCl containing 10 mM sodium phosphate, pH 7.2) and the cells were fixed in 3.7% formaldehyde in saline for 5 min at room temperature. After washing in PBS, they were permeabilized in 1% Triton X-100 in PBS for 5 min. After a further PBS wash they were incubated with 25 μ l of antiserum diluted 25-fold in PBS for 30 min at 37°C in a humid atmosphere. Preimmune sera were used in control experiments. After another wash in PBS they were treated in the same way with fluorescein-conjugated second antibody diluted 50-fold in PBS. The coverslips were then washed and mounted. They were observed in a Leitz Ortholux II microscope with a Ploemopak 2.2 fluorescence vertical illuminator, using a Zeiss Planapo 63 \times oil immersion objective (numerical aperture 1.4), and Leitz filter block type H. Cells were photographed using Kodak Tri-X pan (ASA 400) film; all fields were photographed with both epifluorescence and phase-contrast optics.

Secretion Experiments: Cells on coverslips were drained against a laboratory tissue, and fresh serum-free medium at 37°C (25 μ l) was added. The coverslips were incubated at 37°C in a moist chamber for 30 min (prewash period). The medium was then replaced with warm medium containing secretagogue (100 μ M veratridine or 10 μ M nicotine) and 25-fold diluted antiserum. This, and other aspects of this procedure, were varied as indicated in Fig. 6. To terminate secretion, we drained the coverslips and rinsed them once with warm medium containing an antagonist (100 μ M tetrodotoxin or 20 μ M *d*-tubocurarine). In many experiments this was followed by a chase period when the coverslips were incubated for a period at 37°C with a further 25 μ l of medium containing antagonist. At the end of this period the coverslips were drained and plunged into ice-cold PBS; after a few minutes they were fixed (5 min) and permeabilized as described above, and then incubated with 50-fold diluted fluorescein-conjugated goat anti-rabbit IgG immunoglobulin for 30 min. The above times were followed exactly in all experiments, even when the order of treatments was varied. When Triton X-100 treatment was omitted (see Fig. 6b), an identical Triton X-100 permeabilization step was included after the final incubation with fluorescent antibody, so that the phase-contrast appear-

ance of the cells would be the same as that in other experiments. In all control experiments care was taken to use photographic conditions that were identical in all parts of the experiment.

Concanavalin A Treatment: Fluorescein-conjugated concanavalin A (Con A) was obtained from Vector Laboratories, Inc. (Burlingame, CA). Cells on coverslips were incubated for 5 min at 37°C in serum-free medium containing fluorescein-conjugated Con A at ~ 0.2 mg/ml. They were then rinsed with warm fresh medium and incubated for further periods before chilling, fixing, and permeabilizing as described above.

RESULTS

Cultures of Chromaffin Cells

Chromaffin cells can be isolated in high yield from bovine adrenal medullae by a procedure involving collagenase perfusion, tissue disruption, and cell purification on a Percoll gradient (8, 14, 29). Such preparations contain >90% chromaffin cells as determined by electron microscopy, staining, and catecholamine fluorescence (29). When the cells adhere to plastic or to glass coverslips, however, $\sim 50\%$ of the cells lose the characteristic enzyme and catecholamine components of chromaffin cells within a few days; they develop dark vacuoles (pinocytic vesicles?) and well developed stress fibres. The remaining cells contain abundant chromaffin granule components, and, as we show below, retain the ability to secrete catecholamines in response to a suitable stimulus. Such cells are easily recognised in phase-contrast micrographs by their rather granular, greyish cytoplasm.

Although originally plated in a serum-containing medium, the cells are transferred to a serum-free medium after 20 h (29). All experiments reported in this paper have been performed in this medium within 48 h of the medium change.

Stimulation in Presence of Anti-DBH

Cells were stimulated to secrete catecholamines by replacing their medium with one containing either 100 μ M-veratridine or 10 μ M-nicotine, at 37°C. The actions of these two agonists on bovine chromaffin cells have previously been characterised at 25°C (14). In Fig. 1 we show the time course of catecholamine release induced by these agents. The change of medium itself results in some loss of catecholamine ($\sim 8\%$ of the total content); this is probably due either to loss of some poorly adhering cells from the culture wells, or to lysis of cells, and it has been subtracted from the data in Fig. 1. It can be seen that there is a relatively long-lasting discharge with veratridine. This is completely inhibited by adding tetrodotoxin (100 μ M) at any time during the release process. In contrast, nicotine-evoked release is complete within 5 min, when only $\sim 10\%$ of the total catecholamine in the culture has been released, i.e., approximately double the background in the medium. Nicotine action is inhibited by 20 μ M *d*-tubocurarine (results not shown).

We performed an experiment similar to that in Fig. 1 by adding pre-warmed veratridine-containing medium to cells growing on coverslips. This medium also contained antiserum to DBH. After 5 min, secretion was halted by plunging coverslips into ice-cold saline; the cells were fixed and then permeabilized with Triton X-100. Anti-DBH remaining bound to the cells was visualized by incubation with fluorescent goat IgG against rabbit IgG (Fig. 2, *a* and *b*). Fig. 2*c* is a control, showing cells that have been permeabilized first and then treated with antiserum for visualization of DBH inside the cells by standard immunofluorescence technique.

The cells contain abundant DBH. It is particularly evident

in the greyish, nonvacuolated cells, but, as seen in Fig. 2c, individual secretory granules are not readily visualized, except occasionally in very thin regions of the cell. This is not surprising, since each cell contains about 20,000 granules (23). In contrast, when cells are stimulated to secrete in the presence of anti-DBH before fixation, the treatment with the second antibody reveals a mass of dots. These are particularly distributed around the cell peripheries, and arise, as shown below, from exposure of the inner surface of the granule membrane during exocytosis. In general, the vacuolated cells

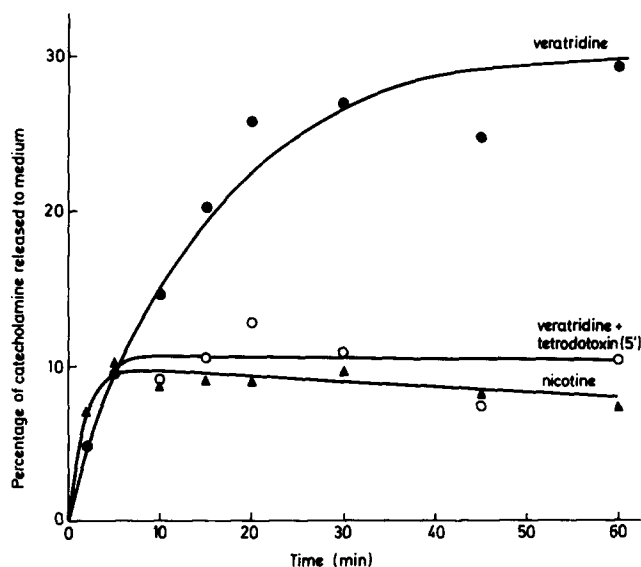


FIGURE 1 Secretion of catecholamine by cultured chromaffin cells. Cells were incubated with 100 μ M veratridine (●) or 10 μ M nicotine (▲) in culture medium at 37°C, and the percentage of catecholamine released to the medium was measured. After 5 min, 100 μ M tetrodotoxin was added to a set of cells with veratridine (○).

referred to above appear to be inactive (Fig. 2, a and b), although sometimes they acquire fluorescent dots (from surface DBH) over parts of their surfaces only. In the absence of secretagogue, intact cells do not bind anti-DBH. Even after 1 min of exposure to the secretion medium, however, the nonvacuolated cells acquire a few very small dots around their peripheries; a maximal number appears after ~5-min exposure, similar results being obtained with both veratridine and nicotine.

It can be seen in Fig. 2b that some cells are well-spread, while others are small and are in clumps. Fluorescent dots in the latter are often out of focus because of the thickness of the cells, and appear instead as large fluorescent patches (Fig. 2a).

If these fluorescent dots represent exposure of the interior of a granule membrane by exocytosis, their appearance should be sensitive to inhibitors of secretion. A variety of inhibitors is available, and their mechanisms of action are known (15, 16). In Fig. 3 it is shown that nicotine-evoked antibody binding is inhibited by *d*-tubocurarine (a cholinergic receptor antagonist) and by EGTA (removing free calcium from the medium). It is not inhibited by tetrodotoxin, a blocker of voltage-sensitive sodium channels. In contrast, veratridine, which opens sodium channels, is antagonized both by tetrodotoxin and by EGTA, since Ca^{2+} entry is required for exocytosis (Fig. 4). In these experiments we identified the small plain cells, shown above to be competent in secretion, by phase-contrast microscopy, and have photographed them under identical conditions to the controls.

Stimulation in the Presence of Other Antisera

The most abundant intrinsic protein in the chromaffin granule membrane is cytochrome *b*₅₆₁ (30), a rather hydrophobic protein that spans the membrane (1). Antisera to this protein react only with determinants located on the outer,

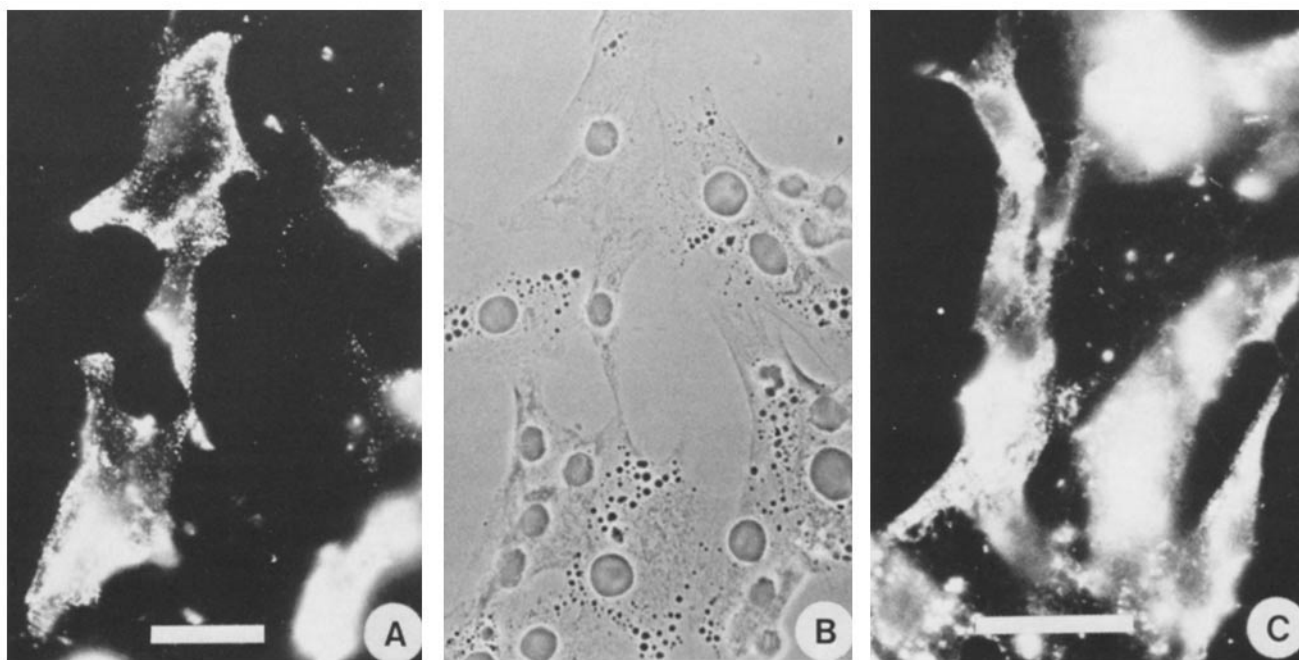


FIGURE 2 (A and B) Chromaffin cells treated with 100 μ M-veratridine in the presence of anti-DBH serum for 5 min at 37°C. Some cells observed in the phase-contrast micrograph (B) fail to become fluorescent (A); these are generally vacuolated cells. Clumps of small cells which are thick and rounded (B) show an intense unfocused fluorescence (A). (C) Control immunofluorescent localization of DBH in chromaffin cells. Bars, 25 μ m.

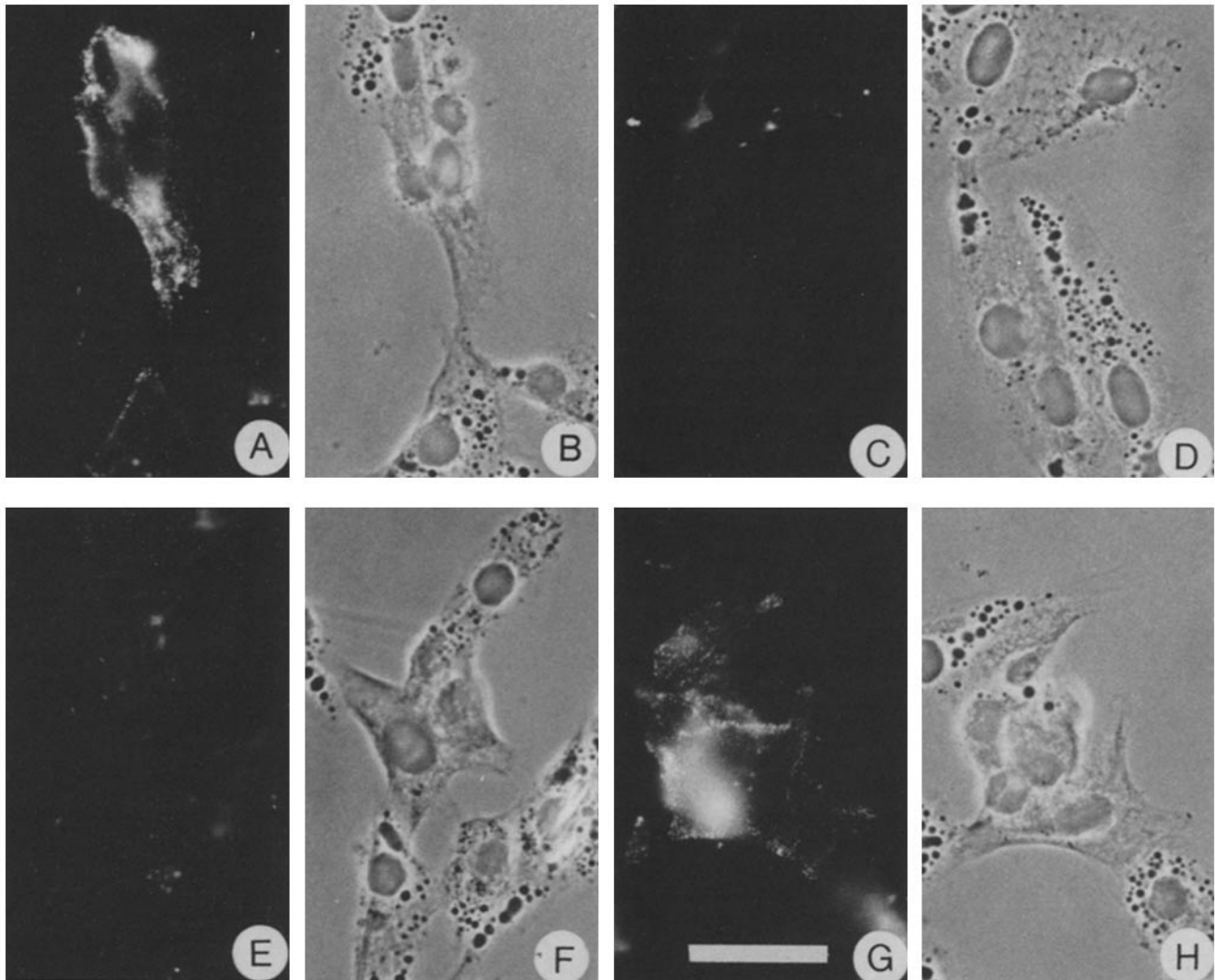


FIGURE 3 Chromaffin cells treated with secretagogue ($10 \mu\text{M}$ nicotine) in the presence of anti-DBH serum for 5 min at 37°C (A and B). The secretion medium was supplemented additionally with $20 \mu\text{M}$ *d*-tubocurarine (C and D), 2 mM EGTA (E and F), or $100 \mu\text{M}$ tetrodotoxin (G and H). Fluorescence and phase-contrast micrographs. Bar, $25 \mu\text{m}$.

cytoplasmic surface of the granule membrane (13). The cytochrome is abundant in cultured chromaffin cells (Fig. 5, *e* and *f*).

We found that a small amount of anticytochrome bound to the surface of most chromaffin cells, so that they can be visualized after treatment as faintly hazy outlines. This occurs on both vacuolated and nonvacuolated cells and may be caused by contamination with cytochrome during the cell preparation, or by a minor component in the antiserum. Stimulation of cells with veratridine or nicotine failed to increase the cell-surface fluorescence (Fig. 5, *a* and *c*), nor did we find the patterns of dots that are so characteristic with antiserum to DBH, even though the cytochrome is easily revealed inside the cells after permeabilization (Fig. 5 *e*).

We also used an antiserum to brain spectrin (fodrin), which is abundant in chromaffin cells (unpublished work). It is thought to form a network on the inner surface of the plasma membrane, in a manner analogous to erythrocyte spectrin (5, 9, 19). It should be readily exposed to the medium if veratridine or nicotine treatment is causing the plasma membrane of the chromaffin cells to become leaky or vesiculated. Stimulation of the cells in the presence of antiserum to brain

spectrin, followed by fluorescent antibody treatment, failed to reveal any spectrin unless the cells were first permeabilized with detergent.

Internalization of DBH/Anti-DBH Complex

We exposed cells to the secretion medium, as in Fig. 2*a*, in the presence of anti-DBH for 5 min, and then incubated the cells for a further period in the presence of an antagonist, before fixing and processing them (Fig. 6, scheme *a*). A selection of typical cells is shown in Fig. 7. It can be seen that initially the cells are covered by minute dots: these tend to be clustered towards the cell peripheries. Some confluent patches of fluorescence are seen, resulting from unfocused parts of the cells. Within 15 min, the dots become larger and are more uniformly located in the cell. After 2 h most of the fluorescent dots are clustered around the cell nuclei, and they are larger and fewer. The total fluorescence decreases somewhat after about an hour, although its distribution after 4 h of chase is similar to that seen in Fig. 7*d* at 2 h.

We assumed that we were visualizing the internalization of surface-exposed DBH/anti-DBH complexes. To confirm this,

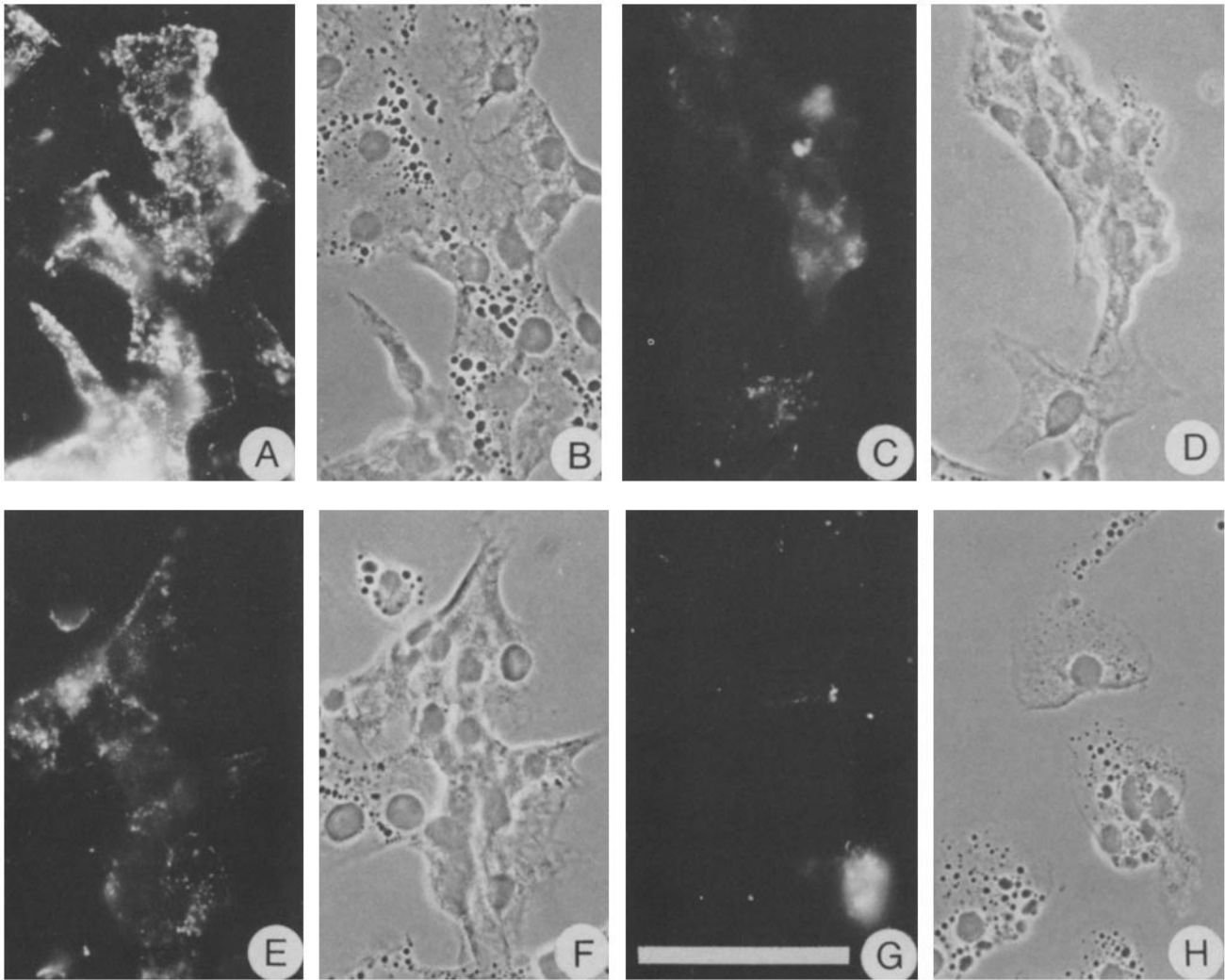


FIGURE 4 (A–F) Chromaffin cells treated with secretagogue (100 μM veratridine) in the presence of anti-DBH serum for 15 min at 37°C. The secretion medium was supplemented additionally with 2 mM EGTA (C and D), or 100 μM tetrodotoxin (E and F). In (G and H) are shown cells incubated in medium containing 100 μM tetrodotoxin with anti-DBH serum, but no secretagogue, for 15 min at 37°C. Fluorescence and phase-contrast micrographs. Bar, 50 μm .

we processed coverslips as described above, but omitted the Triton-permeabilization step that normally preceded treatment with fluorescent goat anti-rabbit IgG (Fig. 6*b*). In this way fluorescent antibody can only bind to rabbit anti-DBH that is located on the cell surface (or, possibly, just below the cell surface: we have shown that slight penetration of some of the cells by antibody is possible following formaldehyde fixation [our unpublished observations]).

Results of such an experiment are shown in Fig. 8. These pictures are of cells treated by procedure *b* of Fig. 6 at the same time as the cells in Fig. 7 were being treated by procedure *a*. It is clear that, with no chase period, the appearance of the cells in Fig. 8*a* is identical to that of the unchased cells in Fig. 7*a*. Thereafter, however, their fluorescence declines, and has nearly disappeared after 30 min (at which time the cells in Fig. 7*c* are brightly fluorescent). This must represent clearance of the DBH/anti-DBH complex from the cell membrane.

The experiment shown in Figs. 7 and 8 was performed using 100 μM veratridine as a secretagogue, followed by incubation in 100 μM tetrodotoxin to inhibit any further secretion. The experiment was also performed using 10 μM -

nicotine and 20 μM -*d*-tubocurarine in case the tetrodotoxin was decreasing the rate of complex internalization. The rate observed, however, was unchanged.

Internalization of DBH

It seemed possible that complex formation between DBH, on the inner surface of a chromaffin granule membrane, and an antibody might affect the rate at which membrane containing the complex could be internalized. We therefore looked for residual DBH on the cell surface, following the procedure shown in Fig. 6*c*, in which a short treatment with anti-DBH is given after the chase period, immediately before processing the cells. (Controls for this experiment, in which anti-DBH is added in the absence of a prior secretion stimulus, fail to reveal any surface fluorescence: cf. Fig. 4*g*.)

Results of an experiment comparable to that in Figs. 7 and 8 are shown in Fig. 9. In this case, identical results were obtained whether or not a Triton-permeabilization step was included after binding anti-DBH, strongly indicating that the fluorescent dots shown on the cells in Fig. 9 are truly on the

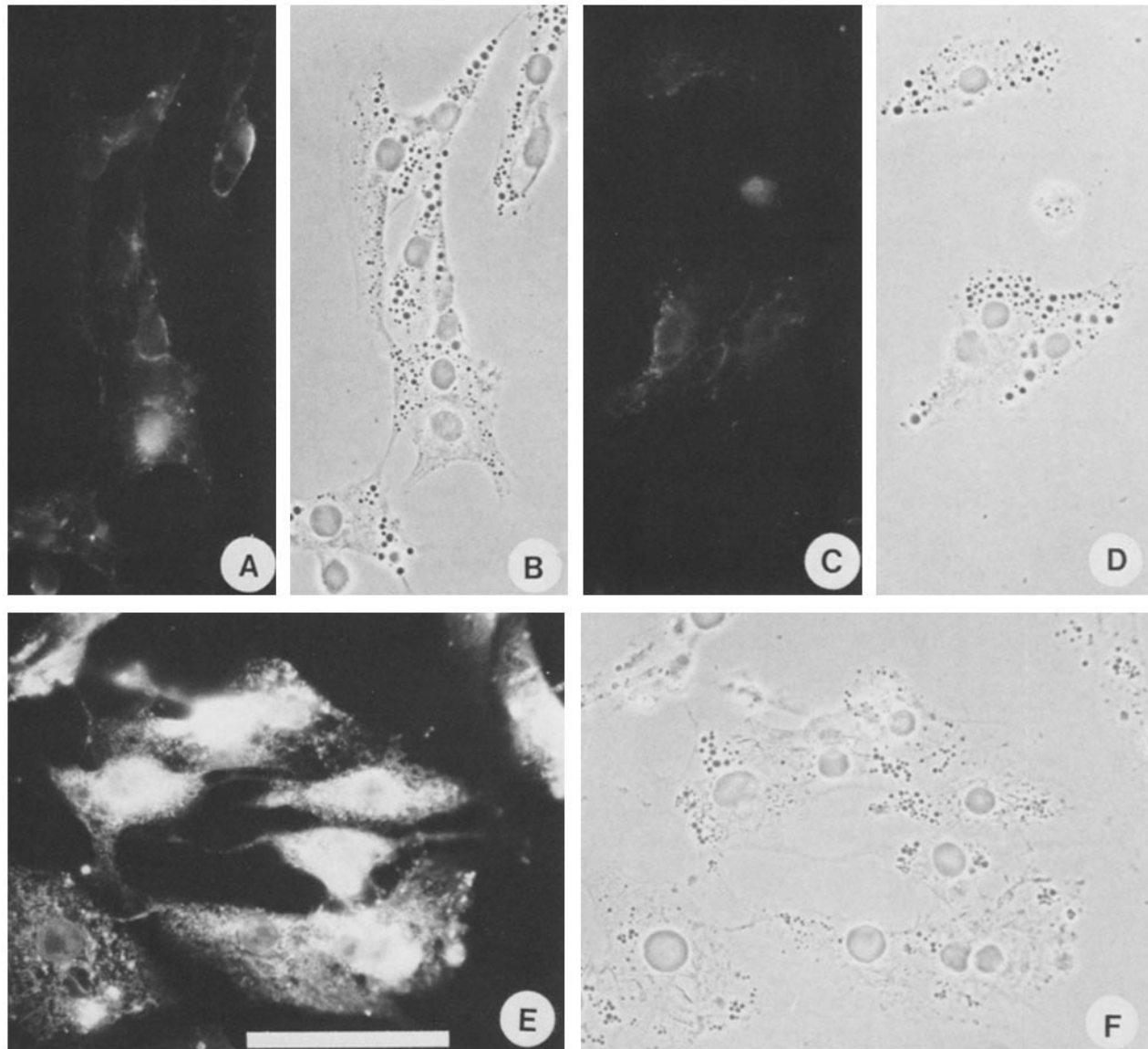


FIGURE 5 Chromaffin cells treated with (a and b) 100 μM veratridine and 100 μM tetrodotoxin in medium containing anti-cytochrome b_{561} serum for 5 min at 37°C. Cells were treated similarly in c and d except that tetrodotoxin was omitted. In e and f the cells were permeabilized before treatment with antiserum in order to reveal cytochrome b_{561} inside the cells. Fluorescence and phase-contrast micrographs. Bar, 50 μm .

cell surface, and represent a visualization of residual cell-surface DBH. It can be seen that there is a considerable loss of fluorescence over 30 min, although faint discrete dots are still visible all over cell surfaces after this time. There is some redistribution of the dots, as well as a reduction in their number: the clustering at the cell peripheries is less marked after a 15-min chase period (Fig. 9b).

Identical results were obtained when the anti-DBH treatment was performed at 37°C or at 0°C to prevent antibody-induced redistribution of DBH in the membrane, and when nicotine was used as secretagogue instead of veratridine.

Internalization of Fluorescein-conjugated Con A

The lectin, Con A, binds to the surfaces of chromaffin cells, presumably to both glycoproteins and glycolipids. It has previously been shown that fluorescent Con A is internalized after prolonged periods of incubation (26), and we decided to

use fluorescein-conjugated Con A as a nonspecific marker for cell-surface internalization.

Con A binds to the carbohydrate moiety of DBH (27), so we incubated cells in media containing both fluorescein-conjugated Con A and *d*-tubocurarine, to prevent DBH exposure at the cell surface. Excess lectin was then washed off the cells, and incubation was continued for various times. Some results are shown in Fig. 10. Initially, Con A binds all over the cell surface, although some clustering is apparent. Within 20 min there is considerable patching on the cell surface, visualized either as dots or as rather jagged patches. During the following hour, redistribution occurs (presumably internalization, although we cannot detect that unequivocally in this experiment): by 90–120 min there is a clustering of fluorescent dots around cell nuclei, possibly representing internalized Con A trapped within lysosomes. By 120 min, little surface Con A fluorescence remains (although, as shown

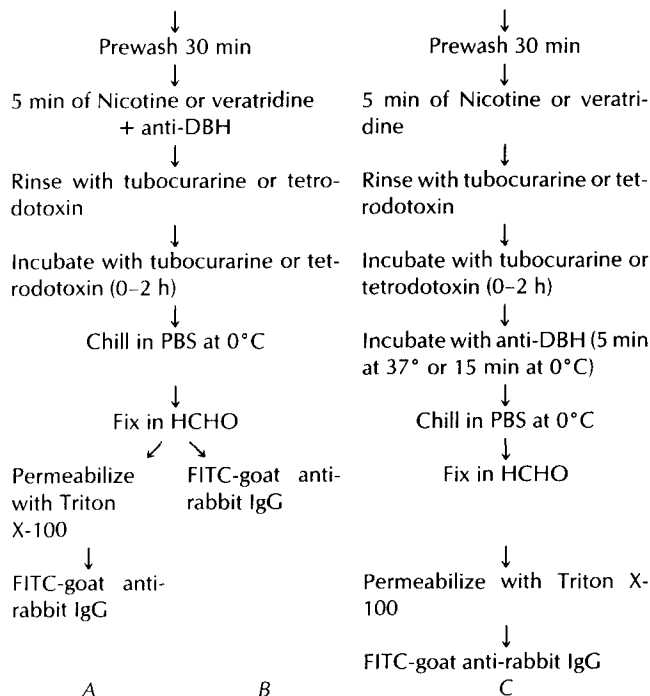


FIGURE 6 Experimental schemes used in chase experiments.

previously [26], some Con A-binding sites are probably located on the cell surface for longer periods).

The fluorescein-conjugated Con A binds to both vacuolated and nonvacuolated chromaffin cells and is internalized by both. Stimulation of the cells with nicotine (10 μ M, 5 min at 37°C) following Con A binding did not appear to affect the rate of Con A receptor redistribution or internalization. We did not observe fluorescein-labeled Con A patches aligning with the stress fibers of vacuolated cells, as has previously been observed in cultures of fibroblasts or kidney cells (2).

DISCUSSION

The phenomenon we have observed appears to represent DBH exposure to the cell surface during exocytosis, followed by its retrieval into the cell. We can draw the following conclusions:

(a) It is possible to visualize sites of exocytosis by fluorescence microscopy as fine dots on the cell surface. These are not arranged in any particular pattern, although, in these cells at least, they are most prolific in the thinnest portions of the cell, away from the nucleus. A similar distribution of DBH within these cells has been noted previously (11), with granule components concentrated at the cell periphery or in neuritic extensions of the cells. The sites of exocytosis in these cultured secretory cells thus resemble sites of insertion of recycled endocytosed membrane in cultured cells, which also occur at cell peripheries (4).

(b) The granule membrane protein DBH does not become uniformly distributed on the cell surface. It never resembles the Con A receptors seen in Fig. 10 immediately after an incubation with the lectin. Even as long as 30 min after the secretory stimulus has been inhibited, DBH remaining on the cell surface is still present as discrete patches (Fig. 9c); it is presumably present in patches of granule membrane, since it can be revealed as dots by subsequent treatment with anti-DBH. There does not seem to be free diffusion of this antigen

within the plane of the membrane. (The discrete dots visualized in Fig. 9 are not a consequence of anti-DBH-induced DBH reorganization, since one obtains the same result if anti-DBH binding is performed at 0°C.)

(c) Free DBH is internalized into the cell at approximately the same rate as DBH/anti-DBH complexes (Figs. 8 and 9). It is impossible to say from these experiments whether this is a first-order process, but about half the cell-surface fluorescence is lost in \sim 15–30 min, whether free DBH is assayed (Fig. 9) or its complex with antibody (Fig. 8). This rate of internalization is approximately the same as the rate that can be inferred for Con A removal from the cell surface. Unfortunately, the fluorescent dots visible in Figs. 8 and 9 are too close to each other to enable quantitative assessments of membrane removal to be made by counting.

(d) Concomitant with DBH/anti-DBH removal from the cell surface, the fluorescent dots seen in Fig. 7 become larger (this occurs over the first 15 min of chase), suggesting that retrieved membranes are incorporated into larger compartments inside the cell. These may well be the same as the vacuoles seen just below the surface of stimulated cells (8) by electron microscopy: such empty profiles have a diameter about two to three times as large as that of a typical chromaffin granule.

(e) Redistribution of the DBH/anti-DBH complex within the cell is relatively slow. After several hours the fluorescence remains largely as dots, although these are clustered away from the cell periphery. This may represent accumulation of the complex within lysosomes.

Freeze-fracture reveals a mass of exocytotic pits in the surface membranes of adrenal medulla cells when the intact organ is perfused with solutions containing secretagogues (25). In a thin-section electron microscopic study of chromaffin granule membrane retrieval by stimulated intact glands, Nagasawa and Douglas (22) showed accumulation of an extracellular marker (Thorotrast), first in coated vesicles that pinched off from the granule membrane, and then in larger structures, presumed to be lysosomes. Other workers have also demonstrated coating and vesiculation of chromaffin granule membranes after discharge of the granule contents (3, 10), although it is not clear whether this could account for all membrane retrieval. It has also been suggested that most of the membranes may be retrieved more or less intact to form electron-translucent vesicles of about the same size as intact granules (17). Our data are consistent with either mechanism: it is clear, however, that, following retrieval into the cell, the membranes fuse with other membranes to form larger structures. Surface Con A receptors may well be internalized by coated vesicles (24), so it is of great interest that Con A internalization takes place at approximately the same rate as granule membrane retrieval. Our data are not compatible with the idea that granule antigens diffuse freely in the plasma membrane and that endocytotic recovery occurs randomly in the membrane, although the redistribution of dots seen in Fig. 9 suggests that, following exocytosis at the periphery, a granule patch may be relocated towards the center of the cell before internalization.

Compound exocytosis (the fusion of granules with the membranes of other granules that have already fused with the plasma membrane) occurs in some secretory cells; although it has never been observed in the intact adrenal medulla, there is one report of multiple fusion in stimulated isolated bovine cells (8). In the intact parotid gland, compound exocytosis

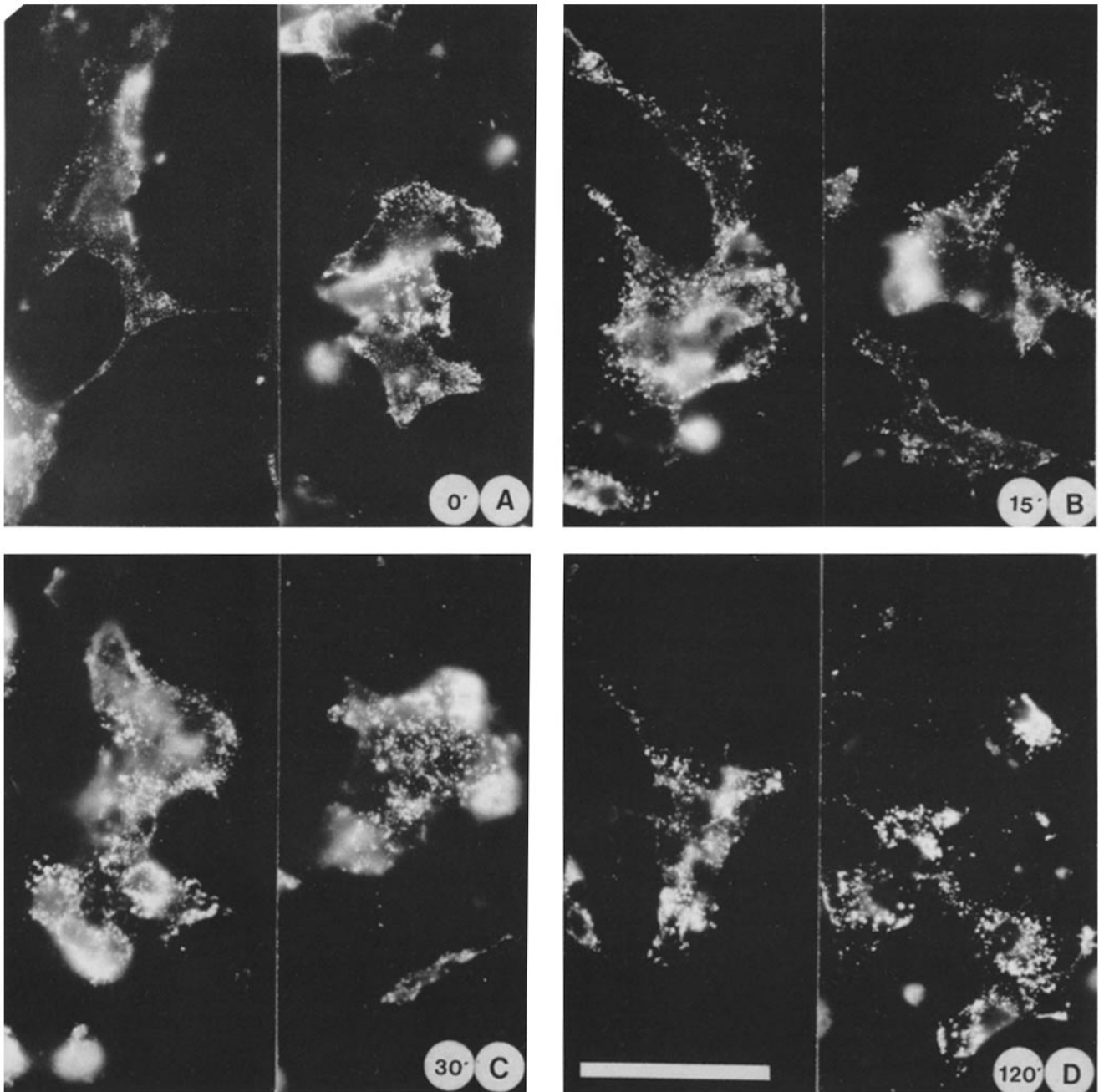


FIGURE 7 Chromaffin cells treated with 100 μM veratridine in the presence of anti-DBH serum for 5 min at 37°C. In A are shown two micrographs of cells fixed after this treatment. Other cells were incubated further in a medium containing 100 μM tetrodotoxin (Fig. 6, scheme A), and pairs of micrographs of these chased cells are shown in each panel. The chase period was (B) 15 min, (C) 30 min, and (D) 120 min. Bar, 50 μm .

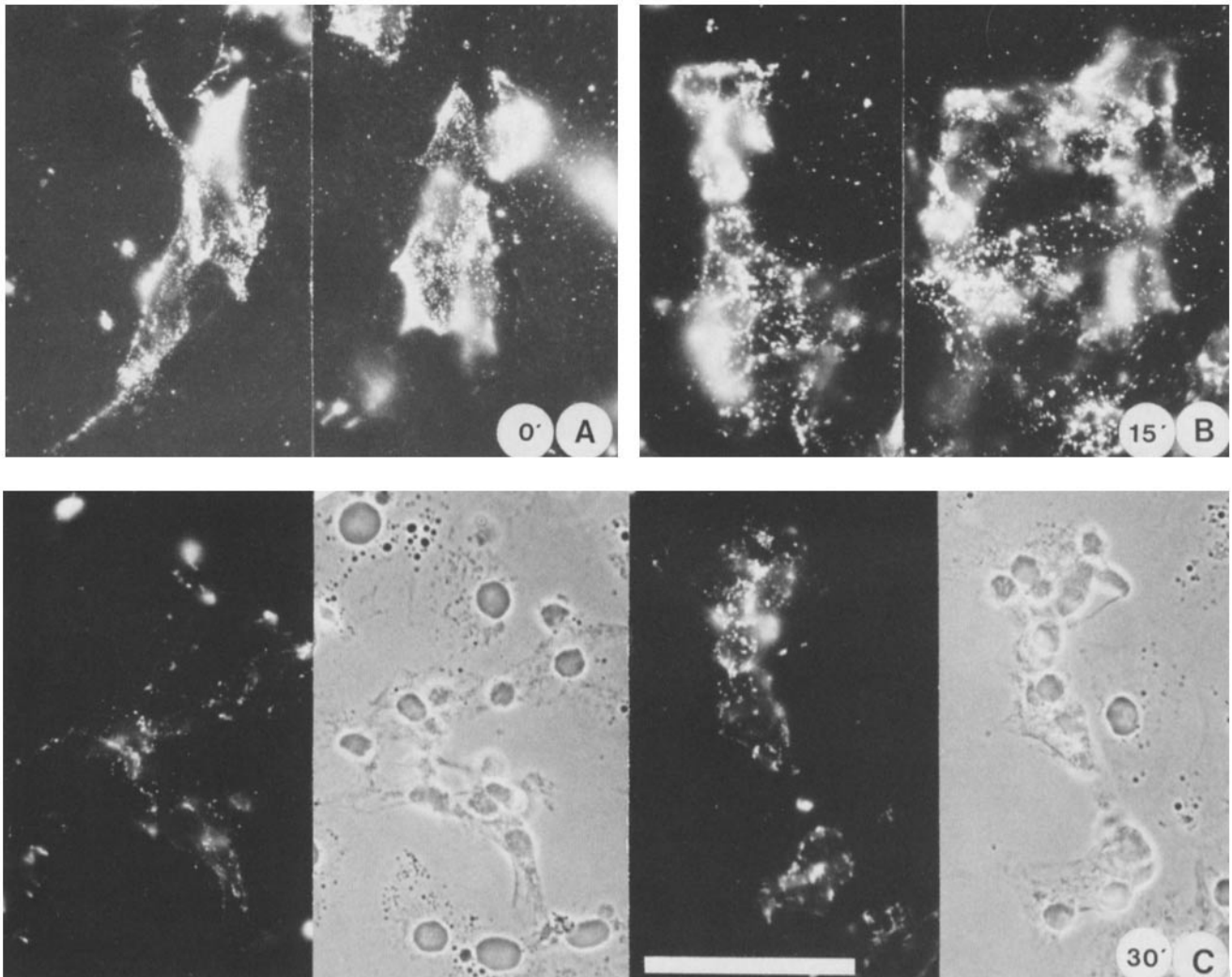


FIGURE 8 Chromaffin cells treated with $100 \mu\text{M}$ veratridine in the presence of anti-DBH serum for 5 min at 37°C , then further incubated in medium containing $100 \mu\text{M}$ tetrodotoxin. Same experiment as Fig. 7, except that Triton permeabilization before second antibody treatment was omitted (see Fig. 6, scheme B). (A) Two fields of cells fixed after veratridine treatment. (B) Two fields of cells incubated for a chase period of 15 min. (C) Two fields (fluorescence and phase contrast micrographs) of cells incubated for a chase period of 30 min. Bar $50 \mu\text{m}$.

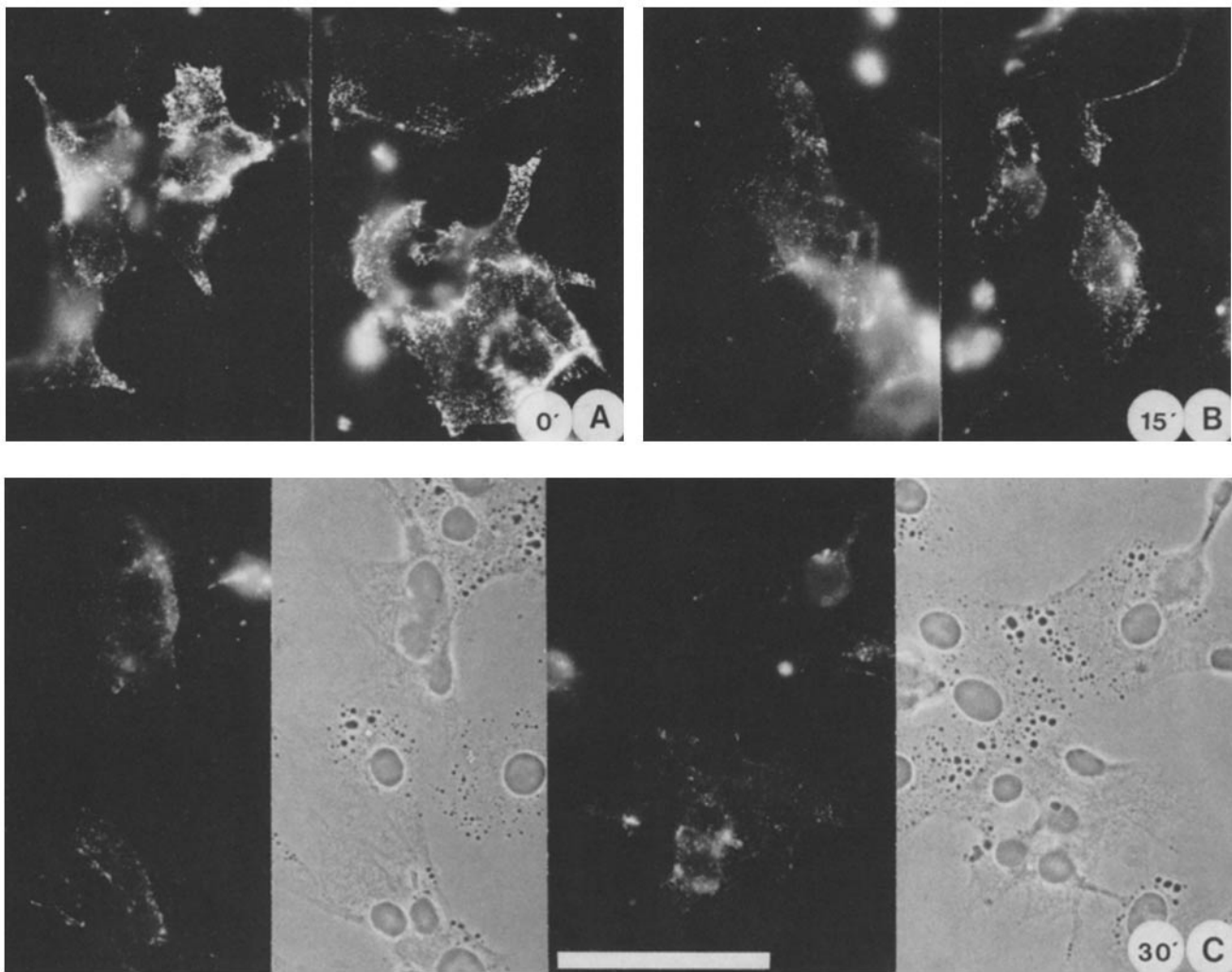


FIGURE 9 Chromaffin cells treated with 100 μM veratridine for 5 min at 37°C, then with medium containing 100 μM -tetrodotoxin plus anti-DBH serum for 5 min at 37°C (A). In B the cells were incubated for 15 min at 37°C with tetrodotoxin-containing medium before anti-DBH serum was added; and in C this chase period was 30 min. (See Fig. 6, scheme C.) Bar, 50 μm .

leads to a great enlargement of the luminal surface membrane of the acinar cells. Freeze-fracture studies show that, following a period of secretion, granule membranes remain distinguishable from the neighboring plasma membrane (they retain a much lower density of intramembrane particles) (6). This is consistent with our observation (Fig. 9) that the patches of DBH-containing membrane remain distinct.

Measurements of the complex luminal profiles of parotid acinar cells show that, following massive secretion induced by isoproterenol, membrane is retrieved into the cell in a process showing first-order kinetics with a half-life of 18.5 min (18). This is very similar to the rate deduced in the present study for isolated cells showing predominantly simple exocytosis. Our observations are also consistent with a recent quantitative assessment of the rate of removal of a different granule membrane antigen from the surfaces of chromaffin cells (20), in which retrieval was shown to be half-complete in ~ 10 min.

It is clear that although 10–30% of the isolated chromaffin cell's store of catecholamines is readily released on stimulation (Fig. 1), the rest may be released only relatively slowly, if at all (14). Assuming that a bovine chromaffin cell has a surface

area of $\sim 800 \mu\text{m}^2$, release of 10% of its content of granules (assuming about 20,000 granules per cell) (23) will increase its surface area by $\sim 50\%$ if retrieval is slow compared with release. It seems quite likely that such a large addition to the surface membrane may overwhelm the capacity of the cell to recover membrane, a process that might, for example, be limited by the availability of clathrin. In vivo, in the intact gland, however, it is probable that many fewer granules are released. Even very prolonged stimulation of the splanchnic nerve has been shown to release only $\sim 5\%$ of the stored catecholamine (7, 23). The cellular machinery for membrane retrieval may well be able to cope rapidly with only a small number of exocytotic pits. It has been suggested that a similar situation, of rapid recovery on a small scale, but slow recovery after massive release, may exist in nerve terminals at the neuromuscular junction (21).

If granule membranes remain inserted in the plasma membrane for prolonged periods it is clear that the cell surface must acquire the permeability properties of these membranes. In the case of chromaffin granules this includes a proton-translocating ATPase that will pump protons out of the cell, and a catecholamine transporter (as well as carriers for ade-

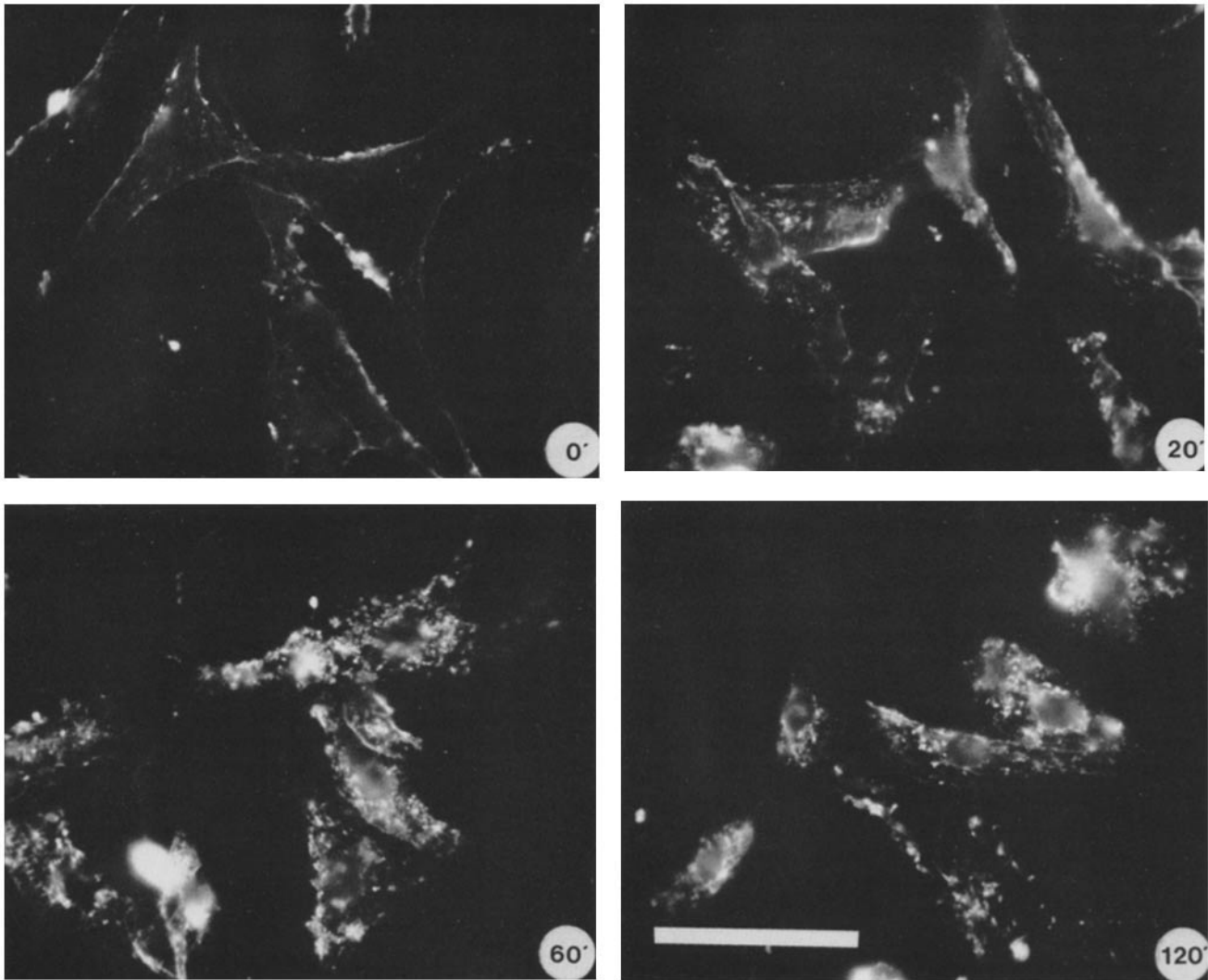


FIGURE 10 Chromaffin cells treated with fluorescein-labeled Con A for 5 min at 37°C. This was followed by incubation in normal medium for 20, 60, or 120 min. Bar, 50 μ m.

nine nucleotides and divalent cations) (23). It is conceivable that, with large extents of secretion *in vitro*, this could have a seriously deleterious effect on cellular metabolism.

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REFERENCES

1. Abbs, M. T., and J. H. Phillips. 1980. Organisation of the proteins of the chromaffin granule membrane. *Biochim. Biophys. Acta* 595:200-221.
2. Ash, J. F., and S. J. Singer. 1976. Concanavalin-A-induced transmembrane linkage of concanavalin A surface receptors to intracellular myosin-containing filaments. *Proc. Natl. Acad. Sci. USA* 73:4575-4579.
3. Benedeczy, I., and A. D. Smith. 1972. Ultrastructural studies on the adrenal medulla of golden hamster: origin and fate of secretory granules. *Z. Zellforsch. Mikrosk. Anat.* 124:367-386.
4. Bretscher, M. S. 1983. Distribution of receptors for transferrin and low density lipoprotein on the surface of giant HeLa cells. *Proc. Natl. Acad. Sci. USA* 80:454-458.
5. Burridge, K., T. Kelly, and P. Mangeat. 1982. Nonerythrocyte spectrins: actin-membrane attachment proteins occurring in many cell types. *J. Cell Biol.* 95:478-486.
6. De Camilli, P., D. Peluchetti, and J. Meldolesi. 1976. Dynamic changes of the luminal plasmalemma in stimulated parotid acinar cells. A freeze-fracture study. *J. Cell Biol.* 70:59-74.
7. Edwards, A. V., P. N. Furness, and K. B. Helle. 1980. Adrenal medullary responses to stimulation of the splanchnic nerve in the conscious calf. *J. Physiol. (Lond.)* 308:15-27.
8. Fenwick, E. M., P. B. Fajdiga, N. B. S. Howe, and B. G. Livett. 1978. Functional and morphological characterization of isolated bovine adrenal medullary cells. *J. Cell Biol.* 76:12-30.
9. Glenney, J. R., P. Glenney, and K. Weber. 1982. Erythroid spectrin, brain fodrin, and intestinal brush border proteins (TW-260/240) are related molecules containing a common calmodulin-binding subunit bound to a variant cell type-specific subunit. *Proc. Natl. Acad. Sci. USA* 79:4002-4005.
10. Grynszpan-Winograd, O. 1971. Morphological aspects of exocytosis in the adrenal medulla. *Phil. Trans. R. Soc. Lond. B. Biol. Sci.* 261:291-292.
11. Hesketh, J. E., J. Ciesielski-Treska, and D. Aunis. 1981. A phase-contrast and immunofluorescence study of adrenal medullary chromaffin cells in culture: neurite formation, actin and chromaffin granule distribution. *Cell Tissue Res.* 218:331-343.
12. Huber, E., P. König, G. Schuler, W. Aberer, H. Plattner, and H. Winkler. 1979. Characterization and topography of the glycoproteins of adrenal chromaffin granules. *J. Neurochem.* 32:35-47.
13. Hunter, A., K. Waldron, and D. K. Apps. 1982. Determination of the proportion of sealed vesicles in a preparation of chromaffin granule membrane 'ghosts.' *FEBS (Fed. Eur. Biochem. Soc.) Lett.* 144:51-56.
14. Kilpatrick, D. L., F. H. Ledbetter, K. A. Carson, A. G. Kirshner, R. Slepets, and N. Kirshner. 1980. Stability of bovine adrenal medulla cells in culture. *J. Neurochem.* 35:679-692.
15. Kilpatrick, D. L., R. Slepets, and N. Kirshner. 1981. Ion channels and membrane potential in stimulus-secretion coupling in adrenal medulla cells. *J. Neurochem.* 36:1245-1255.
16. Kilpatrick, D. L., R. Slepets, and N. Kirshner. 1981. Inhibition of catecholamine secretion from adrenal medulla cells by neurotoxins and cholinergic antagonists. *J. Neurochem.* 37:125-131.
17. Koerker, R. L., W. E. Hahn, and F. H. Schneider. 1974. Electron translucent vesicles in adrenal medulla following catecholamine depletion. *Eur. J. Pharmacol.* 28:350-359.
18. Koike, H., and J. Meldolesi. 1981. Post-stimulation retrieval of luminal surface membrane in parotid acinar cells is calcium-dependent. *Exp. Cell Res.* 134:377-388.

19. Levine, J. and M. Willard. 1981. Fodrin: axonally transported polypeptides associated with the internal periphery of many cells. *J. Cell Biol.* 90:631-643.
20. Lingg, C., R. Fischer-Colbrie, W. Schmidt, and H. Winkler. 1983. Exposure of an antigen of chromaffin granules on cell surface during exocytosis. *Nature (Lond.)*, 301:610-611.
21. Meldolesi, J., and B. Ceccarelli. 1981. Exocytosis and membrane recycling. *Phil. Trans. R. Soc. Lond. B Biol. Sci.* 296:55-65.
22. Nagasawa, J., and W. W. Douglas. 1972. Thorium dioxide uptake into adrenal medullary cells and the problem of recapture of granule membranes following exocytosis. *Brain Res.* 37:141-145.
23. Phillips, J. H. 1982. Dynamic aspects of chromaffin granule structure. *Neuroscience.* 7:1595-1609.
24. Salisbury, J. L., J. S. Condeelis, N. J. Maihle, and P. Satir. 1981. Receptor-mediated endocytosis by clathrin-coated vesicles: evidence for a dynamic pathway. *Cold Spring Harbor Symp. Quant. Biol.* 46:733-741.
25. Smith, U., D. S. Smith, H. Winkler, and J. W. Ryan. 1973. Exocytosis in the adrenal medulla demonstrated by freeze-etching. *Science (Wash. DC)*. 179:79-82.
26. Trifaro, J. M., and G. W. Bourne. 1981. Differential effects of concanavalin A on acetylcholine and potassium-evoked release of catecholamines from cultured chromaffin cells. *Neuroscience.* 6:1823-1833.
27. Wallace, E. F., and W. Lovenberg. 1974. Studies on the carbohydrate moiety of dopamine β -hydroxylase: interaction of the enzyme with concanavalin A. *Proc. Natl. Acad. Sci. USA.* 71:3217-3220.
28. Wilson, S. P., K.-J. Chang, and O. H. Viveros. 1982. Proportional secretion of opioid peptides and catecholamines from adrenal chromaffin cells in culture. *J. Neurosci.* 2:1150-1165.
29. Wilson, S. P., and O. H. Viveros. 1981. Primary culture of adrenal medullary chromaffin cells in a chemically defined medium. *Exp. Cell Res.* 133:159-169.
30. Winkler, H., and E. Westhead. 1980. The molecular organization of adrenal chromaffin granules. *Neuroscience.* 5:1803-1823.