Exocytotic Exposure and Retrieval of Membrane Antigens of Chromaffin Granules: Quantitative Evaluation of Immunofluorescence on the Surface of Chromaffin Cells

ALEXANDRA PATZAK, GÜNTHER BÖCK, REINER FISCHER-COLBRIE, KONRAD SCHAUENSTEIN, WOLFGANG SCHMIDT, GEBHARD LINGG, and HANS WINKLER

Department of Pharmacology and Department of General and Experimental Pathology, University of Innsbruck, A-6020 Innsbruck, Austria

ABSTRACT The exocytotic exposure of antigens of chromaffin granule membranes was studied with chromaffin cells isolated from bovine adrenal medulla. Antigens on the cell surface were visualized by indirect membrane immunofluorescence employing antisera against glycoprotein III and dopamine β-hydroxylase. With unstimulated cells, only weak immunofluorescence on the cell surface was observed, whereas stimulated cells (with carbachol or Ba^{2+}) exhibited much stronger reactions. In all cases the staining appeared as dots and patches. To quantitatively prove these observations, we analyzed the immunostained cells using a fluorescence-activated cell sorter. After stimulation, the average fluorescence intensity of the cell population was enhanced. This increase correlated with the degree of catecholamine secretion. The fluorescence intensity of stimulated cells varied over a broad range indicating that individual cells reacted variably to the secretagogues. When stimulated cells were incubated at 37°C for up to 45 min after stimulation, a decrease of membrane immunofluorescence approaching that of unstimulated control cells was observed. Apparently, the membranes of chromaffin granules, which had been incorporated into the plasma membrane, were retrieved by a specific and relatively fast process. This retrieval of the antigen from the cell surface was blocked by sodium azide, but not influenced by colchicine, cytochalasin B, and trifluoperazine. The quantitative methods established in this paper should prove useful for further study of the kinetics of the exo-endocytotic cycle in secretory tissues.

Secretion of catecholamines from adrenal medulla occurs by exocytosis (23, 27). During this process the membranes of the secretory organelles, i.e., the chromaffin granules, are incorporated into the plasma membrane. In order to prevent enlargement of the cell surface, membrane retrieval has to accompany exocytotic activity (17). In adrenal medulla, evidence for endocytotic processes subsequent to secretion has been obtained by studying the uptake of exogenous markers (10, 16, 26). These investigations have not established whether the membranes of chromaffin granules are retrieved specifically and, if so, how fast this specific endocytosis proceeds. To answer these questions, specific components of the membranes of chromaffin granules have to be followed through the exo-endocytotic cycle. The biochemical composition and topology of these membranes has already been characterized in great detail (33, 35). Suitable markers for exocytosis are membrane constituents, which are present on the inner surface of the granule membrane, since such components become exposed on the cell surface during exocytosis. The glycoproteins of chromaffin granules (1, 11, 13) fulfill this criterion. In a qualitative immunohistochemical study, an increased reaction for dopamine β-hydroxylase was observed on the surface of chromaffin cells during catecholamine secretion (29). In a first attempt to obtain more quantitative data, we subjected isolated chromaffin cells to a cytotoxicity test using an antiserum against a glycoprotein of the granule membrane (14). It was demonstrated that an increased amount of granule antigen appeared on the cell surface during secretory activity. Within 30 min after stimulation this exposed antigen had disappeared again.

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In the present study we have investigated the exocytotic exposure of two membrane antigens (glycoprotein III and dopamine β-hydroxylase) of chromaffin granules by indirect membrane immunofluorescence with isolated adrenal medullary cells in suspension. Employing a fluorescence-activated cell sorter for quantitative analysis, we approached the following two questions: (*a*) How do stimulated and unstimulated cells quantitatively compare in the surface expression of granule membrane antigens? and (*b*) is there any correlation between catecholamine secretion and the exposure of granule membrane antigens on the cell surface? Furthermore, we used this technique to monitor the retrieval of granule membrane constituents occurring after stimulation and to study the effect of several drugs on this process.

MATERIALS AND METHODS

Isolation of Chromaffin Cells: Chromaffin cells were isolated from bovine adrenal medulla with a slight modification of the procedure of Fenwick et al. (7). Bovine glands were perfused retrogradely with Krebs Henseleit buffer (with 0.05% collagenase) for 30 min at 37°C. After removal of the cortex, the medulla was minced and incubated twice for 15 min with collagenase in the same buffer. The suspension was filtered through a nylon mesh and then centrifuged for 7 min at 50 *g* at 4°C. The sediment of cells was resuspended and washed twice with buffer containing 0.5% BSA and left standing for 1–2 h at room temperature. This suspension was centrifuged at room temperature. The cells were resuspended in BSA buffer. Aliquots of this suspension were used for further experiments. During all incubations the cell suspensions were gassed with 95% O_2/5% CO_2. The viability of these cells was tested with trypan blue (final concentration: 0.06%) and usually found to be 98–100%. The percentage of chromaffin cells present was determined with neutral red (final concentration: 0.003%) (25). Chromaffin cells represented 84.3% (± 1.3, SE; n = 8) of the total cell populations used for our experiments. Their catecholamine content was 96.2 nmol/10^6 cells (± 6.3, SE; n = 5). Cell preparations with <75% of chromaffin cells were discarded.

Preparation of Antisera: Glycoprotein III was isolated from the soluble lysate of chromaffin granules with sequential immuno- and lectin-affinity chromatography as already described in detail (9). Soluble dopamine β-hydroxylase was purified by immunoaffinity chromatography (9). Antisera against these antigens raised in rabbits gave one precipitation line for both the membrane and the soluble lysate of chromaffin granules. In immunoblotting after two-dimensional electrophoresis, only one spot corresponding to the injected antigen was stained (9). Antisera were adsorbed with pig liver acetone powder (200 mg powder/2.5 ml 1:20 diluted antiserum) for 1 h at room temperature.

Immunofluorescence: Cells (2×10^6 cells/ml) were incubated in 2.4 ml BSA buffer for 10 min at 37°C. Stimulation was performed either with carbachol (2 mM) or with Ba^{2+} (5 mM, in the absence of Ca^{2+}). After incubation the cell suspensions were chilled and 1.2 ml of PBS, pH 7.4, containing sodium azide (final concentration 20 mM) was added. Cells were sedimented at 50 *g* and washed three times at 0°C with BSA buffer. Other cell aliquots were washed only twice without azide and were then incubated again for 15 min to 45 min at 37°C. After this incubation they were sedimented and washed with PBS containing azide. In all subsequent steps sodium azide was present. Cell suspensions were then treated with antisera (anti-glycoprotein III, 1:200 dilution; anti-dopamine β-hydroxylase, 1:100 dilution) at 0°C for 30 min. This was followed by three washes and incubation with fluorescein isothiocyanate-conjugated goat antibodies against rabbit immunoglobulins (1:80 dilution) for 30 min at 0°C. The cells were then fixed in suspension with 4% formaldehyde for 20 min at 0°C in 0.1 M cacodylate buffer (pH 7.0).

Micrographs of the cell suspensions were taken with a fluorescence microscope (Leitz Ortholux II, Wetzlar, Federal Republic of Germany [FRG]) on Agfachrome 50 S professional film. Quantitative evaluation of the immunofluorescence staining was performed with a fluorescence-activated cell sorter (FACS III, Becton & Dickinson, Sunnyvale, CA). 10^4 cells were evaluated for one histogram. In some experiments cells were fixed with 1.3% formaldehyde (30 min at 0°C) immediately after the first incubation and then subjected to indirect membrane immunofluorescence as described.

The effect of drugs (colchicine, 200 μM; cytochalasin B, 200 μM; trifluoperazine, 15 μM; sodium azide, 20 mM) on membrane retrieval was studied by adding them to cells washed after stimulation. After 15 min at 0°C these cells were incubated for 30 min at 37°C. Drugs were dissolved in BSA buffer with the exception of cytochalasin B (stock solution of 50 mg/ml dimethylsulfoxide).

Chemical Assays: Catecholamines were determined by high performance liquid chromatography with electrochemical detection (24).

Materials: Collagenase Type I, BSA fraction V, neutral red, carbamylcholine chloride (carbachol), colchicine, cytochalasin B, trifluoperazine, and pig liver acetone powder were obtained from Sigma Chemical GmbH, Munich, FRG. Goat anti-rabbit IgG–fluorescein isothiocyanate (AIA) was purchased from Medac GmbH, Hamburg, FRG.

RESULTS

Qualitative Immunofluorescence Results

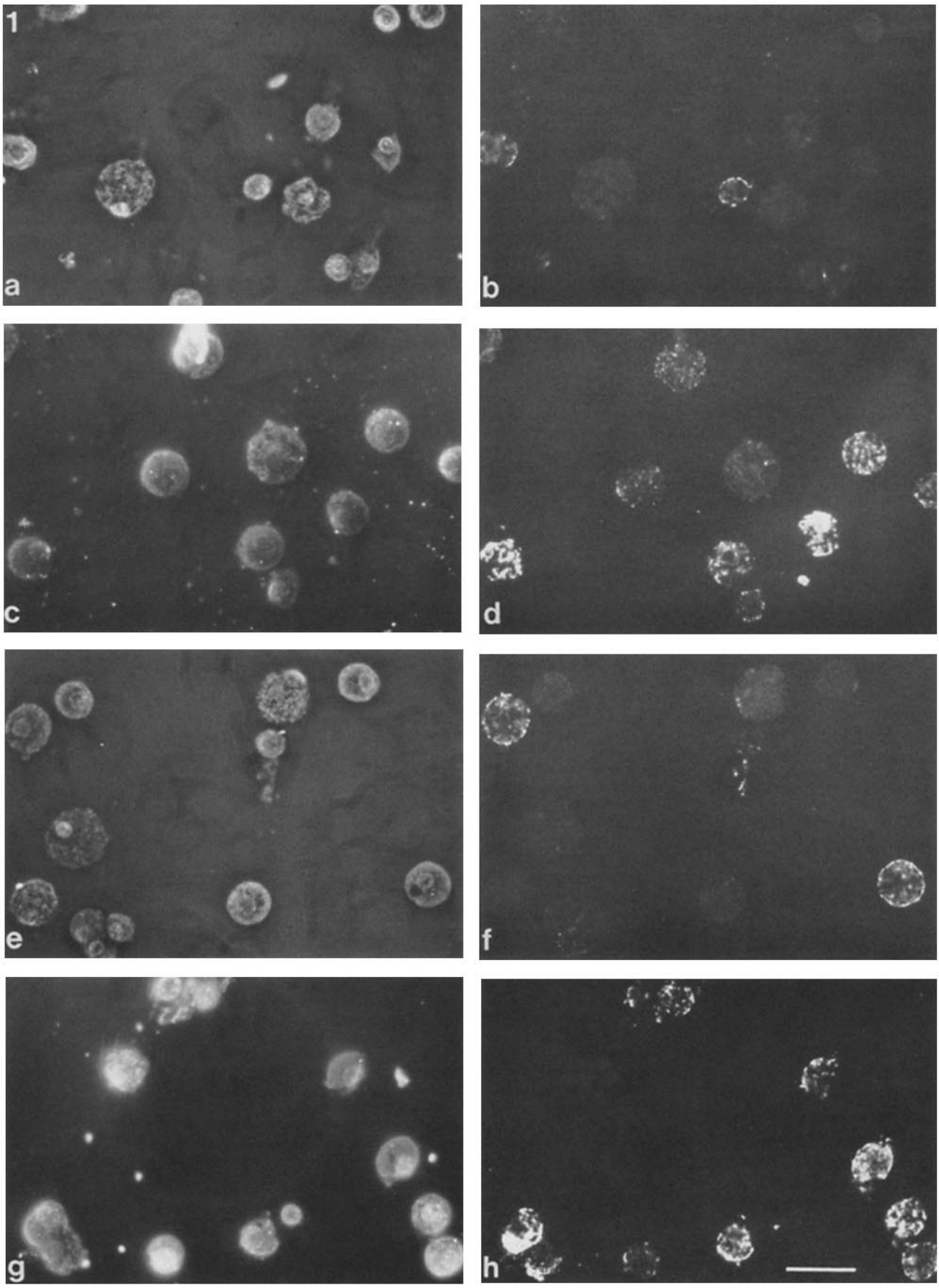
Aliquots of cells (control and stimulated) were subjected to indirect membrane immunofluorescence in the presence of 20 mM sodium azide. Results obtained with the antiserum against glycoprotein III are shown in Fig. 1. In unstimulated control preparations only a few cells exhibited a weak specific staining. After stimulation with carbachol a more intense staining was observed and this was even more pronounced for cells stimulated with Ba^{2+}. However, the degree of immunostaining varied considerably among individual cells (Fig. 1). A few cells were without staining, probably representing nonchromaffin cells. Specific immunostaining (Fig. 2) was confined to the cell surface in tiny spots or larger patches. This was true for all cell preparations irrespective of pretreatment, including experiments where the cells were fixed with formaldehyde prior to immunofluorescence. Analogous results (not shown) were obtained with an antiserum against dopamine β-hydroxylase. With preimmune serum no staining was observed.

When stimulated cells were washed and incubated again for 30 min, the degree of staining decreased. Apparently the number of fluorescent patches on the cell surface became reduced. There was no evidence that the size of the remaining patches differed from those of unincubated cells. Since the decrease of fluorescence did not occur uniformly for all cells, it was difficult to evaluate this in a reliable way.

Quantitative Evaluation of Immunofluorescence Results

Aliquots of cells stained immunohistochemically with antiserum against glycoprotein III were evaluated by a fluorescence-activated cell sorter. Control (unstimulated) cells (Fig. 3) show a fluorescence profile that is different from cells only treated with preimmune serum. This fluorescence profile was well reproducible, varying little from one cell preparation to

FIGURE 1 Membrane immunofluorescence of isolated chromaffin cells. Aliquots of cells were subjected to indirect immunofluorescence with an antiserum against glycoprotein III (diluted 1:200) and with goat anti-rabbit fluorescein isothiocyanate (diluted 1:80). Micrographs show chromaffin cells, as they appear in dark field (*left*) and under fluorescence illumination (*right*, corresponding fields). (*a* and *b*) Control cells (10 min, 37°C); (*c* and *d*) stimulated cells (carbachol for 10 min, 37°C); (*e* and *f*) stimulated cells (carbachol for 10 min, 37°C), washed and then incubated for 30 min, 37°C; (*g* and *h*) stimulated cells (Ba^{2+} for 10 min, 37°C). Photographs of fluorescent cells were taken with the same exposure time (2 min) for stimulated and control cells. Bar, 20 μm. × 600.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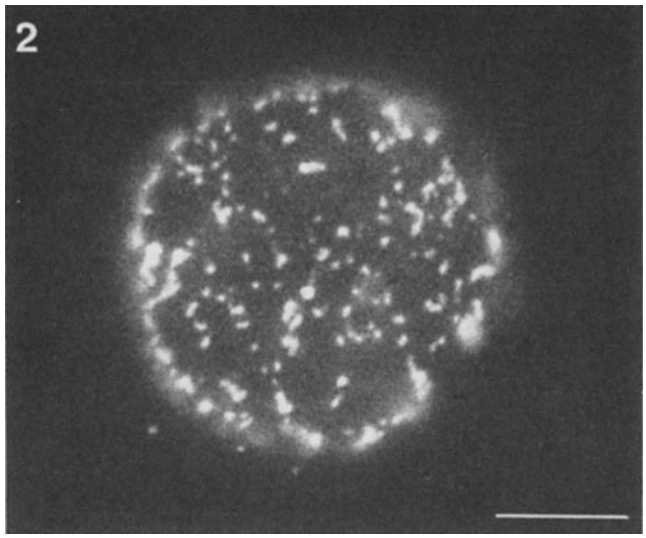

FIGURE 2 Membrane immunofluorescence of isolated chromaffin cells. Chromaffin cell, stimulated (Ba^{2+} for 10 min, 37°C) and fixed (1.3% formaldehyde for 30 min, 0°C) before the immunofluorescence procedure (for details see legend to Fig. 1). Note the patchy distribution of fluorescence. Bar, 5 μm. × 3,400.

another (see SE bars in Fig. 3*A*). Stimulation with either carbachol or Ba^{2+} resulted in a marked increase of cells exhibiting high fluorescence intensities shifting the fluorescence profile to the right. Furthermore, as evident from the width of the profiles, the individual variation in staining intensity was much more pronounced with carbachol-stimulated cells as compared with controls. This was less marked for Ba^{2+}. The antiserum against dopamine β-hydroxylase gave comparable results (Fig. 3*B*).

Catecholamine secretion during stimulation was 4.5 (± 0.8, SE; $n = 6$) times control (4.4 nmol/10 min per 10^6 control cells [± 0.5, SE; $n = 5$]) for carbachol and 5.7 (± 1.2, SE; $n = 6$) times control for Ba^{2+}. Up to 19% of the total catecholamines was released during stimulation. When cells were fixed with formaldehyde prior to immunostaining, quantitative evaluation of these cells by the cell sorter became impossible since a high degree of background fluorescence interfered. A quantitative immunofluorescence analysis of cells, which had been incubated for up to 45 min after stimulation, is shown in Fig. 4. Stimulation of the cells with carbachol shifted the fluorescence profile to the right (compare Fig. 3). When these cells were washed, incubated for further 30 min, and then assayed for specific membrane staining, a marked reduction of fluorescence intensity was observed. After 45 min the

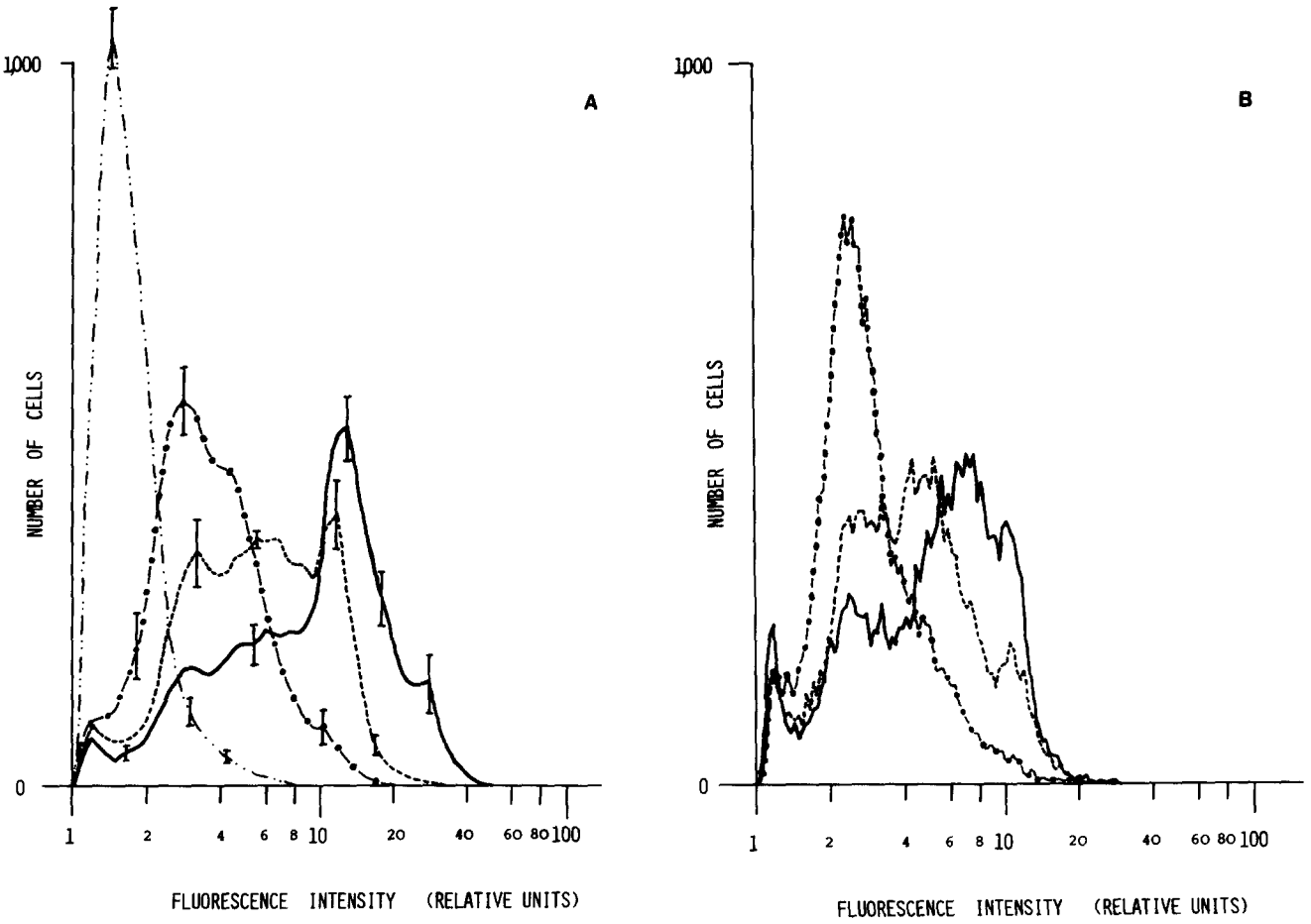

FIGURE 3 Quantitative evaluation of membrane immunofluorescence with antisera against GP III (*A*) and dopamine β-hydroxylase (*B*). Aliquots of cell preparations were analyzed with the fluorescence-activated cell sorter. The abscissa gives the intensity of the fluorescence in relative units (logarithmic scale). The ordinate indicates the number of cells. Each profile corresponds to 10^4 cells investigated. Cells treated with preimmune serum ($n = 6$) (· · · ·). The following preparations were treated with specific antiserum: control cells, 10 min, 37°C (- - -); cells stimulated with carbachol (- - -); cells stimulated with Ba^{2+} (——). The histograms in *A* represent mean value curves obtained from six different cell preparations. SE are only given for some representative points. *B* shows the results from a single experiment.

degree of fluorescence was quite similar to that of unstimulated control cells (Fig. 4*A*). In another experiment, this level was already reached within 30 min of incubation after stimulation (Fig. 4*B*). The two experiments shown in Fig. 4 are representative of six experiments.

In a further series of experiments, stimulated cells were incubated with various drugs in order to study their influence on the disappearance of antigen from the cell surface. As shown in Fig. 5, colchicine had no effect on this process. The same negative results were obtained with cytochalasin B and trifluoperazine. Since all the fluorescence profiles resulting from these drugs were practically identical, only one is presented in Fig. 5. On the other hand, in the presence of sodium azide the decrease in immunofluorescence of previously stimulated cells was completely prevented (Fig. 4*B*). Similar results for all these drugs were obtained in three experiments.

DISCUSSION

During exocytosis membrane components of chromaffin granules should become exposed on the surface of isolated chromaffin cells. We have studied this process in a quantitative manner using specific antisera against two membrane components. Both are glycoproteins having at least some of their antigenic groups on the inner surface of the chromaffin granule membranes. Dopamine β-hydroxylase is the major glycoprotein of the membrane of chromaffin granules. A significant concentration of this enzyme (5% of total protein) is, however, also found in the soluble lysate (31). The other antigen, glycoprotein III, is the second major glycoprotein of the granule membrane (8, 9). This glycoprotein is firmly bound to the membrane since it is not removed by washes with hypertonic solutions or with low concentrations of detergents (9). In the soluble granule content only a very small concentration of glycoprotein III (0.25% of total protein) is found (9). Therefore, this protein is a more useful membrane marker than dopamine β-hydroxylase. We thus focused our attention on glycoprotein III, although the experiments with dopamine β-hydroxylase gave essentially identical results.

Qualitative evaluation of immunofluorescence revealed both antigens to be present on the plasma membrane in tiny spots and/or patches irrespective of the pretreatment of the cells. It seems unlikely that this patchy pattern was induced by the reaction with antibodies causing aggregation of antigens originally randomly distributed in the plasma membrane. Antibody treatment was performed at 0°C in the presence of sodium azide. This is known to inhibit active processes depending on intact cell metabolism, i.e., capping and subsequent stripping of membrane antigens (2, 20), but does not prevent passive antigen redistribution in the membrane. However, the same patchy immunofluorescence appeared when cells were fixed with 1.3% formaldehyde before immunostaining, which is known to preserve the original distribution of antigens in the membrane (30).

After stimulation the intensity of specific membrane fluorescence was observed to increase, whereas further incubation at 37°C after stimulation reduced it again. However, it was difficult to ascertain these changes by conventional fluorescence microscopic observations, since apparently not all cells were affected by the stimulation to the same degree. Therefore, quantitative cytofluorometry was introduced to determine changes in mean fluorescence intensity as well as in proportions of cells exhibiting different fluorescence signals. Unstimulated cells showed some specific fluorescence when compared with cells treated with preimmune serum. It seems likely that even in the absence of stimulants the basal secretion of catecholamines leads to some exposure of granule membranes. During stimulation a significant increase of specific staining occurred. There was a good correlation between the increase in catecholamine secretion (four to five times) and the increase in relative fluorescence (up to five times, see Fig. 3). Thus this method appears suitable to monitor the antigen exposure.

Additional information was obtained by evaluating the degree of fluorescence of single cells. It became obvious that stimulation does not affect all cells to the same degree, since the fluorescence profile of the stimulated cells was much broader than that obtained by unstimulated ones. With Ba^{2+}, as a secretagogue, most cells were apparently stimulated strongly since a large part of the fluorescence profile was shifted to higher intensity. With carbachol the fluorescence profile was rather broad, indicating that a smaller portion of the cells exhibited fluorescence intensities significantly different from control cells. Apparently carbachol, depending on the presence of receptors, cannot stimulate all cells to the same degree. Treatment of the cells during isolation with collagenase (containing traces of proteases) might degrade some receptors making the cell less sensitive to carbachol. Ba^{2+}, on the other hand, is thought to stimulate cells by displacing calcium from the plasma membrane (4), a process that may be less sensitive to the preparation procedures.

What is the further fate of the antigen exposed on the cell surface during exocytosis? This question was studied with cells stimulated with carbachol, since this compound is the more physiological secretagogue of the two used. When stimulated cells were washed and incubated again for up to 45 min at 37°C, glycoprotein III exposed on the cell surface disappeared gradually. These originally stimulated cells exhibited fluorescence profiles practically identical with that of unstimulated control cells. It should be emphasized that throughout this incubation the antigen on the cell surface was present in spots or patches, even on those cells that had been prefixed with formaldehyde. The most likely underlying mechanism for the disappearance of the antigen from the cell surface is a specific membrane retrieval. Morphological evidence for this process has been obtained by the detection of coated vesicles that appear along exocytotic profiles of adrenal medullary cells (3, 16). It has been proposed for adrenal medulla (26, 32, 34) and other secretory tissues (6, 15) that the retrieved membranes return to the Golgi region for reuse in another secretory cycle.

According to our results the specific membrane retrieval seems relatively fast and quite efficient. Within 30 min membrane antigens corresponding to up to 19% of the granule population have been removed. Such a specific retrieval is consistent with the fact that the plasma membrane of chromaffin cells has a composition different from that of the membranes of chromaffin granules (32). In rat parotid gland, poststimulation retrieval of luminal surface membranes occurs at a rate similar to that shown here (12).

Several compounds were tested for their effect on the removal of the granule antigen from the cell surface. Three of the tested drugs (colchicine, cytochalasin B, trifluoperazine) had no influence on this process. It is difficult to compare these results with data from the literature, since only limited and conflicting data on the influence of these compounds on

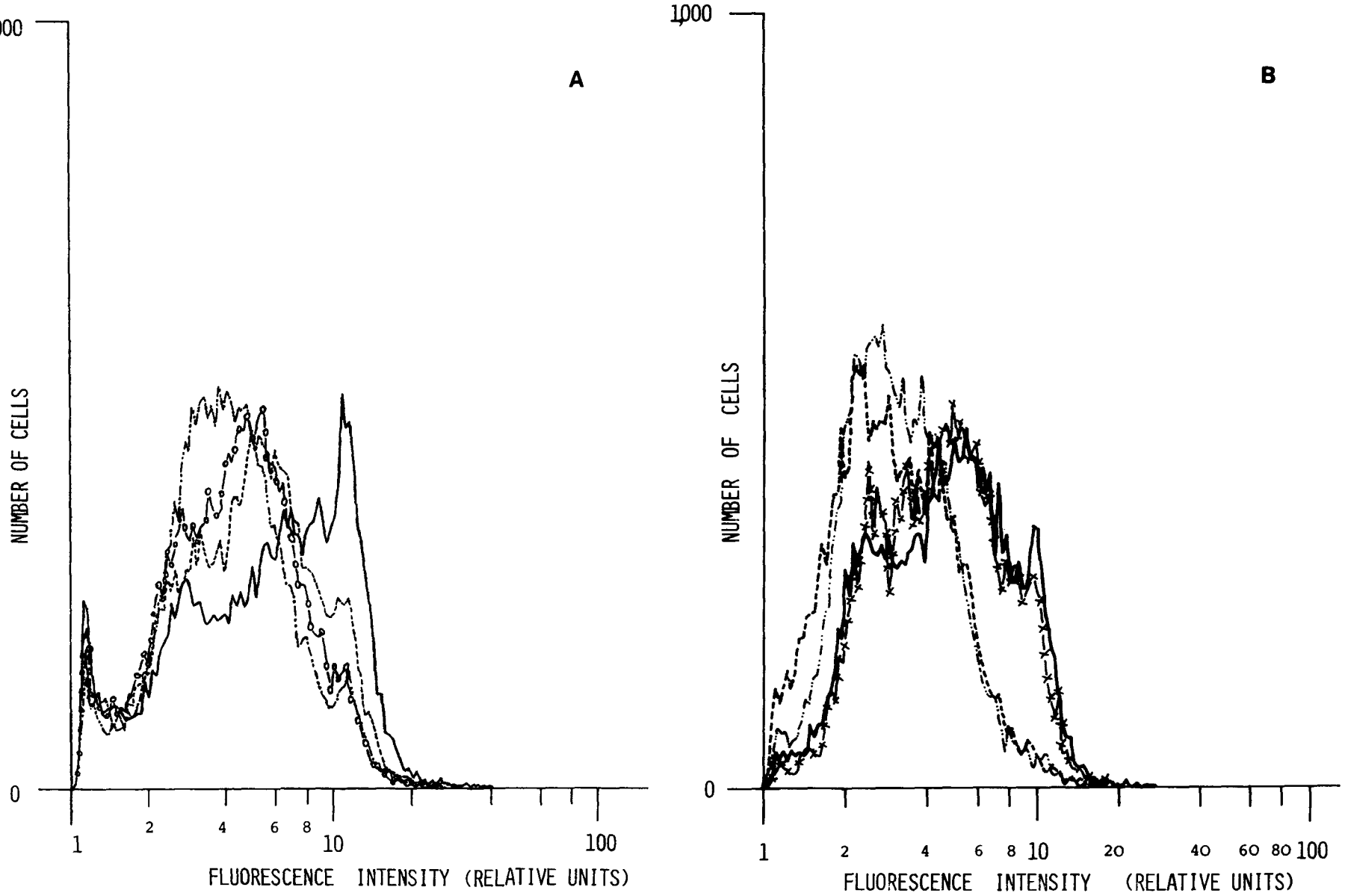

FIGURE 4 Quantitative immunofluorescence results with cells incubated after stimulation. Aliquots of cell preparations were treated for 10 min at 37°C without or with carbachol, washed, and then further incubated for 30 or 45 min at 37°C. These cells were then subjected to immunofluorescence with the antiserum against glycoprotein III and analyzed by the fluorescence-activated cell sorter. Two experiments (*A* and *B*) are shown. Unstimulated control cells (- · · -); stimulated cells (——); cells incubated (30 min) after stimulation (---); cells incubated (45 min) after stimulation (-o-); cells incubated (30 min) after stimulation in the presence of 20 mM sodium azide (glucose omitted from the Krebs Henseleit buffer) (-x-).

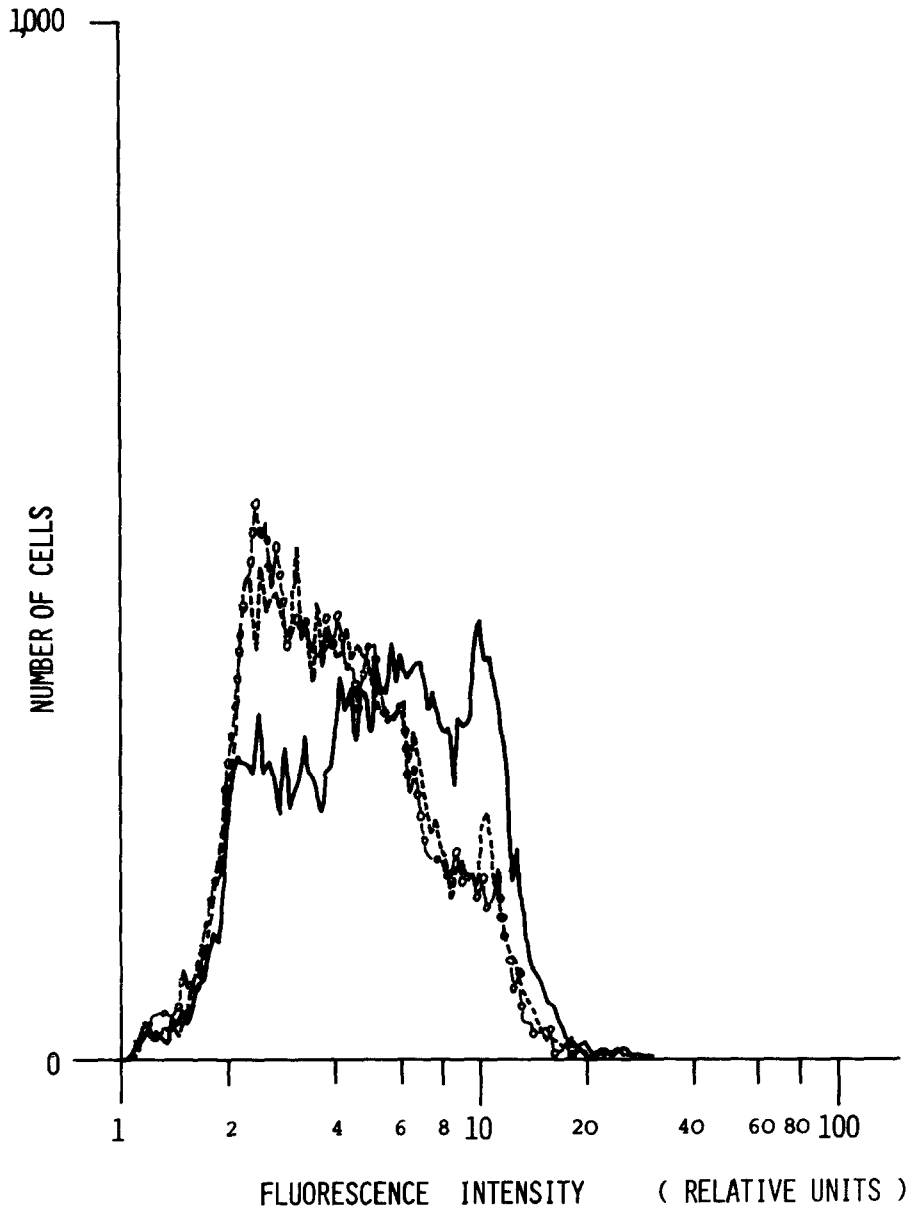

FIGURE 5 The influence of drugs on the decrease of membrane immunofluorescence of cells incubated after stimulation. Immunofluorescence staining was performed with the antiserum against glycoprotein III (compare legend to Fig. 4). Cells stimulated with carbachol (——); cells incubated (30 min) after stimulation (---); cells incubated (30 min in the presence of colchicine 200 μM) after stimulation (-o-). Fluorescence profiles of cells in the presence of cytochalasin B (200 μM) and trifluoperazine (15 μM) were identical to that of cells incubated for 30 min without drugs. The profiles obtained with these drugs are therefore not shown in the figure.

endocytotic uptake have been published. Colchicine, interfering with microtubules, has been shown to reduce endocytotic uptake of lysosomal enzymes (28) and to inhibit receptor-mediated uptake of prolactin and transfer into the Golgi region (19). In the latter case, colchicine may have interfered less with uptake than with the subsequent transfer step. Cytochalasin B, inactivating microfilaments, was shown to inhibit formation of coated vesicles in lymphoblastoid cells (reference 22, including earlier references reporting negative results). Trifluoperazine, an inhibitor of calmodulin, was shown to interfere with recruitment of clathrin to the cell surface (21). In our experiments only sodium azide prevented membrane retrieval. This clearly indicates that this process is energy dependent.

After the submission of this manuscript, two papers on this topic were published (5, 18). With qualitative immunofluorescence the exposure of dopamine β-hydroxylase on the surface of chromaffin cells during stimulation was demonstrated. During incubation after stimulation the exposed antigen gradually disappeared.

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