Exocytotic Exposure and Recycling of Membrane Antigens of Chromaffin Granules: Ultrastructural Evaluation After Immunolabeling

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Abstract. The exocytotic exposure and retrieval of an antigen of chromaffin granule membranes were studied with chromaffin cells isolated from bovine adrenal medulla. Cells were incubated with an antiserum against glycoprotein III followed by fluorescein- or gold-labeled anti-IgG. Immunofluorescence on the cell surface was present in a patchy distribution irrespective of whether bivalent antibodies or Fab fragments were used. During subsequent incubation these fluorescent membrane patches were internalized within 45 min. At the ultrastructural level immunogold-labeled patches were present on the surface of stimulated cells. During incubation (5 min to 6 h) these immunolabeled membrane patches became coated, giving rise to coated vesicles and finally to smooth vesicles. These latter vesicles were found spread throughout the cytoplasm including the Golgi region, but Golgi stacks did not become labeled. Part of the immunolabel was transferred to multivesicular bodies, which probably represent a lysosomal pathway. 30 min after incubation immunolabel was also found in electron-dense vesicles apparently representing newly formed chromaffin granules. After 6 h of incubation immunolabel was found in vesicles indistinguishable from mature chromaffin granules. These results provide direct evidence that after exocytosis membranes of chromaffin granules are selectively retrieved from the plasma membrane and are partly recycled to newly formed chromaffin granules, providing a shuttle service from the Golgi region to the plasma membrane.

SECRETION from adrenal medulla occurs by exocytosis (2, 21, 26). This process, which adds membranes of chromaffin granules to the plasma membrane, is followed by endocytosis, as shown in early studies (11, 15). In 1972 we demonstrated (34) that the synthesis rate of the membrane proteins of chromaffin granules was significantly lower than that of the secretory proteins (chromogranins) of the contents (for similar results in exocrine pancreas see reference 4). Based on this study we proposed that the membranes of chromaffin granules are reused for several secretory cycles (see also the discussion in reference 31). This concept gained considerable support when it was shown that in stimulated endocrine cells exogenous markers were taken up by endocytosis and were subsequently found in newly formed secretory vesicles (see references 5, 8, 9, and 24). These experiments demonstrated recycling of membranes but did not yet establish the specific nature of the recycled membranes. To achieve this, specific antigens of the secretory vesicle membrane have to be followed throughout the exo/endocytosis cycle. Two antigens of the chromaffin granules, i.e., dopamine, β-hydroxylase and glycoprotein III, proved suitable for such an approach. In a first series of immunofluorescent studies it was established that these granule antigens become exposed on the cell surface of isolated chromaffin cells during exocytotic activity (3, 13, 18, 19, 27). By quantitative evaluation we were able to show that after stimulation antigens were again removed from the cell surface within 45 min (13, 18). Apparently after exocytosis an efficient and specific process of membrane retrieval operates that is a prerequisite for a specific recycling of secretory vesicle membranes.

In the present study we have analyzed this exo/endocytosis cycle at the ultrastructural level, employing the immunogold technique. On the surface of stimulated cells small patches can be labeled by this method. During subsequent incubation these specifically marked membranes are retrieved via coated vesicles. Finally, immunogold label turns up in lysosomal structures, and also in newly formed chromaffin granules, providing direct evidence for a specific recycling process.

Materials and Methods

Isolation of Chromaffin Cells

Chromaffin cells were isolated from bovine adrenal medulla as described previously (18). After isolation they were resuspended in medium RPMI 1640 (with 0.5% bovine serum albumin [BSA]) gassed with 95% O_2/5% CO_2. After 1–2 h at room temperature this suspension was centrifuged at room temperature at 50 *g* for 10 min. The sedimented cells were resuspended in BSA/Krebs Henseleit buffer, and aliquots of this suspension were used for further experiments.

Preparation of Antisera

Glycoprotein III was isolated from the soluble lysate of chromaffin granules with sequential immuno- and lectin-affinity chromatography (6). The antiserum against glycoprotein III 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 (6). For preparation of the Fab-fragments purification of the rabbit immunoglobulins on a protein A-Sepharose column (7) was followed by papain digestion and purification over a carboxymethyl cellulose column (20). The purity of the Fab-fragments was analyzed by SDS slab gel electrophoresis (12).

Immunostaining

Cells (2×10^6 cells/ml) were stimulated in 2.4 ml BSA/buffer for 1 or 10 min at 37°C with carbachol (2 mM). After incubation the cell suspensions were chilled and centrifuged at 50 *g* for 10 min. The following immunocytochemical procedures were done at 0°C with Krebs Henseleit as the buffer. Incubation (30 min) with the first antiserum (anti-glycoprotein III, 1:100 dilution) was followed by three washes and incubation with the second antibody (fluorescein isothiocyanate-conjugated antibodies, 1:80 dilution; colloidal gold-conjugated antibodies, 1:12 dilution) for 30 min at 0°C. After three washes part of the cells were fixed immediately with 1.25% glutaraldehyde in 0.1 M cacodylate (pH 7.0) for 1 h (10 min at 0°C and 50 min at room temperature). Other aliquots of immunostained cells were resuspended in medium RPMI 1640, reincubated at 37°C for from 5 min to 6 h, and then fixed. After fixation cells were washed three times in 0.1 M cacodylate for 1 h at room temperature. After two subsequent washes in cacodylate buffer they were dehydrated through graded series of acetone and embedded in Spurr's resin (23), then the resin was polymerized for 55 h at 72°C. Ultrathin sections were cut on an OM U3 ultramicrotome (Reichert, American Optical Scientific Instruments, Buffalo, NY), poststained with 2% uranylacetate and lead citrate (25), and examined on an EM 10 electron microscope (Carl Zeiss, Oberkochen, FRG) at 80 kV.

Chemical Assays

Catecholamines were determined by high-performance liquid chromatography with electrochemical detection (22).

Materials

Collagenase type I, BSA fraction V, carbamylcholine chloride (carbachol), papain type IV, glutaraldehyde, and sodium cacodylate were purchased from Sigma Chemical GmbH, Munich; protein A-Sepharose from Pharmacia Fine Chemicals, Uppsala, Sweden; carboxymethyl cellulose Cellex-CM from Bio-Rad Laboratories, Richmond, CA; medium RPMI 1640 from Gibco Laboratories, Grand Island, NY; and the epoxy resin components from Serva Feinbiochemica, Heidelberg. Goat anti-rabbit IgG-fluorescein isothiocyanate was purchased from Medac GmbH, Hamburg, FRG; goat anti-rabbit Fab-fluorescein isothiocyanate was obtained from Cappel Laboratories, Cochranville, PA; and goat anti-rabbit IgG-5-nm colloidal gold was purchased from Janssen Life Sciences Products, Beerse, Belgium.

Results

Immunofluorescence Studies

Isolated bovine chromaffin cells were stimulated for 10 min at 37°C and then subjected to indirect immunofluorescence with antibodies against an antigen of chromaffin granules i.e., glycoprotein III. As shown in Fig. 1 this granule antigen was present on the cell surface in discrete patches irrespective of whether IgG antibodies or Fab fragments were used.

When cells that had been immunolabeled were incubated again at 37°C the immunofluorescent patches were apparently retrieved since the fluorescence was now found in the cell interior (see Fig. 1; compare reference 19).

Immunogold Labeling Studies

Stimulated cells were treated with either control sera or an antiserum against glycoprotein III followed by anti-IgG gold-labeled immunoglobulins. On the surface of cells treated with control serum gold label was only infrequently observed and, if present, consisted only of a few gold particles (see Fig. 2). On stimulated cells gold-labeled patches were consistently present (see Fig. 2). Their diameters ranged from 150 to 790 nm (347 ± 33.6 nm, mean ± SE; $n = 33$). An average cell section exhibited 10 such patches (± 1.36 SE; $n = 14$).

When cells already treated with the antisera were incubated again at 37°C for up to 6 h these labeled membrane patches became internalized. In Fig. 2 a representative example of such a cell is shown which has eight gold-labeled vesicles in its interior. The various stages of this membrane retrieval during reincubation are demonstrated by representative examples in Fig. 3. After the shortest interval investigated (5 min) we observed gold-labeled membrane patches, which were coated; various stages of endocytosis of labeled patches, and, finally, labeled coated vesicles within the cytoplasm. In an apparent further step smooth vesicles of varying sizes (65–340 nm) were found deeper in the cytoplasm. There was no evidence that these smooth vesicles were formed by pinching off from coated membranes still in connection with the plasma membrane, as suggested by Willingham and Pastan (28). After 30 min of reincubation smooth vesicles of various sizes and forms were seen close to the Golgi region. However, labeled membrane pieces were never seen in the stacks of the Golgi cisternae. Already after 30 min labeled vesicles with electron-dense contents were observed. At shorter intervals the size of these vesicles and the structure of their contents resembled those of chromaffin granules, but their membranes appeared smoother than those of typical chromaffin granules (Fig. 3, *k* and *l*). After 6 h of incubation immunolabel was found in vesicles (see Fig. 3, *m* and *n*) that appeared identical to chromaffin granules.

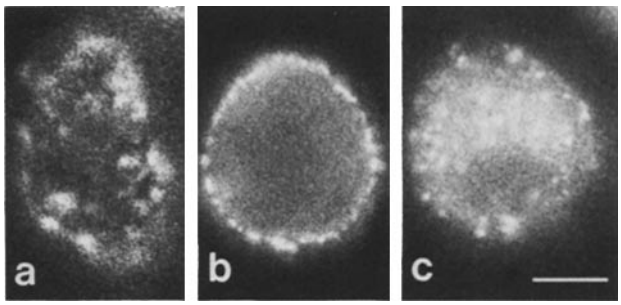

Figure 1. Immunofluorescence of isolated chromaffin cells. Cells were stimulated (2 mM carbachol, 10 min, 37°C) and subjected to indirect immunofluorescence. (*a*) Stimulated cell, stained with anti-glycoprotein III-Fab and goat-anti rabbit-Fab-fluorescein isothiocyanate, focused on the surface. Granule antigens are present as patches on the surface of this cell. (*b*) Stimulated cell, stained with anti-glycoprotein III and goat anti-rabbit IgG-FITC, equatorial plane in focus. In this case the patchy distribution of the antigen can only be seen along the equator. The antigens on the surface are out of focus and therefore give a diffuse staining. (*c*) Cell treated as in *b* and reincubated for 45 min at 37°C after immunostaining, equatorial plane in focus. The immunofluorescent patches are now in focus and therefore seen in the cell interior. The area of the nucleus is free of immunofluorescence. Bar, 6.2 μm.

Throughout this process of membrane retrieval the gold label was present in close contact with the membrane. However, in some electron-dense vesicles gold label was apparently also present in the granule content.

Gold label was also found (Fig. 3, *o* and *p*) in heterogenous-looking vesicles (e.g., multivesicular bodies), some of which probably represent lysosomal structures.

Discussion

In a previous study (18) we already demonstrated by immunofluorescence that antigens of chromaffin granules that become exposed on the cell surface during exocytosis are present as discrete patches. Since this patchy distribution was also

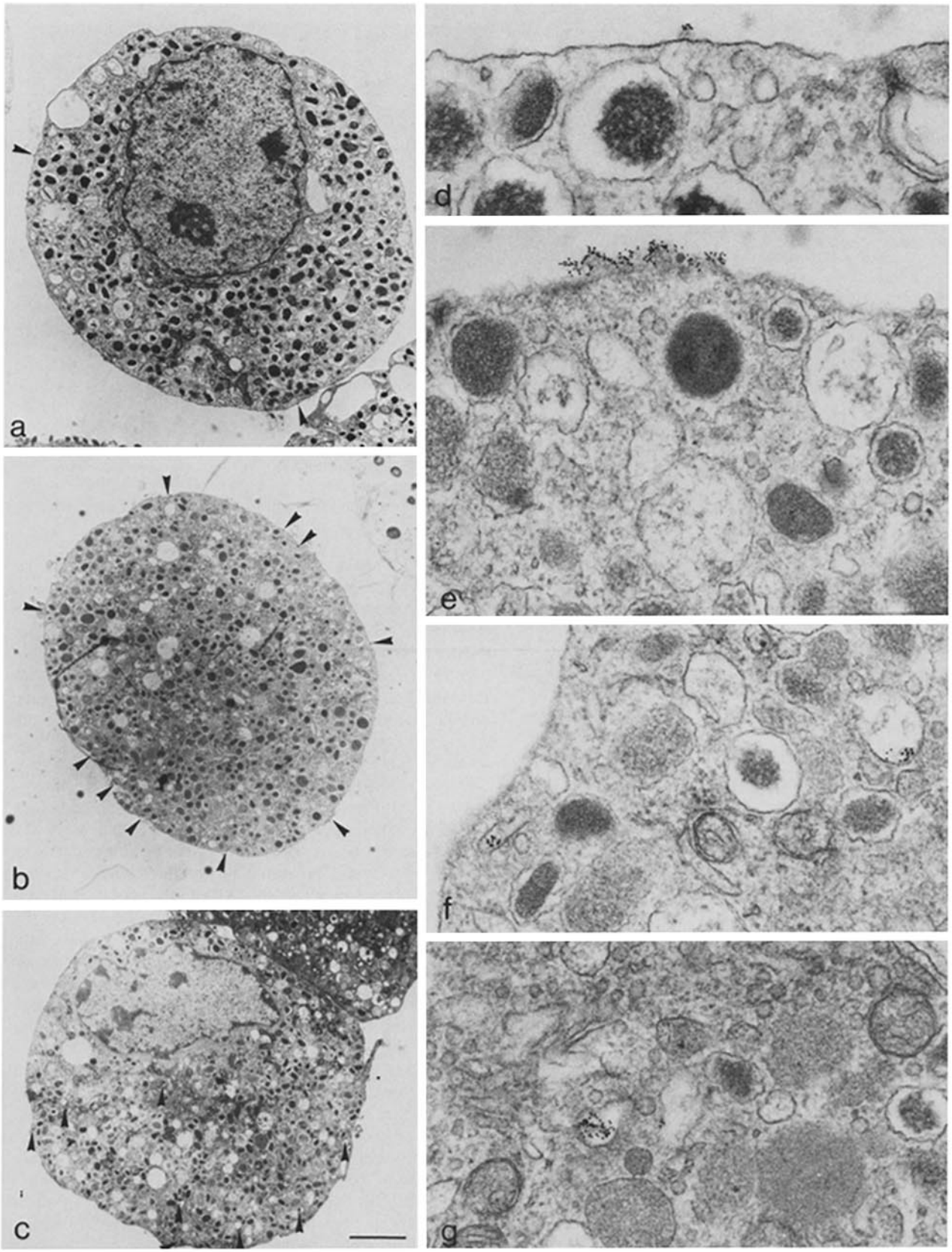

Figure 2. Isolated chromaffin cells, stimulated (2 mM carbachol, 10 min, 37°C) and immunogold labeled. (*a*) Adrenaline cell, treated with preimmune serum and 5-nm gold-conjugated goat anti-rabbit IgG. There are two spots (see arrowheads) of unspecific staining with a few gold particles (see *d*). (*b*) Adrenaline cell treated with antiglycoprotein III (diluted 1:100) and 5-nm gold-conjugated goat anti-rabbit IgG. There are 10 patches (marked with arrowheads) of gold staining on this cell (compare *e*). (*c*) Adrenaline cell treated with anti-glycoprotein III (diluted 1:100) and 5-nm gold-conjugated goat anti-rabbit IgG and after immunostaining reincubated for 15 min at 37°C. Arrowheads reveal membranes with immunogold label within the cell (compare *f* and *g*). The gold-labeled patches in *a–c*, identified at a magnification of 55,000, are marked by arrowheads. For representative details of these cells see *d–g*. (*d*) Detail of the cell shown in *a* demonstrating an immunogold spot seen on the upper left of this cell. (*e*) Detail of the cell shown in *b*, with immunolabel in bottom right corner. (*f* and *g*) Details of the cell shown in *c* demonstrating immunogold labeling in the left (*f*) and middle (*g*) of the cell within the Golgi region. Bars: *a–c*, 1.7 μm; *d–g*, 180 nm.

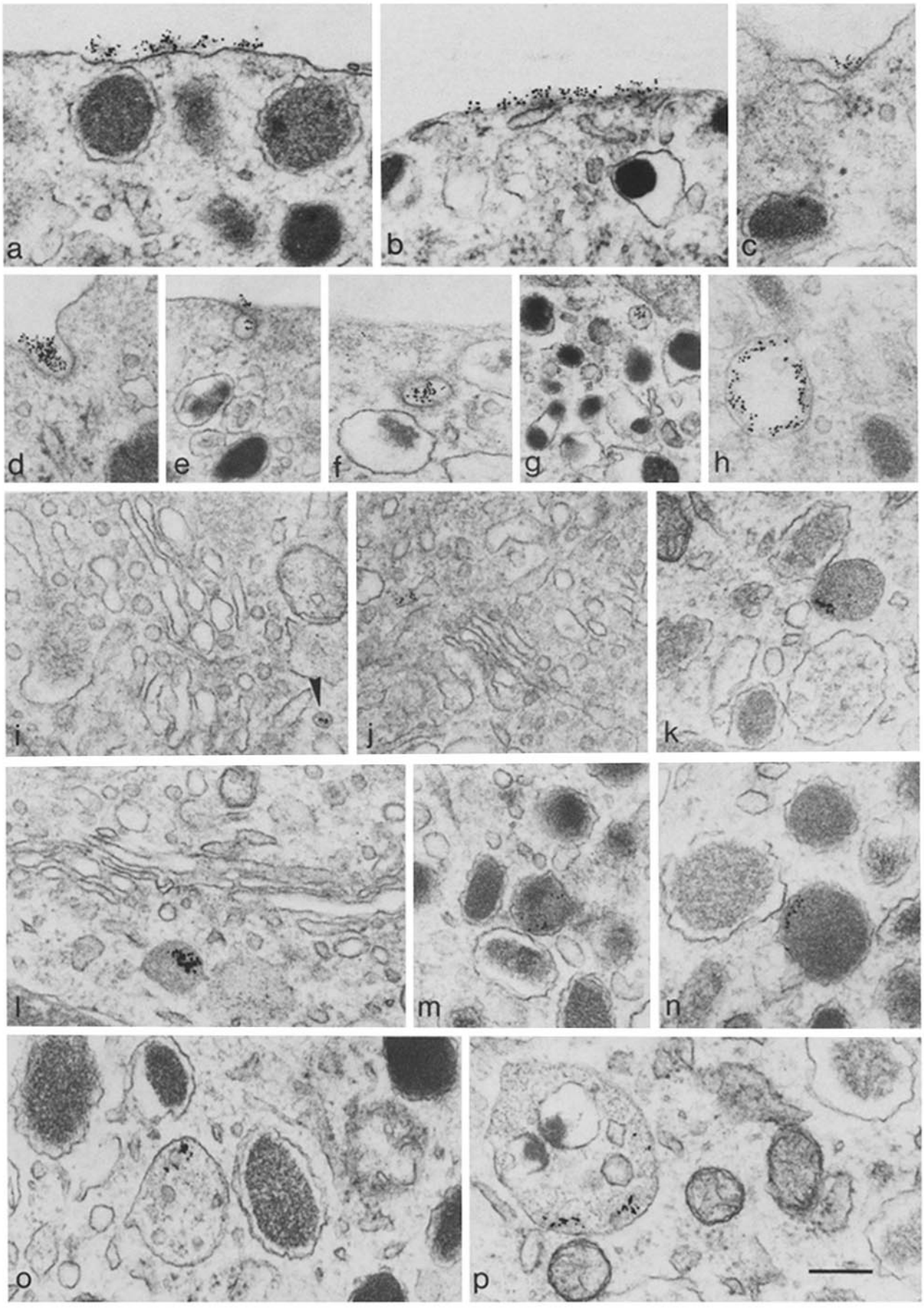

Figure 3. Isolated chromaffin cells, stimulated (2 mM carbachol, 1 min, 37°C) and immunostained with anti-glycoprotein III (diluted 1:100) and 5-nm gold-conjugated goat anti-rabbit IgG. (*a* and *b*) Typical patches of immunogold labeling on the surface of stimulated cells. The following pictures were obtained from cells that were reincubated after immunostaining: (*c–e*) labeled membrane patches with coating at various stages of membrane retrieval (5-min incubation); (*f*) coated vesicle in the cytoplasm (5- to 15-min incubation); (*g* and *h*) smooth vesicles in the cytoplasm (5 to 15 min); (*i* and *j*) immunogold stain appearing in a vesicle (see arrowhead) and a tubular structure close to the Golgi region (30- to 45-min incubation); (*k* and *l*) labeled vesicles with an electron-dense content (30 to 45 min incubation); (*m* and *n*) immunogold stain within chromaffin granules (6-h incubation); (*o* and *p*) immunogold stain in multivesicular bodies. Bar, 0.2 μm.

found in prefixed cells it seemed unlikely that it was due to antigen aggregation induced by the treatment with bivalent antibodies. On the other hand, Dowd et al. (3) reported that granule antigens are spread diffusily over the cell surface when instead of bivalent antibodies Fab fragments are used for immunofluorescence. In the present study we could not confirm this observation and we therefore conclude that antigens of secretory vesicles exposed during exocytosis are present on the cell surface as discrete patches. Such a distribution seems a prerequisite for a specific and fast membrane retrieval by endocytosis, and in fact we have previously established that after a strong secretion stimulus the granule antigens disappear from the cell surface within 45 min (13, 18).

To study this process further and to establish the final fate of the retrieved membranes we have immunolabeled stimulated chromaffin cells with antibodies against a granule antigen (glycoprotein III) and then incubated them at 37°C to allow membrane retrieval. At the light microscopic level cells that had been immunolabeled but not incubated further showed the already established (18, 19) patchy immunofluorescence on the cell surface. After renewed incubation (45 min) the immunolabeled membranes were found within the cell. Apparently the granule membrane patches are removed from the cell surface (see also reference 19) within 45 min, the same time course as found for the retrieval of unlabeled granule antigens (13, 18). This model seemed therefore suitable to use to study this process at the ultrastructural level. On the surface of stimulated cells the specific immunogold label was found in discrete patches. Their size ranged from 150 to 790 nm. The circumference of a chromaffin granule is 870 nm (diameter, 280 nm; see reference 33), which probably represents the maximal diameter of an exocytotic site. Taking section thickness into account it is reasonable that we found only patches below this size.

During subsequent incubation of immunolabeled cells the patches of the granule membranes within the plasma membrane became coated and were finally retrieved in coated vesicles, which apparently shed their coats and became smooth vesicles of varying sizes. On the other hand it was suggested (28) that coated vesicles are stable structures always associated with the cell surface and that smooth vesicles pinch off after the ligand has been transferred into them by lateral diffusion. Thus this mechanism removes membrane proteins that have been transferred from one patch of the lipid bilayer into an adjacent one. We did not observe any images supporting this concept. We should also emphasize that in chromaffin cells retrieval should remove both the membrane proteins and the specific lipid patch of the secretory vesicle. Chromaffin granules are rich in lysolecithin (1, 32), whereas the plasmamembrane is not (16). If only the membrane proteins are removed and not the lipids, this difference in the lipid composition is difficult to explain. Thus, our present results, suggesting specific removal of total membrane patches of chromaffin granules, are consistent with the different lipid composition of these two membrane compartments.

Already after 30 min of incubation immunolabel was found in multivesicular bodies, probably indicating lysosomal structures. Apparently some retrieved membranes of chromaffin granules take this pathway, which allows the cell to renew its compartment of secretory vesicle membranes. This is consistent with the previous observation that an exogenous marker like horseradish peroxidase is endocytosed by stimulated chromaffin cells and is finally found in lysosomes (11; for a discussion of other endocrine and exocrine tissues see reference 5). However, our specific immunolabel, which is firmly bound to the membrane, allowed us to establish a further pathway for these granule membranes. A significant part of the labeled membranes was found in electron-dense vesicles that resembled chromaffin granules in size and in the appearance of their contents, but in early stages their membranes appeared smoother than those of the total population of granules. However, after 6 h labeled vesicles were observed that also had the typical membrane appearance of chromaffin granules and were indistinguishable from them. Thus our results provide direct evidence that granule membranes that had been retrieved from the plasma membrane after exocytosis were recycled to newly formed chromaffin granules. Two observations require further comment:

(*a*) The gold label was usually found in close apposition to the membranes; however, in some of the newly formed granules it sometimes appeared to be dissociated from them. Two explanations can be offered: The interior of chromaffin granules is known to be acidic (pH 5.5; see reference 17), which might help to dissociate the antibody/antigen or the gold/antibody complex. Furthermore, a small amount of glycoprotein III is also found among the soluble proteins of chromaffin granules (6). The presence of this soluble antigen may have led to dissociation of the antibody from the membrane. In this context one has to consider the possibility that the immunolabel reached newly formed granules already dissociated from the antigen, thus giving the wrong impression of membrane recycling. This seems unlikely, since the immunolabel was usually found to be bound to the membranes of newly formed granules (see Fig. 3, *k* and *n*). Furthermore, previous studies on several endocrine tissues (see reference 5) have shown that markers such as cationized ferritin are recycled into newly formed vesicles. If such markers, which are only bound to the membrane by charge effects, are not dissociated from the membranes during their passage through the cell, it is even more unlikely that a specifically bound antibody should be removed from its binding site on the antigen.

(*b*) Immunolabel was consistently observed in vesicles and tubular structures close to the Golgi region, but it was never found within the Golgi cisternae. On the other hand Farquhar (4) and Herzog (10) demonstrated that in endocrine and exocrine cells at least part of exogenously added cationized ferritin or dextran is recycled via the Golgi stacks before it enters new vesicles. Apparently two pathways for a recycling of membranes are possible, one via the Golgi stacks and another bypassing the Golgi cisternae and probably involving the so-called transreticular portion of Golgi region (29, 30). It has been shown that recycling of transferrin receptor to the plasma membrane can occur through this part of the Golgi stacks (30, 35). In our experiments the chromaffin cells were freshly isolated. It seems likely that during this procedure significant catecholamine release occurs, providing the Golgi region with a considerable amount of retrieved membranes. One might therefore speculate that under such conditions recycled membranes can bypass the Golgi cisternae and become directly incorporated into newly formed vesicles. Further studies employing immunological markers also for other secretory cells are necessary to establish the exact role of the Golgi region for recycling under different conditions of secretory activity.

In any case, employing specific immunological markers we have presented direct evidence that membranes of secretory vesicles are specifically retrieved after exocytosis and partly recycled to new chromaffin granules. Thus these membranes represent a recyclable container that ensures an economical use of a membrane that contains such specific proteins as carriers for nucleotides and amines, a proton-pumping ATPase, cytochrome *b-561*, and dopamine β-hydroxylase, and a high concentration of a particular phospholipid, lysolecithin (see reference 32).

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