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The GTPase Rab3a is associated with large dense core vesicles in bovine chromaffin cells and rat PC12 cells

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SUMMARY

Small GTPases of the rab family control intracellular vesicle traffic in eukaryotic cells. Although the molecular mechanisms underlying the activity of the Rab proteins have not been elucidated yet, it is known that the function of these proteins is dependent on their precise subcellular localization. It has been suggested that Rab3a, which is mainly expressed in neural and endocrine cells, might regulate exocytosis. Recently, direct experimental evidence supporting this hypothesis has been obtained. Consistent with such a role for Rab3a in regulated exocytosis was the previously reported specific association of Rab3a with synaptic vesicles and with secretory granules in adrenal chromaffin cells. Since the latter result, based on subcellular fractionation, has been controversial, we have re-investigated the subcellular localization of this GTP-binding protein by using a combination of morphological techniques. Bovine chromaffin cells were labelled with an affinity-purified polyclonal anti-Rab3a antibody and analyzed by confocal microcopy. Rab3a was found to colocalize partially with dopamine β-hydroxylase, a chromaffin granule marker. In agreement with this observation, immunoelectron microscopy revealed a specific staining of chromaffin granules. In addition to large dense core vesicles, some small vesicles were labelled. To eliminate the possibility that the staining was due to a Rab3a-related protein, we investigated by immunoelectron microscopy the localization of an epitope-tagged Rab3a expressed in rat PC12 cells. Secretory granules were specifically labelled, whereas clear microvesicles were not. These results provide further evidence supporting a specific association of the GTPase Rab3a with large dense core secretory vesicles.

Key words: chromaffin cell, exocytosis, GTP-binding protein, Rab, secretory vesicle

INTRODUCTION

Eukaryotic cells are compartmentalized in a number of specialized organelles. Along the secretory and the endocytic pathways communication between different compartments is achieved by vesicles which bud from a donor membrane and fuse with an acceptor membrane. Recently, it has been proposed that interaction between proteins of the synaptobrevin family (the v-SNAREs) and members of the syntaxin family and SNAP25 (the t-SNAREs) would provide a molecular basis for the specific docking of a vesicle with its target membrane (Söllner et al., 1993; Rothman and Warren, 1994). In addition to the SNAREs, low molecular mass GTP-binding proteins of the rab family have also been shown to play a key role in regulating intracellular membrane traffic (for review, see Bourne, 1988; Pfeffer, 1992; Simons and Zerial, 1993; Lledo et al., 1994). Evidence supporting this hypothesis came first from the yeast secretory mutants sec4 and ypt1 (Salminen and Novick, 1987; Goud et al., 1988; Segev et al., 1988). Both *SEC4* and *YPT1* encode small GTPases closely related to the rab proteins of higher eukaryotes. In mammalian cells, members of the rab family are associated with specific subcellular compartments (Pfeffer, 1992; Simons and Zerial, 1993). This specific localization seems to be conferred on the Rab proteins by their C-terminal hypervariable domain (Chavrier et al., 1991), suggesting that they interact with proteins located on specialized organelles. Post-translational addition of geranylgeranyl residues on the cysteines located at the C terminus is thought to stabilize the membrane association of the Rab proteins (Musha et al., 1992).

Rab3a was originally cloned from rat brain (Touchot et al., 1987), bovine brain (Matsui et al., 1988), and human pheochromocytoma cells (Zahraoui et al., 1989). It is mainly expressed in neurons, but also in endocrine tissues such as pituitary (Stettler et al., 1995) or adrenal medulla (Olofsson et al., 1988; Mizoguchi et al., 1989; Burstein and Macara, 1989; Darchen et al., 1990, Fischer von Mollard et al., 1990; Regazzi et al., 1992), suggesting a role in regulated secretion. Recently, direct

evidence for Rab3a being involved in regulated secretion has been obtained (Holz et al., 1994; Johannes et al., 1994; Lledo et al., 1994). In addition, the closely related protein Rab3b controls Ca^{2+}-induced hormone release in anterior pituitary cells (Lledo et al., 1993).

In agreement with this function in regulating exocytosis, Rab3a has been shown to be associated with synaptic vesicles in brain (Fischer von Mollard et al., 1990; Kim et al., 1989). In cultured chromaffin cells, subcellular fractionation experiments showed that Rab3a was associated with chromaffin granules (Darchen et al., 1990). Similar results were obtained in PC12 cells (Regazzi et al., 1992). However, this conclusion has been challenged by another study, mainly based on immunopurification of organelles which indicated a localization of Rab3a on synaptic-like vesicles, and its absence from chromaffin granules (Fischer von Mollard et al., 1990). The latter finding was given as supporting the hypothesis that secretory granules and synaptic vesicles, which differ in their biogenesis, also possess distinct regulatory protein components.

In the present work, the subcellular localization of Rab3a in bovine chromaffin cells and in rat PC12 cells has been investigated by several morphological techniques.

MATERIALS AND METHODS

Cell culture

Primary dissociated chromaffin cells from bovine adrenal medulla were prepared by retrograde collagenase perfusion and cultured as described (Bittner et al., 1986). PC12 cells were cultured in DME medium supplemented with 10% horse serum (Gibco-BRL) and 5% fetal bovine serum (Hyclone Laboratories, Inc.), and passaged by treating the cells with PBS containing 0.05% trypsin and 0.53 mM EDTA.

Transfection of PC12 cells

The vector used for expressing epitope-tagged *Rab3a*, pCH-Rab3a, was constructed by inserting an oligonucleotide sequence corresponding to the influenza hemagglutinin peptide sequence HA1 (amino acids 98-106, YPYDVPDYA), immediately downstream from the start codon. Transfections were carried out by the electroporation method. In each cuvette, 6×10^6 PC12 cells were transfected with 10 μg of supercoiled plasmid pCH-Rab3a diluted in 0.8 ml of 137 mM NaCl, 5 mM KCl, 0.7 mM Na_2HPO_4, 6 mM dextrose, and 20 mM HEPES (pH 7.05). The cuvettes had a gap of 0.4 cm (Bio-Rad Laboratories) and received a pulse of 400 V at 500 μF by an electroporator device from Bio-Rad Laboratories. Approximately 10 to 20% of the cells surviving the electroporation pulse transiently expressed Rab3a.

Production of an anti-Rab3a antiserum

Briefly, *H-Rab3a* was expressed in *Escherichia coli*, purified as described by Zahraoui et al. (1989), and coupled to keyhole limpet haemocyanin with 0.05% glutaradehyde. Serum was collected after 3 subcutaneous injections into rabbits. Specific anti-Rab3a antibodies were purified by passing the serum onto an affinity chromatography column prepared by coupling purified Rab3a protein with Affigel-10 (Bio-Rad) according to the manufacturer's instructions.

Two-dimensional gel electrophoresis

Bovine chromaffin cells were homogenized in 0.3 M sucrose, 10 mM NaOH-Hepes, pH 7.0, 1 mM PMSF, 5 μg of aprotinin per ml, 5 μg of leupeptin per ml. The cell debris and the nuclei were eliminated by centrifugation at 750 ***g*** for 10 minutes. The homogenate was dissolved in 9 M urea, 4% (w/v) Nonidet P-40, 6% (v/v) ampholines, pH 3.5-10 (Pharmacia LKB), 0.4 M dithiothreitol, 10% (w/v) glycerol. IEF gels were run at 400 V for 16 hours. The pH gradient after electrophoresis ranged from 4.0 to 7.5. The second dimension was performed on a 13% SDS-PAGE gel. Molecular masses and isoelectric points were determined by comparison with SDS-PAGE molecular mass standards and 2-D gel standards (Bio-Rad). Proteins were then transferred onto nitrocellulose filters and bound to [^{35}S]GTPγS (New England Nuclear; 1000 Ci/mmol), as described previously (Darchen et al., 1990). [^{35}S]GTPγS binding was visualized by autoradiography (48 hours at −70°C) using Kodak Xomat AR films. The same nitrocellulose sheet was then processed for immunostaining with anti-Rab3a antiserum and alkaline phosphatase-conjugated anti-rabbit IgG, as described previously (Darchen et al., 1990).

Confocal microscopy

Bovine chromaffin cells, which had been purified by differential plating (Waymire et al., 1983) and maintained on collagen-coated coverslips, were permeabilized for 5 minutes at room temperature with 20 μM digitonin in 139 mM potassium glutamate, 5 mM EGTA, 20 mM Pipes (pH 6.6), 2 mM MgATP and 5 mg/ml BSA. Cells were fixed with 4% paraformaldehyde in phosphate buffered saline (PBS) for 30 minutes at room temperature, rinsed with PBS and then incubated with 50 mM NH_4Cl in PBS for 20 minutes to react with residual paraformaldehyde. Dopamine β-hydroxylase (DBH) was detected with goat anti-DBH (1:1500) followed by porcine anti-goat FITC (1:50). Rab3a was detected with affinity-purified rabbit anti-Rab3a (1:30) followed by donkey anti-rabbit-lissamine rhodamine (1:50). Cells were incubated with primary antibodies for 1 hour at 4°C, rinsed 5 times with Tris-buffered saline, incubated with secondary antibodies at room temperature for 30 minutes followed by 5 rinses with Tris-buffered saline. Cells were fixed again with 4% paraformaldehyde for 10 minutes at room temperature followed by 2 rinses with Tris-buffered saline and then mounted in glycerol:PBS (9:1), pH 9, containing 1 mg/ml *p*-phenylenediamine. Non-immune goat antiserum and non-immune rabbit IgG at the same concentrations as the specific primary antibodies were used to determine nonspecific staining. Images were obtained with a Bio-Rad MRC600 Laser Confocal Scanning Microscope with a 100× objective and had a resolution of 0.3-0.4 μm in horizontal plane and 0.5-1 μm in the vertical axis.

Electron microscope immunocytochemistry

Pre-embedding procedure for immunoelectron microscopy

Cultured bovine chromaffin cells in primary culture were quickly rinsed in cold PBS, fixed with 2% paraformaldehyde-2% glutaraldehyde in PBS at 4°C for 30 minutes, rinsed in PBS and processed for immunocytochemistry. The cells were pre-treated with blocking solution containing 10% of horse normal serum (HNS) in PBS for 30 minutes and then incubated overnight at 4°C with affinity-purified rabbit anti-Rab3a antibodies (1/50 in 5% HNS/PBS) or with preimmune rabbit serum for control. After several washes in HNS/PBS, the cells were incubated for 2 hours at room temperature with peroxidase-coupled anti-rabbit IgG antibodies (Cappel, 1/100 in HNS/PBS) and then rinsed in PBS. The peroxidase activity was revealed with 0.025% 3,3′-diaminobenzidine (Sigma) in 0.05 M Tris-Cl buffer, pH 7.6, containing 0.01% hydrogen peroxide. The cells were then prepared for standard electron microscopy by post-fixation in 1% osmium tetroxide/phosphate buffer, dehydration in progressive alcohols and embedding in Spurr (Taab). Areas of interest, selected by light microscope, were trimmed out and thin sectioned with an ultramicrotome. Ultrathin sections were examined under the electron microscope without conterstaining.

Post-embedding procedure for immunoelectron microscopy

PC12 cells were transfected with pCH-Rab3a as described above, plated onto polylysine-coated Lab-Tek culture slides (VWR Scientific) and treated with 50 ng/ml of human recombinant nerve growth factor (kindly provided by Tony Evans, Genentech, Inc., So. San Francisco). Four days after the transfection, the cells were washed

once with PBS, fixed for 60 minutes at room temperature with 2% paraformaldehyde-0.1% glutaraldehyde in PBS. Following fixation, the cells were rinsed with PBS, and then with PBS containing 0.05 M NH_4Cl for 30 minutes to quench free aldehyde groups. Finally, the cells were rinsed with PBS and stored overnight in PBS at 4°C. The next day, the cells were dehydrated and embedded in situ in the hydrophilic resin Lowicryl K4M (Carlemalm et al., 1982) at low temperature according to the following protocol: 30% ethanol, 15 minutes on ice; 50% ethanol, 30 minutes on 3 parts ice:1 part NaCl (–20°C); 80% ethanol, 30 minutes at –35°C; 100% ethanol, 2 changes of 30 minutes each at –35°C; 1 part 100% ethanol:1 part Lowicryl K4M, 60 minutes at –35°C; 1 part 100% ethanol:2 parts Lowicryl K4M, 60 minutes at –35°C; pure Lowicryl K4M, 60 minutes at –35°C; pure Lowicryl K4M, overnight at –35°C. The next morning, the Lowicryl K4M was replaced with freshly prepared resin and allowed to infiltrate the cells for a further 6 hours. Finally, the Lowicryl K4M resin was decanted out of the flaskettes and replaced with enough fresh Lowicryl K4M to form a layer of approximately 3 mm thick over the monolayer of cells. Because of the relatively large surface area of the cells to be covered (the chambers are approximately 5 cm long × 2 cm wide), nitrogen gas was bubbled into the chambers for 2 minutes, followed by quick capping of the flask. This was performed to assure that minimal amounts of oxygen would be present in the chamber, since oxygen interferes with the polymerization of the Lowicryl resin. The Lowicryl K4M resin was polymerized by exposure to long wave ultraviolet light for 2 days at –35°C, followed by 3 days at room temperature.

For ultrathin sectioning of embedded PC12 cells parallel to the monolayer, small blocks of the polymerized monolayer were cut out with a saw and glued onto plastic stubs which fit into the specimen holder of the microtome. Ultrathin sections (50-70 nm thick) were cut with glass knives and retrieved onto 150 mesh nickel grids coated with parlodion and carbon. For serial sections, ultrathin sections were cut with glass knives and placed onto 2× 0.5 mm slot Synaptek grids (Electron Microscopy Sciences) coated with 0.25% Butvar-B-98 (Electron Microscopy Sciences) in chloroform. The sections were used for incubations within a week from the time of sectioning.

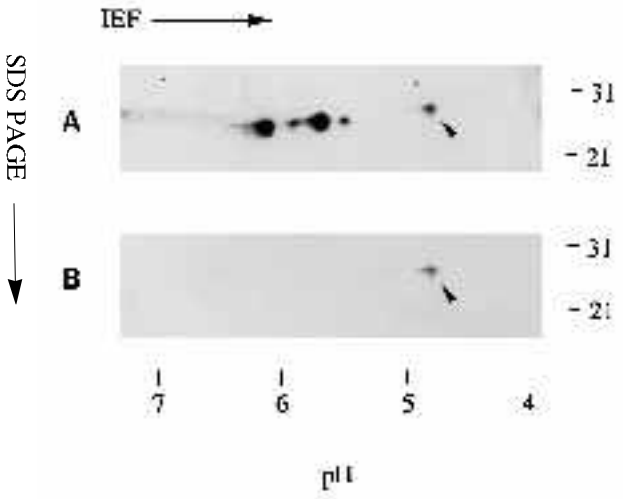

Fig. 1. Analysis of small GTP-binding proteins from bovine chromaffin cells by 2-D gel electrophoresis. 50 μg protein from chromaffin cell homogenate was resolved by two-dimensional PAGE and transferred to nitrocellulose paper. (A) The small GTP-binding proteins were labelled by [^{35}S]GTPγS binding and visualized by autoradiography. (B) Immunoblotting of the same nitrocellulose sheet with anti-Rab3a antiserum. Rab3a labelling is indicated by arrowheads in A and B. Position of molecular mass standards is indicated on the right.

Antigenic sites were visualized on thin sections using the Protein A-gold technique (Roth et al., 1978, 1989). Briefly, grids were floated section side down on drops of 0.5% ovalbumin in PBS for 20 minutes, followed by transfer to droplets of primary antibody and incubated overnight at 4°C. Mouse monoclonal antibody 12CA5 (Berkley Antibody Company) was used at a dilution of 1:20,000 in PBS containing 1% bovine serum albumin, and mouse monoclonal antibody SY38 raised against synaptophysin (BioGenex Laboratories) was used at a dilution of 1:200 in PBS containing 1% bovine serum albumin. After rinses on drops of PBS (3× 5 minutes), the sections were incubated for 30 minutes at room temperature on droplets of affinity-purified rabbit anti-mouse IgG (10 μg/ml; Jackson ImmunoResearch Laboratories, Inc.). The grids were then washed on drops of PBS (2× 5 minutes), followed by transfer to droplets of Protein A-gold and incubated for 1 hour at room temperature. Colloidal gold particles were prepared with an average diameter of 15 nm according to the citrate reduction method of Frens (1973) and complexed with Protein A as described by Roth and coworkers (1978). For incubation with sections, the Protein A-gold complex was diluted with PBS containing 1% bovine serum albumin, 0.075% Triton X-100 and 0.075% Tween-20 to yield an optical density of 0.3 at 525 nm. Finally, the grids were washed with PBS and distilled water, allowed to air dry, and then contrasted with 3% aqueous uranyl acetate (5 minutes) and lead citrate (45 seconds).

RESULTS

Characterization of an anti-Rab3a antibody

In order to determine the subcellular localization of Rab3a, we used an antibody which was raised against purified recombinant H-Rab3a and purified by affinity chromatography. Its specificity was assessed by immunoblotting; no cross-reactivity could be detected with the following members of the rab family that we have tested: Rab1, Rab2, Rab4, Rab5, and Rab6 (data not shown). Moreover, when the small GTPases of bovine adrenal medulla were analyzed by 2-D electrophoresis and [^{35}S]GTPγS overlay, the anti-Rab3a antibody revealed only one spot with the characteristics of Rab3a (Fig. 1). These

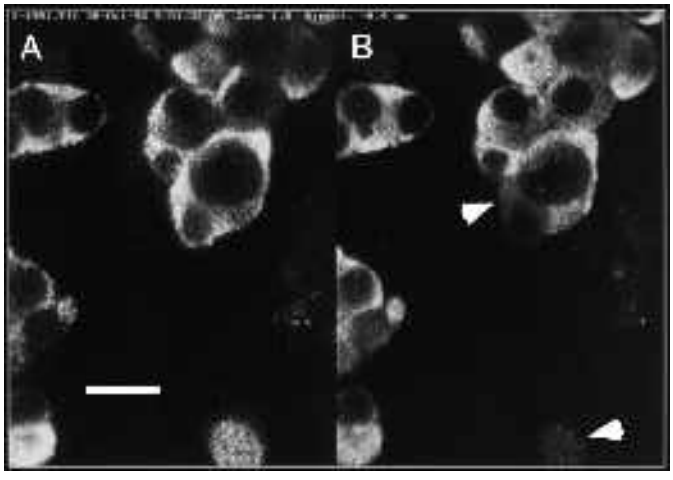

Fig. 2. Low magnification, confocal immunofluorescence localization of dopamine β-hydroxylase (DBH) and Rab3a. (A) DBH immunofluorescence. (B) Rab3a immunofluorescence. DBH and Rab3a were detected in most cells. Occasionally, single cells which strongly stained for DBH showed little Rab3a immunofluorescence. Bar, 10 μm.

results argued in favour of the specificity of the antibody but did not exclude the possibility of a cross-reactivity with another member of the rab3 subfamily. Rab3b, one of the Rab3 isoforms with biochemical properties very similar to those of Rab3a, could not be distinguished from Rab3a by 2-D electrophoresis. However, Rab3b produced by in vitro translation was not significantly labelled by the anti-Rab3a antibody (P. Vernier and F. Darchen, unpublished experiments). In addition, Rab3b, c, and d are very poorly expressed in chromaffin cells, as revealed by northern blots and PCR experiments (P. Vernier and F. Darchen, unpublished experiments). Therefore, the specificity of this antibody allowed its use for morphological localization of Rab3a.

Confocal microscopy study of Rab3a localization

The distribution of Rab3a has been compared to that of DBH (dopamine β-hydroxylase), a marker of chromaffin granules (Laduron and Belpaire, 1968; Aunis et al., 1980). Immunocytochemistry was performed on non-stimulated cells in which the chromaffin granule membrane is not recycling from the plasma membrane. Under these conditions the major fraction of the DBH is associated with chromaffin granules (Viveros et al., 1971). The experiments were performed on cells permeabilized with digitonin to reveal intracellular antigenic sites. Strong staining for DBH and Rab3a was found in most cells (Fig. 2). A few DBH-positive cells (less than 5%) showed much weaker staining for Rab3a (Fig. 2, indicated by arrow-

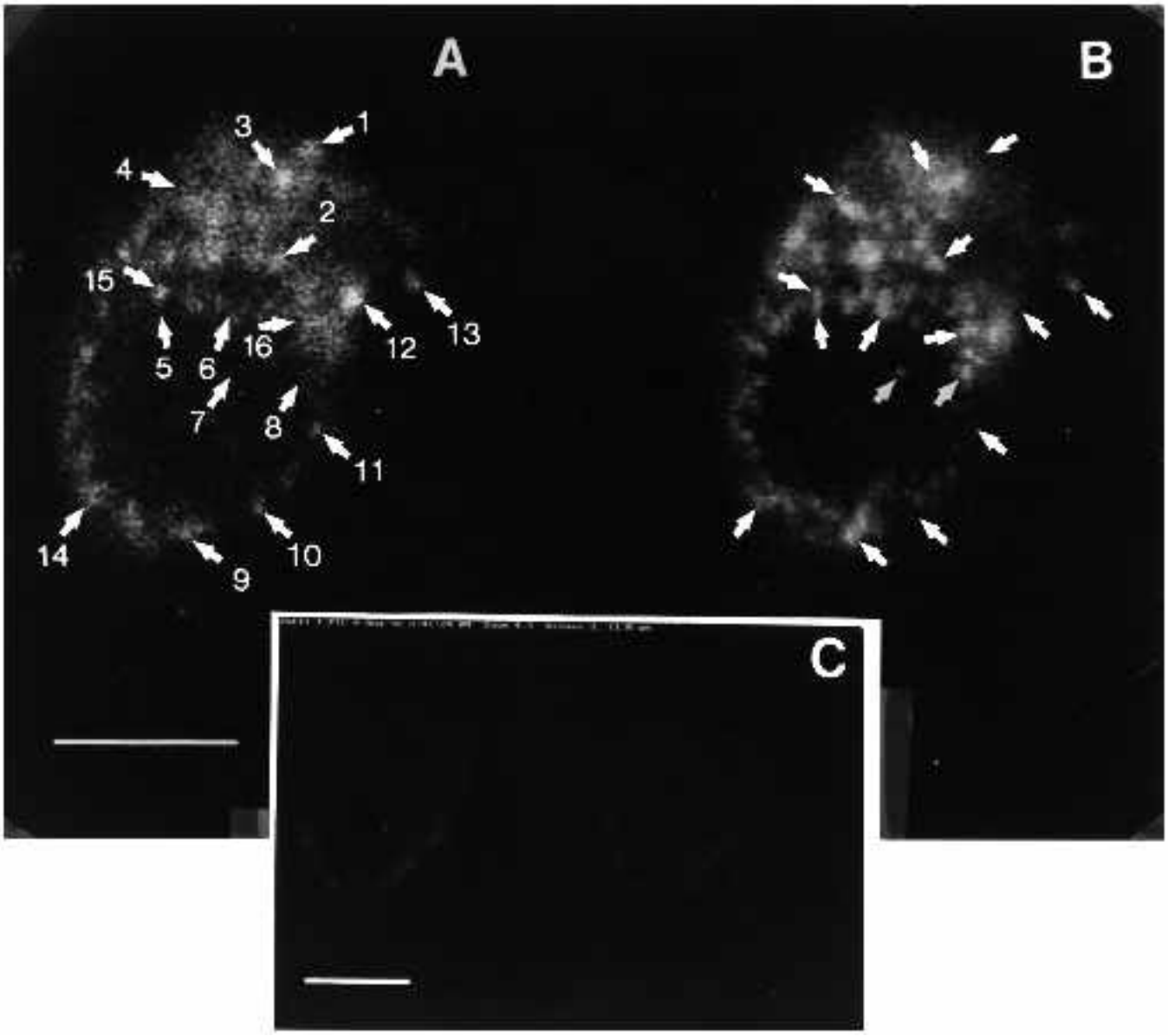

Fig. 3. High magnification, confocal immunofluorescence localization of dopamine β-hydroxylase (DBH) and Rab3a in a single cell. (A) DBH immunofluorescence. (B) Rab3a immunofluorescence. (C) Cells treated in the same manner as in A and B except that normal goat serum and nonimmune rabbit IgG were used. Discrete punctate structures were observed in A and B. Some of the punctate structures of DBH and Rab3a precisely coincided (indicated by numbered arrows 2, 3, 9, 13, 14, and 15). Others were only positive for DBH (numbered arrows 1, 10, 11, 12). Others were only positive for Rab3a (numbered arrows 4, 5, 6, 7, 8, 16). Bar, 10 μm.

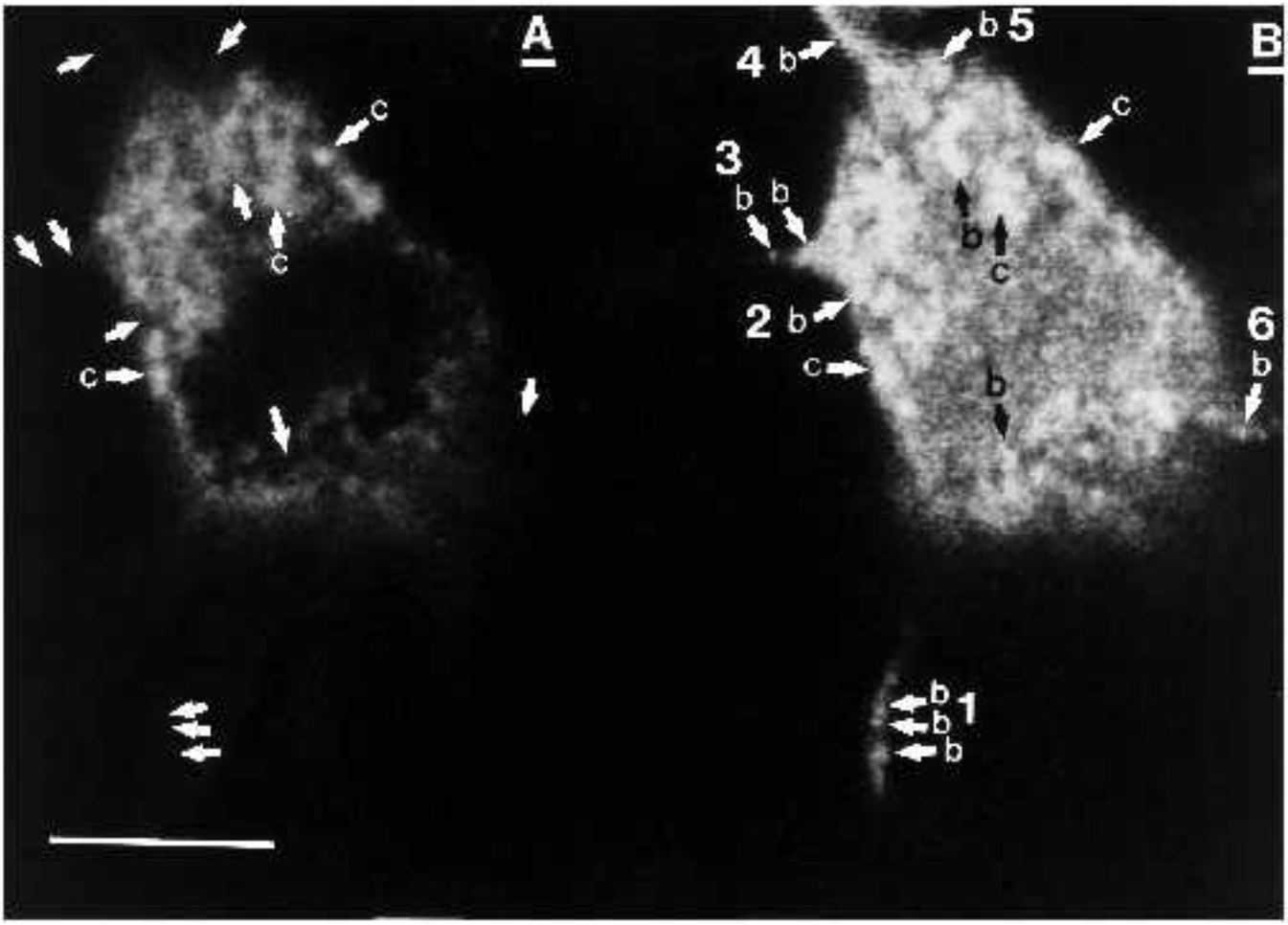

Fig. 4. High magnification, confocal immunofluorescence localization of dopamine β-hydroxylase (DBH) and Rab3a in a single cell. (A) DBH immunofluorescence. (B) Rab3a immunofluorescence. This cell demonstrates more examples of Rab3a localization at sites devoid of DBH (structures labelled as 'b') and of structures in which DBH and Rab3a colocalize (labelled as 'c'). Bar, 10 μm.

heads). These particular cells were also observed using conventional fluorescence microscopy and, therefore, were not an artefact of the section visualized by the confocal microscope. At higher magnification both DBH and Rab3a had a punctate appearance (Figs 3,4). The precise positions of the DBH- and Rab3a-positive structures were determined. Rab3a staining coincided with some of the chromaffin granules; some chromaffin granules were devoid of Rab3a staining; some punctate structures with Rab3a did not stain for DBH and therefore were not chromaffin granules. Serial sections (not shown) confirmed the common staining of DBH and Rab3a in some but not all punctate structures. Clearly visualized punctate structures in three chromaffin cells were evaluated for expression of one or both DBH and Rab3a (Table 1). Approximately one-half of the chromaffin granules (DBH positive structures) also stained for Rab3a; approximately two-thirds of the Rab3a positive structures were negative for DBH.

Ultrastructural study of Rab3a localization

The subcellular localization of Rab3a in bovine cultured chromaffin cells was investigated by immunoperoxidase with a pre-embedding approach. Rab3a was detected specifically on the membrane of some dense-core secretory granules (Fig. 5A,B, and Fig. 6A,C). The labelling could not be correlated with any spatial arrangement or morphological feature of the granules. The plasma membrane was completely unstained. The nucleus, the mitochondria, and membranes from endoplasmic reticulum or Golgi apparatus were not labelled either. This was taken as an evidence for the specificity of the labelling of chromaffin granules. Some immunoreactivity was

Table 1. Summary of punctate structures with either or both DBH and Rab3a in chromaffin cells

Cells	DBH+/Rab3a+	DBH+/Rab3a–	DBH–/Rab3a+
1	9	6	14
2	10	9	25
3	10	13	22
Totals	29	28	61

DBH and Rab3a were localized by immunocytochemistry and confocal microscopy in 3 cells. Punctate structures, which contained both DBH and Rab3a or either DBH or Rab3a alone, were determined. Cell 1 corresponds to the cell in Fig. 3 and cell 3 corresponds to the cell in Fig. 4 (more punctate structures were analyzed than indicated by arrows in the Figures). Note that Rab3a colocalized with approximately 50% of the DBH positive structures (chromaffin granules).

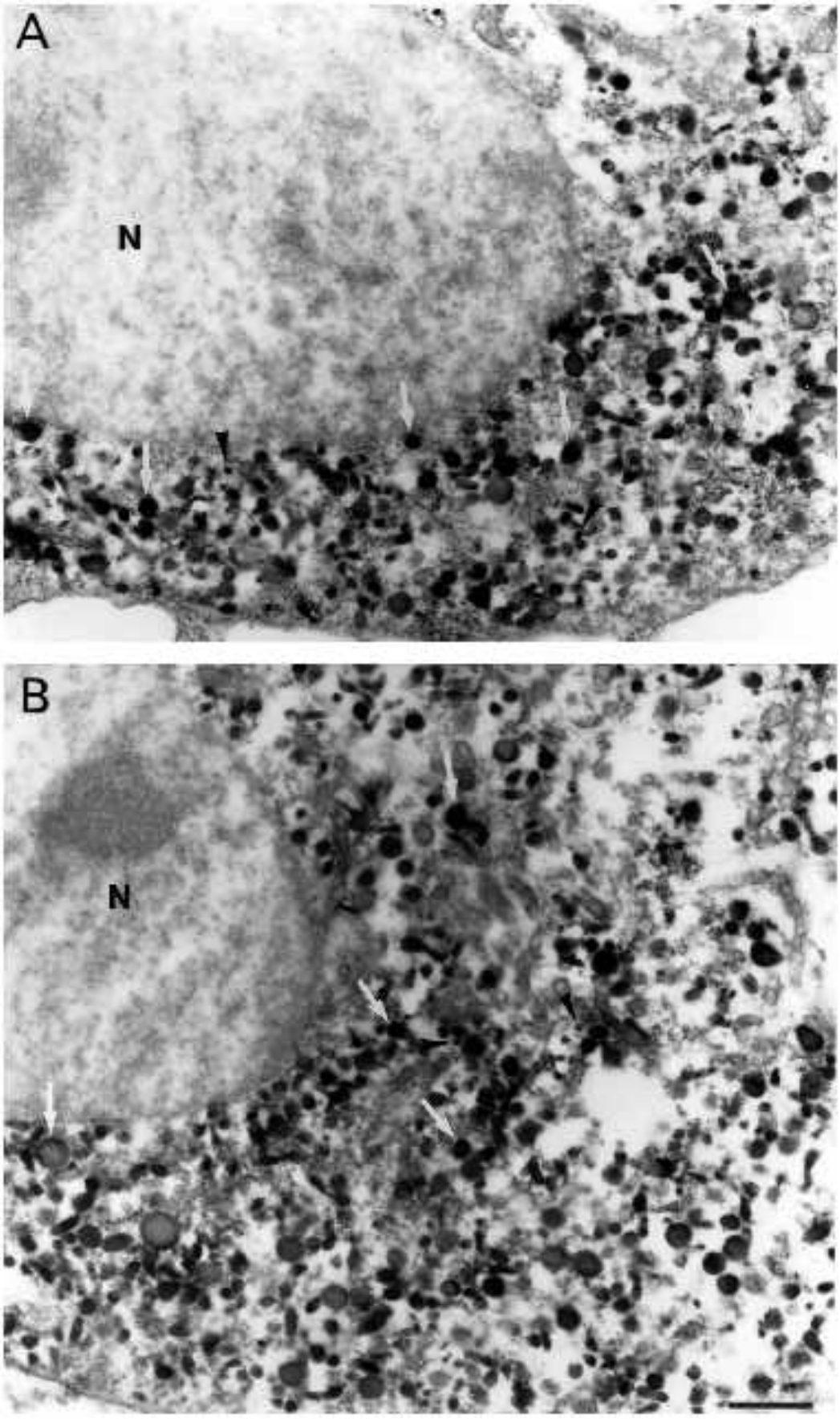

Fig. 5. Electron micrographs showing immunoperoxidase staining of Rab3a in chromaffin cells at low magnification. Many chromaffin granules are labelled. Some of them are indicated by arrows. Some small vesicles are also stained (indicated by arrowheads). N, nucleus. Note the absence of staining on the plasma membrane, the nucleus, and the membrane of other subcellular compartments. Bar, 1 μm.

also found in the cytoplasm, in agreement with previous biochemical studies indicating that Rab3a is partially cytosolic (Burstein and Macara, 1989; Darchen et al., 1990). In control incubation without anti-Rab3a antibodies, chromaffin granules were completely unstained (Fig. 6B). In addition to chromaffin granules, some labelled vesicles with 50-60 nm diameter, a size corresponding to that of synaptic-like microvesicles (Navone et al., 1986; Cameron et al., 1991; Annaert et al., 1993) were occasionnally seen (Figs 5, 6, indicated by arrowheads).

Localization of epitope-tagged Rab3a expressed in PC12 cells

It is difficult to exclude the possibility that antibodies directed to endogenous Rab3a could detect, in addition to Rab3a, proteins closely related to Rab3a. Thus, to be certain that our techniques were detecting Rab3a, we designed experiments using related PC12 cells which were transiently transfected with a vector that expressed Rab3a with a 9 amino acid epitope tag (HA1) at its amino terminus. A monoclonal antibody (12CA5) specific for the epitope was used to stain the tagged

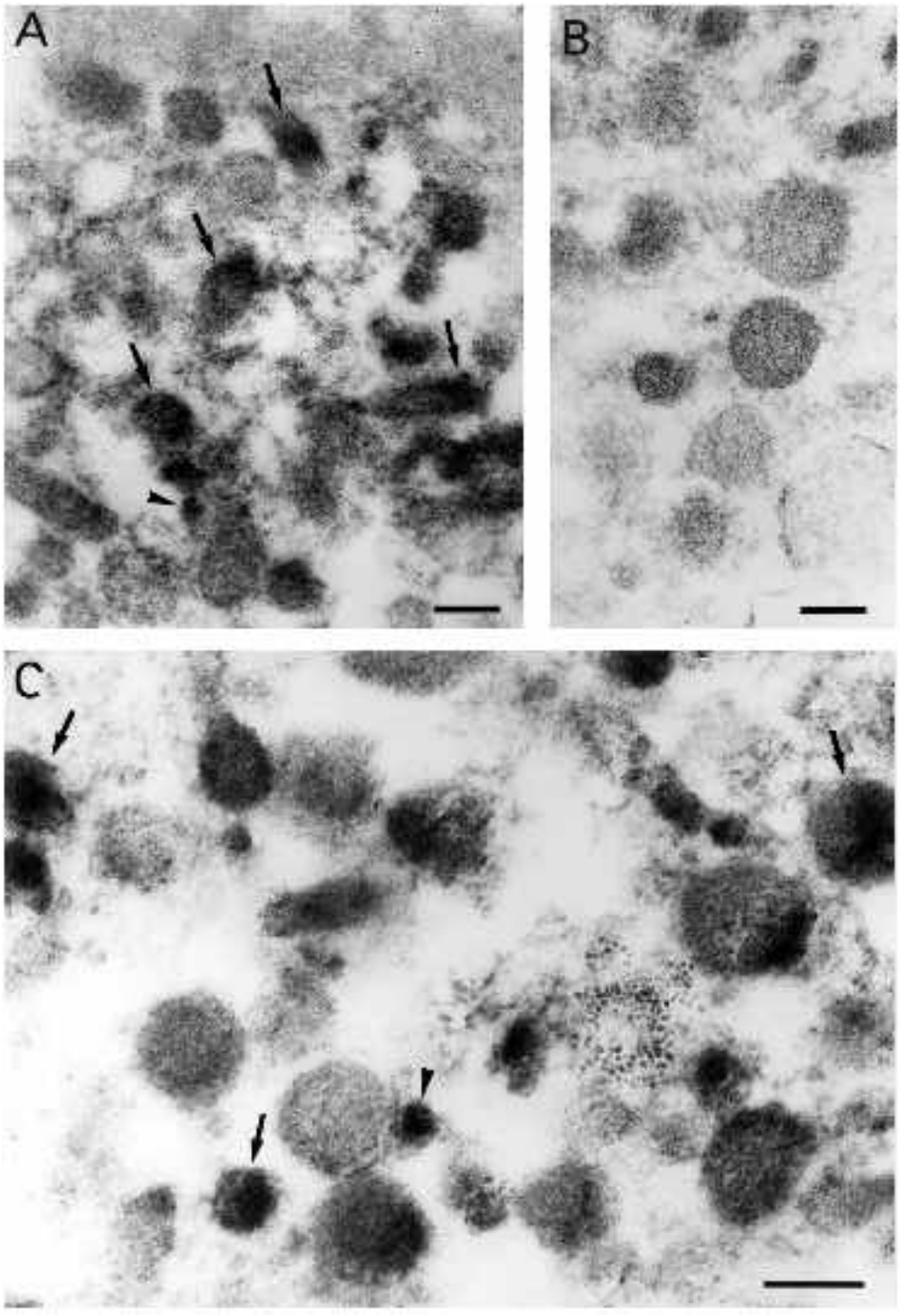

Fig. 6. Electron micrographs of immunoperoxidase staining of Rab3a in chromaffin cells at high magnification. Immunoperoxidase reaction is localized predominantly on dense core secretory granules (some of them are indicated by arrows in A and C). Smaller labelled vesicles are also seen (arrowheads). (B) Control incubation without anti-Rab3a antibody. Bar, 0.2 μm.

Rab3a in a post-embedding Protein A-gold protocol. Gold particle label indicative of monoclonal antibody 12CA5 binding to Rab3a was found in approximately 10 to 20% of the transfected PC12 cells. This percentage correlated well with the known transfection efficiency of the PC12 cells, as established by a parallel electroporation using v-ras as a marker. In this case, neurite outgrowth induced by oncogenic Ras provided a visible marker for successful transfection. The staining of tagged Rab3a was primarily distributed in association with spherical to irregularly shaped dense core granules (Fig. 7). These granules were generally restricted to the cell periphery, often occurring in a line directly below the plasma membrane, as described previously (Pozzan et al., 1984). A much lesser amount of immunoreactivity was associated with the general cytoplasm. Control incubations of sections with rabbit anti-mouse IgG followed by Protein A-gold, or with Protein A-gold alone resulted in no immunolabelling.

Immunoreactivity for synaptophysin was found dispersed throughout the cytoplasm of all PC12 cells examined (Fig. 8), with no preferential association with distinct cytoplasmic structures. We were unable to detect the microvesicles with which synaptophysin comigrates in equilibrium gradients of PC12 cells extracts (Clift-O'Grady et al., 1990; Cutler and Cramer, 1990; Schweitzer and Paddock, 1990; Cameron et al., 1991; Linstedt and Kelly, 1991). To investigate whether epitope-tagged Rab3a and synaptophysin immunoreactivity codistribute in association with the dense core granules, we collected serial sections of PC12 cells embedded in Lowicryl K4M and incubated consecutive sections with 12CA5 or anti-synaptophysin antibody. The diameters of the dense core granules appeared to be within the thickness of the ultrathin sections (50-80 nm thick), such that it was rare to visualize the same granule on consecutive sections. Fig. 8 shows two consecutive thin sections of the same PC12 cell incubated with monoclonal antibody 12CA5 (Fig. 8A) or monoclonal antibody SY38 raised against synaptophysin (Fig. 8B). When the same dense core granule appeared in both sections, the granule was labelled by the 12CA5 antibody but not by the anti-synaptophysin antibody. Although some immunoreactivity for synaptophysin was in close proximity to the dense core granules (Fig. 8B), the granules themselves were not stained.

DISCUSSION

In addition to dense core granules, PC12 cells and chromaffin cells contain microvesicles with biochemical characteristics similar to neuronal synaptic vesicles. The exact subcellular localization of Rab3a in chromaffin cells has been controversial. Darchen et al. (1990) found that Rab3a copurifies with dense core granules in sucrose density gradient and immunoadsorption experiments. In contrast, Fischer von Mollard et al. (1990) suggested that Rab3a in adrenal medulla localizes to the synaptic-like microvesicles and not to the dense-core granules by using a combination of differential centrifugation and immunoadsorption of synaptophysin-containing vesicles. More recently, Matteoli et al. (1991) presented evidence by using confocal immunofluorescence that Rab3a in PC12 cells and bovine chromaffin cells colocalizes with synaptophysin, which is associated predominantly with synaptic-like microvesicles (Navone et al., 1986; Johnston et al., 1989; Clift-O'Grady et al., 1990; Cutler and Cramer, 1990; Schweitzer and

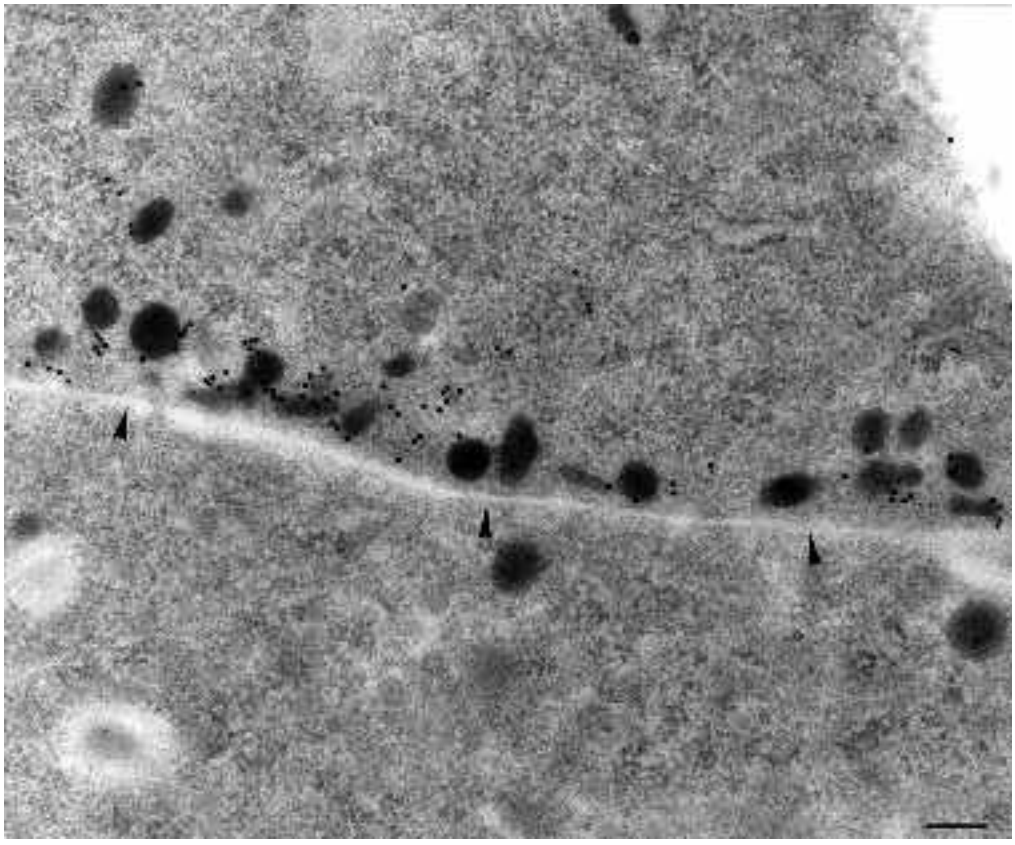

Fig. 7. Immunoelectron microscopic localization of Rab3a in transfected PC12 cells embedded in Lowicryl K4M. Peripheral cytoplasmic region of two cells is shown, separated by the plasma membrane (arrowheads). Gold particles indicative of Rab3a are localized predominantly to dense core granules lined up beneath the plasma membrane. Note that immunoreactivity is restricted to only one of the cells, suggesting that the other cell was not transfected. Bar, 0.2 μm.

Paddock, 1990; Cameron et al., 1991; Linstedt and Kelly, 1991; Régnier-Vigouroux et al., 1991; Annaert et al., 1993). To resolve the controversial question of the subcellular localization of Rab3a, we combined different morphological techniques that should minimize possible artefacts that might occur during homogenization and fractionation of the cells. The results provide compelling evidence for the association of Rab3a with dense core granules (chromaffin granules) in bovine chromaffin cells and in PC12 cells.

Confocal microscopy was used to visualize secretory granules within chromaffin cells with DBH immunocytochemistry. The anti-Rab3a antibody also detected punctate structures in chromaffin cells. The ability to resolve punctate Rab3a-positive structures was probably enhanced by the permeabilization method used. Because cells were digitonin-permeabilized before fixation and incubation with antibodies, soluble Rab3a probably diffused out of the cells, thereby minimizing diffuse cytosolic staining. Rab3a staining coincided with approximately one-half of the chromaffin granules. The confocal microscope does not have the resolution to determine whether Rab3a was actually inserted in the chromaffin granule membrane or was part of a small structure in very close proximity with chromaffin granules. In either case, the images strongly suggest that Rab3a is a component of the chromaffin granule membrane or is closely associated with it for a significant fraction of the chromaffin granules.

Immunoelectron microscopy allowed direct identification of the structures bearing the label. Some chromaffin granules were clearly labelled, thus excluding the possibility that Rab3a could be located on a structure in proximity with chromaffin granules. Consistent with the confocal experiments was the fact that some granules were not stained, indicating either an heterogeneity of the granule population, or a lack of accessibility of the epitopes.

Thus, confocal microscopy and immunoelectron microscopy indicate the presence on chromaffin granules of Rab3a or a closely related protein. This view is supported by the localization of epitope-tagged Rab3a in PC12 cells which demonstrates staining of dense core secretory granules. Our use of an epitope-tagged system eliminates the possibility that the staining was due to a Rab3a-related protein. It is recognized that overexpression of Rab3a might result in an artefactual distribution of the protein. However, the results obtained are fully consistent with experiments with antibody against endogenous Rab3a and therefore strengthen the conclusion that Rab3a is associated with dense core secretory granule membranes in neuroendocrine cells.

Subcellular fractionation experiments in chromaffin cells (Darchen et al. 1990) indicated the presence of a fraction of the Rab3a protein in the cytosol and in an unidentified compartment of light density. Localization at the level of synaptic-like vesicles has been observed in some experiments, but not systematically by all approaches. Confocal microscopy clearly indicated the presence of Rab3a on structures which were not positive for DBH, but which could not be identified. At the ultrastructural level, some positively labelled vesicles were

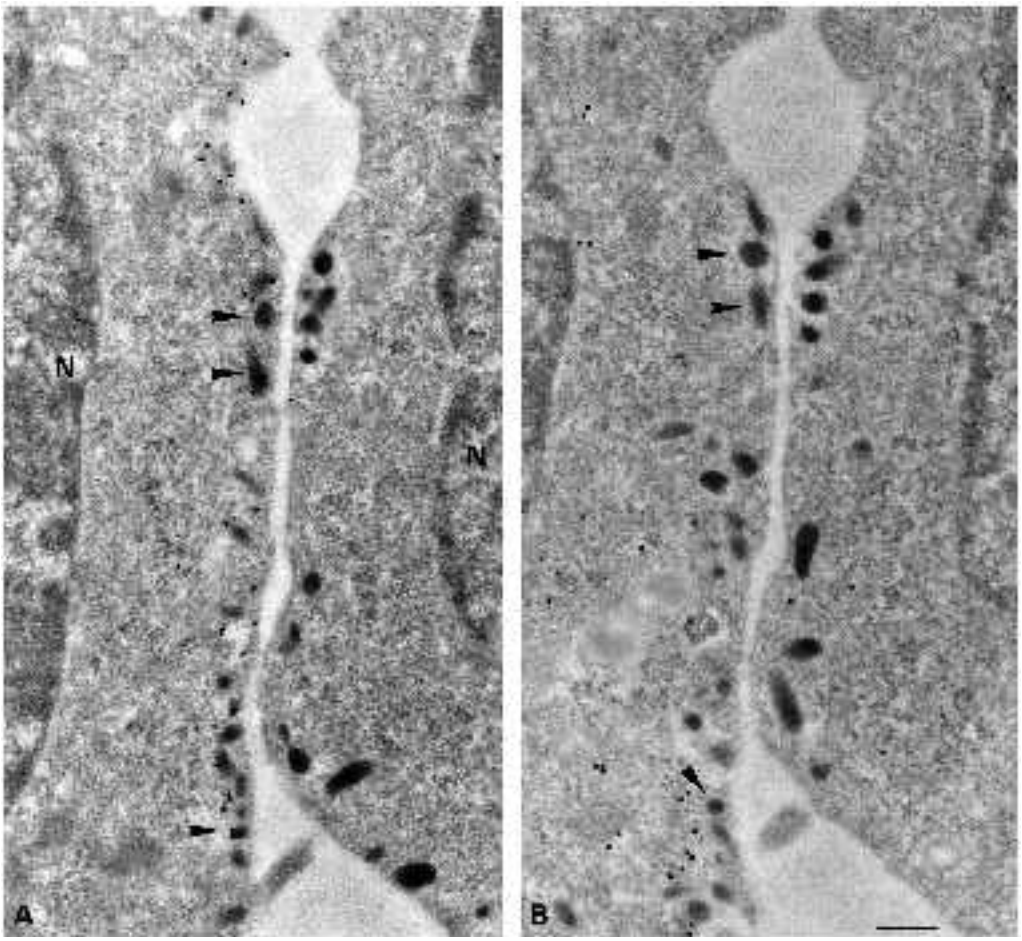

Fig. 8. Immunoelectron microscopic localization of Rab3a and synaptophysin on serial sections from PC12 cells embedded in Lowicryl K4M. Two PC12 cells are shown sectioned en face. While immunoreactivity for Rab3a is associated with dense core granules (A), synaptophysin immunoreactivity is randomly dispersed throughout the cytoplasm (B). By examining the same dense core granules (arrowheads) on consecutive sections, the lack of coincidental staining for Rab3a and synaptophysin is apparent. Similar to Fig. 7, note that the cell on the left in (A) displays immunoreactivity for Rab3a, while the cell on the right side does not. N, nucleus. Bar, 0.24 μm.

observed using the pre-embedding approach (Figs 5,6), but not with the post-embedding one in PC12 cells overexpressing Rab3a (Figs 7,8).

That Rab3a is associated with dense-core vesicles is also supported by functional data. It was reported that peptides homologous to the effector domain of Rab3a enhance Ca^{2+}-dependent secretion in chromaffin cells (Senyshyn et al., 1992), in acinar pancreatic cells (Padfield et al., 1992), and in mast cells (Oberhauser et al., 1992). However, several reports have challenged the specificity of these peptides: (i) they have a mastoparan-like effect on heterotrimeric GTP-binding proteins (Law et al., 1993) and stimulates phospholipase C activity (Zeuzem et al., 1994); (ii) the stimulatory effect persisted when the amino acid sequence was substantially modified (MacLean et al., 1993); (iii) the most effective peptide, Rab3AL, carried mutations that, in Rab3a, abolish the interaction with all known interacting proteins (McKiernan et al., 1993). Using mutant proteins and antisense oligonucleotides, we have now provided strong evidence for Rab3a being involved in regulated exocytosis of secretory granules in chromaffin cells (Holz et al., 1994; Johannes et al., 1994). Moreover, results obtained with other members of the Rab3 subfamily also suggest an association with peptide-containing vesicles: Rab3b controls exocytosis in anterior pituitary cells (Lledo et al., 1993), the *D. ommata* o-Rab3 protein, when expressed in AtT-20 cells, is targeted to large dense-core vesicles (Ngsee et al., 1993), and a Rab3-like protein is associated with zymogen granules in exocrine pancreas (Jena et al., 1994).

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