Nerve Growth Factor–induced Changes in the Intracellular Localization of the Protein Kinase C Substrate B-50 in Pheochromocytoma PC12 Cells

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Abstract. High levels of the neuron-specific protein kinase C substrate, B-50 (= GAP43), are present in neurites and growth cones during neuronal development and regeneration. This suggests a hitherto nonelucidated role of this protein in neurite outgrowth. Comparable high levels of B-50 arise in the pheochromocytoma PC12 cell line during neurite formation. To get insight in the putative growth-associated function of B-50, we compared its ultrastructural localization in naive PC12 cells with its distribution in nerve growth factor (NGF)– or dibutyryl cyclic AMP (dbcAMP)–treated PC12 cells.

B-50 immunogold labeling of cryosections of untreated PC12 cells is mainly associated with lysosomal structures, including multivesicular bodies, secondary lysosomes, and Golgi apparatus. The plasma membrane is virtually devoid of label. However, after 48-h NGF treatment of the cells, B-50 immunoreactivity is most pronounced on the plasma membrane. Highest B-50 immunoreactivity is observed on plasma membranes surrounding sprouting microvilli, lamellipodia, and filopodia. Outgrowing neurites are scattered with B-50 labeling, which is partially associated with chromaffin granules. In NGF-differentiated PC12 cells, B-50 immunoreactivity is, as in untreated cells, also associated with organelles of the lysosomal family and Golgi stacks. B-50 distribution in dbcAMP-differentiated cells closely resembles that in NGF-treated cells.

The altered distribution of B-50 immunoreactivity induced by differentiating agents indicates a shift of the B-50 protein towards the plasma membrane. This translocation accompanies the acquisition of neuronal features of PC12 cells and points to a neurite growth-associated role for B-50, performed at the plasma membrane at the site of protrusion.

B-50 (= GAP43) is a neuron-specific protein. Various studies suggest its involvement in the process of neuronal development, axonal regeneration, and the modulation of synaptic function (De Graan et al., 1986; Benowitz and Routtenberg, 1987). In the rat, the protein is expressed at high levels during development of the central nervous system (Jacobson et al., 1986; Zwiers et al., 1987) and during regeneration of peripheral nerves (Verhaagen et al., 1986, 1988; Snipes et al., 1987). After the growth phase has ceased, B-50 content decreases to remain present at moderate concentrations in adult or recovered nervous tissue. Its relatively high expression throughout adulthood in discrete associative regions of adult human brain (Neve et al., 1988), the absence of B-50 immunoreactivity in neuritic plaques in Alzheimer brains (Clark et al., 1988), and its reexpression during transient, clinically curable injury (Ng et al., 1988) underscores its presumed importance in neuronal plasticity and extends the role of B-50 to the human brain.

Several studies have been designed to localize the protein in the nervous system. In developing and regenerating neurons B-50 is detected along the entire length of outgrowing neurites, but particularly in growth cones (Oestreicher and Gispen, 1986; Meiri et al., 1986; Verhaagen et al., 1986; Gorgels et al., 1987). In adult rat brain, strong B-50 immunoreactivity in neuropil-rich regions (Oestreicher et al., 1981; Oestreicher and Gispen, 1986; Benowitz et al., 1988) appears to represent immunoreactive presynaptic terminals (Gispen et al., 1985*a*). These studies strongly suggest the involvement of B-50 in neurite outgrowth.

In addition, evidence has been obtained that B-50 plays a feedback role in phospholipid-mediated transmembrane signal transduction. In rat brain, the protein is a prominent substrate of protein kinase C (PKC)[1] in presynaptic membranes (Zwiers et al., 1976, 1980) as well as in nerve growth cone membranes (De Graan et al., 1985; Van Hooff et al., 1988). In both these membrane fractions the degree of B-50 phos-

1. *Abbreviations used in this paper*: dbcAMP, dibutyryl cyclic AMP; GAR, goat anti-rabbit IgG; NGF, nerve growth factor; PA, protein A; PKC, protein kinase C; PVP-10, polyvinyl-pyrrolidone-10.

phorylation is presumed to impose a feedback regulation on the amount of phosphatidylinositol 4,5-bisphosphate, available for receptor-mediated breakdown (Gispen et al., 1985*b*; Van Hooff et al., 1988). These studies indicate the possible importance of posttranslational modifications, such as phosphorylation, for B-50 function.

Rat pheochromocytoma cells, clone PC12, have been used extensively as a model system for neuronal differentiation, since in the presence of nerve growth factor (NGF) these cells acquire a phenotype resembling sympathetic neurons (Greene and Tischler, 1976). Recently, we have demonstrated that PC12 cells express a protein that is indistinguishable from rat brain B-50 (Van Hooff et al., 1986). During NGF-induced differentiation of the cells, the levels of B-50 increase (Van Hooff et al., 1986) due to elevated levels of its mRNA (Karns et al., 1987; Basi et al., 1987). In further analogy to B-50 in rat brain, B-50 in PC12 cells can be phosphorylated by exogenous PKC (Van Hooff et al., 1986) and is a substrate for endogenous PKC in these cells (Van Hooff, unpublished results).

The prominent association of B-50 with growth cones and its role in phospholipid-mediated transmembrane signal transduction suggests that B-50 functions at the level of the plasma membrane. The PC12 cell system provides an attractive model system to study these features of B-50, since the cell line can be cultured in the presence of NGF, thereby resembling sympathetic neurons, but also in the absence of NGF, thereby resembling chromaffin cells. To obtain a better insight in the putative role of B-50 in neurite formation, we have studied the ultrastructural localization of B-50 in both proliferating and differentiating PC12 cells using cryoultramicrotomy in combination with immunogold labeling. The results indicate that in untreated cells B-50 is mainly found in lysosomal structures and the Golgi apparatus, while differentiation is accompanied by a strong manifestation of B-50 immunoreactivity at the plasma membrane. Its prominent association with protruding plasma membranes is in line with its presumed involvement in the generation of these extensions.

Materials and Methods

Cell Culture

Rat pheochromocytoma PC12 cells (passage 15–35) were cultured routinely in DME, supplemented with 10% FCS and 5% heat-inactivated horse serum (Flow Laboratories, Inc., McLean, VA), in a humidified incubator at 37°C and 7.5% CO_2. Cells were dislodged from the tissue-culture plastic (Costar, Cambridge, MA) by resuspension, passaged once a week, and seeded as single cells by passing them through a needle. For cryoultramicrotomy, single cells were seeded on poly-L-lysine (50 μg/ml)/gelatin (0.1%)-coated, 150-cm^2 plastic tissue-culture dishes (Costar) or on poly-L-lysine-coated, 12-mm Millicell-CM culture plate inserts (Millipore Corp., Bedford, MA) at a density of 25,000–70,000 cells per cm^2. Medium was replaced 24 h after plating with fresh serum containing medium or chemically defined N1 medium (5 μg/ml transferrin, 100 μM putrescine, 30 nM selenium, 20 nM progesterone, 0.01% BSA, and 5 μg/ml insulin; Bottenstein, 1983) with either 50 ng/ml NGF (Boehringer GmbH, Mannheim, Germany) or 1 mM dibutyryl cyclic AMP (dbcAMP) (Sigma Chemical Co., St. Louis, MO). Cells were then cultured 48 h before fixation.

Fixation and Cryosectioning of Scraped PC12 Cell Pellets

Cells were fixed for 60 min at room temperature in 0.1% acrolein/2% paraformaldehyde in PBS, pH 7.4, or in 0.5% glutaraldehyde/2% paraformaldehyde in PBS. Cells were subsequently incubated in 50 mM glycine in PBS for 60 min and were collected by scraping from the culture dish with a single-edged razor blade. Scraped cells were pelleted and embedded in 10% gelatin in PBS, followed by fixation overnight at 4°C in the previously mentioned fixative. Gelatin blocks were cryoprotected by immersion in PBS containing 0.3 M sucrose, 3.7% polyvinyl-pyrrolidone-10 (PVP-10, Sigma Chemical Co.) for 30 min; 0.6 M sucrose, 7.5% PVP-10 for another 30 min; and 1.15 M sucrose, 15% PVP-10 overnight at 4°C. 70-nm cryosections were prepared from the gelatin blocks and mounted on nickel grids covered with a carbon-coated parlodion film. Grids with thawed sections were placed upside down on droplets during immunogold labeling.

Fixation and Cryosectioning of PC12 Cell Monolayers on Millicell-CM

PC12 cells, grown on polylysine/gelatin-coated cellulose filters (Van Buskirk et al., 1988), were fixed in 2% paraformaldehyde/0.1% acrolein in PBS for 60 min, incubated for another 60 min in 50 mM glycine in PBS, and embedded in 20% gelatin in PBS. Cellulose filters, sandwiched between two differently colored gelatin layers, were excised from the insert and cut in small squares. These gelatin blocks were incubated in graded sucrose/PVP-10 and prepared for cryosectioning as described for scraped cell pellets. All these steps were performed at room temperature. The sections were rinsed for 60 min with warm PBS (37°C) to remove the gelatin matrix.

Immunogold Labeling of Cryosections

Grids with thawed cryosections were washed once in PBS containing 50 mM glycine for 15 min and three times in PBS with 0.2% gelatin and 0.5% BSA for 30 min. Grids were incubated overnight at 4°C with affinity-purified anti-B-50 IgGs (antiserum 8420; dilution 1:300; cf. Oestreicher et al., 1983; Oestreicher and Gispen, 1986; Verhaagen et al., 1986), followed by a 2-h incubation at room temperature with protein A (PA) coated with colloidal gold (PA-gold; diameter, 10.5–12 nm) or goat anti-rabbit IgG (GAR)-gold complexes (GAR-gold; diameter, 9.3 nm; Van Bergen en Henegouwen and Leunissen, 1986). Each incubation was followed by four washes in PBS/gelatin/BSA for 30 min. After immunoincubation, sections were fixed for 10 min in 1% glutaraldehyde in PBS and rinsed four times in bidistilled water. Subsequently, sections were counterstained for 10 min in 2% neutralized uranyl acetate, pH 7.0, and rinsed three times with bidistilled water, followed by incubation for 12–14 min in 1.1% tylose/0.5% uranyl acetate, pH 4.0, on ice. Sections were air dried and B-50 immunoreactivity was examined in Philips Electronic Instruments, Inc. (Mahwah, NJ) EM 201 and EM301 electron microscopes.

Immunofluorescence Light Microscopy

PC12 cells were grown for 48 h on polylysine (50 μg/ml)-coated glass coverslips in control, serum-containing medium or in 50 ng NGF/ml N1 medium (see cell culture section). Cells were rinsed with PBS and either exposed to affinity-purified anti-B-50 IgGs (2 μg/ml) in PBS or fixed in 2% paraformaldehyde/50 mM phosphate buffer, pH 7.4, containing 0.1 M lysine and 0.2% sodium periodate for 1 h. During antibody incubation some cells detached, but their morphology did not change. Subsequently, the immunolabeled cells were fixed, while the fixed cells were incubated with the anti-B-50 antibody solution for 1 h. Anti-B-50 antibodies were visualized using fluorescein-conjugated GAR in PBS (1:60, DAKOPATTS, Copenhagen, Denmark). All steps were performed at room temperature and followed by three washes in PBS.

Results

Ultrastructure of PC12 Cells

Cryosections of control and NGF-treated PC12 cells reveal striking morphological differences (Fig. 1), comparable to those described by Tischler et al. (1983) and Luckenbill-Edds et al. (1979). Control cells cultured in serum-containing medium have round appearances and contain large nuclei which are surrounded by cytoplasm with some electron-dense core vesicles identified as chromaffin granules (Luckenbill-Edds et al., 1979; Roda et al., 1980) and larger dark spots reminiscent of lysosomes (Fig. 1 *A*). Their plasma

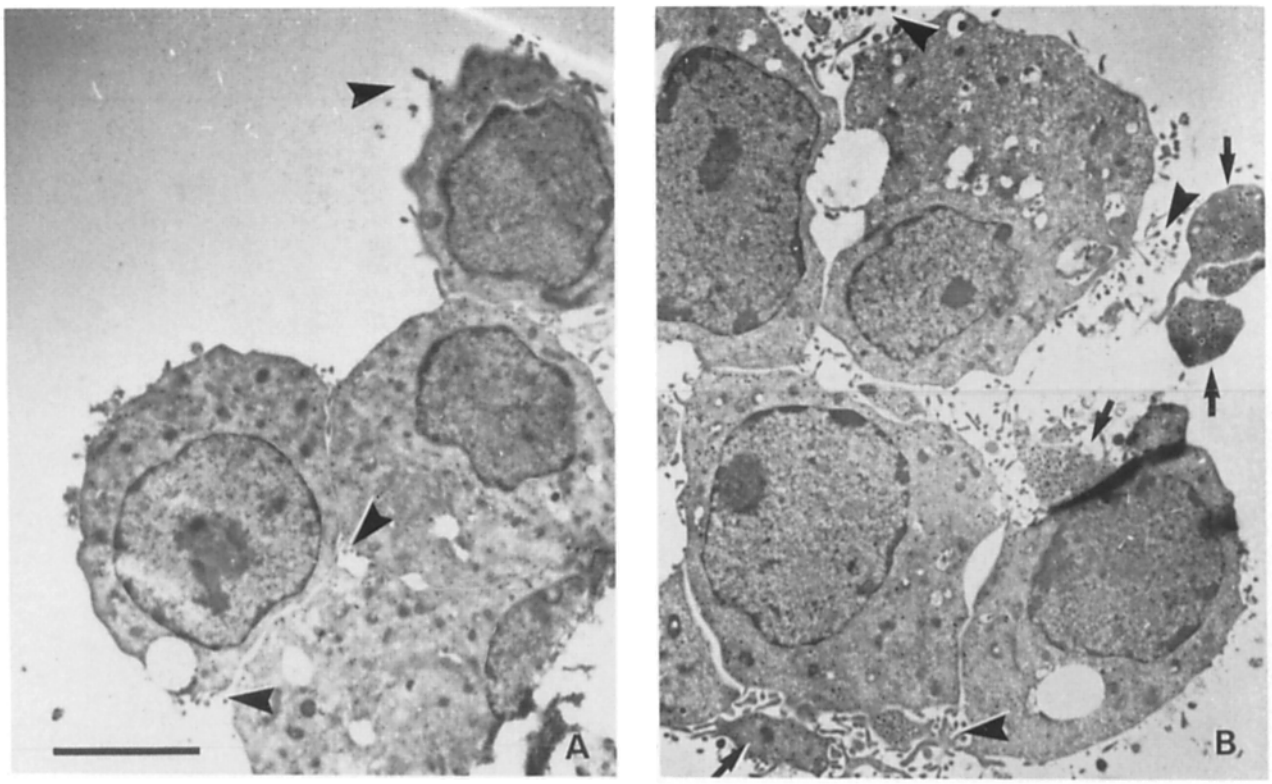


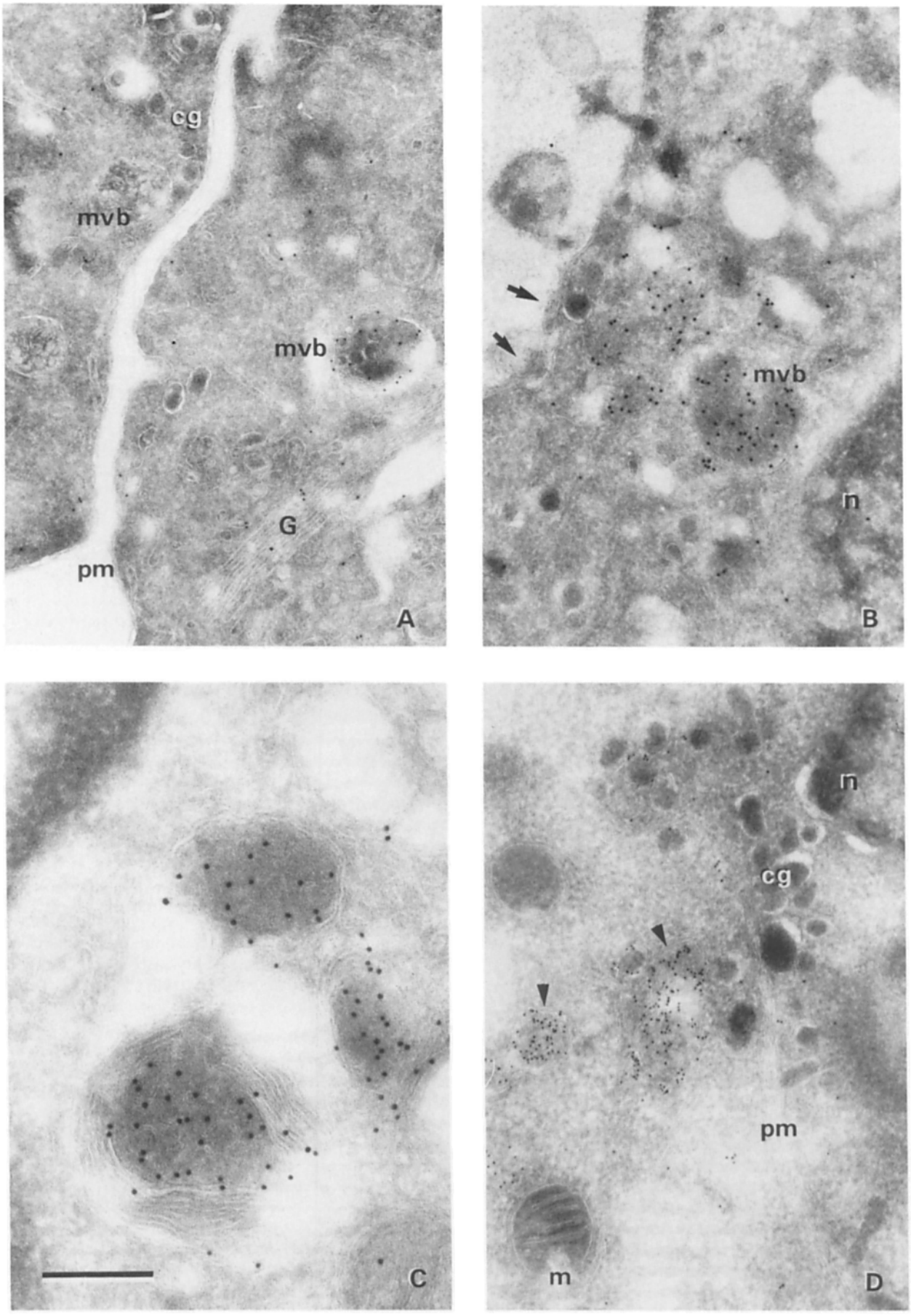
Figure 1. Survey micrograph of cryosectioned, scraped-off PC12 cell pellets, after culturing for 48 h in control medium (*A*) or in 50 ng/ml NGF (*B*). Note the smooth plasma membranes of untreated control cells with rare microvilli (*A*, *arrowheads*). NGF-treated cells display irregularly folded surfaces from which numerous microvilli are sprouting (*B*, *arrowheads*). Cross-sectioned neurites (*B*, *arrows*) are characterized by densely packed chromaffin granules. Bar, 7 μm.

membranes form regular smooth cell surfaces with only a few short microvilli. When PC12 cells are exposed to NGF for 48 h, they become irregularly shaped and exhibit numerous cytoplasmic extensions (Fig. 1 *B*), comparable to those described in monolayer cultures (Luckenbill-Edds et al., 1979). These protrusions range from microvilli to longer neuritic extensions that are characterized by dense packages of chromaffin granules. These protrusions often seem to emanate clusterwise from a restricted peripheral site of the cell body and may therefore represent initiating sprouts that precede the development of neurites.

B-50 Localization in PC12 Cell Pellets

Immunolabeling of the cryosections with affinity-purified anti-B-50 antibodies is visualized with GAR or PA both complexed to colloidal gold. In proliferating untreated PC12 cells, B-50-associated immunogold is concentrated in intracellular organelles of the lysosomal family, such as multivesicular bodies (Fig. 2, *A* and *B*) and secondary lysosomes (Fig. 2 *C*). The Golgi apparatus is only slightly labeled, whereas nuclei and mitochondria are not. Chromaffin granules are virtually devoid of label, although sometimes gold particles were found on their surrounding membranes (Fig. 2 *D*). By comparison of the labeling of undifferentiated and differentiated PC12 cells, the most striking difference is seen at the plasma membrane. Plasma membranes of control cells are scarcely labeled (Fig. 2), whereas membranes of NGF-differentiated cells reveal strong B-50 immunoreactivity (Fig. 3). Although sometimes high label intensity is observed on smooth parts of the NGF-treated cell surfaces (Fig. 3 *A*), the majority of the gold particles is concentrated on the plasma membrane of microvilli emerging from the cell body (Fig. 3 *C*). The plasma membrane surrounding sprouts of a neurite (Fig. 3 *D*) and long cytoplasmic protrusions (Fig. 3 *B*) particularly reveal high label intensities. Labeling is much higher on the microvilli when compared to adjacent nonsprouting regions of the plasma membrane (Fig. 3, *C* and *D*). In neurites, B-50 immunoreactivity is found at the plasma membrane but also diffusely spread throughout the cytoplasmic compartment (Fig. 3 *E*). Part of the diffuse gold labeling appears to be associated with chromaffin granules.

PC12 cells have been fixed either in glutaraldehyde for optimal conservation of the ultrastructure or in acrolein to protect immunoreactive sites (Boonstra et al., 1985). Light microscopic comparison of B-50 immunofluorescence in glutaraldehyde- and acrolein/paraformaldehyde-fixed preparations did not show any qualitative difference in labeling (results not shown). The shift in ultralocalization of B-50 immunoreactivity of control and NGF-exposed cells is consistent in both glutaraldehyde- and acrolein-fixed preparations (cf. Fig. 2, compare *A* to *B–D*; and Fig. 3, compare *C* and *E* to *A*, *B*, and *D*). Association of gold label with intracellular organelles in NGF-differentiated cells is essentially the same as in control, untreated PC12 cells (Fig. 3 *C*). Multivesicular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bodies and lysosomes are labeled, Golgi stacks are weakly labeled, but mitochondria and nuclei remain free of label. In addition, relatively high, diffuse staining for B-50 was observed in the cytoplasm of NGF-induced neurites, in which large numbers of chromaffin granules accumulate (Fig. 3, *D* and *E*). Some chromaffin granules appear to carry gold particles on their surrounding membranes although most of them remain unlabeled. Control incubations with preimmune IgGs (not shown) are devoid of gold particles, except slight background labeling of the mitochondrial matrix and the cytosolic compartment of glutaraldehyde-fixed cells. When cultured cells of nonneuronal origin, i.e., fibroblasts, were processed similarly for ultrastructural B-50 immunostaining, no labeling for B-50 could be detected (not shown). Considering the monospecificity of the polyclonal anti-B-50 IgGs for rat brain B-50 (Oestreicher et al., 1983; Oestreicher and Gispen, 1986), for B-50 in peripheral nerves (Verhaagen et al., 1986), and for B-50 in PC12 cells (Van Hooff et al., 1986), it is reasonable to assume that the gold particles are specifically associated with B-50 molecules. We therefore conclude that the pronounced labeling of the plasma membrane during NGF-induced differentiation of PC12 cells represents an altered localization of B-50 in these cells.

B-50 Localization in PC12 Cells Cultured on Cellulose Filters

Growth cones could not be identified with certainty in the scraped-off cell pellets. It is likely that many are ripped off during the scraping procedure. To study B-50 distribution in extending neurites with intact growth cones, PC12 cells have been cultured on Millicell cellulose filters in the presence of NGF. Cryosections from whole filter mounts, sectioned in the plane of the filter, show spreading PC12 cells that extend microvilli and neurites into the filter and towards each other (Fig. 4 *A*). Many chromaffin granules migrate into neuritic extensions all the way into the growth cone, while a few are seen in the cell bodies (Fig. 4 *B*). Some B-50 immunoreactivity is associated with the plasma membrane of the neurite, but strongest labeling is found in thin filopodia extending from the growth cones (Fig. 4, *C* and *D*) and in microvilli extending from the cell body (Fig. 4 *D*). Labeling of the lysosomal organelles, as described for scraped-cell pellets, is relatively low in these preparations.

PC12 cells grown on cellulose filters in control, serum-containing medium (not shown) exhibit abnormal sprouting and extend some neurite-like processes in contrast to the scraped-off control cell population as shown in Fig. 1 *A*. This morphological differentiation may be induced by the cellulose filter substrate, as recently described (Van Buskirk et al., 1988). These protrusions sometimes resemble neurites and are also strongly labeled for B-50. Labeling is consistently most pronounced at the distal, thinnest parts of the extensions.

B-50 Localization in dbcAMP-differentiated PC12 Cells

Exposure of PC12 cells to 1 mM dbcAMP induces the formation of neurite-like extensions similar to NGF-stimulated neurite outgrowth, albeit via a different mechanism (Heidemann et al., 1985; Boonstra et al., 1987). Immunolabeling of PC12 cells in cryosections of scraped-off cell pellets after 48 h exposure to dbcAMP, reveals a similar distribution of B-50 immunoreactivity (Fig. 5) as compared to NGF-differentiated cells. The plasma membrane on microvilli displays the highest label density. Multivesicular bodies and lysosomes are labeled and diffuse labeling is seen throughout the neuritic extensions (Fig. 5 *A*). Furthermore, a lamellipodium-like protrusion from which filopodia extend is decorated with gold particles on its plasma membrane (Fig. 5 *B*). Some diffuse labeling is seen, sometimes associated with large clear vesicles that have been suggested to represent a membrane pool for insertion at growth sites (Pfenninger, 1987). Apparently, irrespective of the inducing agent, cytoplasmic extensions that precede or accompany neurite outgrowth bear strong B-50 immunoreactivity.

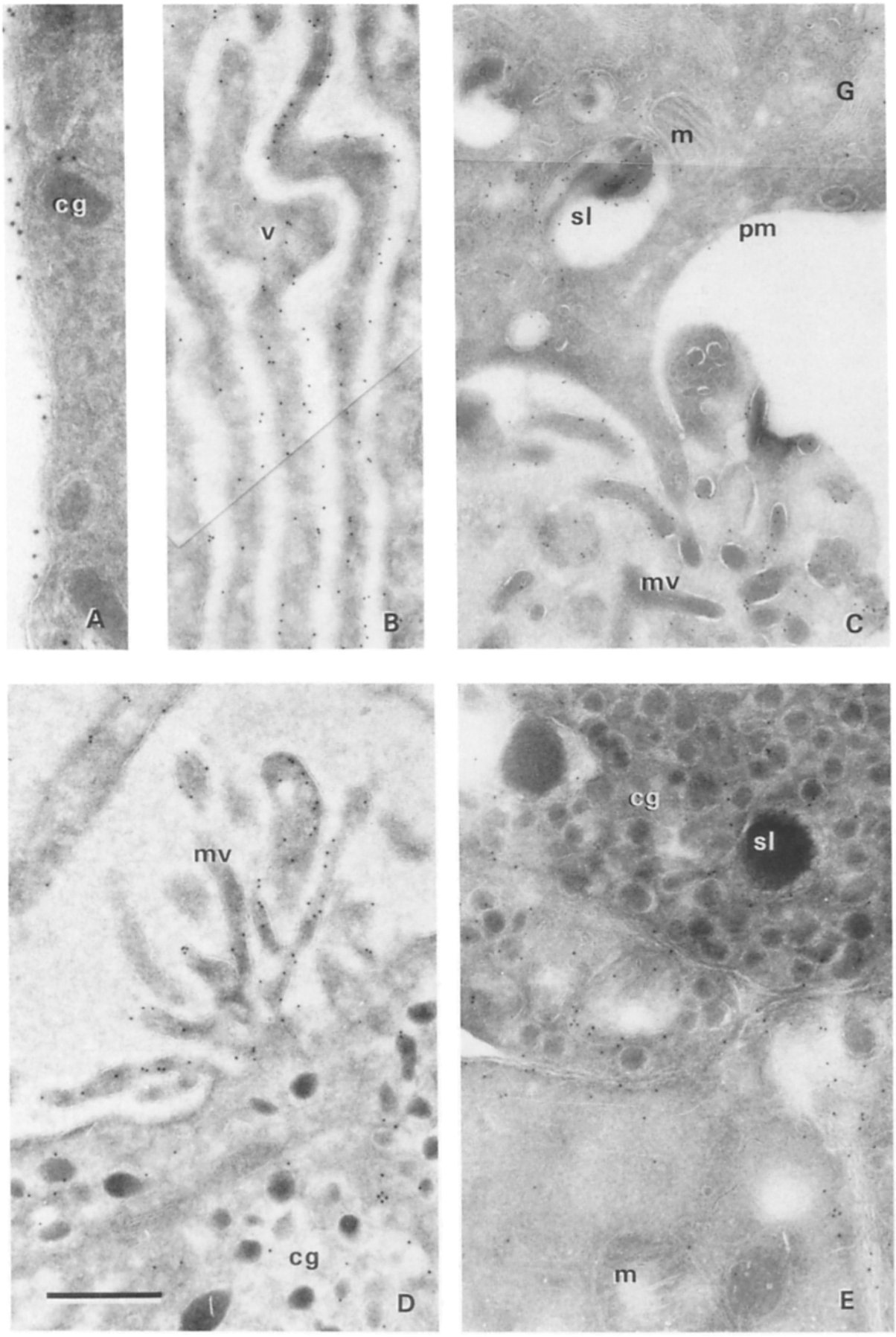
B-50 at the Inner Face of the Plasma Membrane

Careful observation of the plasma membrane labeling of NGF-differentiated cells shows a considerable portion of the gold particles to be localized at the exterior of the cell surface (Fig. 3 *A*). This could imply that part of the B-50 molecules is exposed at the outer face of the membrane. However, analysis of strongly labeled membranes by stereo electron micrography revealed that most of the gold particles float above the section surface of the plasma membrane. In a second approach, living PC12 cells were incubated with anti-B-50 antibodies in PBS, fixed, and probed with a fluorescein-conjugated secondary antibody. No immunofluorescence could be detected, neither in control nor in NGF-differentiated cells (Fig. 6, *A* and *B*, *pre*). In contrast, probing fixed, and thereby permeabilized, PC12 cells with the same antibody solution clearly demonstrates high B-50 immunofluorescence (Fig. 6, *C* and *D*, *post*). Apparently, at least most of the B-50 molecules are located at the inner side of the plasma membrane. These results are in agreement with the observation that B-50 is located at the inner face of the presynaptic membrane (Gispen et al., 1985*a*).

Discussion

In this paper we present the ultrastructural localization of B-50 in PC12 cells, using cryoultramicrotomy in combina-

Figure 2. B-50 immunoreactivity in untreated PC12 cells. Cryosections of scraped-off cell pellets, fixed in glutaraldehyde (*A*) or in acrolein (*B*, *C*, and *D*), followed by immunogold labeling with anti-B-50 IgGs and PA-gold (*A*, *B*, and *C*) or GAR-gold (*D*). (*A*) Two apposed cells with plasma membranes (*pm*) and chromaffin granules (*cg*) free of gold label. One of the multivesicular bodies (*mvb*) is strongly labeled for B-50 and some gold is associated with the Golgi apparatus (*G*). (*B*) Strong immunolabeling of a multivesicular body (*mvb*). Note the unlabeled microvilli (*arrows*). (*C*) Gold particle concentration in secondary lysosomes. (*D*) High label concentration is associated with a cluster of vesicular structures with amorphous content (*arrowheads*). Note that the chromaffin granular (*cg*) membranes are rarely labeled and that the nucleus (*n*), mitochondrion (*m*), and the plasma membrane (*pm*) are devoid of label. Bar: (*A*) 0.54 μm; (*B*) 0.48 μm; (*C*) 0.24 μm; and (*D*) 2.3 μm.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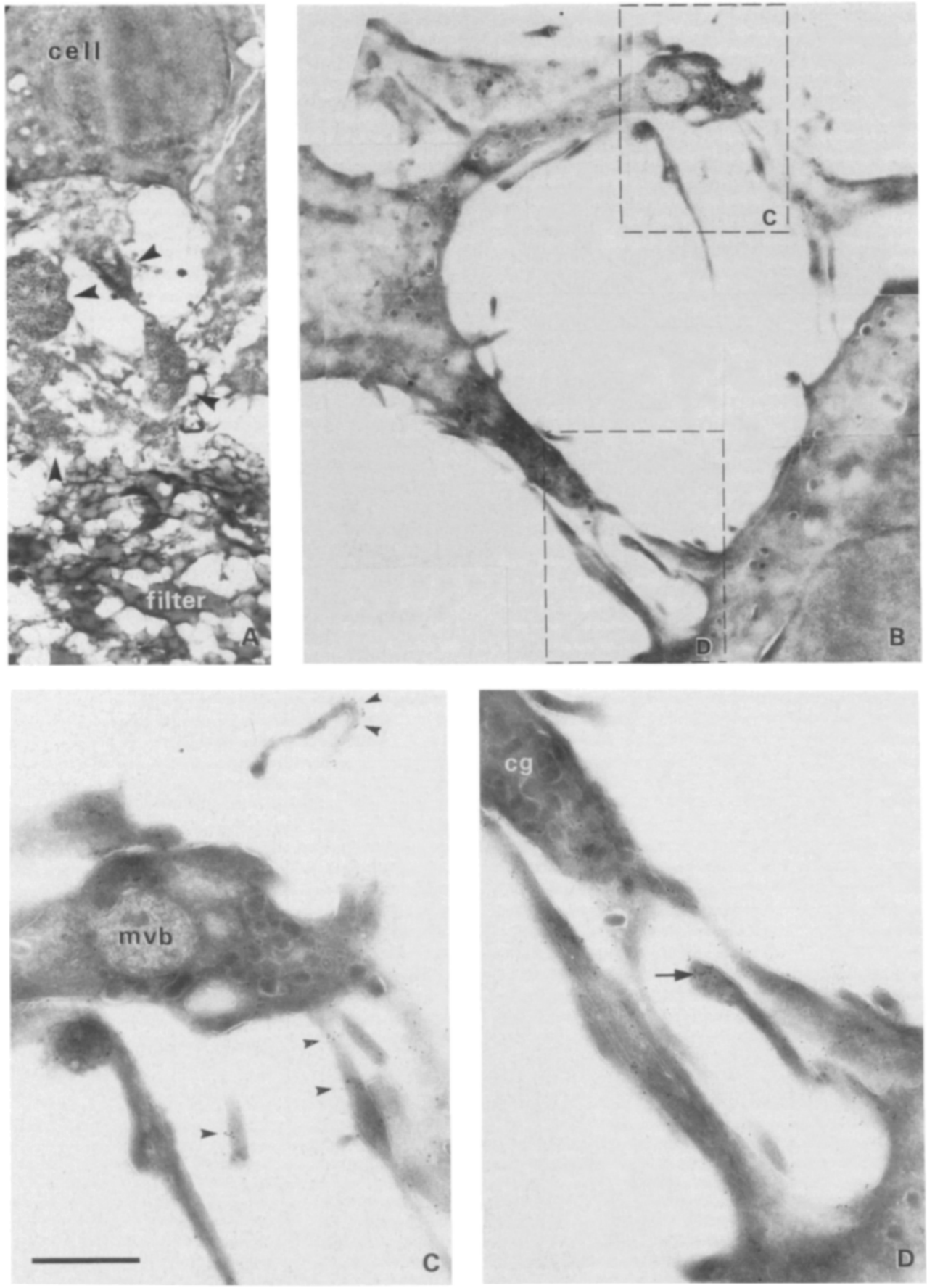
tion with immunogold labeling. In undifferentiated PC12 cells B-50 immunoreactivity is mainly associated with lysosomal structures, such as multivesicular bodies and secondary lysosomes. Interestingly, the plasma membrane exhibits virtually no B-50 immunoreactivity, in contrast to earlier observations using immunofluorescence microscopy that yields a lower resolution (Van Hooff et al., 1986; Fig. 6 *C*). Furthermore, immunoreactivity is observed in the Golgi apparatus. B-50 may either pass the Golgi apparatus through its synthesis pathway or on membrane recycling. One possibility would be that B-50 arrives via the Golgi stacks at the plasma membrane, from which it is removed by membrane recycling and subsequent breakdown in lysosomes. The virtual absence of B-50 immunoreactivity from the plasma membrane could then point at a very short residence time at the plasma membrane. Alternatively, its synthesis pathway could be a short-cut from the Golgi apparatus to lysosomal structures; undifferentiated PC12 cells would not require B-50 at the plasma membrane.

Upon exposure of PC12 cells to NGF, their plasma membranes become irregularly folded, many sprouts emerge, and finally neurites extend, bearing growth cones at their tips. Concomitant with neuritogenesis, B-50 protein levels (Van Hooff et al., 1986) and B-50 mRNA levels (Karns et al., 1987; Basi et al., 1987) both increase. Interestingly, this increase in B-50 expression is accompanied by a shift in its localization. Part of the B-50 immunolabel is associated with the cytoplasmic organelles as in undifferentiated cells, but the majority becomes associated with protruding regions of plasma membranes (Fig. 3). B-50 is consistently most pronounced at the most distal and thinnest parts of neurites. After 120 h NGF treatment, PC12 cells exhibit more neurites, but the distribution of the B-50 immunolabel is comparable to 48-h-treated cells (results not shown). Neurites grow by insertion of new membrane elements at the growth cone (Pfenninger, 1987; Letourneau, 1985), specifically by sending out lamellipodia and filopodia (Aletta and Greene, 1988). These are the most densely B-50-labeled structures in differentiating PC12 cells (Figs. 3 *B* and 5 *B*). Apparently, the increased amount of B-50 is incorporated in newly inserted membrane regions that become highly enriched in B-50. In lamellipodia and filopodia B-50 is sometimes associated with intracellular clear vesicles. It is assumed that these vesicular structures represent membrane pools to be inserted during neurite elongation (Pfenninger, 1987). In neurites, a rather diffuse B-50 labeling is observed throughout the cytoplasm and on the plasma membrane. Subcellular fractionation studies on peripheral neurons (Snipes et al., 1987), central neurons (Cimler et al., 1987), and also on PC12 cells, report that part of the B-50 is recovered in high speed supernatants. Maybe the diffuse labeling represents B-50 molecules associated with very small membranous organelles not detectable by the methods used, but it can not be excluded that B-50 is freely floating in the cytoplasm. The great abundance of chromaffin granules in neurites complicates the exact label localization. Part of the label is associated with chromaffin granules. However, not all granules are consistently labeled for B-50.

Karns et al. (1987) report that dbcAMP exposure, like NGF-exposure, also increases the expression of GAP43 (B-50), while highest expression is accomplished by the combined treatment of NGF and dbcAMP. This synergism is in agreement with the reported synergistic effects of NGF and dbcAMP on neurite formation (Heidemann et al., 1985; Richter-Landsberg and Jastorff, 1986; Boonstra et al., 1987). Interestingly, the mechanism underlying NGF-induced outgrowth seems to parallel dbcAMP-mediated outgrowth only partly (Greene et al., 1986; Richter-Landsberg and Jastorff, 1986). However, both agents induce a qualitatively comparable translocation of B-50 into sprouting membranes and neurites. Furthermore, PC12 cells grown on cellulose filters display moderate neuritic sprouting in the absence of NGF (results not shown). This sprouting is presumably induced by the adhesive and differentiative properties of these filters (Van Buskirk et al., 1988). These neurite-like extensions that grow into the filter also carry B-50 labeling on their plasma membranes. Thus, NGF, dbcAMP, and cellulose filters, that accomplish neurite outgrowth partly via different mechanisms, all induce a shift of B-50 to the plasma membrane. We therefore conclude that the appearance of B-50 immunoreactivity on the plasma membrane is a typical phenomenon associated with morphological differentiation in PC12 cells.

Most of the B-50 immunolabel at the plasma membrane is located at the cytoplasmic face. This is consistent with early immunoelectron microscopy, which shows B-50 to be located at the inner face of presynaptic membranes isolated from adult rat brain (Gispen et al., 1985*a*). However, part of the B-50 immunogold particles apparently extends into the extracellular space (Fig. 3). In search of possible externally exposed B-50 molecules, we incubated living PC12 cells with the B-50 antibody. These prefixation incubations of control and NGF-differentiated cells revealed no immunoreactive sites at the external face of the plasma membrane in contrast to the pronounced immunofluorescence in fixed, permeabilized cells (Fig. 6). Thus, the apparent localization of part of the gold particles on the outer cell surface must be due to "plane of section" artifacts (Boonstra et al., 1985) or to extension of the antibody–PA–gold complex. If B-50 is

Figure 3. B-50 immunoreactivity in NGF-treated PC12 cells. PC12 cells were exposed to NGF for 48 h, fixed in acrolein (*A*, *B*, and *D*) or glutaraldehyde (*C* and *E*), and immunolabeled with anti-B-50 IgGs and PA-gold. (*A*) Smooth plasma membrane with intense gold labeling. (*B*) Thin protrusions, virtually devoid of cellular organelles, that run along the somatic plasma membrane. Note the high label intensity along their plasma membranes and some gold associated with clear vesicles (*v*). (*C*) Intracellular labeling is associated with secondary lysosomes (*sl*) and some with the Golgi apparatus (*G*), but not with mitochondria (*m*). Plasma membranes of the numerous microvilli (*mv*), that extend from the cell body into the extracellular space are strongly labeled. The somatic plasma membrane (*pm*) is only weakly labeled. (*D*) Dense labeling of microvilli (*mv*) emerging from a neurite that contains numerous chromaffin granules (*cg*). Note that gold particles are diffusely spread throughout the neurite. (*E*) Cross section through a neurite adjacent to a cell body. Diffuse labeling is seen in the neurite, amongst numerous chromaffin granules (*cg*) and secondary lysosomes (*sl*). Bar: (*A*) 0.23 μm; (*B*) 0.59 μm; (*C*) 0.74 μm; (*D*) 0.51 μm; and (*E*) 0.58 μm.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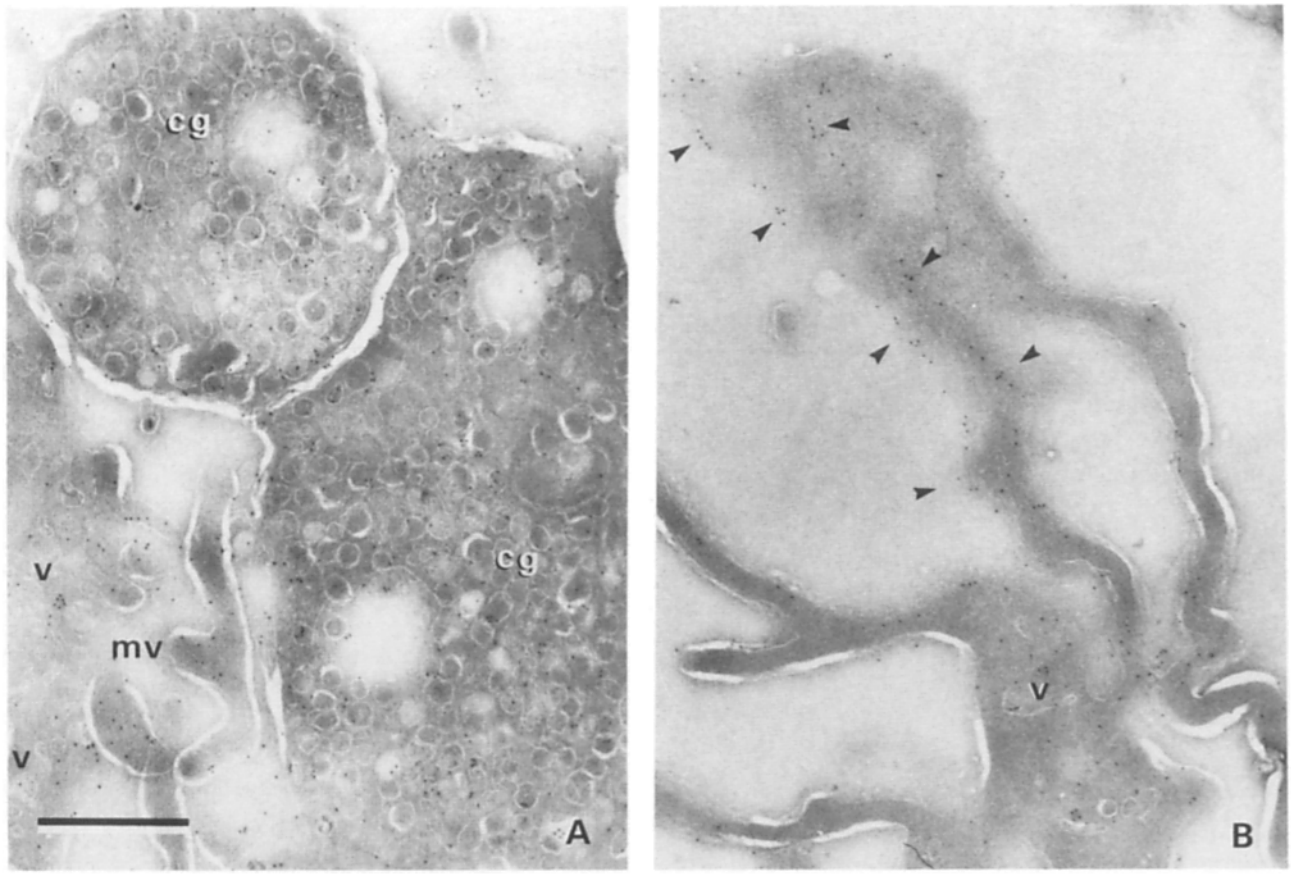

Figure 5. B-50 immunoreactivity in PC12 cells exposed for 48 h to 1 mM dbcAMP. Cells were fixed in acrolein and immunolabeled for B-50 using PA-gold. (*A*) Two closely apposed neurites scattered with B-50 labeling. Some of the gold is associated with chromaffin granules (*cg*). Membranes of microvilli (*mv*) are also strongly labeled. (*B*) Lamellipodium-like structure with filopodia that are heavily decorated with gold label (*arrowheads*). Note some diffuse intracellular labeling associated with clear vesicles (*v*). Bar: (*A*) 0.79 μm; (*B*) 0.88 μm.

anchored in the membrane by some plasma membrane component, this could also contribute to the distance of the label from the antigenic site. Purification studies (Zwiers et al., 1980; Oestreicher et al., 1983) and analysis of the primary structure of the protein (Nielander et al., 1987) have shown that B-50 is not an intrinsic membrane protein, but a very hydrophilic protein with hardly any hydrophobic domains for association with membranes. How B-50 is anchored to the membrane awaits further investigation.

Strong evidence exists that the increased turnover of polyphosphoinositides (Traynor, 1984; Burstein et al., 1985) and PKC phosphorylation (Hall et al., 1988) are essential mediators of NGF-induced neurite formation in PC12 cells. Since B-50 is a substrate for PKC in these cells (Van Hooff et al., 1986), its phosphorylation may be involved in transducing the NGF–receptor signal to neurite extension. In rat brain membranes, the degree of B-50 phosphorylation is presumed to be involved in transmembrane signal transduction (Gispen et al., 1985*b*): breakdown of phosphatidylinositol 4,5-bisphosphate generates the second messengers inositol 1,4,5-trisphosphate, which mobilizes intracellular calcium, and diacylglycerol which stimulates PKC. The activated PKC increases the degree of B-50 phosphorylation. In its turn, phospho–B-50 inhibits the formation of phosphatidylinositol 4,5-bisphosphate and hereby decreases its availability for further receptor-mediated breakdown. It is tempting to speculate that such a relationship between B-50 phosphorylation and the polyphosphoinositide cascade also exists in PC12 cells. Vicentini et al. (1985) presented evidence that feedback regulation of the receptor-coupled polyphosphoinositide response is mediated by PKC phosphorylation in PC12 cells.

Figure 4. B-50 immunoreactivity in 48-h NGF-differentiated PC12 cells, cultured on Millicell cellulose filters and acrolein fixed in situ. Specimens, cryosectioned parallel to the filter, have been stained for B-50 using PA-gold. (*A*) Overview micrograph, showing filter grains (*filter*), invaded by neurites (*arrowheads*) that extend from an overgrowing PC12 cell (*cell*). (*B*) Composite micrograph of a cell border from which two neurites extend. Note that chromaffin granules tend to accumulate in the neurites. (*C*) Higher magnification of the growth cone on the upper neurite in *B*, illustrating that gold labeling of the plasma membrane is highest in filopodia (*arrowheads*). (*D*) Higher magnification of the lower neurite in *B*. Some diffusion labeling is found in the growth cone body around chromaffin granules (*cg*). High gold particle density is seen on filopodia that emerge from the growth cone, and on microvilli that protrude from the membrane of the adjacent cell body. Note the intracellular label in these thin protrusions, associated with clear vesicles (*arrow*). Bar: (*A*) 5.76 μm; (*B*) 1.7 μm; (*C*) 0.72 μm; and (*D*) 0.88 μm.

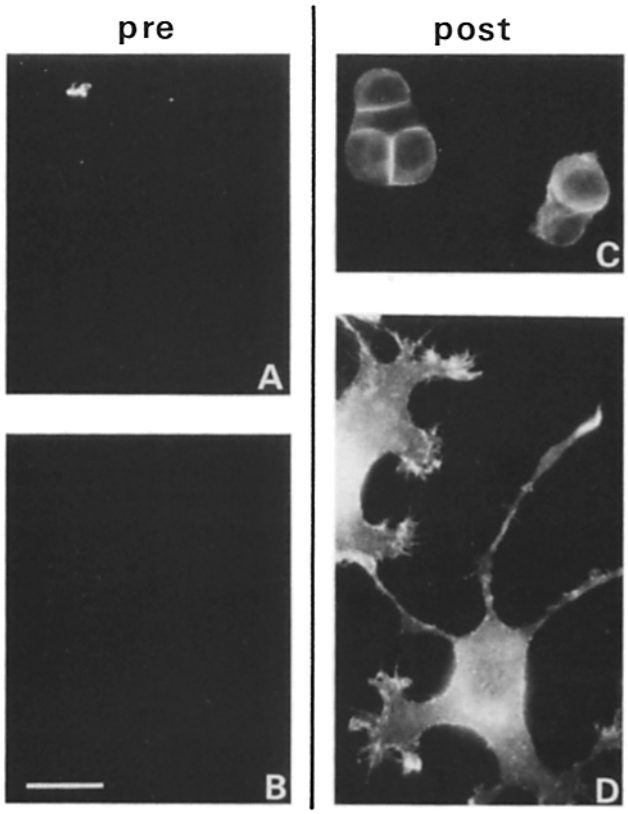

Figure 6. B-50 immunofluorescence in control (*A* and *C*) and 48-h NGF-differentiated (*B* and *D*) PC12 cells. Living cells were incubated with anti-B-50 antibodies before fixation (*pre*). Fixed, permeabilized cells were incubated with the same antibody solution (*post*). Both preparations were probed with fluorescein-conjugated GAR. Note that the cells do not stain for B-50 unless permeabilized. Bar, 4.4 μm.

During preparation of this manuscript, it came to our attention that an atypical calmodulin-binding protein P-57, is identical to GAP43 (= B-50, Cimler et al., 1987). It is suggested that P-57 may act as a local concentrator of calmodulin within neurons. Calmodulin is a calcium-binding protein that regulates free calcium levels and mediates calcium/calmodulin-dependent kinase and phosphatase activities. Calmodulin binds to P-57 under low calcium conditions in vitro, but is released from P-57 by increases in calcium levels (Andreassen et al., 1983). This unexpected feature of B-50 sheds new light on its presumed function. This calmodulin-binding property may indicate that B-50 is expressed at the plasma membrane during neurite outgrowth to "fine tune" the concentration of free calmodulin and calcium. This might represent an important contribution to neurite formation, for critical regulation of free calcium levels is a prerequisite for effective neurite extension (Kater et al., 1988). Since the B-50 antibody also recognizes the calmodulin-associated form of B-50 (Oestreicher and De Graan, unpublished results), the immunoreactivity represents the whole B-50 population, whether (de-)phosphorylated or bound to calmodulin. In the PC12 cells little vesicular organelles, so-called "calsiosomes", have been proposed as intracellular calcium stores and as targets for inositol trisphosphate-mediated calcium mobilization (Volpe et al., 1988). By the methods used we were not able to identify these, but it is possible that the apparent cytoplasmic localization of B-50 in growing neurites is in fact associated with this type of organelle. This would certainly be interesting in view of a possible implication of B-50 and its phosphorylation in the regulation of free cellular calcium. If B-50 is indeed present free in the cytosol of neurites, then what regulates its membrane association–dissociation dynamics? Two prominent presynaptic phosphoproteins, the acidic 87-kD PKC substrate and synaptic vesicle-associated synapsin I, are both released from membranes upon phosphorylation (Aderem et al., 1988; Schiebler et al., 1986). A similar mechanism for B-50 association with the plasma membrane being regulated by PKC phosphorylation would be an exciting speculation. However, in this study we used polyclonal anti-B-50 antibodies, which do not distinguish between phospho- and dephospho-B-50. Thus, the immunoreactivity described here does not does inform us about the phosphorylation state of B-50.

Taken together, we have shown that the acquisition of neuronal features by PC12 cells is accompanied by a translocation of B-50 to the plasma membrane. High B-50 levels, especially at sites of protrusion, emphasize its putative role in neuritogenesis. Considering the presumed involvement of PKC in neurite outgrowth, phosphorylation of its growth-associated substrate B-50 during this process should certainly merit further investigation.

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