Three-dimensional, Computer-tomographic Analysis of Membrane Proteins (TrkA, caveolin, clathrin) in PC12 Cells

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Signaling of nerve growth factor (NGF) and its receptor (TrkA) promotes neuronal differentiation, synapse formation and survival. It has been known that the complex of NGF and TrkA is internalized into the cytoplasm and transported for further signal transduction, but the ultrastructural information of this process is virtually unknown. In order to clarify the relationship between the internalization of TrkA and the membrane-associated proteins (caveolin and clathrin), the localization and three-dimensional structures of those proteins were examined with computer tomography of high voltage electron microscopy in PC12 cells. TrkA immunoreactivity was found only at definite areas in the plasma membrane, as ring and cluster structures. Its 3D image indicated that those cluster structures contained small pits, which did not appear to be typical caveolae in size and shape. 3D images of clathrin and caveolin-1 immunoreactivities indicated that the formation of those small pits was associated with clathrin, but not with caveolin-1. Caveolin-1 immunoreactivity was found as a mesh-like structure just beneath the plasma membrane. These results suggest that clathrin rather than caveolin is mainly involved in the process of TrkA internalization, at least in differentiated PC12 cells.

Key words: TrkA, caveolin, clathrin, computer tomography, electron microscope

I. Introduction

Nerve growth-stimulating factor (NGF), the neurotrophin first identified by Cohen and Levi-Montalcini, promotes neuronal survival, neurite outgrowth and synaptic plasticity [3]. In addition to NGF, the family of neurotrophins includes several factors, such as brain-derived neurotrophic factor (BDNF), neurotrophin-3 (NT-3), and NT-4/5. They bind to two distinct classes of receptors: the low affinity receptor p75NTR and the high affinity Trk receptors. The latter Trk (tyrosine kinase receptor) family contains three members, TrkA, TrkB and TrkC, which are widely and variably expressed throughout the central and peripheral nervous systems. NGF acts on TrkA, BDNF and NT-4/5 on TrkB, and NT-3 on TrkC, although NT-3 also acts on TrkA and TrkB with low affinity.

In general, it is thought that neurons receive trophic information from their target cells by receptors located on plasma membrane, and then ligands-binding receptors are internalized into the cytoplasm and transported retrogradely for further signal transduction. A recent study [18] reported that Trk A might be localized in caveolae of plasma membrane of undifferentiated PC12 cells. Caveolae are small flask-like pits (50–100 nm in diameter) of plasma membrane and are found in numerous cell types, but are relatively rare in neurons [15, 25]. Caveolins, 20–25 kDa integral membrane proteins, are structural components of caveolae. Three members of caveolin family have been identified from different tissues. Caveolin-1 and -2 are found in various cell types such as endothelial cells, adipocytes and fibroblasts [8, 13, 19, 22]. Caveolin-3 is found only in cardiac and skeletal muscle cells [21, 23]. In addition, caveolae have been char-

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acterized biochemically as regions enriched in cholesterol and glycosphingolipids [3, 14].

PC12 cells, a cell line of pheochromocytoma artificially obtained from the rat adrenal medulla, express both TrkA and p75NTR receptors and have been extensively used as a model for studies of neuronal cell differentiation [7]. Although caveolin-1 and -2 were found in rat brain and PC12 cells [6, 9], it is still unclear whether caveolins are associated with the formation of caveolae in neurons.

In this study, in order to clarify the relationship between the internalization of TrkA and the membrane-associated proteins (caveolin and clathrin), the localization and three-dimensional structures of those proteins were examined with computer tomography of high voltage electron microscopy (HVEM) in PC12 cells. The advantages of HVEM are as follows: 1) it is capable of observation of thick specimens such as whole cells without ultrathin sectioning; and 2) 3D images from computer tomography provide valuable information about intracellular structures of membrane-associated protein complex.

II. Materials and Methods

Materials

Dulbecco's modified Eagle's medium (DMEM) and fetal bovine serum (FBS) was purchased from GIBCO. Twenty-five cm^2 cell culture flasks were purchased from Iwaki. Four well plates were purchased from NUNC™. NGF 2.5S (Tissue Culture grade) was purchased from Chemicon International (Temecula, CA). Mouse monoclonal anti-clathrin antibody was a kind gift from Prof. S. Maekawa (Kobe University, Japan). Rabbit polyclonal anti-TrkA antibody (No. 763, sc-118) was from Santa Cruz Biotechnology (Santa Cruz, CA). Mouse monoclonal anti-caveolin-1 antibody was from Research Diagnostics (Flanders, NJ). Specificity of immunoreaction was checked as follows: the specific staining was abolished after the cells were incubated with normal sera instead of the specific primary antibodies at the light microscopic level. Biotinylated anti-rabbit IgG, biotinylated anti-mouse IgG and avidin-biotin-peroxidase complex (ABC) kit were from Vector Laboratories (Burlingame, CA). PC12 cells were a kind gift from the late Prof. H. Hatanaka (Osaka University, Japan).

Cell culture and cell differentiation

PC12 cells were maintained in DMEM supplemented with 4.4 mM sodium hydrogen carbonate, 1 mM pyruvic acid sodium salt, 50 µg/ml kanamycin monosulfate, and 10% FBS, at 37°C in a humidified atmosphere containing 5% CO$_2$. Confluent cells were harvested and plated onto type IV collagen coated 4 well plates or gold grids coated with formvar in the presence of NGF (50 ng/ml) for 4 days [6].

Immunostaining and electron microscopy

PC12 cells were fixed in 4% paraformaldehyde and 0.1% glutaraldehyde in 0.1 M phosphate buffer (pH 7.2) for

1 hr at room temperature, rinsed in PBS, and subjected to cryoprotection by immersing in 30% sucrose solution overnight at 4°C. Cryoprotected cells were frozen in a deep-freeze at −80°C and then thawed in PBS [11]. After this freeze-thaw process was repeated at least 4 times, the cells were blocked for 1 hr in 1.5% normal goat or horse serum. Fixed and permeabilized cells were then incubated with primary antibodies overnight at 4°C: rabbit anti-TrkA antibody (1:1000), mouse anti-caveolin-1 antibody (1:200) or mouse anti-clathrin antibody (1:1000). After three washes with PBS (10 min each), cells were incubated with secondary antibodies for 1 hr at room temperature: biotinylated goat anti-rabbit or horse anti-mouse IgG (1:200). After washing three times, cells were incubated with avidin-biotin-HRP (ABC) complex for 1 hr at room temperature. Cells were rinsed in PBS and visualized by using a diaminobenzidine substrate. After HRP reaction, cells were postfixed in 2.5% glutaraldehyde in 0.1 M phosphate buffer for 30 min. Cells were then rinsed in 0.1 M phosphate buffer, and postfixed in 1% osmium tetroxide for 1 hr at room temperature. Cells were dehydrated through a graded ethanol series, and embedded in Epon 812 (TAAB Laboratories). Ultrathin sections were obtained by using a diamond knife on an ultramicrotome (Leica ULTRACUT R). Sections were mounted on 200 mesh copper grids, stained with lead citrate, and examined on a JEM1220 electron microscope at accelerating voltage of 80 kv.

HVEM and computer tomography

Almost similar immunostaining procedure was used for HVEM specimens. Cells were grown on the 0.5% formvar-coated and carbon-coated gold grids for 4 days in the presence of NGF (50 ng/ml). Cells were then fixed, permeabilized with freeze-thaw method and incubated with the primary antibodies at 4°C for overnight. Incubations with secondary antibodies and ABC reagents were performed at room temperature for 1 hr, respectively. HRP reactions were done with diaminobenzidine substrate containing 0.3% ammonium nickel (II) sulfate hexahydrate to intensify the contrast. After being postfixed with 2.5% glutaraldehyde and 1% osmium tetroxide, and dehydrated through a graded ethanol series, cells were dried in a critical point CO$_2$ dryer (Hitachi HCP II). Electron micrographs were obtained using H-1250M (Hitachi) at accelerating voltage of 1000 kv and H-3000 (Hitachi) at accelerating voltage of 2000 kv. The specimen was rotated from −60° to +60°, and the images were captured at 2° intervals. The images were digitized and 3D models constructed using Midas and IMOD software.

III. Results

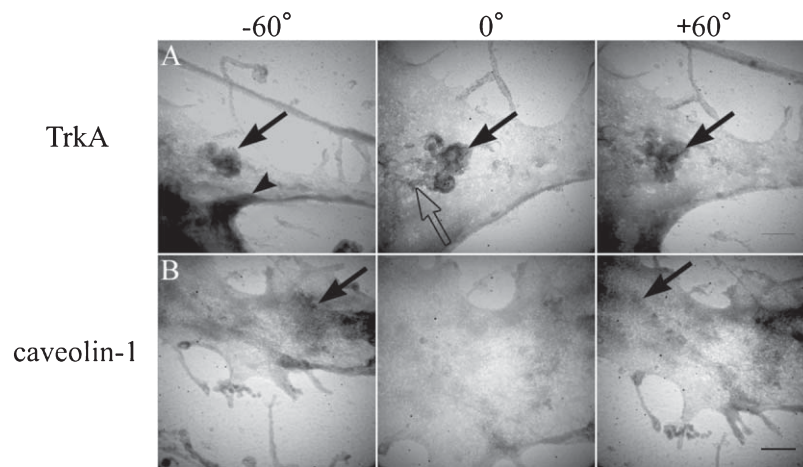### Subcellular localization of TrkA and caveolin-1 by HVEM

To examine the localization of TrkA and caveolin-1 in differentiated PC12 cells using HVEM, PC12 cells were cultured on the formvar-coated gold grids for 4 days under the NGF treatment (50 ng/ml). In this study, the freeze-thaw

method for permeabilization of plasma membrane was used to improve the preservation of intracellular structure, instead of detergent treatments such as Triton X-100 and Tween-20. Figure 1 shows the stereomicrographs obtained by changing the tilt angle of −60°, 0° and +60°, respectively. The upper panels of Figure 1A indicated that the immunoreactivities of TrkA were found in some lysosomes, plasma membrane and neurites. TrkA in the plasma membrane was found only in a definite area and appeared to be a ring-like structure. The lower panels of Figure 1B indicated that the immunoreactivities of caveolin-1 were found only near the plasma membrane and appeared to be an amorphous mesh-like structure.

Localization of TrkA and caveolin-1 by computer tomography

The tomographic slice images showed that the immunoreactivities of TrkA were present in different planes under the plasma membrane (Fig. 2A–C). TrkA immunoreactivities were found in a small definite area just beneath the plasma membrane (Fig. 2B). In addition, several lysosomes or endosomes that contained strong TrkA immunoreactivities were aggregated in the relatively shallow cytoplasm of cell body (Fig. 2A–C arrows). In the neurites, the immunoreactivities of TrkA were found only in the plasma membrane (Fig. 2C arrowheads).

The immunoreactivities of caveolin-1 in the cell body

Fig. 1. Subcellular localization of TrkA and caveolin-1 in differentiated PC12 cells by HVEM. The three numbers on the HVEM images indicate their respective tilt angles. In this study, we performed "freeze-thaw methods" instead of detergent treatment to protect the membrane structure. **A:** The three panels in **A** show that the localization of TrkA is found in the lysosome (arrows) and a part of the plasma membrane (arrowhead). TrkA immunoreactivities are also seen as a ring-like structure (blank arrow). **B:** The three panels in **B** show the localization of caveolin-1 in the plasma membrane. Strong caveolin-1 immunoreactivities are seen only in a part of the plasma membrane (arrows). Bar=2 μm.
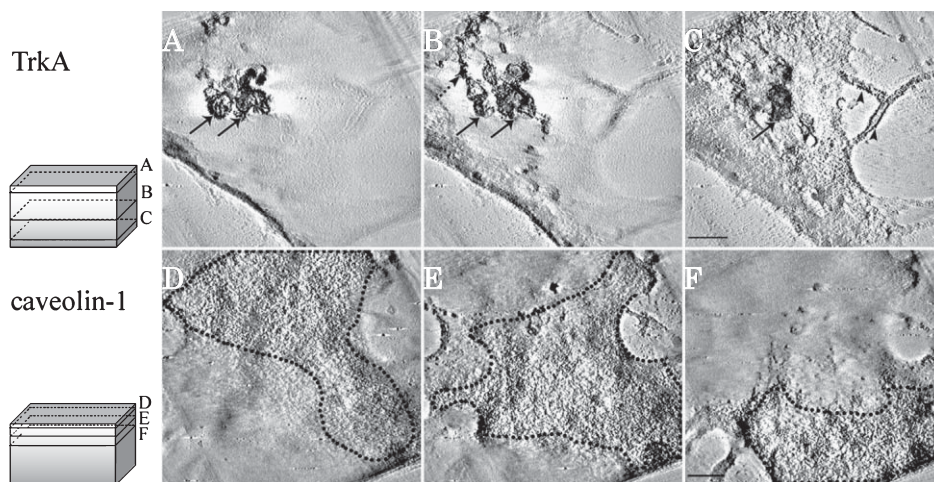
Fig. 2. Immunoreactivities of TrkA (**A–C**) and caveolin-1 (**D–F**) in PC12 cells by computer tomography. In the lefthand column, schematic illustrations and capital letters indicate the regions corresponding to planes of tomographic slice images. TrkA immunoreactivities in the plasma membrane are seen only in a definite area in PC12 cell (dotted arrow). TrkA is also found in some lysosomes (arrows) and processes membrane (arrowheads). Caveolin-1 immunoreactivities (dotted line region inside) in the plasma membrane are found in the cytoplasm as well as just beneath the plasma membrane. Bar=2 μm.
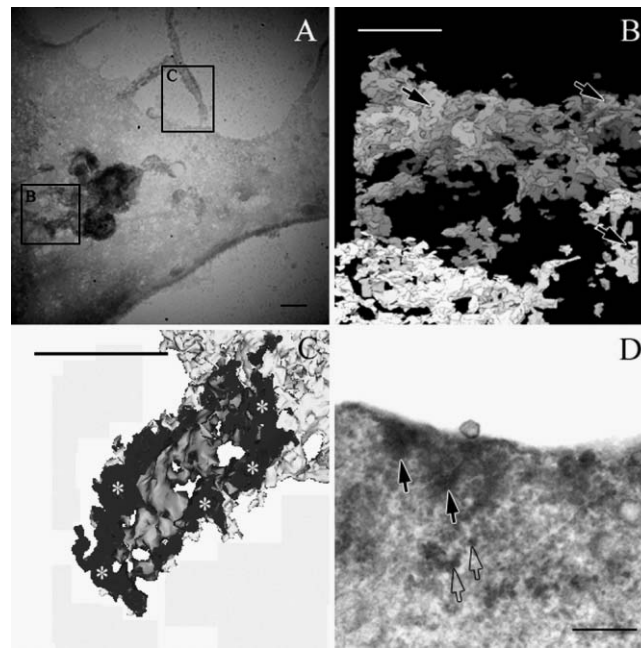
Fig. 3.   Structure of TrkA-labeled plasma membrane in differentiated PC12 cell by 3D imaging. **A:** HVEM image indicated that localization of TrkA is found in a part of the plasma membrane of the cell body and process. **B:** TrkA immunostaining based-3D image indicated that a few invaginations with a diameter of 50 nm or less (arrows) are to be found in plasma membrane, but their morphological features are not the typical caveolae structure (boxed area **B** in **A**). Furthermore, localization of TrkA in the cytoplasm is found as a three-dimensional network. **C:** The vertical 3D sectional view of the process shows that TrkA immunoreactivity (asterisks) is found as a thickness of 20–40 nm (boxed area **C** in **A**). **D:** TEM image indicate that small vesicles with a diameter of 50 nm or less (blank arrows), which contain TrkA immunoreactivity, are found just beneath the TrkA-labeled plasma membrane (solid arrows). Bars=1 μm (**A**), 500 nm (**B, C**), 200 nm (**D**).

were found beneath the plasma membrane (Fig. 2D, E) and in a relatively deep area of the cytoplasm (Fig. 2F). Caveolae or caveolin-associated membrane invaginations were not found on the cell surface.

Three-dimensional reconstruction of TrkA immunoreactivities

HVEM-tomographic slice images (Fig. 2A–C) indicated that TrkA immunoreactivities were distributed not only in the plasma membrane but also just beneath the plasma membrane. To reveal the subcellular localization of TrkA in differentiated PC12 cells, 3D models were constructed from a combination of tomographic slice images. The 3D image showed that a few invaginations in a diameter of about 50 nm were found in the membrane domain but their morphological features were not the typical caveolae structure (Fig. 3B). TrkA in the cytoplasmic site was seen as three-dimensional network structures.

In the neurites, TrkA immunoreactivities were distributed as a relatively thick layer beneath the plasma membrane, the thickness of which was measured as a range between 20–180 nm (Fig. 3C). Network structures were not seen in the neurites.

Under transmission immunoelectron microscopy (TEM), TrkA immunoreactivities were found in a part of plasma membrane and neighboring cytoplasm (Fig. 3D black arrows). In these sites, typical caveolae invagination

structures were not seen. TrkA immunoreactive vesicles in a diameter of about 50 nm were scattered near the plasma membrane (Fig. 3D white arrows).

Three-dimensional reconstruction of caveolin-1 immunoreactivities

HVEM showed that caveolin-1 immunoreactivities were present in a relatively small area of the plasma membrane (Fig. 4A), wherein caveolin-1 immunoreactivities were seen as mesh-like structures (Fig. 4A inset). In the 3D models, caveolin-1 immunoreactivities were seen as small clusters near the cell surface (Fig. 4B dotted line). Caveolae-like pits were not found in the 3D image of caveolin-1. In these surface areas, caveolae-like invaginations were not seen.

The tilt view of the 3D image (Fig. 4C) indicated that caveolin-1 immunoreactivities formed a relatively large mass in the inner cytoplasm, compared to those of the cell surface (arrows).

HVEM and conventional immunoelectron microscopy of clathrin and caveolin

The image of clathrin immunoreactivities under HVEM (Fig. 5A) indicated that many clathrin-immunoreactive vesicles (Fig. 5A arrows) were widely distributed in the cytoplasm, and that some of them were associated and fused to endosomes or lysosomes (Fig. 5A asterisk). Under con-
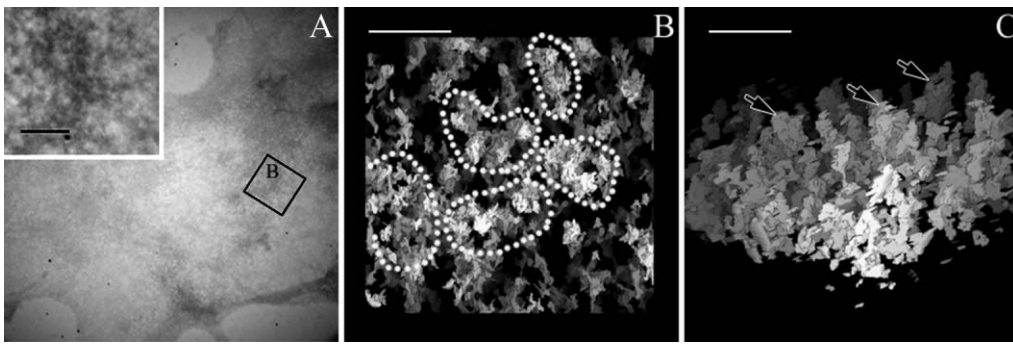
Fig. 4. HVEM and 3D images of caveolin-1 in PC12 cells. **A:** Immunoreactivities of caveolin-1 are seen as mesh-like structures (inset is higher magnification of boxed area **B**). **B:** 3D image of boxed area in **A**. Caveolin-1 near the cell surface (dotted line) forms clusters in relatively small areas. **C:** Tilted view of **B**. Caveolin-1 is a relatively large mass in the inner cytoplasm, compared to those of the cell surface (arrows). Bar=500 nm.
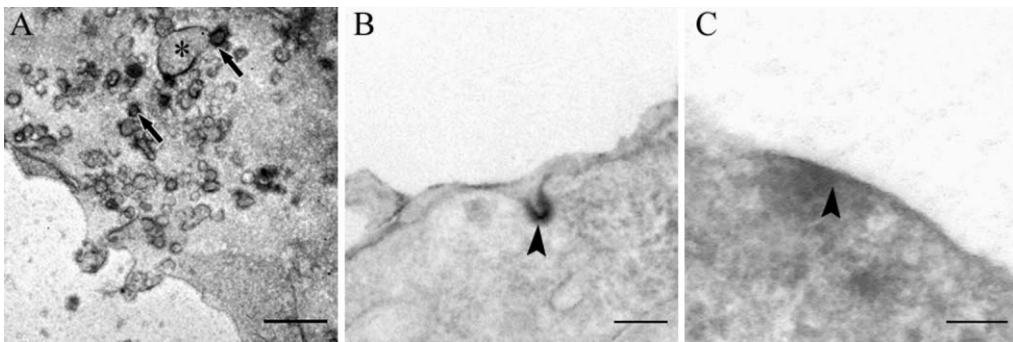
Fig. 5. Immunoreactivities of caveolin-1 and clathrin by HVEM. **A:** Many clathrin-immunoreactive vesicles (arrows) and early endosomes (asterisk) are found in the cytoplasm. **B:** Membrane invagination is coated by clathrin immunoreactivity (arrowhead), indicative of membrane internalization. **C:** Immunoreactivity of caveolin-1 (arrowhead) in plasma membrane. There is not related in formation of caveolae. Bars=1 µm (**A**), 200 nm (**B**), 100 nm (**C**).

ventional transmission immunoelectron microscopy, typical clathrin-immunoreactive pits were often observed in the plasma membrane (Fig. 5B). Caveolin-immunoreactivities were also found around the plasma membrane, but those reactivities were located beneath the flat cell surface and distributed as a relatively diffused mass in the cytoplasm (Fig. 5C).

IV. Discussion

In the present study using HVEM and computer tomography, we demonstrated that the TrkA immunoreactivities of differentiated PC12 cells were localized only at a definite area of the plasma membrane and in some of the endosomes or lysosomes, where the typical caveolae invaginations were not seen. Although clathrin-associated invaginations, indicative of membrane retrieval, were often found, caveolin-1 immunoreactivities were not involved in those structures. These results suggest that TrkA in cultured neurons may be located at specific areas of plasma membrane, where it is not specialized into caveolae, and are internalized into the cytoplasm by the clathrin-mediated process.

Our results were partially inconsistent with the recent report [18], in which it was stated that TrkA was located at caveolae in PC12 cells. Although the reasons for this discrepancy are unknown at present, there are several possibilities as follows: 1) Differences in the physiological conditions of PC12 cells used might explain this discrepancy. In this study, differentiated cells by NGF treatment were used. Although there were few caveolae in our specimens, the caveolae might be rich in the undifferentiated state of neurons. 2) Differences of the antibodies used might be related to the discrepancy. In this study, we used an antibody that could recognize the cytoplasmic domain of TrkA. 3) Differences of immunocytochemical methods might also be related. In any case, further studies are needed to examine how TrkA is related to other membrane structures and proteins in the different stages of neuronal development.

A previous study [6] reported that the expression of caveolin-1 was up-regulated on day 4 of NGF treatment in PC12 cells, whereas caveolin-2 was transiently up-regulated early in the differentiation process and then rapidly down-regulated. In the cultured neurons of dorsal root ganglion, different expression patterns of caveolin-1 and -2 were also

found [6]. Thus, the expression of caveolin-family proteins does not always correspond to the membrane structure of caveolae in different types of neurons.

Parton [16], using human keratinocyte A431 cell line, reported that the ganglioside (GM1) was concentrated in caveolae and also in trans-Golgi network. Kanzaki and Pessin [10], using murine adipocyte 3T3L1 cell line, found that caveolin and lipid raft microdomains were clustered as relatively large rosette-like structures. Parton *et al.* [17], using the same cell line, stated that the rosette-like structures were termed "caves", which were a complex of many elements including clathrin-coated pits, lipid raft markers and non-raft markers, rather than simple caveolar domain.

In the present study, TrkA immunoreactivities were found as ring-like structures, which were thought to correspond to the structures previously termed "rosette-like" or "caves". It is likely that those areas of plasma membrane containing lipid rafts, receptors, clathrin and caveolins are specific sites of signal transduction.

Mundy *et al.* [12], using CHO cells expressing caveolin-1-GFP, demonstrated that the fluorescent caveolin proteins moved actively from the plasma membrane to the cytoplasmic vesicles and endosomes, and that their movements were affected by perturbation of microtubules and actin filaments. Another study suggested that transmembrane proteins anchored to the actin cytoskeleton act as membrane compartmentalization units [5]. Although we could not find the vesicular structures of caveolin-1 in PC12 cells, the mesh-like structure of caveolin-1 immunoreactivities in the cytoplasm were thought to be involved in the dynamic functions of caveolins for intracellular trafficking.

Our HVEM showed that there were many clathrin-coated vesicles in PC12 cell bodies, but TrkA was seen in a relatively small number of those vesicles. Several studies have reported that the clathrin-independent pathways are involved in the endocytosis of membrane receptors [1, 4, 20]. Although the exact mechanisms are unknown, the lipid raft membrane domains might be associated with such pathways. EGF receptor, one of the tyrosine kinase receptors, is endocytosed through both clathrin-dependent and -independent pathways [20]. In addition, P75NTR and TrkB are contained in lipid raft fraction [2, 24]. From these findings, it is likely that the internalization of TrkA in PC12 cells occurs in a similar clathrin-dependent and -independent manner.

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