Functional Compartmentalization of the Plasma Membrane of Neurons by a Unique Acyl Chain Composition of Phospholipids*

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Background: In neurons, the plasma membrane is separated into functional segments.

Results: A phospholipid with a unique acyl chain composition is concentrated at the tips of neuronal projections, which is necessary for the confinement of proteins at the site.

Conclusion: The neuronal plasma membrane is compartmentalized by a unique phospholipid species.

Significance: This reveals a novel mode of membrane compartmentalization.

In neurons, the plasma membrane is functionally separated into several distinct segments. Neurons form these domains by delivering selected components to and by confining them within each segment of the membrane. Although some mechanisms of the delivery are elucidated, that of the confinement is unclear.

We show here that 1-oleoyl-2-palmitoyl-phosphatidylcholine (OPPC), a unique molecular species of phospholipids, is concentrated at the protrusion tips of several neuronal culture cells and the presynaptic area of neuronal synapses of the mouse brain. In PC12 cells, NGF-stimulated neuronal differentiation induces a phospholipase A1 activity at the protrusion tips, which co-localizes with the OPPC domain. Inhibition of the phospholipase A1 activity leads to suppression of phospholipid remodeling in the tip membrane and results in disappearance of the OPPC at the tips. In these cells, confinement of dopamine transporter and Gαo proteins to the tip was also disrupted. These findings link the lateral distribution of the molecular species of phospholipids to the formation of functional segments in the plasma membrane of neurons and to the mechanism of protein confinement at the synapse.

In neurons, the plasma membrane is functionally separated into the somatodendritic membrane and several distinct segments of the axon. These membrane compartments are essential for the directional propagation of action potential from the soma to the axon tip and for formation of neural circuits among neurons; however, the mechanisms underlying this compartmentalization are unclear. These compartments maintain distinct distributions of membrane proteins without obvious structural barriers at their boundaries (1). In addition, although the movement of membrane proteins between different compartments across the boundary is slower than that within a compartment, these membrane proteins cross the boundary at a measurable rate (2, 3). These findings suggest that, in neurons, a different confinement mechanism other than a physical diffusion barrier operates to maintain the distinct compartments.

Biochemically, the main constituents of biological membrane are the glycerophospholipids. Besides variety in their hydrophilic portions, phospholipids comprise various molecular species with different fatty acid moieties (4, 5). Recent advances in imaging mass spectrometry have shown characteristic distributions of some of the molecular species among different tissues or even in the different areas of the brain (6, 7), indicating strict control of their composition in the tissues. However, their distribution at subcellular levels and the biological significance of the variety remain unclear. The variety in the molecular species of phospholipids is produced by the remodeling process or Lands cycle (8, 9). In this process, one of the acyl chains of a phospholipid already present in membranes is cleaved by the activity of phospholipase A, and subsequently, a different fatty acid is attached to the lysophospholipid product of the lipase by either acyltransferase or transacylase activity (9, 10). Here we describe our findings linking the remodeling process of phospholipids to the functional compartmentalization of the presynaptic area in the neuronal plasma membrane.

**EXPERIMENTAL PROCEDURES**

Cell Cultures—PC12 cells were cultured in DMEM (Wako) with 10% heat-inactivated horse serum and 5% heat-inactivated FBS on collagen (0.3 g/liter, Nitta)-coated tissue culture plastic dishes for cell preparation or glass bottom dishes (Iwaki) for immunostaining studies. The cells were treated with 50 μg/liter NGF (Sigma, N0513) for 48 h or specified periods to induce neuronal development. Neuro-2a cells were cultured in DMEM with 10% FBS on poly-L-lysine (Sigma)-coated glass bottom dishes (Iwaki). COS-7 cells were cultured in DMEM with 10% FBS on the plain glass bottom dishes.

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Production of Monoclonal Antibody—A recombinant adenovirus with masculine complementary DNA was produced by inserting the NsiI-SspI fragment of the complementary DNA into the Ad5 vector through the PstI-EcoRI site of pHM5 intermediate vector as described (11). PC12 cells were infected with the recombinant adenovirus at a multiplicity of infection of 5 and cultured for 2 days to isolate 1% Triton X-resistant membrane (detergent-resistant membrane; DRM) fraction (12). The fraction (1 mg of protein) was injected into BALB/c mice without adjuvant to produce monoclonal antibody, using PAI cells as described (13). 250 clones were screened by comparing the reactivity to DRM fraction from virus-infected PC12 cells against that from the control PC12 cells by dot blots. 15 clones that reacted more than 3-fold more strongly to the DRM fraction from the virus-infected PC12 cells than to that from the control PC12 cells were further analyzed by immuno-chemistry. One of the clones, named monoclonal antibody #15 (mAb#15), stained the tips of the protrusions of PC12 cells after NGF treatment and was characterized further in this study.

Immunoelectron Microscopy of PC12 Cells—PC12 cells were fixed with 4% paraformaldehyde in 0.1 M sodium phosphate, pH 7.2, for 15 min. After washing with PBS, cells were blocked with 1% BSA in PBS for 1 h before adding 3 mg/liter purified mAb#15 IgM in 5% skim milk and 1% BSA in PBS for 12 h at 4 °C. After washing away the primary antibody with PBS, the cells were stained with 200-fold diluted HRP-labeled antibody to mouse IgM (Santa Cruz Biotechnology, Inc., SC2064) in 1% BSA in PBS for 2 h. The localization of the antigen was revealed by a DAB substrate kit (Nacalai Tesque, 25985-50). These samples were fixed again with 1% glutaraldehyde in 0.1 M phosphate buffer, pH 7.2, for 15 min. After washing away the primary antibody with PBS, the cells were blocked with 10,000-fold diluted Alexa 488-labeled antibody to mouse IgG. The cells were observed with a JEM-1400-Plus transmission electron microscope (JEOL, Tokyo, Japan).

Immunofluorescence Staining—PC12, Neuro-2a, and COS-7 cells were fixed with 4% paraformaldehyde in 0.1 M sodium phosphate, pH 7.2, for 15 min. For immunostaining with mAb#15 antibody, cells were blocked with 1% BSA in PBS for 1 h before adding 0.3 mg/liter IgM concentration of mAb#15 antibody, cells were blocked with 1% BSA in PBS for 1 h before adding 0.3 mg/liter IgM concentration of mAb#15 antibody, cells were blocked with 1% BSA in PBS for 1 h before adding 0.3 mg/liter IgM concentration of mAb#15 antibody, cells were blocked with 1% BSA in PBS for 1 h before adding 0.3 mg/liter IgM concentration of mAb#15 antibody, cells were blocked with 1% BSA in PBS for 1 h before adding 0.3 mg/liter IgM concentration of mAb#15 antibody, cells were blocked with 1% BSA in PBS for 1 h before adding 0.3 mg/liter IgM concentration of mAb#15 antibody, cells were blocked with 1% BSA in PBS for 1 h before adding 0.3 mg/liter IgM concentration of mAb#15 antibody, cells were blocked with 1% BSA in PBS for 1 h before adding 0.3 mg/liter IgM concentration of mAb#15 antibody, cells were blocked with 1% BSA in PBS for 1 h before adding 0.3 mg/liter IgM concentration of mAb#15 antibody, cells were blocked with 1% BSA in PBS for 1 h before adding 0.3 mg/liter IgM concentration of mAb#15 antibody, cells were blocked with 1% BSA in PBS for 1 h before adding 0.3 mg/liter IgM concentration of mAb#15 antibody, cells were blocked with 1% BSA in PBS for 1 h before adding 0.3 mg/liter IgM concentration of mAb#15 antibody, cells were blocked with 1% BSA in PBS for 1 h before adding 0.3 mg/liter IgM concentration of mAb#15 antibody, cells were blocked with 1% BSA in PBS for 1 h before adding 0.3 mg/liter IgM concentration of mAb#15 antibody, cells were blocked with 1% BSA in PBS for 1 h before adding 0.3 mg/liter IgM concentration of mAb#15 antibody, cells were blocked with 1% BSA in PBS for 1 h before adding 0.3 mg/liter IgM concentration of mAb#15 antibody, cells were blocked with 1% BSA in PBS for 1 h before adding 0.3 mg/liter IgM concentration of mAb#15 antibody, cells were blocked with 1% BSA in PBS for 1 h before adding 0.3 mg/liter IgM concentration of mAb#15 antibody, cells were blocked with 1% BSA in PBS for 1 h before adding 0.3 mg/liter IgM concentration of mAb#15 antibody, cells were blocked with 1% BSA in PBS for 1 h before adding 0.3 mg/liter IgM concentration of mAb#15 antibody, cells were blocked with 1% BSA in PBS for 1 h before adding 0.3 mg/liter IgM concentration of mAb#15 antibody, cells were blocked with 1% BSA in PBS for 1 h before adding 0.3 mg/liter IgM concentration of mAb#15 antibody, cells were blocked with 1% BSA in PBS for 1 h before adding 0.3 mg/liter IgM concentration of mAb#15 antibody, cells were blocked with 1% BSA in PBS for 1 h before adding 0.3 mg/liter IgM concentration of mAb#15 antibody, cells were blocked with 1% BSA in PBS for 1 h before adding 0.3 mg/liter IgM concentration of mAb#15 antibody. After washing the plates with PBS, the secondary antibody (HRP-conjugated antibody to rabbit IgG and Alexa 488-labeled antibody to mouse IgG. Antibody to Go, (Santa Cruz Biotechnology, sc-13532) was used in 25-fold dilution with 2500-fold diluted Alexa 488-labeled antibody to mouse IgG. The cells were observed with a confocal fluorescent microscope with Nomarski optics (Olympus FV10).

Phospholipase Treatment—PC12 cells were fixed with 4% paraformaldehyde for 15 min before treatment with phospholipases. Cells were treated with 0.5% (v/v) phospholipase A1 (PLA1, from Thermomyces lanuginosus; Sigma, L3295) in 100 mM Tris-HCl, pH 6.8, 200 kilounits/liter phospholipase A2 (PLA2, from honey bee venom, Sigma, P9279) in TBS, or 150 kilounits/liter phospholipase D (PLD, from cabbage; Sigma, P8398) in 100 mM MES, pH 6.8, 50 mM CaCl2 for 12 h at 25 °C. After the treatments, cells were immunostained with mAb#15 antibody as described above.

Isolation of the Tip Membrane Fraction—The tip membrane fractions (TMFs) from NGF-treated PC12 cells or from the untreated cells were purified from 0.5 g each of cell pellet as described (14, 15).

Lipid Analysis of the Tip Membrane Fraction—Lipid was extracted from the TMF pellet with chloroform/methanol (1:1). The remaining precipitate was dissolved in SDS-sample buffer for protein analysis. The lipids were separated on a C8 reversed-phase column (OS12503-1546WT, YMC) at 40 °C by isocratic elution with 10 mM triethylamine acetate, pH 4, in H2O/methanol (7:93) at a flow rate of 1 ml/min by a HPLC system (Shimazu, LC-20).

ELISA—Lipid samples were dissolved in 50 μl of methanol and applied to 96-well ELISA plates (Immunon 1B, Thermo). The samples were dried at 80 °C for 30 min. For assays on the HPLC samples, the plates were blocked with 1% BSA in PBS for 1 h and then incubated for 1 h with 3 mg/liter mAb#15 antibody in 5% milk, 1% BSA/PBS. After washing with PBS, the secondary antibody (horseradish peroxidase (HPR)-conjugated antibody to mouse IgM (sc-2064, Santa Cruz Biotechnology), 30,000-fold dilution in 1% BSA, PBS) was added, followed by incubation for 1 h. After washing the plates with PBS, immunoreactivity was quantified with a chemiluminescent HRP substrate (Millipore) measured with a luminometer (Fluoroskan, Thermo). For binding measurements to lipid standards, the plates were blocked with 0.1% ovalbumin (Wako) in PBS for 1 h and then incubated for 30 min with 10 μg/liter mAb#15 antibody in 5% milk, 3% normal goat serum, PBS. After washing with PBS, the secondary antibody (HPR-conjugated F(ab)2 antibody fragment to mouse IgM (ab5930, Abcam), 2,000-fold dilution in 1% BSA, PBS) was added, followed by incubation for 30 min. After washing the plates with PBS, immunoreactivity was quantified as above. Dioleoylphosphatidylcholine (DOPC), distearoylphosphatidylcholine, dipalmitoylphosphatidylcholine (DPPC) and sphingomyelin from bovine brain (S7004) were from Sigma. Dilinoleoylphosphatidylcholine, 1-palmitoyl-2-oleoyl-phosphatidylcholine (POPC), 1-oleoyl-2-
palmitoyl-phosphatidylycerine (OPPC), dioleoylphosphatidylethanolamine (DOPE), dioleoylphosphatidylserine and dioleoylphosphatidylinositol were from Avanti Polar Lipids.

**MALDI-TOF-MS Analysis**—For the analysis of the tip membrane fractions, the lipid samples were mixed with equal volumes of 12.5 g/liter 2,5-dihydroxybenzoic acid (DHB) (Sigma)/DHB-Li (Wako) 9:1 mix or 12.5 g/liter DHB/DHB-Na (Wako) 9:1 mix in 1:1 methanol/H$_2$O as described (16). One microliter of the mix was spotted onto a sample plate. The solution of matrix and analyte on the sample plate was allowed to dry for a few min. The crystal that formed was analyzed with an ABI SCIEX TOF-TOF 5800 mass spectrometer in positive ion mode. For quantitative analysis, 6.25 pmol of dimyristoylphosphatidylycholine was mixed to each spot as a calibration standard. For MS/MS analysis, collision-induced dissociation fragmentation was performed with a collision energy of 2 kV (17).

For the analysis of 7-nitrobenzofurazan (NBD)-labeled standards in negative ion mode, 0.17 mM p-nitroaniline (Wako) in 1:1 methanol/H$_2$O was used as a matrix.

**Liposome Binding Assay**—To make liposome preparations, lipid mixtures containing 0.125 mg (20% mol/mol of PC) cholesterol and total 1 mg of phosphatidylcholine consisting of 90% mol/mol DPPC and 10% mol/mol of either DPPC, 1-palmitoyl-lyso-PC (Sigma, L5254) or 1-oleoyl-lyso-PC (Sigma, L1881) were dried in a nitrogen gas stream. The dried lipid membranes were sonicated in a bath type sonicator in a 1-ml solution of PBS; and then centrifuged at 200,000 $g$ for 10 min and 5% BSA in PBS for 1 h and then incubated with mAb#15 culture supernatant (3 mg/liter) for 12 h. These samples were then incubated with 3-fold diluted 10-nm immunogold-labeled antibody to mouse IgM (Aurion, 810.033) for 2 h. These were postfixed with 2% glutaraldehyde in PBS for 2 min and washed with water. These sections were observed with an H-7100 transmission electron microscope (Hitachi, Japan).

**In Situ Phospholipase A Assay**—PC12 cells treated with NGF for specified periods were washed extensively with DMEM without serum and phenol red (DMEM—SP) (Wako, 040-30095). The cells were cultured with DMEM—SP in the continuous presence of 50 $\mu$g/liter NGF during the assay.

For the PLA$_1$ assay of live cells, 1 $\mu$m PED-A1 substrate (Invitrogen) was added to the medium. The cells were immediately observed for less than 30 min with a confocal fluorescence microscope with the excitation wave length at 488 nm and the emission band pass between 500 and 600 nm.

For the PLA$_2$ assay, 1 $\mu$m PC-A2 substrate (Invitrogen) was added instead to the medium. The rest of the procedure was similar to that used for the PLA$_1$, assay of live cells.

For the simultaneous staining of mAb#15 and PED-A1, PC12 cells treated with NGF for 36 or 48 h were fixed with 4% paraformaldehyde in 0.1 mM sodium phosphate, pH 7.2, for 15 min. At first, cells were processed as described for the immunostaining with mAb#15, except the secondary antibody was 10,000-fold diluted Alexa 555-labeled antibody to mouse IgM (Invitrogen) in 1% BSA in PBS. After washing the secondary antibody, 3 $\mu$m PED-A1 in PBS was applied. The cells were immediately observed with a confocal fluorescent microscope, using an excitation wavelength at 488 nm for PLA$_1$ and at 545 nm for mAb#15 with sequential scan mode.

**Remodeling Assay**—1-Palmitoyl-2-oleoyl-phosphatidylethanolamine (POPE)-NBD, dipalmitoyl-phosphatidylethanolamine (DPPE)-NBD, and DOPE-NBD were synthesized by mixing either 1.6 mM POPE, DPPE, or DOPE (Avanti Polar Lipids) with 25 mM 4-fluoro-7-nitrobenzofurazan (Dojin) in methanol with 15 mM EDTA, 37.5 mM sodium borate, pH 8, at 60 °C for 5 min. Excess NBD was removed with Presep RPP reversed-phased columns (Wako, 294-36851). The products were further purified on a C8 reversed-phase column by isocratic elution with 10 mM triethylamine acetate, pH 4, in H$_2$O/methanol (8:92) at a flow rate of 1 ml/min. Elusion was monitored by the fluorescence of NBD at excitation/emission $=460/535$ nm. For 1-acyl-2-hydroxy-phosphatidylethanolamine-NBD (lyso-PE-NBD) standards, 500 pmol of either DPPE-NBD or DOPE-NBD dissolved in 250 $\mu$l of hexane was hydrolyzed by 3 units of PLA$_2$ (Sigma, P9279) in 250 $\mu$l of 10 mM Tris-HCl, pH 8.9, at 25 °C for 12 h with rotation. Each lyso-PE-NBD was recovered from the aqueous phase by Presep RPP columns. These products were further purified on a C18 reversed-phase column (Imtakt, CD036) at 65 °C by isocratic elution with 50 mM triethylamine acetate, pH 4, in H$_2$O/methanol (17:83) at a flow rate of 0.75 ml/min. The identity of the products was verified by MALDI-
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FIGURE 1. mAb#15 recognized phospholipids present on the plasma membrane and some of the vesicular structures at the tips of PC12 processes. A–D, distribution of mAb#15 antigen in NGF-stimulated PC12 cells was determined by immunoelectron micrograph analysis. A and B, NGF-treated PC12 cells were stained with mAb#15. In A, a NGF-induced process is crossing under the other adjacent cell. The area of the rectangle in A is magnified in B. The large and small arrows show the staining at the plasma membrane and vesicular structures, respectively. C and D, cells were stained with the secondary antibody alone. The area of the rectangle in C is magnified in D. Bars, 5.0 μm (A and C) and 500 nm (B and D). E–J, the distribution of the antigen in PC12 cells was determined by immunofluorescent micrograph analysis. PC12 cells were fixed with paraformaldehyde (except in G) and then immunostained with mAb#15 (shown in green). E, PC12 without NGF stimulation. F–J, PC12 with NGF stimulation for 48 h. G, cells were fixed with cold methanol. H, cells were treated with PLA2. I, cells were treated with PLA2. Bar, 40 μm.

TOF-MS analysis as described above. The purified lipids were evaporated with an N2 gas stream and dissolved in ethanol.

For remodeling experiments, 1 × 107 PC12 cells in 25 × 25 cm square culture plates were treated with NGF for 48 h or left alone. For inhibition study, either 100 μM RHC80267 (Sigma) or vehicle ethanol was preincubated with the cells for 30 min in the continuous presence of NGF before adding the substrate. Then cells were washed with PBS extensively, followed by the addition of the substrate DPPE-NBD (1 μM in DMEM—SP) and incubated for 20 min at 37 °C in a CO2 incubator. For the inhibition study, either 100 μM RHC80267 or vehicle ethanol was added with the substrate. Cells were washed once with PBS, once with PBS containing 5 g/liter BSA (fatty acid free; Nacalai Tesque, 08587-26), and twice with PBS. From these cells, the lipids were extracted by the Bligh-Dyer method (19). The lipid fractions were evaporated, dissolved in 200 μl of methanol, and centrifuged at 12,000 × g for 5 min, and supernatants were recovered. The supernatants were evaporated and dissolved in 30 μl of methanol, and 20 μl of each of the samples was injected for analysis with HPLC. The lipids were separated on a C8 reversed-phase column as described above. Each area of the corresponding peaks in the chromatogram was quantified, and oleic acid content at the sn-1 site was calculated with the equation, oleic acid content at the sn-1 site = 1-oleoyl-lyso-PE-NBD (mol)/(1-oleoyl-lyso-PE-NBD + 1-palmitoyl-lyso-PE-NBD) (mol). The p values were examined by two-tailed Student’s t test (n = 6).

RHC80267 Treatment—For immunocytochemistry or the preparation of TMF in the presence of the inhibitor, PC12 cells treated with NGF for 48 h were treated with 100 μM RHC80267 (Sigma) or vehicle ethanol for 3 h in the continuous presence of NGF.

Dopamine Uptake Assay—PC12 cells treated with NGF for 3 days or unstimulated control cells were washed with Hanks’ balanced salt solution with 1% BSA. The uptake of a fluorescent dopamine analog (Neurotransmitter Transporter Uptake Assay Kit, Molecular Devices, R8173) was observed by the addition of the analog in Hanks’ balanced salt solution with 1% BSA to the cells following the manufacturer’s instructions. After the addition of the reagent, cells were incubated for 15 min at 37 °C before observation with a confocal fluorescent microscopy at excitation/emission = 440/520 nm for up to 15 min.

RESULTS

Antibody to 1-Oleoyl-phosphatidylcholine—We produced monoclonal antibodies against the DRM fraction (12) of PC12 cells (a rat pheochromocytoma cell line) into which the masculine gene had been introduced. One of them, designated mAb#15, recognized an antigen present on the plasma membrane and on some of vesicular structures, both at the protrusion tips of PC12 cells after NGF stimulation (Fig. 1, A–F). The antigen of mAb#15 was extracted from the cells with cold methanol (Fig. 1G), and the antigen in the methanol extract was resistant to a protease K digestion (data not shown), indicating the lipid nature of the antigen. In addition, digestion of phospholipids in the fixed cells with PLA2 or PLD removed the anti-
gen at the tips (Fig. 1, H and J). In contrast, treatment with PLA2 enhanced the staining (Fig. 1I). These results indicated that the antigen is a kind of phospholipid and that the structure at the sn-1 position is important for the recognition.

To identify the antigen molecule, we purified the membrane of the tips of processes (TMF) by applying a method for isolation of the growth cone membrane (14, 15). The efficacy of the purification was verified by co-purification of a protein, Gά/H9251o, which is localized at the tips of processes (Figs. 2A and 7J) (15).

From this tip fraction of NGF-treated cells and the corresponding fraction of unstimulated cells, lipids were extracted and fractionized by reversed-phase HPLC (RP-HPLC). Then the distribution of the antigen over the fractions was assayed by ELISA with mAb#15. The majority of the antigen that depends on NGF stimulation was eluted in fractions 29–33 (Fig. 2B).

MALDI-TOF-MS analysis of these fractions using DHB with lithium ion additive as a matrix (16) identified that the monocations with m/z 760.5, 766.6, 782.5, and 798.5 were present only in the NGF-stimulated tip fraction (Fig. 2C and D). By comparing this ion profile with the ion profile with sodium ion additive, these were assigned to proton, lithium ion, sodium ion, and potassium ion adducts, respectively, of the molecule with the mass of 759.5 Da (Fig. 2D and E).

To analyze positioning of the acyl chains, the fragmentation profile (Fig. 2F) was compared with corresponding phosphatidylcholine standards: 1-oleoyl-2-palmitoyl-phosphatidylcholine (OPPC (18:1/16:0)) (Fig. 2G) and 1-palmitoyl-2-oleoyl-phosphatidylcholine (POPC (16:0/18:1)) (Fig. 2I). By comparing the product-ion spectra of OPPC and POPC, preferential loss of the fatty acid group at the sn-1 site was observed (21). The product-ion spectrum (Fig. 2H) matched up to that of OPPC (Fig. 2G). These analyses identified OPPC as the antigen of mAb#15 that was present in the NGF-stimulated tip fraction.

To verify the identity of the antigen, we examined immunoreactivity of mAb#15 to a variety of phosphatidylcholine standards with different acyl chain compositions by ELISA. Among those we tested, DOPC (18:1/18:1) and OPPC (18:1/16:0) had a
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**FIGURE 3.** mAb#15 selectively binds 1-oleyl-phosphatidylcholine. A, binding of mAb#15 to phosphatidylcholine standards with different acyl chain compositions was measured by ELISA (binding of mAb#15 to dioleoylphosphatidylinositol (DOPC). mAb#15 did not bind to dioleoylphosphatidylserine or dioleoylphosphatidylcholine standards with different headgroups or to sphingomyelin was measured by ELISA (Fig. 3B). These results showed that mAb#15 distinguishes the acyl chain structures of phosphatidylcholine and that it selectively binds to dioleoylphosphatidylcholine (18:0/18:0), or dilinoleoylphosphatidylcholine (18:2/18:2) (Fig. 3B). B, binding of mAb#15 to 1-oleoyl-lyso-PC in liposome was examined by a flow cytometer (green, 80% DPPC, 20% cholesterol liposome (the base liposome) with mAb#15; red, the base liposome plus 10% 1-oleoyl-2-lyso-PC with no mAb#15; green, the base liposome plus 10% 1-oleoyl-2-lyso-PC with mAb#15. C, binding of mAb#15 to dioleoyl phospholipid standards with different headgroups or to sphingomyelin was measured by ELISA (red, DOPC; black, DOPE; blue, dioleoylphosphatidylcholine; green, dioleoylphosphatidyldserine; yellow, sphingomyelin). D, mAb#15 binding to a panel of phospholipids (100 pmol each) was evaluated with the lipids spotted on a membrane. LPA, lysophosphatidic acid; LPC, lysophosphatidylcholine; PI, phosphatidylinositol; PI(3)P, phosphatidylinositol 3-phosphate; PI(4)P, phosphatidylinositol 4-phosphate; PI(3,4)P2, phosphatidylinositol 3,4-bisphosphate; PI(3,5)P2, phosphatidylinositol 3,5-bisphosphate; PI(4,5)P2, phosphatidylinositol 4,5-bisphosphate; PI(3,4,5)P3, phosphatidylinositol 3,4,5-trisphosphate; PA, phosphatidic acid; PS, phosphatidyldserine. Error bars, S.D. of triplicate, arbitrary units.

A comparable reactivity to mAb#15. In contrast, mAb#15 did not bind to DPPC (16:0/16:0), POPC (16:0/18:1), dioleoylphosphatidylcholine (18:0/18:0), or dilinoleoylphosphatidylcholine (18:2/18:2) (Fig. 3A). In addition, mAb#15 selectively binds to 1-oleoyl-lyso-PC in DPPC/cholesterol liposome (Fig. 3B). These results showed that mAb#15 distinguishes the acyl chain structures of phosphatidylcholine and that it selectively binds to the phosphatidylcholine with oleic acid (18:1) moiety at the sn-1 site.

To examine the effect of the hydrophilic headgroups on immunoreactivity to mAb#15, we next tested a variety of dioleoylphospholipids with different headgroups by ELISA. Among the main phospholipid classes, DOPC had the highest reactivity, and DOPE had about 2.5-fold less reactivity than DOPC. mAb#15 did not bind to dioleoylphosphatidyldserine or dioleoylphosphatidylcholine (Fig. 3C). Sphingomyelin, the sphingolipid that has the same headgroup as phosphatidylcholine, showed little reactivity to mAb#15 (Fig. 3C). We also evaluated the binding of mAb#15 to an array of phosphatidylinositol phosphates, which show polarized distributions in cell membranes (22). Although OPPC on the membrane was detected by mAb#15, none of the phosphatidylinositol phosphate varieties, lysophosphatidic acid, phosphatidic acid, or sphingosine 1-phosphate was immunoreactive to mAb#15 (Fig. 3D). These results showed that mAb#15 distinguishes both the structures of the fatty acid moiety and the structures of the headgroup in phospholipids and that it selectively binds to the neutral phospholipids with oleic acid moiety at the sn-1 site. In combination with the immunostaining analyses (Fig. 1) and the identity of the antigen purified from the tip membrane fractions (Fig. 2), we concluded that mAb#15 recognized OPPC at the tips of the protrusions of PC12 and that OPPC became concentrated there in response to NGF. These results revealed a previously unrecognized structure of phospholipid distribution in a confined area of the plasma membranes of a neuronal cell.

**OPPC Domain at the Neuronal Synapse**—To examine whether the formation of the OPPC domain at protrusion tips is a peculiarity of PC12 cells, we next investigated several other cell types. mAb#15 antigen was also localized at the tips of processes in Neuro-2a cells (a murine neuroblastoma cell line) but not in COS-7 cells (a fibroblast cell line) (Fig. 4A). These results suggested that the formation of the OPPC domain at the tips is not restricted to PC12 cells but is a more general characteristic of neuronal cells. We next analyzed the adult mouse brain by immunoelectron microscopy with mAb#15. mAb#15 antigen was detected at the presynaptic area on the plasma membrane (arrows). Bar, 33 nm.

**PLA_2 Activity at the Tips**—One of the possible mechanisms leading to the confined distribution of OPPC at the tips is the remodeling process of phospholipids. To evaluate this possibl-
ity, we used PC12 cells and analyzed the activities of two key enzymes for this process: PLA₁ toward the sn-1 site and PLA₂ toward the sn-2 site. To visualize the activities of these enzymes in live PC12 cells, we utilized fluorogenic substrate analogues: PED-A₁ and PC-A₂ for PLA₁ and PLA₂, respectively (23, 24).

Following the addition of one of the substrates to the medium, cells incorporate it, and the distribution of activity of each phospholipase A was observed by the fluorescence of its cleaved product. After 48 h of treatment with NGF, PLA₁ activity was detected mostly at the tips of processes in similar locations to mAb#15 antigen (Fig. 5A). In contrast, PLA₂ activity was detected mainly in the cytoplasmic area throughout the cell (Fig. 5B). At higher magnifications, we observed the major activity of PLA₁ on the plasma membrane at the very tips of processes and weaker activities at vesicular structures inside the tips (Fig. 5C). These results indicated the involvement of PLA₁ in the formation of the OPPC domain through the remodeling process at the sn-1 site of phosphatidylcholine.

To further evaluate the involvement of PLA₁ activity in formation of the OPPC domain, we investigated association of PLA₁ activity with mAb#15 antigen expression over time after NGF treatment (Fig. 5D). Before NGF treatment, the cells had few processes and little mAb#15 antigen or PLA₁ activity. After 24 h with NGF, the cells began to grow processes, and PLA₁ activity was detected weakly at the tips, although little mAb#15 antigen was present there. After 36 h, both PLA₁ activity and mAb#15 antigen were detected at the tips of processes. The appearance of mAb#15 antigen occurred after process formation and closely followed the detection of the PLA₁ activity. In addition, when we analyzed these two items simultaneously using fixed PC12 cells, distribution of the PLA₁ activity and that of mAb#15 antigen coincided with each other in the tips of processes both at 36 h (data not shown) and at 48 h (Fig. 5E) after NGF stimulation. These correlations in the expression

FIGURE 5. PLA₁ activity was induced at the tips of processes by NGF treatment and it co-localized with mAb#15 antigen. A, live PC12 cells that were treated with NGF for 48 h were stained with fluorogenic substrate for PLA₁ activity (PED-A₁ in green; left) or for PLA₂ activity (PC-A₂ in green; right). Bar, 40 μm. B, major activity of PLA₁ was localized on the plasma membrane at the very tips of processes (thick arrow) and weaker activities were detected at vesicular structures inside the tips (thin arrows). Bar, 10 μm. C, after treatment with NGF for 30 h, live PC12 cells were stained with PED-A₁. Although the activity was weaker than that of later time points, PLA₁ activities were detected at the very tips of filopodia (arrows). Bar, 25 μm. D, time course examinations of PLA₁ activity (top lane) and of mAb#15 immunoreactivity (bottom lane) after NGF treatment. Activities are shown in green. Bar, 40 μm. E, simultaneous staining of PLA₁ activity and mAb#15 antigen with fixed PC12 cells (green, PED-A₁; red, mAb#15; yellow, the merge of PED-A₁ and mAb#15). Bar, 10 μm.
time courses and in the localizations of the PLA1 activity with mAb#15 antigen are consistent with the role of PLA1 in the formation of the OPPC domain at the tips.

Remodeling at the Protrusion Tips—In addition to the cleavage of fatty acid moiety, the remodeling process involves attachment of a new fatty acid to the lysophospholipid product of PLA. To examine this process quantitatively, we developed an assay that measures conversion of the acyl chains of a fluorescently labeled (NBD-labeled) 16:0/16:0 DPPE-NBD (Fig. 6A) at the protrusion tips of PC12 cells. After incubation of the substrate with the cells for 20 min, TMFs were isolated (Fig. 2A). Then their lipids were extracted to analyze the molecular species of the remodeled products by RP-HPLC, using the fluorescence of the NBD moiety as a tracer. In contrast to the simple profile of the substrate (Fig. 6E), the recovered products consisted of a variety of molecular species, which showed the acyl chain remodeling at the tips of cell protrusions (Fig. 6, F and G). Among them, the peaks 3 at the retention time 12.5 min, which corresponded to either 16:0/18:1 POPE-NBD or 18:1/16:0 OPPE-NBD, were recovered and further analyzed to determine the content of OPPE-NBD in them by PLA2 treatment. By this treatment, the acyl chain at the sn-2 site was removed to yield 1-acyl-2-hydroxy-phosphatidylethanolamine-NBD. The acyl chain identity at the sn-1 site of these products was analyzed with RP-HPLC by comparing their retention time with the standards (Fig. 6, B, C, H, and I).

FIGURE 6. NGF stimulates remodeling at the sn-1 site of phospholipids by tip membrane. A–C, the purity and identity of each of DPPE-NBD (A), 1-palmitoyl-2-hydroxyphosphatidylethanolamine-NBD (B), and 1-oleoyl-2-hydroxyphosphatidylethanolamine-NBD (C) standard was verified with MALDI-TOF-MS analysis in negative ion mode with PNA as a matrix. D–F, after incubation of DPPE-NBD with the cells, its remodeled products in TMF were analyzed by RP-HPLC with a C8 column. D, standard molecules (1-palmitoyl-2-hydroxy-PE-NBD (green); 3.2 min), POPE-NBD (12.5 min), and DOPE-NBD (13.6 min). E, the substrate DPPE-NBD (11.6 min). F, the remodeled products recovered in TMF fraction. Black, −NGF; red, +NGF. G, amounts of each remodeled product were quantified, and the percentages in total phospholipid-NBD (retention time between 7.5 and 20 min) were shown (white, substrate DPPE; black, TMF lipid without NGF stimulation; red, TMF lipid with NGF stimulation). H–J, the peaks 3 at the retention time 12.5 min in F were recovered and further analyzed by PLA2 treatment. The acyl chains at the sn-1 site of PLA2-treated products were analyzed with RP-HPLC by a C18 column. H, standard molecules (1-palmitoyl-2-hydroxy-PE-NBD (green); 7.0 min) and 1-oleoyl-2-hydroxy-PE-NBD (purple; 7.9 min)). I, the PLA2 digests of the peaks 3 in F (black, −NGF; red, +NGF). J, amount of each lysophospholipid was quantified, and the contents of 1-oleoyl-phospholipids in peak 3 in F are shown (black, −NGF; red, +NGF). K, OPPE-NBD content per protein in TMF and in whole cell extract. Data in G, J, and K are means from six independent experiments evaluated using two-tailed Student’s t test (*, p < 0.02). Error bars, S.D. a.u., arbitrary units.
Although NGF stimulation had little effect on the portion of peak 3 (19% without NGF, 17% with NGF) (Fig. 6, F and G), NGF treatment increased the oleic acid content at the sn-1 site of phospholipids in peak 3 from 62% (−NGF) to 84% (+NGF) (Fig. 6f). These results showed that the TMF from NGF-stimulated PC12 cells had a significantly increased activity, converting DPPE-NBD to OPPE-NBD, compared with the TMF from unstimulated cells. In addition, when it was normalized with protein content, OPPE-NBD was concentrated 29-fold in TMF over the whole cell extract (Fig. 6K), illuminating the production of OPPE-NBD by NGF stimulation at the tips. These results support the hypothesis that the OPPC domains are formed by the acyl chain remodeling at the tips of protrusions.

**Protein Localization to the Lipid Domain**—The above results showed correlations between the remodeling activity and the formation of the OPPC domain. To assess the necessity of the remodeling process for the domain formation, we used an inhibitor, RH80267, to inhibit the remodeling. Although RH80267 is known as an inhibitor for diacylglycerol lipase (25), which cleaves the sn-1 site of diacylglycerol (26), the treatments of PC12 with this inhibitor diminished the activity of PLA1 at the tips (Fig. 7A). This inhibitor also significantly...
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decreased the remodeling of DPPE-NBD to OPPE-NBD in the TMF to 36% of that of untreated cells (Fig. 7, B and C). Furthermore, RP-HPLC and MALDI-TOF-MS analysis showed that OPPC was absent from the TMF from the RHC80267-treated cells (Fig. 7, D and E). In accord with this, mAb#15 antigen was undetectable at the tips of the processes (Fig. 7F) in these cells. These results showed that the activity of remodeling is necessary for the formation of the OPPC domains at these locations. In combination with the localization of PLA1 activity (Fig. 5) and the remodeling assays (Fig. 6), these results imply that the OPPC domain at the tips of protrusions is produced or maintained by the remodeling process of phospholipids.

One of the possible functions of the lipid domain in the plasma membrane is protein sorting to the domains. To determine whether the OPPC domain at the tips has such a role, we determined the distribution of DAT. DAT is an integral membrane protein that locates on the plasma membrane at the perisynaptic zone of synapses (27) and participates in the recovery of released dopamine from synaptic clefts to terminate postsynaptic responses (28). In PC12 cells, DAT protein (Fig. 7F) and its activity (Fig. 7I) were localized at the tips of processes after NGF stimulation. In parallel to the disruption of the OPPC domain, the treatment of the cells with RHC80267 abolished the confinement of DAT protein to the tips (Fig. 7H). The alteration of DAT distribution was also confirmed by its activity assay, which showed the loss of the confined incorporation of a fluorescent dopamine analog to the tips. Instead, the dopamine analog was incorporated mainly into the cell bodies (Fig. 7I). The treatment, however, had no obvious effects on the distribution of a membrane protein, Thy-1, which is present throughout the cell surface, including the tips (Fig. 7G). We further analyzed the distribution of another protein, Goαo, which is a membrane protein anchored by myristoyl (29) and palmitoyl (30) attachments to the inner leaflet of the plasma membrane and localized at the tips of processes in PC12 cells after NGF stimulation (15) (Fig. 7J). The localization of Goαo to the tips was also abolished by the treatment with RHC80267 (Fig. 7J). These results indicate a necessary role of the lipid domain in the selective localization of proteins to the tips and its significance in neuronal cell functions.

DISCUSSION

Here we show a lateral segmentation of the plasma membrane by the confined distribution of OPPC (Figs. 1, 2, 4, 5, and 7). This observation represents a new aspect of the functions of phospholipids, regulated in the lateral dimension at the level of acyl chain structures by localized remodeling. In PC12, this distribution is regulated by NGF stimulation (Figs. 1 and 5), indicating its functional roles in neuronal differentiation. The localizations of OPPC at the protrusion tips (PC12 and Neuro-2a), at the perisynaptic plasma membrane and at presynaptic vesicles (in the mouse brain) (Figs. 1 and 4), which coincides with the previously reported localization of DAT protein in the brain (26), are all consistent with the OPPC domain playing a role in neuronal synapses.

The confined distribution of OPPC at the tips in PC12 was produced by regulated remodeling at the sn-1 site of phosphatidylcholine (Figs. 5–7). Although cellular functions involving the remodeling at the sn-2 site are widely documented (31), those for the sn-1 site are rarely described (32). In Caenorhabditis elegans, a PLA1, (ipla-1) (33) and three acyltransferases (acl-8, acl-9, and acl-10) (34) are involved in the remodeling of the sn-1 site of phosphatidylinositol and play crucial roles in the asymmetric cell divisions of stem cells (34). These studies and the present study consistently demonstrate the importance of the remodeling process at the sn-1 site of phospholipids, especially in the determination of cell polarity.

Based on inhibition studies (Fig. 7), we propose that the OPPC domain controls the localization of protein to itself. By attracting a subset of proteins via specific interactions with their transmembrane domains, a specific lipid composition works as a selective scaffold to form functional domains in the plasma membrane. Indeed, some membrane proteins interact selectively with the lipids that have defined acyl chain structures (35, 36), and the transmembrane domains of several membrane proteins determine their own allocation on cell surface (37). From this point, the peculiar structure of OPPC has significance for the recognition by mAb#15 and probably by selected proteins in the membrane. This model, the membrane compartmentalization by the lateral localization of phospholipid molecular species, has several characteristic features distinguishing it from the diffusion barrier model. First, it provides more flexibility to a cell in forming a compartment as to its location, its size, and its plasticity (Fig. 5C). Second, it enables selective recruitment of proteins to a compartment at the same time it permits movement of general membrane proteins in and out of the domain. Third, it is also easily applicable to intracellular compartments (Fig. 5B). Finally, it explains the significance of variety in the composition of phospholipid molecular species among different cell types. These features fit the characteristics of membrane domains found in neurons and other cell types (1–3, 38, 39).

Our study connects a localized distribution of a molecular species of phospholipid with the mechanism of protein allocation in the plasma membrane of neurons. Because phospholipids are the main constituent of the biological membrane, this mode of membrane partitioning is applicable not only to the plasma membrane of neurons but possibly to other membrane structures and to other cell types as well. Interestingly, phospholipase A and lysophospholipid acyltransferase enzymes play important roles in protein sorting at the Golgi complex (40), indicating that a sorting mechanism dependent on the acyl chain remodeling of phospholipids also operates in the Golgi complex. Future studies on the precise distribution of each molecular species of phospholipid among cellular structures will reveal key information about how neurons and other cells coordinate the distribution of membrane components on their surface and inside.

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