A Novel Phospholipase C, PLCγ2, Is a Neuron-specific Isozyme*

Twelve phospholipase C (PLC) isozymes have been cloned so far, and they are divided into six classes, β, γ, δ, ε, ζ, and η-type, on the basis of structure and activation mechanisms. Here we report the identification of a novel PLC isozyme, PLCγ2. PLCγ2 is composed of conserved domains including pleckstrin homology, EF-hand, X and Y catalytic, and C2 domains and the isozyme-specific C-terminal region. PLCγ2 consists of 1164 amino acids with a molecular mass of 125 kDa. The PLC activity of PLCγ2 was more sensitive to calcium concentration than the PLC activity of the PLCδ-type enzyme, which is thought to be the most calcium-sensitive PLC. Immunofluorescence analysis showed that PLCγ2 was localized predominantly to the plasma membrane at resting state via the pleckstrin homology domain. This observation was supported by Western blot analysis of cytosol and membrane fractions. In addition, expression of PLCγ2 was detected after birth and showed a restricted distribution in the brain; it was particularly abundant in the hippocampus, cerebral cortex, and olfactory bulb, beginning of PLCγ2 was expressed abundantly in neuron-containing primary culture but not in astrocyte-enriched culture. These results indicate that PLCγ2 is a neuron-specific isozyme that may be important for the formation and/or maintenance of the neuronal network in the postnatal brain.

Phosphoinositide metabolism contributes an important intracellular signaling system that is involved in a variety of cell functions such as hormone secretion, neurotransmitter signal transduction, cell growth, membrane trafficking, ion channel activity, and regulation of the cytoskeleton (1–4). Phospholipase C (PLC)1 is a key enzyme in this system and acts by hydrolyzing phosphatidylinositol 4,5-bisphosphate (PIP2) to generate two second messengers, inositol 1,4,5-trisphosphate (IP3) and diacylglycerol. Diacylglycerol mediates the activation of protein kinase C, and IP3 releases Ca2+ from intracellular stores (5, 6). Twelve mammalian PLC isozymes have been cloned so far, and they are divided into six classes on the basis of structure and activation mechanisms: β(1–4), γ(1, 2), δ(1, 3, 4), ε(1), ζ(1)–ζ(2), and η(1)-type (1, 3, 7–13). All of the PLC isozymes contain catalytic X and Y domains as well as various regulatory domains, including the protein kinase C conserved region 2 (C2) domain, EF-hand motif, and pleckstrin homology (PH) domain. Subtype-specific domains contribute to specific regulatory mechanisms. These domains include the Src homology domain in PLCγ (3) and the Ras-associating domain and Ras-GTPase exchange factor-like domain in PLCε (14, 15).

Recent data base information suggests the existence of an additional PLC-like protein in the mouse and human genomes. Therefore we attempted to isolate the cDNA for PLC-like protein, PLCγ2, from mouse brain by reverse transcription-PCR. We found PLCγ2 to be much enriched in the brain, particularly in the hippocampus, cerebral cortex, and olfactory bulb, beginning after birth. In addition, PLCγ2 was abundant in primary cultured neurons but not in astrocytes. These results suggest that PLCγ2 may be important for the formation and maintenance of the neuronal network in the postnatal brain.

**MATERIALS AND METHODS**

**Isolation and Construction of Mouse PLCγ2 cDNA—**The open reading frame of PLCγ2 was amplified by reverse transcription-PCR from mouse brain RNA. Primers used were: full-length PLCγ2, forward, 5'-agatctgtcctgccacagcctg-3', and reverse, 5'-ctggagctcatcctactactacgccagc-3'; N-terminal deletion mutant (ΔN), forward, 5'-agatctgtcctgccacagcctg-3', and reverse, 5'-ctggagctcatcctactactacgccagc-3'; PH-truncated mutant (ΔPH), forward, 5'-agatctgtcctgccacagcctg-3', and reverse, 5'-ctggagctcatcctactactacgccagc-3'. βIII and Sall sites in the forward and reverse primers, respectively, are underlined. PCR was carried out for 35 cycles of 15 s at 94 °C, 10 s at 55 °C, and 3.5 min at 68 °C. Correct sequences were confirmed with a BigDye Terminator sequencing kit (Applied Biosystems). For an analysis of subcellular localization, PLCγ2 and ΔPH mutants were cloned into the pcDNA 3.1 vector (Invitrogen), and the PH fragment was cloned into the pFLAG-CMV2 vector (Sigma).

**Antibodies and Western Blot Analysis—**A rabbit polyclonal antibody against a His6-tagged region between the X and Y domains (X-Y region) of PLCγ2 was developed. Affinity-purified anti-PLCγ2 antibody was prepared by passing the serum over a glutathione S-transferase (GST)-tagged antigen-coupled HiTrap™ NHS-activated HP column (Amer sham Biosciences). Anti-FLAG antibody was purchased from Sigma, and fluorescent secondary antibodies and Alexa 594-phalloidin were from Molecular Probes. Anti-microtubule-associate protein (MAP2), anti-myelin basic protein (MBP), anti-glial fibrillary acidic protein (GFAP), and anti-actin antibodies were from Neomarkers, BD Bio-

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EBI Data Bank with accession number(s) AY968876.

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‡ The abbreviations used are: PLC, phospholipase C; PIP2, phosphatidylinositol 4,5-bisphosphate; IP3, inositol 1,4,5-trisphosphate; PH, pleckstrin homology; C2, protein kinase C-conserved region 2; GST, glutathione S-transferase; DMEM, Dulbecco’s modified Eagle’s medium; MAP2, microtubule-associated protein 2; MBP, myelin basic protein; GFAP, glial fibrillary acidic protein; FBS, fetal bovine serum; ADM, astrocyte-defined medium.
sciences, DAKO, and Sigma, respectively.

Brain and other tissues were homogenized in lysis buffer containing 20 mm HEPES-KOH, pH 7.0, 120 mm KCl, complete protease inhibitor mixture (Roche Applied Science), and 0.1% sodium deoxycholate and centrifuged for 30 min at 100,000 × g to remove insoluble debris. The resulting supernatants were subjected to SDS-PAGE and transferred electrophoretically onto polyvinylidene difluoride membranes (Millipore). Membranes were probed with antibodies against PLCγ2 or actin (Chemicon) and incubated with appropriate horseradish peroxidase-conjugated secondary antibodies (DAKO), and immunocomplexes were visualized by enhanced chemiluminescence (ECL, Amersham Biosciences).

**Immunoprecipitation and Measurement of PLC Activity in Vitro**—Brain was homogenized as described above. Lysates were centrifuged at 100,000 × g for 30 min at 4 °C, and the supernatants were used for immunoprecipitation with nonimmune IgG (Santa Cruz Biotechnol-ogy), and PLCγ1, 2, or actin (Chemicon) antibodies and incubated with the appropriate protein A-Sepharose CL-4B (Amersham Biosciences). PLC activity of the precipitates was measured as described elsewhere (17) with some modifications. In brief, precipitates were incubated in a reaction mixture of 50 μM phosphatidylthanolamine (Doosan Serdary Research Laboratories), 40 μM PIP2 (Sigma), 1 μCi/ml (100 nm) [3H]IP3 (PerkinElmer Life Sciences), 76 μM KCl, 10 mM CaCl2, and 0.5 mg/ml bovine serum albumin at 37 °C for 5 min, and the reaction was terminated by adding chloroform:methanol (2:1, v/v). Resulting [3H]IP3 was extracted by adding 1 n HCl and measured with a liquid scintillation counter. For mutation analysis and assay of calcium dependence and pH dependence, GST-tagged PLCγ2 and GST-tagged PLCγ1 were prepared with a baculovirus/Sf9 cell expression system (Invitrogen). Mutations were analyzed and expressed as wild-type and the appropriate mutants. The expressed mutants were performed in the presence of 10 μM CaCl2 and then the amounts of [3H]IP3 production by these enzymes in vitro were measured.

**Northern Blot Analysis**—Mouse Northern blot membrane was purchased from Clontech. The membrane was probed with radiolabeled probe comprising 426 bp of the following region of the C2 open reading frame of PLCγ2 or actin (Chemicon) and incubated with appropriate horseradish peroxidase-conjugated secondary antibodies. Cells were permeabilized with 0.2% Triton X-100, and blocked with 5% skim milk. After hybridization and washing, the membrane was exposed to X-OMAT film for an additional 48–96 h. Cultures were fixed in 3.7% formaldehyde, permeabilized with 0.2% Triton X-100, and blocked with 5% skim milk. Then the cells were incubated with anti-PLCγ2 polyclonal antibody or anti-FLAG polyclonal antibody and treated with the appropriate Alexa 488-conjugated goat secondary antibody. To visualize the actin cytoskeleton, Alexa 594-conjugated phallodin (Molecular Probes) was used.

Hippocampal primary culture and cortical astrocyte-enriched culture were carried out as described previously (18, 19). In brief, the hippocampal cells were prepared from embryonic day 18 or 19 Wistar rats by enzymatic treatment and plated onto 12-mm polyethylene- mine-coated coverslips in DMEM supplemented with 10% fetal bovine serum (FBS, Invitrogen) at 37 °C under 5% CO2. For immunostaining, the cells were transfected with various plasmids with the use of Lipofectamine 2000 transfection reagent (Invitrogen) according to the manufacturer’s protocol. Twenty-four h after transfection, the cells were fixed at room temperature for 5 min, permeabilized with 0.2% Triton X-100, and blocked with 5% skim milk. Then the cells were incubated with anti-PLCγ2 polyclonal antibody or anti-FLAG polyclonal antibody and treated with the appropriate Alexa 488-conjugated goat secondary antibody. To visualize the actin cytoskeleton, Alexa 594-conjugated phallodin (Molecular Probes) was used.

**Distribution of PLCγ2 in Various Tissues by Western and Northern Analysis**—We examined the distribution of PLCγ2 in various mouse tissues. First, Western blot analysis with PLCγ2 antibody was carried out. As shown in Fig. 2A, a 125-kDa protein was specifically detected in mouse brain. We occasionally detected a degraded protein of ~95 kDa. No bands were detected in heart, skeletal muscle, kidney, liver, small intestine, lung, testis, or spleen. Actin was used as a loading control. Northern blot analysis also showed a specific signal of ~6.5 kb in brain (Fig. 2B), indicating that expression of PLCγ2 is limited to the brain.

**Activity of PLCγ2 Is Very Sensitive to the Calcium Concentration**—Two PLC-like proteins (PLC-L-type) that lack PLC activity (PIP2-hydrolyzing activity) have been reported (22, 23). Thus, we examined whether PLCγ2 possesses PLC activity. Endogenous PLCγ2 immunoprecipitated from mouse brain with anti-PLCγ2 antibody showed relatively high activity (Fig. 3A). The activity of PLCγ2 was similar to that of PLCδ4 and lower than that of PLCδ1, which possesses high PLC activity. Immunoprecipitate with normal IgG (Fig. 3A, N) showed little activity.

**RESULTS**

**Isolation of Mouse PLCγ2 cDNA**—The mouse genome-sequencing project has recently been completed. We searched the Celera, Inc. data base and found two genes on mouse chromosomes 3 (mCG6375) and 4 (mCG3920) that encode PLC-like proteins. The former gene was recently reported as PLCγ (21). The latter gene encoded five PLC-specific functional domains, including PH, EF-hand, PLCX, PLCY, and C2, and a long extension at the C terminus, but unfortunately this gene lacked the N-terminal sequence. We then carried out a BLAST search analysis of mCG3920 and found a clone that might contain the N-terminal region of this protein (RIKEN cDNA A98002K705). With this putative full-length sequence, a complete open reading frame region was isolated from mouse brain by reverse transcription-PCR, and correct nucleotide and amino acid sequence were confirmed (Fig. 1A). The five PLC-specific domains, PH, EF-hand, PLCX, PLCY, and C2, are shown boxed in Fig. 1A. Because there were two N-terminal methionine residues that could be used as the first methionine, we attempted to determine which was the correct one. First, we developed a rabbit polyclonal antibody against this PLC-like protein. The antigen was designed against the linker region between PLCX and PLCY (underlined in Fig. 1A). This antibody showed no cross-reaction with other PLC isozymes (data not shown). With this antibody, we immunoprecipitated the endogenous protein from mouse brain, and the molecular mass was compared with the molecular mass of ectopically expressed short-type (ΔN) or long-type (ΔNL) novel PLC-like protein. The molecular mass of the endogenous protein coincided with that of long-type PLC-like protein. The estimated number of amino acids was 1164, and the predicted molecular mass was 125 kDa. The domain structures of PLC subtypes PLCα, γ, δ, ε, η, and θ and the novel PLC-like protein are illustrated in Fig. 1C. Although the novel polypeptide appeared to be a member of the PLCβ family with a long C terminus, amino acid comparison showed that it is similar to PLCδ and has the highest similarity to PLCγ2 (Fig. 1D). Therefore, we named the protein PLCγ2 (GenBankTM accession number AM968687). We could not find any similarity against the PH domain and EF-hand motif of PLCβ and ε. A dendrogram based on total amino acid similarity is shown in Fig. 1E, in which PLCγ2 locates closer to the PLCδ than to the PLCβ family.

**Identification of a Novel Phospholipase C, PLCγ2**

Isolation of Mouse PLCγ2 cDNA—The mouse genome-sequencing project has recently been completed. We searched the Celera, Inc. data base and found two genes on mouse chromosomes 3 (mCG6375) and 4 (mCG3920) that encode PLC-like proteins. The former gene was recently reported as PLCγ (21). The latter gene encoded five PLC-specific functional domains, including PH, EF-hand, PLCX, PLCY, and C2, and a long extension at the C terminus, but unfortunately this gene lacked the N-terminal sequence. We then carried out a BLAST search analysis of mCG3920 and found a clone that might contain the N-terminal region of this protein (RIKEN cDNA A98002K705). With this putative full-length sequence, a complete open reading frame region was isolated from mouse brain by reverse transcription-PCR, and correct nucleotide and amino acid sequence were confirmed (Fig. 1A). The five PLC-specific domains, PH, EF-hand, PLCX, PLCY, and C2, are shown boxed in Fig. 1A. Because there were two N-terminal methionine residues that could be used as the first methionine, we attempted to determine which was the correct one. First, we developed a rabbit polyclonal antibody against this PLC-like protein. The antigen was designed against the linker region between PLCX and PLCY (underlined in Fig. 1A). This antibody showed no cross-reaction with other PLC isozymes (data not shown). With this antibody, we immunoprecipitated the endogenous protein from mouse brain, and the molecular mass was compared with the molecular mass of ectopically expressed short-type (ΔN) or long-type (ΔNL) novel PLC-like protein. The molecular mass of the endogenous protein coincided with that of long-type PLC-like protein. The estimated number of amino acids was 1164, and the predicted molecular mass was 125 kDa. The domain structures of PLC subtypes PLCα, γ, δ, ε, η, and θ and the novel PLC-like protein are illustrated in Fig. 1C. Although the novel polypeptide appeared to be a member of the PLCβ family with a long C terminus, amino acid comparison showed that it is similar to PLCδ and has the highest similarity to PLCγ2 (Fig. 1D). Therefore, we named the protein PLCγ2 (GenBankTM accession number AM968687). We could not find any similarity against the PH domain and EF-hand motif of PLCβ and ε. A dendrogram based on total amino acid similarity is shown in Fig. 1E, in which PLCγ2 locates closer to the PLCδ than to the PLCβ family.
The PLCX and PLCY domains, which confer PLC activity, are highly conserved among PLC isozymes. It is well known that a histidine residue in the PLCX domain is essential for PLC activity (Fig. 3B); substitution of histidine with alanine resulted in a dramatic reduction of enzyme activity (24). To determine whether histidine 341 in the PLCX domain of PLC\( ^{\gamma 2} \) is essential for PLC activity, we constructed a mutant in which this histidine is substituted with alanine (H341A). GST-fused recombinant PLC\( ^{\gamma 2} \) expressed in a baculovirus system showed specific activity of 54 nmol/min/mg protein, whereas the H341A mutant showed activity similar to that of the GST control (Fig. 3C), indicating that histidine 341 in the PLCX domain of PLC\( ^{\gamma 2} \) is essential for PLC activity.

PLC\( ^{\gamma 2} \) resembles PLC\( ^{\delta} \), which is very sensitive to \([Ca^{2+}]\) (25). Therefore, we analyzed the dependence of PLC activity on \([Ca^{2+}]\). PLC activity was measured at \([Ca^{2+}]\) between 10^{-8} and 10^{-4} M for PLC\( ^{\gamma 2} \) and PLC\( ^{\delta} \) 1 (Fig. 3D). Whereas PLC\( ^{\delta} \) 1 showed maximal PLC activity at 10–100 \(\mu M\) \(Ca^{2+}\), PLC\( ^{\gamma 2} \) showed maximal PLC activity at 1 \(\mu M\) \(Ca^{2+}\) and was sensitive to \(Ca^{2+}\) at a concentration as low as 10 nm, indicating that the PLC activity of PLC\( ^{\gamma 2} \) is more sensitive to \([Ca^{2+}]\) than is the PLC activity of PLC\( ^{\delta} \). We also measured PLC activity under...
Identification of a Novel Phospholipase C, PLC\(\gamma\)2

PLC\(\gamma\)2 shows calcium-dependent PIP\(_2\) hydrolyzing activity in vitro. A, PIP\(_2\) hydrolyzing activity of endogenous PLC\(\gamma\)2. B, conserved sequence within the catalytic PLCX domain. Histidine residues (boxed) are conserved among all PLC isozymes. C, histidine at position 341 of mouse PLC\(\gamma\)2 is essential for PLC activity. D, specific localization of PLC\(\gamma\)2. E, dependence of PLC activity of PLC\(\gamma\)2 on pH. Enzymatic activity of purified GST-tagged PLC\(\gamma\)2 was measured in buffers under various pH conditions. Values of specific activity are presented as the mean ± S.D. of three experiments.

PLC\(\gamma\)2 Is Abundant in the Hippocampus, Cerebral Cortex, and Olfactory Bulb—To further evaluate the distribution of PLC\(\gamma\)2, we examined the specific localization of PLC\(\gamma\)2 in the brain. PLC\(\gamma\)2 was detected at a high level in the hippocampus, cerebral cortex, and olfactory bulb and weakly in the telencephalon, diencephalon, mesencephalon, and cerebellum. No expression was observed in the pons, medulla oblongata, or spinal cord (Fig. 5D). Comparison of the expression pattern of PLC\(\gamma\)2 with that of MAP2 (a marker of neurons), GFAP (a marker of astrocytes), and MBP showed that the expression pattern of PLC\(\gamma\)2 was very similar to that of MAP2, implying that PLC\(\gamma\)2 expression is correlated with neural function. Olfactory organs function in odor discrimination, pheromone perception, and odor and pheromone memory. To determine whether PLC\(\gamma\)2 is coupled to the odor or pheromone receptor, we separated olfactory organs into regions and determined the distribution of PLC\(\gamma\)2 (Fig. 5E). We could not detect PLC\(\gamma\)2 in the olfactory epithelium or vomeronasal organs, where odor and pheromone receptors exist. However, PLC\(\gamma\)2 was abundant in the main olfactory bulb and subolfactory bulb, indicating that PLC\(\gamma\)2 is not coupled to odor or pheromone receptors but is involved in odor and pheromone signal transduction.

In situ hybridization supported the expression pattern of PLC\(\gamma\)2 detected by Western blot analysis (Fig. 6). Antisense signals for PLC\(\gamma\)2 were observed in the olfactory bulb (Fig. 6, A–C), cerebral cortex (G–I), and hippocampus (M–O). The enlarged image of the hippocampus (Fig. 6, N and O) showed that pyramidal cells strongly express PLC\(\gamma\)2. The diencephalon, mesencephalon, pons, and medulla oblongata were not stained.

Various pH conditions. Maximal PLC activity of PLC\(\gamma\)2 and PLC\(\delta\)1 was obtained at pH 7.0 (Fig. 3E), similar to that observed for other PLC isozymes.

The N-terminal Region of PLC\(\gamma\)2 Contains a Localization Signal for the Plasma Membrane—Most PLC isozymes exist in the cytosol and translocate to the plasma membrane in response to cell stimulation through isozyme-specific anchoring mechanisms (26–29). PLC\(\delta\)1 expressed in HeLa S3 cells was localized predominantly in the cytosol (Fig. 4B, j). However, PLC\(\gamma\)2 was localized predominantly at the plasma membrane (Fig. 4B, a). Because the PH domain of PLC\(\delta\) provides a trigger for membrane targeting (26, 29), we next examined whether membrane binding of PLC\(\gamma\)2 occurs via the PH domain. A deletion mutant of PLC\(\gamma\)2 PH domain (Fig. 4A, ΔPH) localized predominantly to the cytosol (Fig. 4B, d), whereas the FLAG-tagged PH region was restricted to the plasma membrane (Fig. 4B, g). Distribution of these proteins to the cytosol or plasma membrane in cell populations is shown in Fig. 4C. Approximately 85% of full-length PLC\(\gamma\)2 and 97% of FLAG-tagged PH protein localized to the membrane, whereas less than 6% of ΔPH localized to the membrane. More than two-thirds of PLC\(\delta\)1 was detected in the cytosol. These results strongly suggest that the N-terminal PH region of PLC\(\gamma\)2 contains a targeting signal for the plasma membrane. To confirm these results, membrane and cytosol fractions were separated, and Western blot analysis was performed (Fig. 4D). Bands of full-length PLC\(\gamma\)2 and FLAG-tagged PH protein were detected in the membrane fraction, whereas bands of ΔPH and PLC\(\delta\)1 were detected mainly in the cytosol.

Increased Expression of PLC\(\gamma\)2 in Postnatal Brain—Although expression of PLC\(\gamma\)2 was abundant in adult brain, we did not detect it in embryos (data not shown). To understand the physiological function of PLC\(\gamma\)2, we examined the change in expression of PLC\(\gamma\)2 during brain development (Fig. 5A). At 1 week after birth, PLC\(\gamma\)2 was barely detected, and expression was first observed at 2 weeks after birth. Expression of PLC\(\gamma\)2 gradually increased during brain development and was similar to that of MBP, a marker of mature oligodendrocytes.
Identification of a Novel Phospholipase C, PLCγ2

PLCγ2

A. schematics of the structures of full-length PLCγ2 (Full) and truncated mutants are shown. EF, EF-hand. B, subcellular localization of full-length PLCγ2, ΔPH, FLAG-PH, and PLCγ1. HeLa S3 cells transiently transfected with PLCγ2 (Full), ΔPH, FLAG-PH, or PLCγ1 were immunostained with anti-PLCγ2, anti-FLAG, or anti-PLCγ1 antibody, respectively, followed by immunodetection with Alexa 488-conjugated secondary antibody. Actin was visualized with Alexa 594-conjugated phalloidin. Merged images are also shown. Scale bar, 8 μm. C, populations of cells with PLCγ2 were localized predominantly in the cytosol (C) or membrane (M), as shown in B. Values are presented as the mean ± S.D. of three experiments. D, subcellular fractionation of cells transfected with plasmids containing PLCγ2 (Full), ΔPH, FLAG-PH, or PLCγ1. At 24 h after transfection, cells were lysed with buffer and centrifuged at 10,000 × g for 60 min. The resultant supernatant (C) and insoluble (M) fractions were subjected to SDS-PAGE and Western blot.

fig. 4. the PH domain acts as a localizing signal for the plasma membrane. A, detailed distribution of PLCγ2 in mouse brain. Western blot analysis was performed with anti-PLCγ2, anti-MAP2, anti-GFAP, and anti-MBP antibodies on protein extracts from the indicated regions of mouse brain and spinal cord. Actin was used as a loading control. ob., oblongata. C, localization of PLCγ2 in olfactory organs. Protein extracts from the vomeronasal organ, subolfactory bulb, olfactory epithelium, and main olfactory bulb were subjected to SDS-PAGE and Western blot with anti-PLCγ2. Actin was used as a loading control.

fig. 5. expression of PLCγ2 by western blot analysis. A, increased expression of PLCγ2 with brain development. Lysates from mouse brain at 1 day (1d), 3 days (3d), 1 week (1w), 2 weeks (2w), 3 weeks (3w), and 4 weeks (4w) after birth were subjected to SDS-PAGE and Western blot for PLCγ2, MAP2, GFAP, and MBP. Actin was used as a loading control. B, detailed distribution of PLCγ2 in mouse brain. Western blot analysis was performed with anti-PLCγ2, anti-MAP2, anti-GFAP, and anti-MBP antibodies on protein extracts from the indicated regions of mouse brain and spinal cord. Actin was used as a loading control. ob., oblongata. C, localization of PLCγ2 in olfactory organs. Protein extracts from the vomeronasal organ, subolfactory bulb, olfactory epithelium, and main olfactory bulb were subjected to SDS-PAGE and Western blot with anti-PLCγ2. Actin was used as a loading control.

PLCγ2 is a Neuron-specific Enzyme—The distinct expression of PLCγ2 in the brain and during development implies that PLCγ2 plays a role in nerve tissues. We attempted to confirm this in primary cultured neurons and astrocytes. Neuron-containing primary cultures in defined medium (B27) and astrocyte-enriched cultures in conventional serum-containing medium (FBS) or ADM were used. Cell population was verified by the expression of MAP2, GFAP, or MBP by immunohistochemistry (Fig. 7A) and Western blot analysis (Fig. 7B). Although astrocytes were observed in all of these cultures, neurons were detected only in primary cultures (B27). As shown in Fig. 7B, PLCγ2 was detected only in primary cultures (B27) but not in astrocyte-enriched cultures (FBS and ADM). Oligodendrocytes were not detected in these cultures. These results indicate that PLCγ2 is expressed specifically in neurons and may have important functions postnatally.

Discussion

Herein we report the isolation of a novel PLC isozyme, PLCγ2, from mouse brain. The primary sequence of PLCγ2 suggests that it should be classified as a PLCβ-type enzyme because it includes the basic domains of PLC and an additional 290 amino acids at the C terminus (Fig. 1C). However, sequence comparison showed a similarity to PLCγ (Fig. 1, D and E). Recently, a novel class of PLC isozyme, PLCγ, was reported (21). Because PLCγ2 has the highest homology to PLCγ, we named this enzyme PLCγ2. One of the specific characteristics of PLCγ2 is the high sensitivity of PLC activity to [Ca2+] [Ca2+]2, which is similar to the sensitivity of PLCα. PLCγ2 is activated at [Ca2+]2 as low as 10 nm, indicating a sensitivity 10 times...
greater than that of PLCδ (Fig. 3D). PLCδ is known as the most calcium-sensitive PLC and is activated only by calcium. Therefore, it is possible that the increased calcium dependence of PLCγ2 allows it to act as a calcium sensor and to be activated by small increases in intracellular calcium concentration under physiological conditions. It is also noteworthy that PLCγ2 is localized predominantly to the plasma membrane (Fig. 4B, a) in contrast to most other PLC isozymes, which commonly exist in the cytosol and translocate to the plasma membrane in response to receptor activation. We showed by analysis of cell staining and subcellular fractionation that the PH domain of PLCγ2 is essential for membrane anchoring. Constitutive PH domain-mediated localization of PLCγ2 to the plasma membrane suggests a novel role of PLCγ2 as an early response molecule.

We further found that the expression of PLCγ2 is restricted to the brain (Fig. 2) and occurs after birth (Fig. 5A). The expression continues throughout the life of the adult (Fig. 5B). Robust synapse formation occurs during a short period of postnatal development. Thus, it is likely that PLCγ2 functions in the formation and maintenance of the neuronal network in the brain. Detailed examination by Western blot and in situ hybridization revealed that PLCγ2 is particularly abundant in pyramidal cells of the hippampus, cerebral cortex, and olfactory bulb. These organs are known to contribute to memory formation, suggesting that PLCγ2 may be involved in this function. PLCγ2 is also expressed in olfactory organs (Fig. 5C). Although PLCγ2 is not apparently coupled to odor or pheromone receptors, we detected expression of PLCγ2 in the main olfactory bulb and subolfactory bulb, in which sensory neurons synapse with projection neurons, indicating that PLCγ2 may be involved in odor and pheromone signal transduction. Finally, we detected PLCγ2 abundantly in neuron-containing primary cultures but not in astrocytes (Fig. 7B). These observations indicate that PLCγ2 may have important functions in neurons, such as neuronal network formation, a

**Fig. 6. In situ hybridization of PLCγ2.** Parasagittal sections of olfactory bulb (A–F), cerebral cortex (G–L), and hippocampus (M–R) were incubated with digoxigenin-labeled antisense (AS) cRNA probe (A–C, G–I, and M–O) and sense (S) cRNA probe (D–F, J–L, and P–R) for PLCγ2. From left to right, figures show low to high magnification. Cells were counterstained with nuclear fast red. Scale bar, 300 μm (A, D, G, J, M, and P); 100 μm (B, E, H, K, N, and Q); 25 μm (C, F, I, L, O, and R).

**Fig. 7. Expression of PLCγ2 in primary cultured hippocampal neurons.** A, rat hippocampal neuronal primary culture and cortical astrocyte-enriched culture were carried out. To confirm proper cell types, cells were co-stained with anti-MAP2 and anti-GFAP antibodies. Staining was visualized with appropriate Alexa 568 (MAP2, red) or Alexa 488 (GFAP, green)-conjugated secondary antibody. B, PLCγ2 distribution by Western blot analysis. Neuron-containing culture (B27) and astrocyte-enriched cultures (FBS and ADM) were subjected to SDS-PAGE and Western blot for PLCγ2, MAP2, GFAP, and MBP. Mouse brain lysate was used as a positive control. Actin was used as a loading control.
process that extends continuously from late embryogenesis to adulthood.

The importance of calcium in the brain has been widely reported, including involvement of axon growth and retraction, growth cone guidance, synapse formation, and responses of various neurotransmitters. Because PLC is a key enzyme in cellular calcium mobilization, PLCγ2 may play a specific role in calcium detection and mobilization in neurons.

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