The Interaction of Phospholipase C-β3 with Shank2 Regulates mGluR-mediated Calcium Signal*

Phospholipase C-β isozymes that are activated by G protein-coupled receptors (GPCR) and heterotrimeric G proteins carry a PSD-95/Dlg/ZO-1 (PDZ) domain binding motif at their C terminus. Through interactions with PDZ domains, this motif may endow the PLC-β isozyme with specific roles in GPCR signaling events that occur in compartmentalized regions of the plasma membrane. In this study, we identified the interaction of PLC-β3 with Shank2, a PDZ domain-containing multimodal scaffold in the postsynaptic density (PSD). The C terminus of PLC-β3, but not other PLC-β isotypes, specifically interacts with the PDZ domain of Shank2. Homer 1b, a Shank-interacting protein that is linked to group I metabotropic glutamate receptors and IP3 receptors, forms a multiple complex with Shank2 and PLC-β3. Importantly, microinjection of a synthetic peptide specifically mimicking the C terminus of PLC-β3 markedly reduces the mGluR-mediated intracellular calcium response. These results demonstrate that Shank2 brings PLC-β3 closer to Homer 1b and constitutes an efficient mGluR-coupled signaling pathway in the PSD region of neuronal synapses.

Phosphoinositide-specific phospholipase C (PLC) plays a pivotal role in transmembrane signaling. In response to various extracellular stimuli, such as numerous hormones, neurotransmitters, metabolic products, and growth factors, PLC catalyzes the hydrolysis of phosphatidylinositol 4,5-bisphosphate (PIP2), thereby generating messengers, diacylglycerol and inositol 1,4,5-trisphosphate (IP3), to the receptor for IP3 generated by PLC (21–23). This finding suggests that each PLC-β isoform has a unique distribution pattern in synaptic clustering the complexes of PLC-β and Shank2. Importantly, disrupting the PLC-β3-Shank2 interaction by injection with synthetic C-terminal heptapeptide of PLC-β3 reduced the mGluR-induced intracellular calcium signal. These results suggest that each PLC-β isoform has a distinct role.

Amino acid sequence analysis has shown that PLC-β isoforms share a consensus sequence (X(S/T)(V/L)-COOH) known as the postsynaptic density (PSD)-95/disc large/ZO-1 (PDZ) domain binding motif. PDZ domains exist in a large number of multifunctional proteins that mediate protein-protein interactions at highly specialized submembraneous sites, such as the postsynaptic density and cellular junctions (9–11). The postsynaptic density is a macroscopic submembraneous multi-protein complex within the dendritic spines and contains a variety of scaffolding and signaling proteins. Shank/ProSAP, a core component of the PSD, is a multimodular protein (~200 kDa) that contains various domains for protein interactions including ankyrin repeats, an SH3 domain, a PDZ domain, a long proline-rich region, and a SAM domain (12–15). The PDZ domain of Shank interacts with the GKAP/SAPAP family of synaptic scaffold proteins. This demonstrates that the Shank family of multidomain proteins (Shank1/2/3) plays an important role in the organization and maintenance of the PSD (16).

The metabotropic glutamate receptor (mGluR) triggers intracellular signaling events by activating PLC-β through the Gαq family (17, 18). mGluR is known to be important in defining the efficacy of mGluR coupling to PLC-β (19, 20). In addition, the Homer proteins, a family of adaptor proteins found at excitatory synapses, interact with mGluR and functionally link to the receptor for IP3 generated by PLC (21–23). This finding led us to postulate that the Homer protein may take part in the signal pathway between membrane mGluR and PLC.

In this study, we identified Shank2 as a PLC-β3-interacting protein. Shank2 specifically interacts with the C terminus of PLC-β3 but not with other isotypes. Furthermore, Homer 1b clusters the complexes of PLC-β3 and Shank2. Importantly, disrupting the PLC-β3-Shank2 interaction by injection with synthetic C-terminal heptapeptide of PLC-β3 reduced the mGluR-induced intracellular calcium signal. These results suggest that the complex of PLC-β3, Shank2 and Homer 1b may participate in organizing the molecules involved in PLC-β3-mediated signaling in the PSD.

**EXPERIMENTAL PROCEDURES**

**Yeast Two-hybrid System**—Yeast two-hybrid screening was performed as described previously (24). pBH3 (LexA fusion vector) carrying DNA oligomers encoding C-terminal heptapeptides of PLC-β were used as the bait in the screening of a rat brain cDNA library in which the cDNAs were inserted into the activation domain of GAL4 in pGAD10 (Clontech, Palo Alto, CA). We screened ~1.5 × 10^6 primary transformants for interacting proteins. Clones specifically interacting with the bait were identified by His growth and X-gal activity assays. The plasmids of positive clones were sequenced, and the sequences were and hippocampus, while PLC-β4 is enriched in the cerebellum. PLC-β3 is expressed throughout the brain (7, 8). These findings suggest that each PLC-β isoform may have a distinct role.

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1. The abbreviations used are: PLC, phospholipase C; PSD, post-synaptic density; GST, glutathione S-transferase; TRITC, tetramethylrhodamine isothiocyanate; ACPD, N-1,3-cyclopentanedicarboxylic acid; X-gal, 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside.
compared with sequences in the data bank using the National Center for Biotechnology Information (NCBI) BLAST system.

Plasmid Constructs—To express the glutathione S-transferase (GST) fusion form of the C-terminal region of PLC-β3 in *Escherichia coli*, the cDNA of C-terminal 337 amino acids was amplified by PCR with primers 5′-GGCAATTCGAGGCTTGACAC-3′ and 5′-GGCTCATGCTAAGGCCTTG-3′. The resultant PCR product was inserted as an EcoRI/XhoI fragment into pGEX-4T-1 (Amersham Biosciences). To express green fluorescent protein (GFP)-tagged PLC-β3 in mammalian cells, PCR products using appropriate primers were digested with EcoRI and BamHI and then ligated into pcDNA3. The cDNA of Shank2 was kindly provided by M. Sheng from the Massachusetts Institute of Technology (25). To express Myc-tagged Shank2 in mammalian cells, the cDNAs of whole Shank2 and PDZ domain-deleted Shank2 amplified by PCR were digested with Small and SalI, and then inserted into EcoR/VHxol-digested pCDNA3/Myc-HisA. The GST-fused PDZ domain of Shank2 was generated by subcloning the Shank2/Hxol fragment of amino acids 37–140 into pGEX-4T-3. The cDNA of Homer 1b was isolated from the rat brain cDNA library by PCR using the sense primer 5′-GAGTCAAAGCTGGGTGTTTTCC-3′ and the anti-sense primer of 5′-ACGCGTCACTTATCTTATGTGGCTG-3′. The GST-fused Homer 1b was generated by subcloning the EcoRI/Sall inserts into pGEX4T-1. We also constructed the HA-tagged Homer 1b gene inserts into the EcoRI/Sall sites of pCDNA3-HA, a HA epitope gene-containing plasmid.

Expression in COS7 Cells and Coinmunoprecipitation—COS7 cells were cultured in Dulbecco’s modified Eagle’s medium supplemented with 50 international units/ml penicillin, 50 μg/ml streptomycin, and 10% fetal bovine serum in a 5% CO2, 95% air incubator at 37 °C. The cDNAs for one of the PLC-β isotypes carrying an N-terminal FLAG epitope and the full-length rodent Shank2 were inserted into the pCIC vector with a C-terminal Myc epitope tag, co-expressed in the cells by transient transfection using Lipofectamine (Invitrogen, Life Technologies, Inc.). After 36 h, the transfected cells were harvested in lysis buffer (20 mM Tris-HCl (pH 7.4), 150 mM NaCl, 1% Triton X-100, 0.1 mM phenylmethylsulfonyl fluoride, 5 μg/ml aprotinin, and 1 μg/ml pepstatin). The lysates were centrifuged at 15,000 × g for 15 min at 4 °C, and 500 μl of supernatant from each sample was loaded with 2 μl of 0.1% Triton X-100, 50 mM Tris (pH 7.4) for 2 h at 4 °C. The pellets were then incubated with 4-methylcoumarinyl-labeled anti-FLAG antibody (1 μg/ml) or anti-HA antibody (1 μg/ml) overnight at 4 °C. Subsequently, the cells were washed with PBS and incubated in blocking buffer (1% goat serum in PBS containing 0.2% Triton X-100) at 4 °C for 1 h. Subsequently, the cells were incubated with anti-Shank2 antibody (1 μg/ml) or anti-HA antibody (2 μg/ml) overnight at 4 °C. After washing three times with PBS containing 0.05% Triton X-100 (10 min each), the cells were incubated with secondary antibodies: either rhodamine-conjugated anti-rabbit or anti-mouse antibody. After washing three times with PBS containing 0.05% Triton X-100, the slides were mounted and observed by a fluorescence microscope (Nikon, Inc., Melville, NY).

Immunocytochemistry on Cultured Hippocampal Neurons—Primary cultured rat hippocampal neuron prepared as described previously (28). In brief, hippocampus was isolated from rat embryos (20-day gestation), dissociated, plated on poly-lysine-coated glass coverslips, and then cultured in MEM with 10% horse serum. After 4 days in culture, the medium was replaced with MEM supplemented with N2 supplement, 1 mg/ml of ovulamin, 1 μM pyruvate, and 5 μM cytochrome arabinoside, cultured for 1 week. For immunofluorescence microscopy, the cultured cells were washed with phosphate-buffered saline, fixed with 4% paraformaldehyde in phosphate-buffered saline, chilled with solution A (20 μM phosphate buffer (pH 7.2), and 0.45 mM NaCl), and then treated with solution containing 20 mM Tris (pH 7.5), 140 mM NaCl, 0.1% Triton X-100, and 20% skimmed milk for 30 min. The samples were incubated with rabbit anti-PLC-β3 (Santa Cruz Biotechnology, Inc. mouse anti-Shank2 (25) and rat anti-Homer 1b antibodies (29) for 2 h. The samples were then washed with solution A, and incubated with fluorescein-conjugated anti-rabbit and Cy5-conjugated anti-mouse, and Cy5-conjugated anti-rat antibodies (Molecular Probes) for 30 min. After incubation, the samples were washed with solution A, embedded, and then viewed using a confocal imaging system (Zeiss LSM 510 Meta, Oberkochen, Germany).

Microinjection of Synthetic C-terminal Heptapeptide of PLC-β Isozymes and Intracellular Calcium Response to mGlur4 Agonist—E18 hippocampal cells were isolated from rat embryos by collagenase digestion. A solution was injected into the cells via a glass micropipette attached to a Microinjector (Eppendorf, Hamburg, Germany). Micropipettes had tip diameters of less than 1 micrometer. The microinjection buffer consisted of 27 mM KH2PO4, 8 mM Na2HPO4, 26 mM KH2PO4, 0.45M NaCl, 0.1% Triton X-100, and 2% Dextran-conjugated with TRITC was dissolved with microinjection buffer and used as a microinjection marker. Preparation for an injection, a micropipette was made and the glass micropipette had touched the cell. Then, by using a semi-automatic button, peptides were injected into the cell. The responsiveness of the intracellular calcium level was monitored after mGlur agonist treatment under a confocal microscope. For the intracellular calcium image, Fluo-3 AM (Molecular Probes) was dissolved (1 mg/ml) in dimethyl sulfoxide and stored at −20 °C until use. Cells were loaded with Fluo-3 AM (50 μM) and then incubated with 2% Dextran-conjugated with TRITC overnight. After washing with HBSS buffer, the cells were further incubated for 15 min in the absence of Fluo-3 AM to completely de-esterify the dye. To exclude possible effects of dye loading, we normalized with saponin at the end of the experiments. To normalize, we measured the residual fluorescence (F0) at the end of the experiment, and subtracted from the fluorescence of experiment conditions (F). To rule out the effect of extracellular calcium, all injection procedures were done in calcium-free solution. Excitation of Fluo-3 AM was provided by the 488-nm line of an argon laser, and the emission range was 515 nm. The confocal pinhole was set to the minimum opening compatible with a good signal-to-noise ratio. The intracellular calcium image was detected on an inverted confocal microscope (Zeiss LSM 510 Meta, Oberkochen, Germany) with an objective (×20). The concentration of the peptides used in our experiment ranged from 10 to 30 mM. Injection pressure and time were adjusted to 100–120 kPa and 0.5 s, respectively. Estimated intracellular concentration of peptides after injection came to ~100 to ~300 nM.

Competition of PLC-β3 Binding with Shank by Adding Synthetic C-terminal Peptide of PLC-β3 in Vitro—Subcellular fractions of hippocampal neuron in the rat brain (600 μg protein) in 400 μl of Nonidet P-40 in a centrifuge were precipitated by 10 mM paraformaldehyde for 10 min at 37 °C. The fixed cells were washed with PBS and incubated in blocking buffer (1% goat serum in PBS containing 0.2% Triton X-100) at 4 °C for 1 h. Subsequently, the cells were incubated with anti-Shank2 antibody (1 μg/ml) or anti-HA antibody (2 μg/ml) overnight at 4 °C. After washing three times with PBS containing 0.05% Triton X-100 (10 min each), the cells were incubated with secondary antibodies: either rhodamine-conjugated anti-rabbit or anti-mouse antibody. After washing three times with PBS containing 0.05% Triton X-100, the slides were mounted and observed by a fluorescence microscope (Nikon, Inc., Melville, NY).

Transfection and Clustering in COS7 Cells—COS7 cells were transfected with a combination of appropriate plasmids, using the Lipofectamine method (Invitrogen, Life Technologies, Inc.) on poly-l-lysine-coated coverslips. 24 h after transfection, the cells were fixed in 3.7% (v/v) paraformaldehyde for 10 min at 37 °C. The fixed cells were washed with PBS and incubated in blocking buffer (1% goat serum in PBS containing 0.2% Triton X-100) at 4 °C for 1 h. Subsequently, the cells were incubated with anti-Shank2 antibody (1 μg/ml) or anti-HA antibody (2 μg/ml) overnight at 4 °C. After washing three times with PBS containing 0.05% Triton X-100 (10 min each), the cells were incubated with secondary antibodies: either rhodamine-conjugated anti-rabbit or anti-mouse antibody. After washing three times with PBS containing 0.05% Triton X-100, the slides were mounted and observed by a fluorescence microscope (Nikon, Inc., Melville, NY).
PLC-β3-Shank2 Regulates mGluR-mediated Signal Pathway

**Table 1**

| pBHA clones | pGAD10 clones | HIS3 | β-Gal |
|-------------|---------------|------|-------|
| C-terminal of PLC-β3 (NTQL) | pGAD10 alone | - | - |
| | NHERF1 PDZ1 | - | | |
| | PSD95 PDZ1 | - | | |
| | NHERF2 PDZ2 | + | + |
| | Shank2 PDZ | + | + |

Data Analysis—The calcium imaging data are expressed as a means ± S.D. Statistical analysis was carried out using SAS 8.01 (SAS Institute Inc., Cary, NC). A difference was taken as significant if p < 0.05. Comparisons between cells were analyzed using repeated measure ANOVA (analysis of variance).

**RESULTS**

Identification of the PLC-β3-interacting Protein—The C-terminal heptapeptide (QQENTQL-COOH) of PLC-β3 was used as bait in the yeast two-hybrid assay for screening for proteins interacting with PLC-β3. One positive clone from a rat brain cDNA library was obtained and sequenced and found to contain the N-terminal 360 amino acids of Shank2, which harbors a PDZ domain. The specificity of the interaction between the C-terminal motif of PLC-β3 and these PDZ domains, including Shank2 PDZ, was examined using the yeast two-hybrid assay (Table 1). His activity and the β-galactosidase responses of the yeast cells transformed with the PLC-β3 motif and the PDZ of Shank2 were similar to those of the PLC-β3 motif and the PDZ2 of NHERF2, a positive control. No responses were exhibited by transformants of the PDZ domain of PSD-95 or NHERF1. These results demonstrate that the PLC-β3 specifically interacts with Shank2.

Interaction Domains between PLC-β3 and Shank2 in Vitro—To confirm the interaction between PLC-β3 and Shank2, GST-Shank2 protein was used in a pull-down assay of COS7 cell lysates transfected with FLAG-PLC-β3 or FLAG-PLC-β3ΔC (deleted C-terminal four amino acids, PDZ binding motif). Full-length PLC-β3, but not the deleted mutant, was pulled down by GST-Shank2 (Fig. 1A). Conversely, Shank2 expressed in COS7 cells was precipitated by the GST-PLC-β3 C-terminal 337 amino acids, but not GST alone (Fig. 1B).

Specific Interaction of PLC-β Isotypes and Shank2 in Vivo—All PLC-β isotypes contain the PDZ binding motif at their C termini that may enable the PLC-β to associate with the PDZ domain-containing proteins. To investigate the interaction between Shank2 and each PLC-β isotype, immunoprecipitation using an anti-FLAG antibody was performed with extracts of COS7 cells expressing Shank2 and each of the FLAG-tagged PLC-β isotypes. Shank2 was detected in the immunoprecipitates only with FLAG-PLC-β3, but not with any of the other isotypes (Fig. 2A), implying that Shank2 specifically interacts with PLC-β3. To characterize this interaction, the lysates of COS7 cells co-expressing FLAG-PLC-β3 and Myc-Shank2 were immunoprecipitated with FLAG antibodies. As shown in Fig. 2, B and C, PLC-β3 was co-precipitated with Shank2, whereas the C-terminally deleted PLC-β3 and the PDZ domain-deleted form of Shank2 were not co-precipitated with Shank2 and PLC-β3, respectively. These results suggest that PLC-β3 interacts with the PDZ domain of Shank2 via the C-terminal PDZ binding motif.

Fig. 1. Interaction of Shank2 with PLC-β3 in vitro. A, PLC-β3 interacts with Shank2 through its C-terminal PDZ binding motif. Extracts of COS7 cells expressing FLAG-tagged wild type or the C-terminally deleted form of PLC-β3 were precipitated with GST-Shank2 PDZ domain, and the precipitated proteins were detected with anti-FLAG antibody. The first two lanes (Lysate) were directly loaded with each transfected cell lysate (5% of input). B, Interaction of GST-PLC-β3 C terminus with Shank2. Conversely, the extracts of COS7 cells expressing Shank2 were precipitated with GST-PLC-β3 C-terminal (337 amino acids). The precipitates were detected with anti-Shank antibody. The first lane (Lysate) was loaded with the transfected cell lysate (5% of input).

Several reports demonstrate that PDZ domains bind to the C-terminal four amino acids of the target proteins (11). To identify critical residues of the PLC-β3 C terminus that are required for the Shank2 interaction, each of the last four amino acid residues of PLC-β3 (NTQL-COOH) were mutated to Ala. The extracts of COS7 cells co-expressing each FLAG-tagged PLC-β3 mutant and Shank2 were incubated with an anti-FLAG antibody, and the precipitates were then probed with an anti-Shank antibody. Substitution of Thr to Ala at the second-ary amino acid or Leu to Ala at the last position resulted in a complete loss of Shank2 binding to PLC-β3. In contrast, the substitution of the other residues to Ala had no effect (Fig. 2D). These results thus indicate that the Thr and Leu residues of PLC-β3 are essential for the interaction with Shank2.

PLC-β3 Associates with Homer 1b Through Shank2—Recently, it was reported that Shank1 and Shank3 associate with the EVH domain of the Homer family (23), and sequence analysis indicates that Shank2 possesses two similar proline-rich Homer binding regions. To verify that Shank2 can also associate with Homer and that Shank2 can link PLC-β3 to Homer 1b, COS7 cells expressing Shank2 and PLC-β3 were used in pull-down assays with GST-Homer 1b. Extracts of COS7 cells co-expressing Shank2 and Homer 1b were used for precipitation with the GST-PLC-β3 C-terminal motif. As shown in Fig. 3, A and B, GST-Homer 1b pulled-down PLC-β3 only in the presence of Shank2. Conversely, the GST-PLC-β3 C terminus pulled down Homer 1b only in the presence of Shank2, suggesting that these three proteins form a ternary complex.

To further explore the ternary complex of PLC-β3, Shank2, and Homer 1b, immunocytochemistry was performed on COS7 cells expressing these proteins. In triply expressed cells, PLC-β3, Shank2, and Homer 1b were distributed in plaque-like clusters (Fig. 4A, panels A1 and B1). In a control experiment, PLC-β1 did not colocalize with Shank2 and Homer 1b (Fig. 4A,
These results are consistent with the lack of interaction between PLC-β1 and Shank2 in coimmunoprecipitation assays (Fig. 2A). When C-terminal-deleted PLC-β3 (Fig. 4A, panel D1) or PDZ-domain-deleted Shank2 (Fig. 4A, panel E1) were co-expressed with the other proteins, PLC-β3 did not exhibit any clustering with Shank2. On the other hand, both the wild-type and the PDZ-deleted form of Shank2 were clustered with Homer 1b, consistent with the previous finding that the Homer binding region resides in the proline-rich region of Shank2 (29).

We further verified the ternary complex of PLC-β3, Shank2, and Homer1b using freshly isolated hippocampal cells. Similar with the result of COS7, PLC-β3, Shank2, and Homer1b were existed as a plaque-like cluster form in these cells also (Fig. 4B). Taken together, these results suggest that...
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Shank2 mediates the formation of a ternary complex containing Homer 1b and PLC-β3.

Coimmunoprecipitation of Shank2-interacting Proteins in Brain—Next we determined the in vivo association of Shank2, PLC-β3, Homer 1b, and IP$_3$ receptor. When the P2 fraction of adult rat brain was immunoprecipitated with Shank2 antibody, PLC-β3, Homer 1b, and IP$_3$ receptor were coimmunoprecipitated, but not with IgG (Fig. 5A). In addition, to determine whether C-terminal heptapeptide of PLC-β3 can compete with the endogenous PLC-β3-Shank2 interaction, we added the synthetic C-terminal peptide of PLC-β3 during the immunoprecipitation reaction. For immunoprecipitation, we incubated Shank2 antibody with P2 fraction of adult rat brain. The amount of coimmunoprecipitated PLC-β3 with Shank2 decreased by synthetic PLC-β3 peptide addition in a dose-dependent manner, whereas the association was unaffected by addition of C-terminal peptides of other isozymes (Fig. 5B). Taken together, these results demonstrate that PLC-β3 and Shank2 may be distributed to synaptic sites and form a complex in brain.

Effect of Microinjection of Synthetic PLC-β3 C-terminal Heptapeptide on mGluR Agonist-induced Intracellular Calcium Release—To gain physiological insights into PLC-β3 binding with Shank2, we investigated the effect of this binding on mGluR agonist-induced intracellular calcium signal. To accomplish this, we used HN33 hippocampal cells, because these cells showed an intracellular calcium increase in response to a mGluR agonist, N-1,3-cyclopentanedicarboxylic acid (ACPD, 20 μM). It has been suggested that Shank may cross-link Homer with the PSD-95 complex in the postsynaptic density and play a role in the mGluR signaling. Such a linkage has important implication in the coupling of mGluR receptors to intracellular calcium pools. Therefore, we hypothesized that the calcium release responding to the mGluR agonist might be changed by disrupting Shank2-PLC-β3 binding. To test this hypothesis, we microinjected a synthetic C-terminal heptapeptide of PLC-β3 into the cytoplasm of the HN33 cells and monitored the calcium signal. As a control, we injected C-terminal heptapeptides of other PLC-β isozymes. Cells injected with PLC-β3 peptide showed a markedly decreased calcium signal (Fig. 6). Injection with peptides of other PLC-β isozymes (PLC-β1, PLC-β2, and PLC-β4) did not cause any detectable changes in the calcium signaling. Altogether, we injected 10 cells at a time and performed three independent experiments. These results were reproduced in every experiment. To rule out the involvement of an extracellular calcium effect, we performed the microinjection procedure under calcium-free buffer condition.

These results demonstrate that microinjection of the C-terminal peptide of PLC-β3 suppresses the mGluR agonist induced calcium release in a dose dependent manner, but C-terminal peptides of other isozymes have no significant effect. Taken together, these results demonstrate that the PLC-β3-Shank2 interaction may play an important role in mGluR-mediated intracellular calcium release.

DISCUSSION

In this report, we identified a modular interaction between the C terminus of PLC-β3 and the PDZ domain of Shank2. In support of this conclusion, the C-terminal four amino acids of PLC-β3 are required for the interaction with Shank2 (Fig. 1), and point mutations at the PLC-β3 C terminus eliminate the Shank2 interaction (Fig. 2D). In addition, our data indicate that Shank2 forms a specific complex with PLC-β3, but not with other PLC-β isoforms (Fig. 2A), despite the fact that other PLC-β isoforms also contain the PDZ domain binding motif at their C termini. These results suggest that PLC-β isoforms may act in different signaling pathways by interacting with distinct PDZ-containing proteins.

Previously, we had found that NHERF2 specifically interacts with and enhances the activation of PLC-β3 by the muscarinic receptor (30). However, other isotypes of PLC-β barely interact with NHERF2. With respect to Shank2, the immunoprecipitation analyses performed in the present study demonstrated that Shank2 specifically interacted with PLC-β3, but not with
the other isotypes. In immunoblot analysis, Shank2 was detected in brain tissues, but NHERF2 was not detected, implying that each of two PDZ proteins may play a role in PLC-β3-mediated signaling in different tissues. It has been known that PDZ-containing proteins mediate the clustering of receptors and signaling molecules and thereby regulate agonist-induced signal transduction (31). Thus, the signaling through PLC-β3 may be spatially regulated in cells via the interaction with PDZ-containing proteins.

Proline-rich motifs of Shank2 have been shown to interact with the Homer family, EVH domain-containing proteins (23). Recently, it has been demonstrated that Homer 1 operates in a negative feedback loop that regulates the structure and function of the synapse (32). Homer proteins normally exist in oligomeric forms and are known to interact with group 1 metabotropic glutamate receptors and the inositol 1,4,5-trisphosphate receptor, and therefore may be involved in the cytosolic calcium release by IP₃, a product of PLC activation (22, 27). Given these findings, we propose that Shank2 may serve as a scaffold for G protein-coupled receptor signaling cascades in the PSD by forming bridges between the Homer family and PLC-β3. Shank2, and PLC-β3 undergo a dramatic

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**FIG. 5.** PLC-β3 is coimmunoprecipitated with Shank2 and Homer 1b in the PSD fraction and is disrupted by adding synthetic PLC-β3 C-terminal heptapeptide in vitro. A, extracts of rat hippocampal synaptosomal membranes were immunoprecipitated with anti-Shank antibodies, or with control immunoglobulin (IgG). Immunoprecipitates were then immunoblotted with anti-PLC-β3, Homer 1b and IP₃ receptor antibodies as indicated. The first lane (lysates) was loaded with the immunoprecipitation extract (10% of input). B, competition of the PLC-β3 binding with Shank by adding the synthetic C-terminal peptide of PLC-β3 in vitro. 600 μg of extracts of rat hippocampal synaptosomal membranes was immunoprecipitated with anti-Shank antibody, while various concentrations of synthetic C-terminal peptides of each PLC-β isozyme were added. Immunocomplexes were analyzed by Western blotting with anti-PLC-β3 antibody.

**FIG. 6.** Microinjection of synthetic C-terminal heptapeptide of PLC-β3 suppresses the mGluR agonist-induced intracellular calcium increase in HN33 cells. The peak increase in fluo-3 fluorescence (F/Fₒ) in response to 20 μM ACPD, mGluR agonist. Isozyme-specific C-terminal peptides were microinjected into the cells prior to ACPD treatment. The calcium signal was obtained by an image software program and viewed as a function of time. Results are representative of at least three individual experiments. Solid lines indicate the non-transfected cells and dotted lines indicate the microinjected cells. p < 0.0001.
re-localization to form plaque-like clusters when they are co-expressed with Homer 1b. The experiment using deletion mutants of Shank2 or PLC-β3 indicates that PLC-β3 is recruited into Shank2/Homer 1b complexes through PDZ domain-mediated interaction.

As previously reported, Shank2 is enriched in the PSD fractions (33). Considering PSD signaling events in neuronal cells harboring mGluR signaling networks (34), it is believed that PLC-β3 may be involved in mGluR-mediated signaling, and Homer 1b actually increases mGluR-evoked calcium mobilization (29). Up to now, the role of scaffolding proteins in mediating tyrosine kinase receptors is well characterized (35). Howver, much less is known about the scaffolding protein’s role in PLC-β3-mediated tyrosine kinase receptor activity. Thus, whether mGluR-evoked calcium response occurs through recruiting signal molecules to the PLC-β3-harboring mGluR signaling networks (34), it is believed that signal molecules are involved in a specific signaling cascade by being recruited to specific compartments. Homer 1b, a type I mGluR-interacting protein, forms clusters with PLC-β3, recruiting them to specific compartments. Homer 1b actually increases mGluR-evoked calcium mobilization (29). Considering PSD signaling events in neuronal cells (33). Considering PSD signaling events in neuronal cells, restricted signaling complexes in the PSD are made possible. This finding may lead us to a better understanding of the role of Shank2 in PLC-β3-mediated mGluR signaling.