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

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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 multimodular 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 IP_3 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)[1] 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 (PIP_2), thereby generating messengers, diacylglycerol and inositol 1,4,5-trisphosphate (IP_3) (1, 2). Mammalian PLC isozymes have been classified into five types: PLC-β, PLC-γ, PLC-δ, PLC-ε, and PLC-ζ (3–6). Immunohistological studies have shown that each isotype has a unique distribution pattern in the brain. PLC-β1 is highly expressed in the cerebral cortex 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 isotype may have a distinct role.

Amino acid sequence analysis has shown that PLC-β isotypes share a consensus sequence (*X*(S/T)*X*(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 submembranous sites, such as the postsynaptic density and cellular junctions (9–11). The postsynaptic density is a macroscopic submembranous multiprotein 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 $G\alpha_q$ 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 IP_3 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). pBHA (LexA fusion vector) carrying DNA oligomers encoding C-terminal heptapeptides of PLC- β3 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

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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′-GCGAATTCCAGCAAGGCTCACAGCTC-3′ and 5′-GCCTCGAGTCAAAGCTGGGTGTTTTCC-3′. The resultant PCR product was inserted as an EcoRI/XhoI fragment into pGEX4T-1 (Amersham Biosciences). To express green fluorescent protein (GFP)-tagged PLC-β3 in mammalian cells, PCR products using appropriate primers were digested with EcoRI/SmaI and then ligated into pEGFP-C1. 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 SmaI/SalI, and then inserted into EcoRV/XhoI-digested pCDNA3.1/Myc-HisA. The GST-fused PDZ domain of Shank2 was generated by subcloning the SmaI/XhoI insert of amino acids 37–140 into pGEX4T-3. The cDNA of Homer 1b was isolated from the rat brain cDNA library by PCR using the sense primers 5′-GCGAATTCATGGGGGAACAACCTATC-3′ and the antisense primers of 5′-ACGCGTCGACTCATTTAATCATGATTGCTG-3′. The GST-fused Homer 1b was generated by subcloning the EcoRI/SalI inserts into pGEX4T-1. We also constructed the HA-tagged Homer 1b gene by subcloning the inserts into the EcoRI/SalI sites of pCDNA3-HA, a HA epitope gene-containing plasmid.

Expression in COS7 Cells and Coimmunoprecipitation—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% CO_2, 95% air incubator at 37 °C. The cDNAs for one of the PLC-β isotypes carrying an N-terminal FLAG-epitope tag, and either the wild-type or PDZ-deleted form of Shank2 with a C-terminal Myc epitope tag, were 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 μM pepstatin). The lysates were centrifuged at 15,000 × *g* for 15 min at 4 °C, and 500 μg of the clarified lysate were incubated with anti-FLAG antibody precoupled to protein A-Sepharose for 3 h at 4 °C for immunoprecipitation. The immune complexes were collected by centrifugation (3 min at 3000 × *g*), washed four times with lysis buffer, and denaturated by heating in SDS sample buffer. Proteins were separated by 8% SDS-PAGE. The separated proteins were then transferred to nitrocellulose membranes (Schleicher & Schuell BioScience, Keene, NH). Membrane blocking to prevent nonspecific binding was done with TTBS buffer (10 mM Tris-HCl (pH 7.6), 150 mM NaCl, 0.1% Tween 20) containing 5% skim milk powder. The blocked membranes were then incubated with the appropriate antibodies for 4 h at room temperature, after which they were washed and incubated with horseradish peroxidase-linked secondary antibody for 1 h at room temperature, washed four times in TTBS buffer, and developed using horseradish peroxidase-dependent chemiluminescence (ECL) (Amersham Biosciences).

For the coimmunoprecipitation assay with synaptosome, the synaptosomal fraction (P2) was extracted in 1% deoxycholic acid and dialyzed overnight into 0.1% Triton X-100, 50 mM Tris (pH 7.4). Concurrently, 2 μg of each antibody was preincubated overnight with 10-μl bed volume of protein A-agarose. After centrifugation at 100,000 × *g* for 1 h, 100 μg of extract was incubated with antibody-protein A in 100 μl of 0.1% Triton X-100, 50 mM Tris (pH 7.4) for 2 h at 4 °C. The pellets were washed four times with 1 ml of incubation buffer, and bound proteins were analyzed by immunoblotting as indicated antibodies, including IP_3 receptor (26).

Expression and Purification of GST Fusion Proteins—Recombinant proteins were expressed in the *E. coli* strain BL21 containing the appropriate cDNA constructs. Expression was induced by isopropyl-β-D-thiogalactopyranoside (0.1 mM) for 4 h at 25 °C. Subsequently, the cells were pelleted and sonicated in lysis buffer. The soluble fraction, after centrifugation at 15,000 × *g* for 20 min at 4 °C, was incubated with glutathione-Sepharose 4B (Amersham Biosciences) for 3 h at 4 °C and washed four times with ice-cold lysis buffer.

Pull-down Assay—COS7 cells transiently expressing the PLC-β isotypes or Shank2 were lysed with lysis buffer and centrifuged. The lysates (500 μg) were then incubated with 5 μg of the GST fusion proteins immobilized on glutathione-Sepharose beads at a final lysis buffer volume of 1 ml for 2 h at 4 °C. The protein complexes were collected by centrifugation and washed with lysis buffer, followed by boiling in SDS sample buffer. Proteins were resolved by SDS-polyacrylamide gel electrophoresis and immunoblotted.

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% (w/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 examined by fluorescence microscopy (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 embryo (20-day gestation), dissociated, plated on poly-L-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 ovalbumin, 1 μM pyruvate, and 5 μM cytosine 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 for 1 h, washed with solution A (20 μM phosphate buffer (pH 7.2), and 0.45 M 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-β 3 (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 isothiocyanate-conjugated anti-rabbit, Texas red-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 mGluR Agonist—Established hippocampal cells, HN33 were chosen for microinjection. 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 solution consists of 27 mM K_2HPO_4, 8 mM Na_2HPO_4, 26 mM KH_2PO_4. Dextran-conjugated with TRITC was dissolved with microinjection buffer and used as a microinjection marker. In preparation for an injection, a micropipette was lowered to ensure the micropipette had impaled 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 2 μl Fluo-3 AM for 40 min at room temperature. 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 the possible effects of dye loading, we normalized with saponin at the end of the experiments. To normalize, we measured the residual fluorescence (F_o) 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 lysis buffer were prepared. For immunoprecipitation, Shank antibody was incubated with hippocampal homogenate. In addition, various concentrations of the synthetic C-terminal heptapeptide of each PLC-β isozymes were co-incubated to compete with endogenous PLC-β3 and Shank binding. After incubation for 4 h at 4 °C, immunocomplexes were separated by incubation with protein-A/G Sepharose. Western blotting was performed with the PLC-β3 antibody by standard procedures and detected by ECL chemiluminescence.

TABLE I
Shank2 interacts specifically with the PDZ domain binding motif of PLC-β3

The C-terminal heptapeptide of PLC-β3 was tested for its ability to bind Shank2 in the yeast two-hybrid assay, where it is revealed by the induction of the yeast reporter genes HIS3 and β-galactosidase. HIS activity (measured as percentage of colonies growing on histidine-lacking medium) was rated as follows: +++(>60%), ++(30–60%), +(<30%), −(no significant growth); β-galactosidase activities (time needed for yeast colonies to turn blue in X-gal filter lift assays at room temperature) were as follows: +++(<20 min), ++(20–60 min), −(no detection). PDZ domain of NHERF2 was used as positive control.

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 (QEENTQL-COOH) of PLC-β3 was used as bait in the yeast two-hybrid assay 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 the various PDZ domains, including Shank2 PDZ, was examined using the yeast two-hybrid assay (Table I). 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. 1*A*). Conversely, Shank2 expressed in COS7 cells was precipitated by the GST-PLC-β3 C-terminal 337 amino acids, but not GST alone (Fig. 1*B*).

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-βs 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. 2*A*), 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.

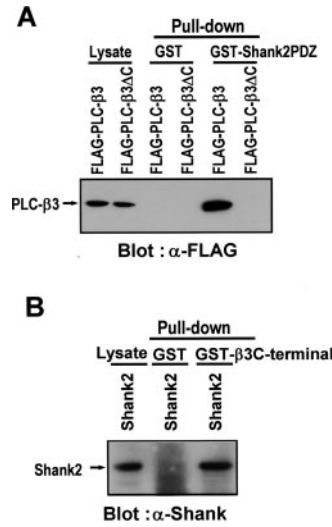

FIG. 1. **Interaction of Shank2 with PLC-β3 *in vitro*.** *A*, PLC-β3 interacts with Shank2 through its C-terminal PDZ binding motif. Extracts of COS7 cells expressing FLAG-tagged wild type or the C-terminally deleted form of PLC-β3 were precipitated with GST-Shank2 PDZ domain, and the precipitated proteins 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 secondary 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. 2*D*). 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 extracts 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. 4*A*, *panels A1* and *B1*). In a control experiment, PLC-β1 did not colocalize with Shank2 and Homer 1b (Fig. 4*A*,

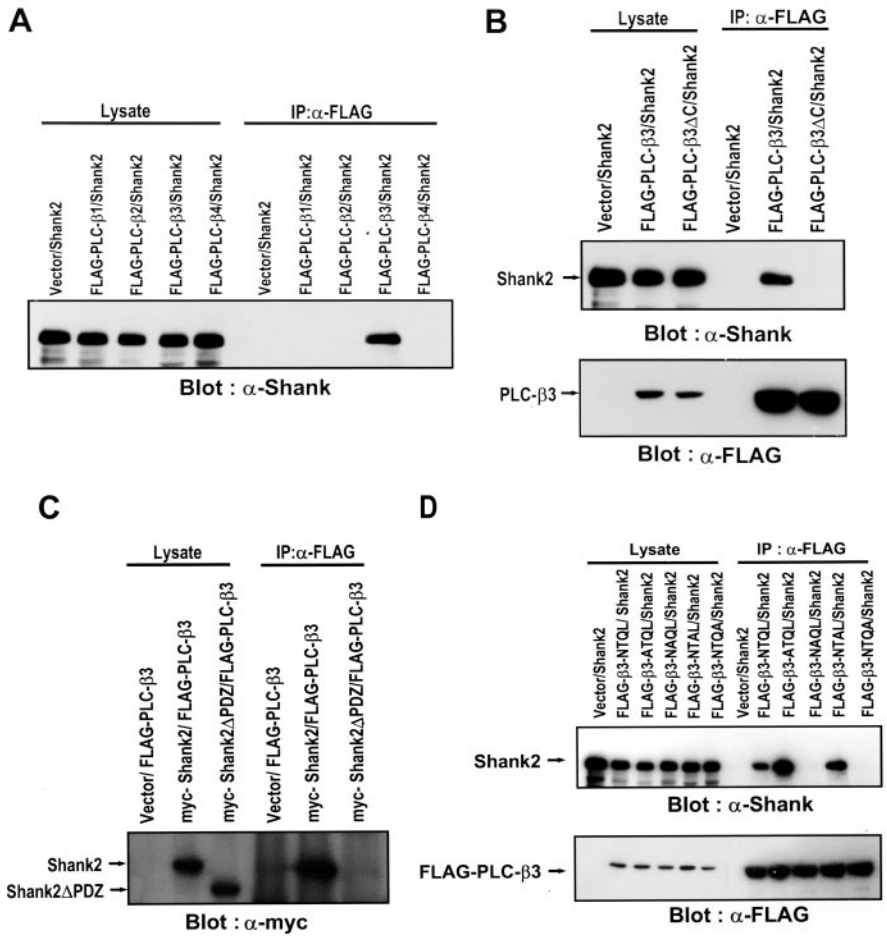

FIG. 2. **Coimmunoprecipitation of Shank2 and PLC-β3 via the PDZ domain from cotransfected COS cells.** *A*, extracts of COS7 cells expressing Shank2 together with one of the PLC-β isotypes (isotypes 1–4) were allowed to immunoreact with anti-FLAG antibody. The precipitates were separated by SDS-polyacrylamide gel electrophoresis, and probed with anti-Shank antibody. *Left lanes* (*lysates*) were each loaded with transfected cell lysates (5% of input). *B*, PLC-β3 interacts with Shank2 through its PDZ binding motif. The extracts of COS7 cells expressing Shank2 together with wild-type or C-terminally deleted FLAG-PLC-β3 were immunoprecipitated using anti-FLAG antibody. The precipitates were then analyzed with anti-Shank antibody (*upper panel*) or anti-FLAG antibody (*lower panel*). The *three lanes* on the *left* (*lysates*) were directly loaded with transfected cell lysates (5% of input). *C*, extracts of COS7 cells expressing FLAG-PLC-β3 with wild-type or PDZ-deleted Myc-Shank2 were incubated with anti-FLAG antibody. The immune complexes were analyzed with anti-Myc antibody. The *left three lanes* (*lysates*) were each loaded with transfected cell lysates (5% of input). *D*, each residue of the PDZ binding motif in PLC-β3 was mutated in turn to Ala by the polymerase chain reaction with mutagenic primers. Extracts of COS7 cells expressing Shank2 with one of the point-mutated forms of PLC-β3 were allowed to immunoreact with anti-FLAG antibody. The precipitated proteins were probed with anti-Shank antibody (*upper panel*) or anti-FLAG antibody (*lower panel*). *Left lanes* (*lysates*) were each loaded with transfected cell lysates (5% of input).

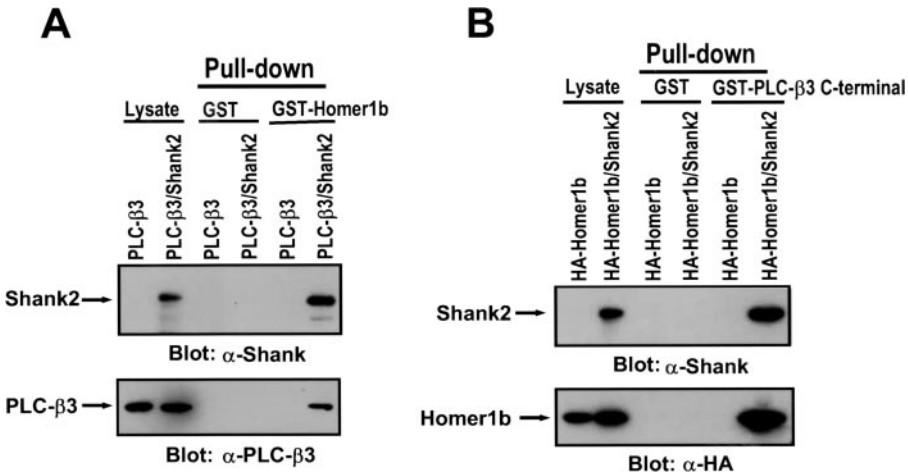

FIG. 3. **Shank2 mediates the association of PLC-β3 and Homer 1b.** *A*, GST-Homer 1b interacts with the PLC-β3-Shank2 complex. The extracts of COS7 cells expressing PLC-β3 alone, or PLC-β3 and Shank2 were incubated with GST-Homer 1b immobilized on glutathione beads. The precipitates were analyzed using anti-Shank antibody (*upper panel*) or anti-PLC-β3 antibody (*lower panel*). *Left two lanes* (*Lysate*) were loaded directly with each transfected cell lysates (5% of input) (*B*). The GST-fused PLC-β3 C terminus interacts with the Homer 1b-Shank2 complex. Extracts of COS7 cells expressing Homer 1b alone, or Homer 1b and Shank2 were incubated with GST-fused PLC-β3-C terminus (337 amino acids). The complexes were analyzed with anti-Shank antibody (*upper panel*) or anti-HA antibody (*lower panel*). The *two left lanes* (*lysates*) were each loaded directly with transfected cell lysates (5% of input). *HA*, hemagglutinin.

panel C1). These results are consistent with the lack of interaction between PLC-β1 and Shank2 in coimmunoprecipitation assays (Fig. 2*A*). When C-terminal-deleted PLC-β3 (Fig. 4*A*, *panel D1*) or PDZ-domain-deleted Shank2 (Fig. 4*A*, *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 Homer1 b using freshly isolated hippocampal cells. Similar with the result of COS7, PLC-β3, Shank2, and Homer 1b were existed as a plaque-like cluster form in these cells also (Fig. 4*B*). Taken together, these results suggest that

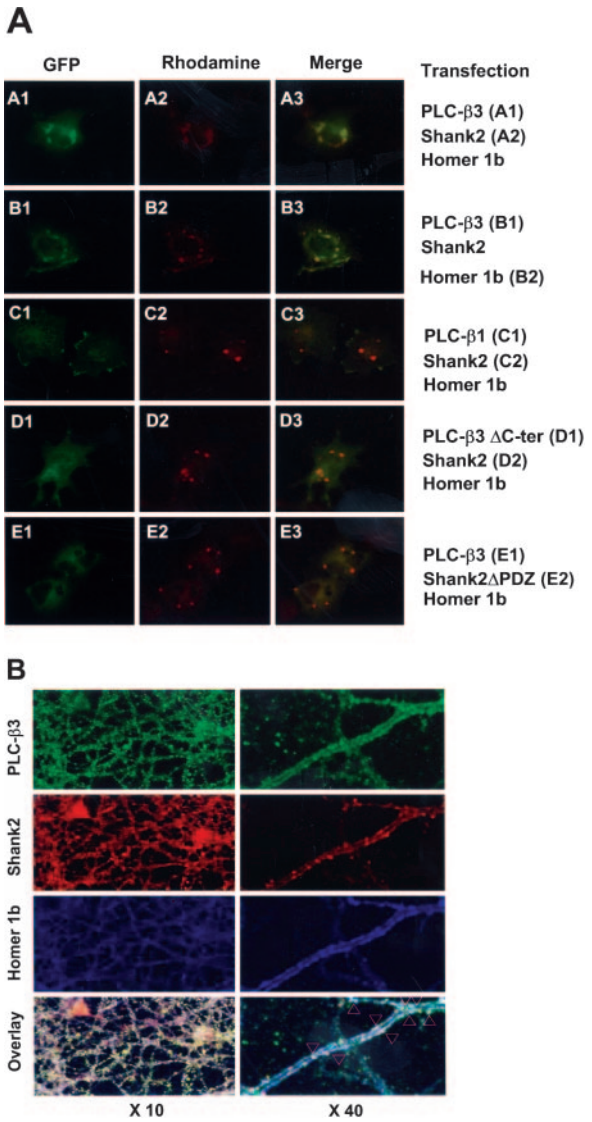

FIG. 4. **Clustering of PLC-β3 with Shank2 mediated by Homer 1b requires PDZ domain-mediated interaction.** *A*, clustering of PLC-β3 with Shank2 is mediated by Homer 1b in heterologous cells. COS7 cells triply transfected with GFP-PLC-β3, Shank2, and Homer 1b were fixed and labeled with anti-Shank antibody (*panel A2*) or anti-HA antibody (*panel B2*). Each protein was visualized as described under "Experimental Procedures." COS7 cells triply transfected with GFP-PLC-β1, Shank2, and HA-Homer 1b were fixed and labeled with anti-Shank antibody (*panel C2*). COS7 cells were triply transfected with C terminus-deleted GFP-PLC-β3, Shank2 and Homer 1b (*panel D*), or GFP-PLC-β3, PDZ-domain-deleted Shank2 and Homer 1b (*panel E*). Shank2 was visualized using rabbit anti-Shank antibodies and rhodamine-conjugated anti-rabbit secondary antibodies. Merged images are shown in the panels on the *right* (*panels A3*, *B3*, *C3*, *D3*, *and E3*). *B*, localization of PLC-β3, Shank2 and Homer 1b in cultured hippocampal cells. Cultured hippocampal cells were triple immunostained with anti-PLC-β3 (*green*, *upper*) and anti-Shank2 (*red*, *middle*) and anti-Homer 1b (*blue*, *lower*). The images were merged in the *bottom panels*. *Left panels* were imaged at low-resolution (×10). *Right panels* were imaged at high-resolution (×40). *Arrowhead* indicates the colocalization of triple complexes.

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. 5*A*). 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. 5*B*). 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. 2*D*). In addition, our data indicate that Shank2 forms a specific complex with PLC-β3, but not with other PLC-β isoforms (Fig. 2*A*), 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

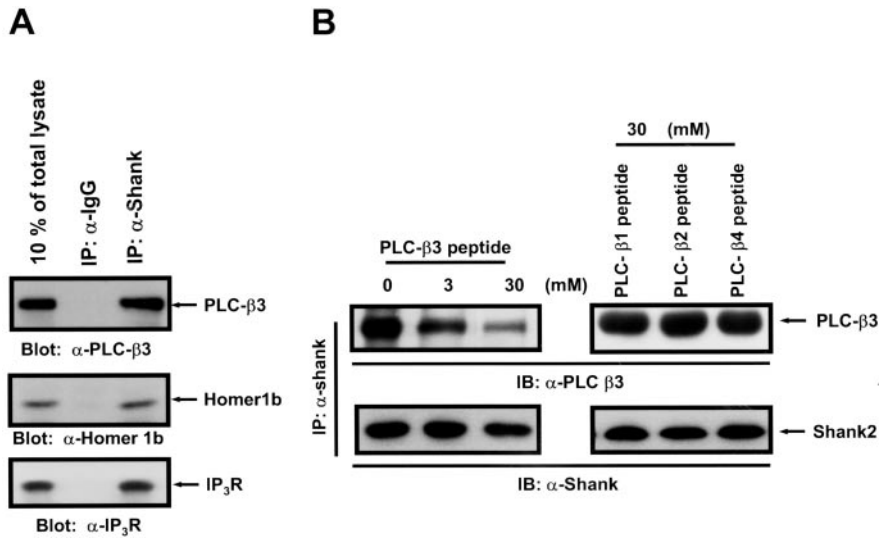

FIG. 5. **PLC-β3 is coimmunoprecipitated with Shank2 and Homer 1b in the PSD fraction and is disrupted by adding synthetic PLC-β3 C-terminal heptapeptide *in vitro*.** *A*, extracts of rat hippocampal synaptosomal membranes were immunoprecipitated with anti-Shank antibodies, or with control immunoglobulin (*IgG*). Immunoprecipitates were then immunoblotted with anti-PLC-β3, Homer 1b and IP_3 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.

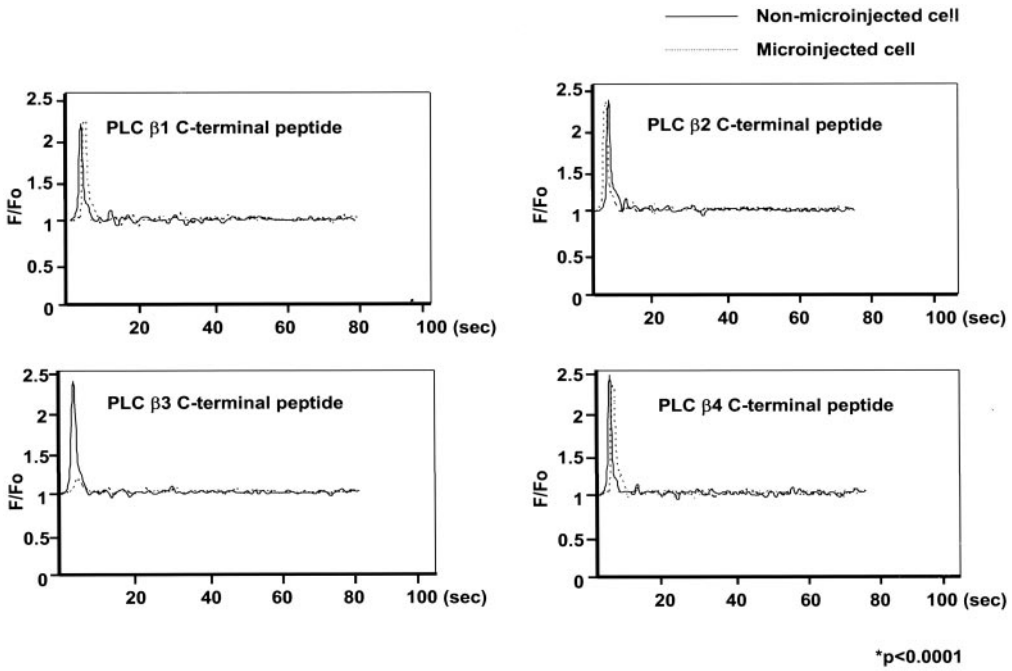

FIG. 6. **Microinjection of synthetic C-terminal heptapeptide of PLC-β3 suppresses the mGluR agonist-induced intracellular calcium increase in HN33 cells.** The peak increase in fluo-3 fluorescence (F/F_o) in response to 20 μM ACPD, mGluR agonist. Isozyme-specific C-terminal peptides were microinjected 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. $p < 0.0001$.

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 IP_3, 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

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). However, much less is known about the scaffolding protein's role in calcium signaling. In synapses, PSD-95, Shank, GRIP, and probably other scaffolds, participate in the assembly of signaling complexes, including the role for calcium signaling. In the present study, we provided the evidence for the role of the binding between Shank2 and PLC-β3 in mGluR-mediated calcium mobilization. We did not check the direct effect of Shank2 on PLC-β activity. Thus, whether mGluR-evoked calcium response occurs through recruiting signal molecules to the mGluR receptor or directly affecting PLC-β activity is yet to be determined.

In conclusion, PLC-β3 interacts with Shank2 via the PDZ binding motif. The isotype-specific interaction of PLC-β3 with Shank2 shows that PDZ-containing proteins allow signaling molecules to be involved in a specific signaling cascade by recruiting them to specific compartments. Homer 1b, a type I mGluR-interacting protein, forms clusters with PLC-β3, which is mediated by Shank2. Upon clustering of the ternary complex consisting of Homer 1b-Shank2-PLC-β3, the spatially restricted signaling complexes in the PSD are made possible. This finding may lead us to a better understanding of the activation mechanism of PLC-β3 by GPCRs in the PSD of neuronal cells.

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