DCC-dependent Phospholipase C Signaling in Netrin-1-induced Neurite Elongation*

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Netrins, a family of secreted molecules, play important roles in axon pathfinding during nervous system development. Although phosphatidylinositol signaling has been implicated in this event, how netrin-1 regulates phosphatidylinositol signaling remains poorly understood. Here we provide evidence that netrin-1 stimulates phosphatidylinositol biphosphate hydrolysis in cortical neurons. This event appears to be mediated by DCC (deleted in colorectal cancer), but not neogenin or Unc5h2. Netrin-1 induces phospholipase Cγ (PLCγ) tyrosine phosphorylation. Inhibition of PLC activity attenuates netrin-1-induced cortical neurite outgrowth. These results suggest that netrin-1 regulates phosphatidylinositol turnover and demonstrate a crucial role of PLC signaling in netrin-1-induced neurite elongation.

Proper wiring in developing brains requires neurite outgrowth and growth cone navigation. Netrins, a family of secreted factors, promote axon outgrowth (1–4). In addition, netrins are able to guide neuronal growth cones and regulate neuronal branching in the developing nervous system (1–4). Netrins act through two classes of receptors: DCC and Unc5. The DCC family includes DCC and neogenin in vertebrates (5), Unc40 in Caenorhabditis elegans (6), and Frazzled in Drosophila (7, 8). DCC is required for the attractive response (9). Unc5 in C. elegans and Unc5A, -5B, and -5C in vertebrates belong to the Unc5 family, which appears to mediate the repulsive response (10–14).

DCC and Unc5 proteins are transmembrane proteins without any obvious catalytic activity, and thus it remains unknown exactly how they initiate downstream signaling to mediate or regulate axonal outgrowth and guidance. Nevertheless, perturbation of Rho family GTPases inhibits netrin-1-induced neurite outgrowth (15). Pharmacological inhibition of extracellular signal-regulated kinase (ERK) attenuates netrin-1-induced neurite outgrowth and growth cone turning (16, 17). Inhibition of focal adhesion kinase (FAK),3 a major tyrosine kinase localized in focal adhesions and implicated in cell spreading and migration, blocks netrin-1-induced neurite elongation and growth cone guidance (18–20). Treatment of wortmannin, an inhibitor of phosphatidylinositol 3-kinase, attenuates netrin-1-induced growth cone turning in Xenopus spinal neurons (27).

Phosphoinositides are quantitatively minor phospholipids of cell membranes, but their metabolism is highly active and tightly regulated. They (e.g., PI(4,5)P2) function either as precursors of second messengers such as inositol 1,4,5-trisphosphate (IP3) and diacylglycerol or by directly interacting with both actin-binding and pleckstrin homology domain-containing proteins to regulate their spatiotemporal distribution and/or activity. In addition, PI(4,5)P2 functions as a cofactor for small GTP-binding proteins (e.g. Arf) and phospholipase D (21, 22). In the nervous system, PI(4,5)P2 plays an important role in membrane trafficking at the synapse. Sypnaptic vesicle exocytosis and endocytosis require phosphatidylinositol 4,5-biphosphate (23–26). Using Xenopus growth cone-turning assays, Poo and colleagues (27) have demonstrated that perturbation of phospholipase Cγ (PLCγ) activity by pretreatment with brain-derived neurotrophic factor (BDNF) attenuates netrin-1-induced neurite outgrowth. Although PLCγ is implicated in netrin-1-induced growth cone guidance, exactly how netrin-1 regulates this signaling event and its role in netrin-1-induced neurite outgrowth remain largely unknown.

In this paper, we have characterized the potential role of PI(4,5)P2 hydrolysis in netrin-1 signaling. We show that netrin-1 stimulates PI(4,5)P2 hydrolysis in cultured cortical neurons. This event appears to be mediated by DCC. In addition, netrin-1 increases tyrosine phosphorylation of PLCγ, a time course similar to that of PI(4,5)P2 hydrolysis induced by netrin-1. Furthermore, inhibition of PLC activity by U73122 attenuated netrin-1-induced neurite elongation. These results demonstrate a crucial role of PLC signaling in netrin-1-induced neurite outgrowth.

MATERIALS AND METHODS

Reagents and Animals—Monoclonal antibodies were purchased form the following sources: anti-FLAG from Sigma, anti-Myc (9e10) from Santa Cruz Biotechnology (Santa Cruz, CA), and anti-PLCγ1 and anti-PLCγ4 from Upstate Biotechnology Inc. (Lake Placid, NY). Goat polyclonal antibodies (anti-DCC and anti-PLCγ-PY783) were purchased from Santa Cruz. Rabbit polyclonal anti-PY861 and anti-PY397 antibodies were from Biosource International, Inc. Rabbit polyclonal anti-neogenin was generated using glutathione S-transferase-neogenin (amino acids 1158–1527) as an antigen. Stable HEK293 cells expressing human netrin-1 and Slit-2 were provided by J. Y. Wu and Y. Rao (Washington University, St. Louis, MO) (28). Unless otherwise indicated, ~200 ng/ml human netrin-1 was used for stimulation. U73122 was obtained from Biomol Research Laboratories, Inc. (Plymouth, PA).

DCC null mutant mice were maintained in B6 background, and DCCflxflx mice were maintained in C. AKR background. Genotyping of both DCCflxflx and DCC null mutant mice was performed by PCR as described previously (18).

Expression Vectors—The cDNAs encoding neogenin, DCC, or DCC mutants were amplified by PCR and subcloned into mammalian expression vectors downstream of a signal peptide and a FLAG epitope tag (MDYKDDDDKGP) or Myc epitope tag under the control of the cyto-
megalovirus promoter (18). Deletion mutations in DCC were generated using the QuickChange kit (Strategene). DCC/SAM is a chimeric protein of DCC with the SAM domain of the EphB1 receptor substitution of the DCC-P3 domain, which was constructed as described (29). The authenticity of all constructs was verified by DNA sequencing.

**HEK293 Cell Culture and Transfection**—HEK293 cells were maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal calf serum and 100 units/ml penicillin G and streptomycin (Invitrogen). Cells were plated at a density of 10^6 cells/10-cm culture dish and allowed to grow for 12 h before transfection using the calcium phosphate precipitation method (30). Thirty-six hours after transfection, cells were lysed in modified radioimmune precipitation buffer (50 mM Tris-HCl, pH 7.4, 150 mM sodium chloride, 1% Nonidet P-40, 0.25% sodium deoxycholate, and proteinase inhibitors) (31). Lyases were subjected to immunoprecipitation or immunoblotting.

**Cortical Neuronal Culture**—Primary cortical neurons were cultured as described previously (18). Briefly, embryos (E17) were removed from anesthetized pregnant Sprague-Dawley rats or mice. Cerebral cortices were dissected out and chopped into small pieces after meninges were completely removed. After incubation in phosphate-buffered saline solution containing 0.125% (w/v) trypsin (Sigma) for 20 min at 37 °C, digested tissues were mechanically triturated by repeated passages through a Pasteur pipette in phosphate-buffered saline solution containing 0.05% (w/v) DNase (Sigma). Dissociated cells were suspended in the neurobasal medium with B-27 supplement (Invitrogen) and 100 units/ml penicillin/streptomycin and were plated on poly-D-lysine-coated dishes (Corning). After a 2-day incubation at 37 °C in a 5% CO_2 atmosphere, 10 μM cytosine arabinoside was added to inhibit the proliferation of glial cells.

**PIP2 Hydrolysis Assay**—HEK293 cells, or mouse embryonic fibroblasts were incubated in inositol-free Dulbecco’s modified Eagle’s medium containing 5 μCi/ml myo-2-[3H]inositol (1 μCi/μl, Amersham Biosciences), and 10% dialyzed fetal calf serum for 20 h. Cells were washed with phosphate-buffered saline and incubated for an additional hour in the same medium lacking isotope. 20 mM LiCl was added to the culture, and incubation continued for another 25 min. [3H]inositol-incorporated cells were stimulated with netrin-1 for different times. Inositol phosphates were extracted in 10 mM formic acid and purified by ion-exchange chromatography using AG1-X8 resin as described previously (32). Each experiment was repeated at least three times.

**Explant Cultures**—Explant assays were carried out as described previously (33–35). Embryos were dissected from the uterine horn of anesthetized pregnant mice. For cortical explants, telencephalic vesicles of E15 embryos were dissected out in L-15 medium (Invitrogen), and the pia mater was removed. The dorsolateral cortex was cut with thin tungsten needles into ~200 × 200-μm pieces that spanned the full thickness of the cortical wall. Explants were embedded in a three-dimensional collagen (Roche Diagnostics) matrix with the ventricular side up. After polymerization, gels were incubated with Ham’s F-12 medium supplemented with 5% heat-inactivated horse serum (Invitrogen) and 100 units/ml penicillin/streptomycin at 37 °C in a 5% CO_2 atmosphere. Neurite outgrowth was analyzed after 19–43 h in culture. Total neurite length for each explant was obtained by adding the lengths of all neurites from each explant (regardless of bundle thickness). Each experiment was repeated three times, and six explants for each treatment were analyzed (n = 2 × 3 = 6).

**Statistical Analysis**—Student’s t test was used to determine significance of effects. Data are presented as the mean of all independent replicates ± S.E.

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**RESULTS**

**Netrin-1 Stimulation of PLC Signaling in a Time-dependent Manner**—To address whether netrin-1 regulates PLC signaling, we measured PLC-mediated PIP_2 hydrolysis in cultured rat cortical neurons in response to netrin-1. Rat cortical neurons (E17, 3 days in vitro) were labeled with [3H]inositol, stimulated with or without netrin-1, and assayed for the production of inositol phosphates, an indicator of PIP_2 hydrolysis. BDNF, a growth factor that stimulates PLC signaling, was used as a positive control. As shown in Fig. 1A, netrin-1 as well as BDNF stimulated PIP_2 hydrolysis, supporting the notion of conserved signaling pathways of netrin-1 and BDNF (27, 36). Interestingly, Slit-2, a repulsive cue that negatively regulates neurite outgrowth (37), failed to stimulate this activity (Fig. 1A). In addition, netrin-1 induction of PIP_2 hydrolysis appeared to be time-dependent, with a maximal activity observed within 15 min of netrin-1 stimulation (Fig. 1B). These results indicate that netrin-1 stimulates PLC signaling in neurons in a time-dependent manner.

**Dependence of Netrin-1 Stimulation of PLC Signaling on DCC**—We next investigated the role of netrin-1 receptors in netrin-1 stimulation of PIP_2 hydrolysis. To this end, we reconstituted netrin-1 regulation of PIP_2 hydrolysis in HEK293 cells, which do not express endogenous DCC (data not shown). Netrin-1 stimulation did not elicit PIP_2 hydrolysis in control HEK293 cells (Fig. 2A, lanes 1 and 2). However, transfection
with DCC rendered HEK293 cells able to respond to netrin-1 with an increase in PIP$_2$ hydrolysis (Fig. 2A, lanes 3 and 4), implying dependence on the DCC protein. Remarkably, netrin-1 stimulation did not induce, or elicited very weakly if at all, PIP$_2$ hydrolysis in HEK293 cells transfected with Unc5h2 or with neogenin, a DCC-related receptor (Fig. 2A, lanes 5–8). These results suggest that DCC, but not neogenin or Unc5h2, may mediate netrin-1-induced PLC signaling.

To determine the role of DCC in vivo in netrin-1-induced PLC signaling, we turned to neurons isolated from DCC knock-out mice (Fig. 2B). As observed with rat cortical neurons, cortical neurons from DCC wild type or heterozygote mice responded to netrin-1 with an increase in PIP$_2$ hydrolysis (Fig. 2B, lanes 1–4). However, the induction of PIP$_2$ hydrolysis was completely eliminated in neurons from DCC homozygote mice (Fig. 2B, lanes 5 and 6), demonstrating a necessity for DCC in netrin-1 activation of PLC signaling in neurons. Note that neogenin was expressed at a similar level in neurons from DCC+/− mice as compared with wild type neurons (Fig. 2B), consistent with the notion that DCC, but not neogenin, mediates netrin-1 induction of PIP$_2$ hydrolysis in neurons.

Requirement of the DCC-P3 Domain for Netrin-1-induced PLC Signaling—The cytoplasmic region of DCC contains no catalytic domain but has three conserved regions: P1, P2, and P3. DCC interacts via the P domains with other receptors to form homo- or heterodimers important for netrin-1 function (9, 29, 38, 39). To identify which domain is necessary for netrin-1-induced PIP$_2$ hydrolysis, we compared the effects of DCC with DCCΔP3, a P3 deletion mutant, because P3 domain is required for netrin-1-stimulated neurite outgrowth and growth cone turning in Xenopus spinal neurons (29, 39). Interestingly, expression of this mutant in HEK293 cells blocked netrin-1 stimulation of PLC signaling, whereas expression of wild type DCC showed a significant response to netrin-1 (Fig. 3A, lanes 3–6). These results suggest a role of the DCC-P3 domain in netrin-1-induced PLC signaling.

We next determined the role of DCC-P3 domain in vivo in netrin-1 induction of PIP$_2$ hydrolysis by taking advantage of DCC$^kanga$ mice. These mice have a spontaneous deletion of exon 29 that encodes the DCC-P3 domain and show abnormal projection of corticospinal tract fibers to the spinal cord (35). Unlike mice homozygous for the targeted allele of DCC, which die perinatally (40), DCC$^kanga$ homozygotes have a relatively normal life span (35). We reasoned that netrin-1-induced PIP$_2$ hydrolysis may be impaired in DCC$^{kanga}$ mice if the DCC-P3 domain plays an important role in this event. Neurons were isolated from DCC$^{kanga}$ mutant and wild type embryos. Wild type mouse cortical neurons responded to netrin-1 with an increase in PIP$_2$ hydrolysis, as did DCC$^{kanga}$ heterozygous neurons (Fig. 3B, lanes 1–4). In contrast, the induction of PIP$_2$ hydrolysis was significantly attenuated in neurons from homozygous embryos (Fig. 3B, lanes 5 and 6). These results suggest a crucial role of the DCC-P3 domain in PIP$_2$ hydrolysis by netrin-1.
Independence of DCC-P3 Homodimerization or FAK for Netrin-1-induced PLC Signaling—DCC-P3 domain not only binds to itself to mediate DCC homodimerization but also interacts with FAK (18, 29). Both events are required for netrin-1 functions (18, 29). We thus examined the effects of DCC dimerization and FAK on netrin-1-induced PIP2 hydrolysis. To test whether self-association of P3 contributes to netrin-1-induced PIP2 hydrolysis, we constructed DCC/SAM, a chimeric protein of DCC with the SAM domain of the EphB1 receptor substituted for the P3 domain. This SAM domain has been demonstrated to mediate self-association of the DCC cytoplasmic domain when it replaces the P3 domain and can substitute fully for P3 in mediating the ligand-regulated chemoattractant function (29). Note that transfection of DCC/SAM blocked netrin-1 response in PIP2 hydrolysis (Fig. 3A, lanes 7 and 8), suggesting a slight role of DCC-P3 domain-mediated dimerization in this event. We next examined the role of FAK in netrin-1 induction of PIP2 hydrolysis, as FAK is a P3 binding partner (18). To this end, FAK-/- fibroblasts were used. As shown in Fig. 3D, netrin-1 was able to elicit PIP2 hydrolysis in both fak+/+ and fak-/- fibroblasts expressing DCC, suggesting a slight role of FAK in this event. In aggregate, our results suggest that neither P3 domain-mediated dimerization nor P3-FAK binding appeared to be required for netrin-1 induction of PLC signaling, thus implicating an involvement of other P3-dependent-binding proteins in this event.

Netrin-1 Induction of Tyrosine Phosphorylation of PLCγ in Rat Cortical Neurons—PLC, a large family of proteins including PLCβ and PLCγ, is a major enzyme that catalyzes the hydrolysis of PIP2 into IP3 and diacylglycerol. To understand further how netrin-1 induces PIP2 hydrolysis, we examined whether netrin-1 activates PLC. Both PLCγ and -β are highly expressed in embryonic rat brain (Fig. 4A). We exami-
PLC in Netrin-DCC Signaling Pathway

Netrin-1-induced PLC$_{\gamma}$ hydrolysis appears to be an important mechanism for netrin-1-induced neurite elongation. This notion is supported by the following observations. First, PLC$_{\gamma}$ hydrolysis is increased in neurons challenged with netrin-1. Netrin-1-induced PLC$_{\gamma}$ hydrolysis occurs rapidly after stimulation, apparently prior to changes in neurite outgrowth. Second, netrin-1 stimulates PLC$_{\gamma}$ in a time-dependent manner. Third, treatment of neurons with U73122, an inhibitor of PLCs, attenuates netrin-1-stimulated neurite outgrowth. Fourth, PLC$_{\gamma}$ signaling has been implicated in nerve growth factor- and netrin-1-induced growth cone guidance of Xenopus spinal neurons (27). Finally, it has been demonstrated that IP$_3$ receptor-mediated calcium release from intracellular stores plays an important role in the axonal extension of chick dorsal root ganglia (42).

Netrin-1 acts through two families of receptors: DCCs and UNC-5s. The DCC family includes DCC and neogenin in vertebrates (5). Although DCC is required for growth cone attraction by netrins (9), the role of neogenin in netrin-1 signaling remains unclear. Interestingly, recent observations indicate that neogenin, but not DCC, appears to be a receptor for RGM (repulsive guidance molecule), which repels retinal axons (43). Our data have indicated a role of DCC, but not neogenin or Unc5h2, in netrin-1-induced PLC$_{\gamma}$ hydrolysis, supporting the notion that different functions could be mediated by DCC and neogenin. DCC, an Ig family receptor, contains a cytoplasmic region with three conserved domains, namely P1, P2, and P3 (7). The P domains are important for DCC functions. For example, P3 is both necessary and sufficient for binding to another molecule of DCC to form a homodimer (29, 39). The homodimerization appears to be necessary for neurite outgrowth and attractive turning in Xenopus spinal neurons (29, 39). In addition, P3 interacts with Robo1, a receptor of the repulsive cue, Slit (39). This interaction is believed to be critical for Slit-2-induced silencing of netrin-1 attraction (39). Furthermore, P3 interacts with FAK and phosphatidylinositol transfer protein $\alpha$, which are required for netrin-1-induced growth cone turning (27), with a different time course than that of netrin-1 induction of PLC$_{\gamma}$ phosphorylation. These results suggest that tyrosine phosphorylation of PLC$_{\gamma}$ by netrin-1 may not be FAK-dependent, which is in line with the observation that FAK is not required for netrin-1-induced PLC$_{\gamma}$ hydrolysis.

Requirement of PLC Signaling for Netrin-1-induced Cortical Neurite Elongation—To explore the involvement of PLC signal transduction in mediating netrin-1 functions, we tested whether inhibition of PLC affects netrin-1-stimulated neurite outgrowth in rat embryonic cortical explants. In the absence of netrin-1, neurites are few and short (Fig. 5A). A significant increase in neurite outgrowth (both in number and length) was observed when explants were stimulated with netrin-1 (Fig. 5, B and I) (33, 34). Importantly, this event was inhibited by a PLC-specific inhibitor, U73122, but not by STI571, an inhibitor for Abl tyrosine kinase (Fig. 5, C–H). Of note is that the inhibitory effect by U73122 appeared to be dose-dependent. Whereas a weak effect was observed at a concentration of 10 nM, treatment with 500 nM U73122 showed significant inhibition (Fig. 5, C–F and I). In addition, this inhibitory effect appeared to be specific and was not associated with necrosis of the explants (Fig. 5, A, C, and E). Furthermore, U73122 had no effect on netrin-1-independent neurite outgrowth (data not shown), suggesting that the observed inhibitory effect by U73122 does not result from a general inhibition of neurite outgrowth but rather from specific inhibition of netrin-1-induced neurite elongation. Taken together, these results support the notion that IP$_3$ hydrolysis may be involved in netrin-1-stimulated cortical neurite outgrowth.

DISCUSSION

Netrin-1-induced PLC$_{\gamma}$ hydrolysis appears to be an important mechanism for netrin-1-induced neurite elongation. This notion is supported by the following observations. First, PLC$_{\gamma}$ hydrolysis is increased in neurons challenged with netrin-1. Netrin-1 induced PLC$_{\gamma}$ hydrolysis occurs rapidly after stimulation, apparently prior to changes in neurite outgrowth. Second, netrin-1 stimulates PLC$_{\gamma}$ in a time-dependent manner. Third, treatment of neurons with U73122, an inhibitor of PLCs, attenuates netrin-1-stimulated neurite outgrowth. Fourth, PLC$_{\gamma}$ signaling has been implicated in nerve growth factor- and netrin-1-induced growth cone guidance of Xenopus spinal neurons (27). Finally, it has been demonstrated that IP$_3$ receptor-mediated calcium release from intracellular stores plays an important role in the axonal extension of chick dorsal root ganglia (42).

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FIGURE 4. Induction of tyrosine phosphorylation of PLC$_{\gamma}$ by netrin-1. A, expression of PLC$_{\beta}$ and PLC$_{\gamma}$ in developing rat brain. Rat brain homogenates (20 $\mu$g of protein) from indicated developmental stages were subjected to Western blotting (IB) analysis using antibodies against the indicated proteins. Actin was used as a loading control. B, netrin-1 induction of phosphorylation of PLC$_{\gamma}$, FAK, and AKT in rat cortical neurons. Cortical neurons (E17, 3 days in vitro) were treated with netrin-1 (+) or control medium (−) for the indicated time. Lysates (10 $\mu$g of protein) were subjected to Western blotting using the indicated antibodies.
FIGURE 5. Requirement of PLC for netrin-1-stimulated neurite outgrowth. Rat cortical explants (E14.5) were cultured in collagen gels with control (A), netrin-1 medium (B), or control/netrin-1 medium containing the indicated doses of U73122 (C–F) or STI571 (G and H) for 24 h. Scale bar, 150 μm. I, quantification of the blocking effects of U73122 and STI571 on netrin-1-induced cortical neurite outgrowth. The total length of neurites growing into the collagen gels was measured in each explant and normalized to the values obtained from explants cultured in the presence of netrin-1 without inhibitor. Values shown are means ± S.E. (n = 6). *, p < 0.05, significant difference from netrin-1-stimulated neurite outgrowth (Kolmogorov-Smirnov test).
duced neurite outgrowth. This is suggested by a similar time course of netrin-1-induced neurite elongation. In addition, it is possible that PIP2 phosphorylation, and stimulates calcineurin-NFAT signaling, which is important for neurite outgrowth and growth cone turning. However, this regulation may be in a time- and space-dependent manner that is crucial for neurite outgrowth and growth cone turning. Consequently, whether netrin-1 stimulates neurite outgrowth through such a mechanism remains to be studied further.

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