Regulation of Neurite Outgrowth in N1E-115 Cells through PDZ-Mediated Recruitment of Diacylglycerol Kinase ζ

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Syntrophins are scaffold proteins that regulate the subcellular localization of diacylglycerol kinase ζ (DGK-ζ), an enzyme that phosphorylates the lipid second-messenger diacylglycerol to yield phosphatidic acid. DGK-ζ and syntrophins are abundantly expressed in neurons of the developing and adult brain, but their function is unclear. Here, we show that they are present in cell bodies, neurites, and growth cones of cultured cortical neurons and differentiated N1E-115 neuroblastoma cells. Overexpression of DGK-ζ in N1E-115 cells induced neurite formation in the presence of serum, which normally prevents neurite outgrowth. This effect was independent of DGK-ζ kinase activity but dependent on a functional C-terminal PDZ-binding motif, which specifically interacts with syntrophin PDZ domains. DGK-ζ mutants with a blocked C terminus acted as dominant-negative inhibitors of outgrowth from serum-deprived N1E-115 cells and cortical neurons. Several lines of evidence suggest DGK-ζ promotes neurite outgrowth through association with the GTPase Rac1. DGK-ζ colocalized with Rac1 in neuronal processes and DGK-ζ-induced outgrowth was inhibited by dominant-negative Rac1. Moreover, DGK-ζ directly interacts with Rac1 through a binding site located within its C1 domains. Together with syntrophin, these proteins form a tertiary complex in N1E-115 cells. A DGK-ζ mutant that mimics phosphorylation of the MARCKS domain was unable to bind an activated Rac1 mutant (Rac1V12) and phorbol myristate acetate-induced protein kinase C activation inhibited the interaction of DGK-ζ with Rac1V12, suggesting protein kinase C-mediated phosphorylation of the MARCKS domain negatively regulates DGK-ζ binding to active Rac1. Collectively, these findings suggest DGK-ζ, syntrophin, and Rac1 form a regulated signaling complex that controls polarized outgrowth in neuronal cells.

Nervous system function depends upon highly specific connections that form between neurons during development. Before these connections can be established, however, neurons must first elaborate cellular processes called neurites that eventually become a single axon and multiple dendrites. Dynamic remodeling of the actin-based cytoskeleton is fundamental to neurite outgrowth (43, 44).

The Rho GTPases are key regulators of actin cytoskeleton organization in neurons and have been shown to play important roles in many aspects of neuronal morphogenesis (43). The best-studied members of this family, RhoA, Rac1, and Cdc42, regulate the formation of distinct actin structures. RhoA controls the formation of stress fibers, whereas Rac1 and Cdc42 control the formation of lamellipodia and filopodia, respectively (13). The latter structures play key roles in the elongation of neurites. Accordingly, Cdc42 and Rac1 are generally considered positive regulators of neurite outgrowth, whereas RhoA normally inhibits neurite extension (13).

Rho GTPases cycle between the inactive GDP-bound and active GTP-bound states (13). Studies in neuronal cell lines have shown that inactive mutants of Cdc42 and Rac1 act as dominant-negative inhibitors of neurite outgrowth (11, 38, 51, 56). Constitutively active mutants of Cdc42 and Rac1 induce the formation of filopodia and lamellipodia in developing growth cones (38), but activation of these GTPases is not sufficient to induce the outgrowth of neurites (11, 51), but see van Leeuwen et al. (56). Thus, the activity of these GTPases appears to be necessary but not sufficient for neurite outgrowth, prompting the question whether there are associated proteins that work in concert with these GTPases to regulate process outgrowth. Another unresolved question is how the activity of these GTPases is correctly localized to the tips of growing neurites (32). For example, in response to nerve growth factor, active Rac1 is rapidly recruited to actin-rich cellular protrusions that are destined to become neurites in PC12 cells (58), but the molecular mechanism underlying this translocation is unclear. Scaffold proteins are likely to play an important role in this process.

Scaffold proteins facilitate the proper intracellular localization of their partner proteins, including establishing interaction with the plasma membrane and targeting proteins to specific membrane domains (8). Many signaling proteins contain C-terminal amino acid sequences that confer binding to PDZ
domain-containing scaffold proteins (52). The Rho GTPases, however, do not appear to contain any obvious PDZ-interaction motifs. Thus, either they are localized by other means or they associate indirectly with scaffolding proteins.

In this regard, Rac1 has been reported to associate with a complex of proteins that includes a phosphatidylinositol-4-phosphate 5-kinase, a Rho guanine nucleotide dissociation inhibitor, and a diacylglycerol kinase (DGK) (53). DGKs phosphorylate the lipid second messenger diacylglycerol, which transiently accumulates in cells following stimulation by growth factors and other agonists (25). The DGK associated with Rac1 had biochemical properties like those of the zeta isoform, but was not identified conclusively (26). We have previously shown that the subcellular localization of DGK-ζ is regulated by the interaction of its C-terminal PDZ-bonding motif with the PDZ domain of syntrphins, scaffolding proteins that interact with the dystrophin family of cytoskeletal proteins (1, 26). Therefore, such interactions may provide a mechanism for localizing Rac1 in cells.

The syntrophin family consists of five structurally related isoforms (α1, β1, β2, γ1, and γ2) that are products of separate genes. Each isoform consists of two tandem pleckstrin homology domains, a single PDZ domain, and a C-terminal region unique to syntrophins (14). The C-terminal half of syntrophin binds to members of the dystrophin family of cytoskeletal proteins, leaving the N-terminal half, including the PDZ domain, available to interact with signaling proteins (3). Since certain dystrophin family members bind directly to actin (36), syntrophins provide a link between signaling proteins and the actin cytoskeleton. Consistent with a potential role in actin organization, we showed that DGK-ζ and syntrophins colocalize with Rac1 in membrane ruffles and at the leading edge of lamellipodia in muscle cells (1).

Here, we sought to identify functional roles for DGK-ζ and syntrophins in actin cytoskeleton regulation in neuronal cells. We found that DGK-ζ expression induces neurite outgrowth in N1E-115 neuroblastoma cells. DGK-ζ-induced neurite outgrowth is largely independent of DGK-ζ catalytic activity and is inhibited by dominant-negative Rac1. DGK-ζ mutants that do not interact with syntrophins not only fail to promote neurite outgrowth, but also act as dominant-negative inhibitors of neurite outgrowth. We show that DGK-ζ interacts directly with Rac1 and provide evidence that the two proteins form a regulated signaling complex with syntrophin that is localized to growth cones and sites of early process formation. Collectively, our results suggest syntrophins function as scaffolds to regulate the subcellular localization of a DGK-ζ/Rac1 complex that catalyzes actin cytoskeleton rearrangements associated with neurite outgrowth.
Pull-down assays. Glutathione S-transferase fusion proteins of Rac1 V12, Rac1 N17 and RhoA were produced as described previously (15, 16, 26) and purified according to the method of Tolia et al. (53). The fusion protein purity was determined by Coomassie blue staining of sodium dodecyl sulfate (SDS)-polyacrylamide gels and the protein concentration was determined by the Bradford method (4). Undifferentiated N1E-115 cells plated on laminin were infected with AdDGK-ζ and grown for 48 h and then extracted with chilled lysis buffer (50 mM Tris, pH 7.5, 50 mM NaCl, 5 mM MgCl2, 1% NP-40) including a protease inhibitor cocktail [antipain, leupeptin, pepstatin A, 4-(2-aminoethyl)-benzene-sulfonfonyl fluoride (AEBSF) HCl and benzamidine HCl]. The lysates were centrifuged at 12,000 rpm for 5 min to remove cell debris and nuclei. An aliquot (5 to 10%) of the supernatant was set aside and constituted the input. Equivalent amounts of GST, GST-Rac1 V12, GST-Rac1 N17 and GST-RhoA fusion proteins bound to glutathione-Sepharose 4B beads were incubated with 600 to 800 μg of supernatant for 2 h at 4°C on a rocker. The tubes were centrifuged at 2,000 rpm for 2 min to collect the beads. The beads were then washed three to five times with 1 ml of lysis buffer, then resuspended in 1× reducing sample buffer and boiled for 5 min. The samples were analyzed by SDS-polyacrylamide gel electrophoresis (PAGE) and immunoblotting.

Blot overlay assays. Rac1 V12 and Rac1 N17 were subcloned into the BamH1 and EcoRI sites of the pEFT-32a expression vector (Novagen, Inc., Madison, WI), which contains sequences encoding thioredoxin, an N-terminal 15-amino-acid S tag, and a hexahistidine nickel-binding motif. The expressed fusion proteins were recognized by the S-protein-horseradish peroxidase conjugate (Novagen) and were purified from the soluble fraction on nickel-Sepharose columns according to the manufacturer’s instructions. Blot overlay assays were carried out as described previously (16). Rac1 V12 and Rac1 N17 were used at a final concentration of 300 nM.

RESULTS

Localization of DGK-ζ and syntrophins in distal neurites and growth cones. DGK-ζ is abundantly expressed in the central nervous system and is present in neurons of the olfactory bulb, hippocampus, and cerebral and cerebellar cortices in the developing and adult brain, suggesting it may be involved in neuronal development and function (12, 21, 26, 28). Syntrophins are also abundantly expressed in the brain, with several isoforms being expressed in neurons in the same regions as DGK-ζ (7, 19, 26, 47).

To begin to investigate the function of DGK-ζ and syntrophins in neurons, we assessed their subcellular distribution in primary mouse cortical neurons double labeled with a pan-specific monoclonal antibody (1351) to syntrophins and affinity-purified anti-DGK-ζ antibodies, followed by Alexa Fluor 488 (green) and Alexa Fluor 594 (red)-conjugated secondary antibodies. Confocal microscopy was used to obtain a Z-series of images, which were subsequently deconvolved as described in Materials and Methods.

Single optical sections show punctate immunoreactivity for DGK-ζ and syntrophins in cell bodies, along neurites, and in growth cones (Fig. 1A to B'). The arrows indicate regions of the neurite shaft (A to A') and of the growth cone (B to B') where punctate accumulations of both proteins overlap.

Additional localization studies were carried out in differentiated mouse N1E-115 neuroblasts, which are commonly used to study aspects of neuronal differentiation in vitro. N1E-115 cells can be transfected with high efficiency (40) and are rapidly induced to spread and extend neurites after serum withdrawal. Western blotting of subcellular fractions from undifferentiated N1E-115 cells confirmed that both DGK-ζ and syntrophins are expressed in these cells (Fig. 2A). No difference in the level or relative distribution of either protein was detected upon differentiation (Y. Yakubchyk and S. Gee, unpublished observations). Figure 1C to C' shows endogenous DGK-ζ and syntrophin concentrated in a patch at the leading edge of a growth cone in a differentiated N1E-115 cell, although elsewhere the signals do not colocalize exactly.

The lack of complete overlap of syntrophin and DGK-ζ immunoreactivity is not surprising because the panspecific syntrophin antibody recognizes at least three different syntrophin isoforms. Moreover, monoclonal antibody 1351 recognizes an epitope within the PDZ domain and competes with the binding of C-terminal peptide ligands (S. Gee, unpublished observations). Therefore, the antibody may only weakly recognize syntrophins with bound ligand. Nonetheless, these results suggest that some DGK-ζ and syntrophins are colocalized in neurites and growth cones.

To confirm and extend these findings, we examined DGK-ζ localization in transiently transfected N1E-115 cells induced to differentiate by serum deprivation. Shortly after serum withdrawal, neuroblastoma cells flatten and produce filopodia and lamellipodia around their circumference, which gradually polarize to one or two discrete regions called neurite buds, which then give rise to neurites (51). In N1E-115 cells differentiated for 48 h, recombinant HA-tagged DGK-ζ accumulated in distal neurite shafts and at neurite tips (Fig. 1D and E). At earlier times after differentiation (<24 h), HA-DGK-ζ was highly concentrated in neurite buds of polarized N1E-115 cells (Fig. 1F and G, arrows). Punctate (endogenous) syntrophin immunoreactivity was also concentrated in these early processes (Fig. 1H, arrow). Collectively, these results show that syntrophins and DGK-ζ are present at the tips of neurites and neurite-like protrusions and raise the possibility that they play a role in process outgrowth.

DGK-ζ induces process outgrowth in N1E-115 cells. To explore potential roles of DGK-ζ in neurite outgrowth, we transiently transfected N1E-115 cells grown in the presence of serum with cDNAs encoding wild-type or mutant versions of DGK-ζ. The constructs are shown schematically in Fig. 2B. Expression of HA-tagged wild-type DGK-ζ in N1E-115 cells caused the cells to flatten and induced the formation of neurite-like extensions (Fig. 2C and H). In contrast, N1E-115 cells transfected with vector alone appeared similar to untransfected cells; they remained round and only a small proportion had processes (Fig. 2H). These findings were obtained with cells plated on laminin, but similar results were obtained for cells plated on other substrates such as polyethyleneimine (not shown). Thus, DGK-ζ expression induces morphological changes in the presence of serum, conditions that normally prevent process outgrowth.

To examine whether the kinase activity of DGK-ζ is necessary for its ability to induce morphological changes, we used a mutant (DGK-ζATP) in which Asp was substituted for Gly 355, a critical residue in the ATP binding site. In vitro lipid kinase analysis of recombinant DGK-ζATP has shown that it is inactive (54). Similar to the wild-type protein, DGK-ζATP expression induced process outgrowth (Fig. 2H), indicating that this effect is mostly independent of DGK-ζ kinase activity.

Recent studies have shown that protein kinase C (PKC)-dependent phosphorylation of the DGK-ζ MARCKS domain induces it to translocate to the plasma membrane (1, 50). Since a mutant that mimics constitutive phosphorylation of the MARCKS domain (DGK-ζM1) shows increased association with the plasma membrane (1), we hypothesized that it might
FIG. 1. Colocalization of DGK-ζ and syntrophins in mouse cortical neurons and N1E-115 neuroblastoma cells. (A to B") Mouse cortical neurons cultured for 2 to 3 days in vitro were fixed and labeled with affinity-purified anti-DGK-ζ antibodies and with the panspecific monoclonal antibody 1351 against syntrophins, followed by Alexa Fluor 594 (red) and Alexa Fluor 488 (green) secondary antibodies. A Z-series obtained by confocal immunofluorescence microscopy was deconvolved as described in Materials and Methods. Single optical sections from the red and green channels are shown. Regions of the neurite where syntrophin and DGK-ζ immunoreactivity overlap are indicated by arrows and by the yellow color.
FIG. 2. DGK-ζ induces the formation of neurite-like extensions in N1E-115 cells. (A) N1E-115 cell lysates were fractionated as described in Materials and Methods and equal amounts of protein from each fraction were analyzed by SDS-PAGE and immunoblotting for DGK-ζ (top panel) and syntrophin (bottom panel). (B) Schematic showing the location of various protein domains within DGK-ζ and the organization of the various DNA constructs used. The N-terminal (open box), C1 (ellipses), MARCKS (solid box) and catalytic domains are indicated. Also shown are the four ankyrin repeats (I to IV) at the C terminus and three mitogen-activated protein kinase phosphorylation sites (gray triangles). The solid inverted triangle indicates a Gly to Asp mutation in the catalytic domain, which completely eliminates DGK-ζ activity (DGK-ζH9004ATP). The open inverted triangle indicates four Ser to Asp mutations in the MARCKS domain, which mimic phosphorylation at these sites. The solid box containing an X represents a FLAG epitope tag, which blocks binding to syntrophins. (C to F) Representative images of N1E-115 cells grown on laminin in serum-containing medium and expressing the indicated DGK-ζ constructs with an N-terminal HA (C, E, and G) or C-terminal FLAG (D and F) epitope tag. The cell shown in panel G was cotransfected with Myc-tagged Rac1N17. After 24 h, the cells were fixed and stained with epitope tag-specific antibodies. In panel G, only the anti-Myc labeling is shown. Comparison of the image in panel E with the corresponding phase contrast image (E') shows untransfected cells in the same field remained round. (H) The graph shows the percentage of cells bearing neurite-like processes, quantified as described in Materials and Methods. Error bars represent the standard error of the mean for at least three independent experiments in which at least 300 cells were counted per condition. Scale bars, 20 μm. An asterisk indicates a statistically significant difference from the vector control (P < 0.05, two-tailed t test).

in the merged images. (C to C') N1E-115 cells differentiated in low-serum conditions for 48 h were fixed and processed as above and visualized by epifluorescence microscopy. The arrows indicate a bright patch of syntrophin and DGK-ζ immunoreactivity at the leading edge of a neuronal process. (D to E) N1E-115 cells transfected with HA-tagged DGK-ζ were differentiated for 48 h in low-serum conditions, then were fixed and stained with anti-HA antibodies. HA-DGK-ζ accumulated in the distal half of the neurite and at the neurite tip. The asterisk in panel D indicates the cell body. (F and G) Transfected N1E-115 cells differentiated for 24 h had high concentrations of HA-DGK-ζ within neurite buds (arrows). (H) Syntrophin immunoreactivity in an untransfected N1E-115 cell differentiated for 24 h. The arrow indicates strong syntrophin immunoreactivity at the tip of a small neuronal process. Scale bars, 20 μm except for panels C to C', 5 μm.
be more biologically active in N1E-115 cells, even though it has only about half the catalytic activity of wild-type DGK-ζ (42). Consistent with this idea, DGK-ζ^{M1} induced a ~2-fold increase in the number of cells with processes compared to wild-type DGK-ζ (Fig. 2E and H). A constitutively phosphorylated but catalytically inactive mutant (DGK-ζ^{M1-ATP}) was less effective than DGK-ζ^{M1} but more effective than wild-type DGK-ζ, confirming that kinase activity is not required for the induction of process outgrowth (Fig. 2H). In addition, these results suggest targeting DGK-ζ to the plasma membrane may contribute to the induction of outgrowth.

**DGK-ζ-induced process outgrowth requires PDZ interactions.** To assess the involvement of syntrophins in DGK-ζ-induced outgrowth, we used a wild-type construct but with a C-terminal FLAG epitope tag (DGK-ζ^{FLAG}) which blocks the interaction with the syntrophin PDZ domain (1, 26). We have previously shown that this DGK-ζ mutant causes aberrant changes in the actin cytoskeleton when expressed in muscle cells (1). When expressed in N1E-115 cells, DGK-ζ^{FLAG} failed to induce neurite-like extensions (Fig. 2D and H). Even the membrane-associated mutant DGK-ζ^{M1} did not induce process outgrowth when its C-terminal PDZ-binding motif was similarly blocked (DGK-ζ^{M1-FLAG}; Fig. 2F and H). These results demonstrate that the C-terminal PDZ-binding motif is necessary for DGK-ζ-induced process outgrowth and suggest DGK-ζ triggers outgrowth by a mechanism that requires interaction with syntrophins.

**DGK-ζ induces neurofilament expression.** To determine if the cellular processes induced by DGK-ζ expression in N1E-115 cells have neurite-like properties, we used an antibody against the phosphorylated 200-kDa neurofilament subunit, which is specifically expressed in differentiated neuronal cells. We first tested the specificity of the antibody by staining undifferentiated N1E-115 cells grown in the presence of serum and N1E-115 cells induced to differentiate by serum deprivation. Undifferentiated cells showed background nuclear and cytoplasmic staining but no filamentous staining (Fig. 3A). In contrast, differentiated N1E-115 cells displayed prominent staining of neurofilaments in cellular processes (Fig. 3B, arrows). For these experiments, N1E-115 cells grown in the presence of serum were infected with an adenoviral vector encoding DGK-ζ^{M1} (AdDGK-ζ^{M1}; see Materials and Methods). Neurite-like extensions induced by expression of DGK-ζ^{M1} showed bright neurofilament staining (Fig. 3C, arrows and C'). Even round, flattened cells with fibroblast-like morphologies had radially oriented bundles of neurofilaments in multiple small cellular extensions (Fig. 3D, arrows and D'). Interestingly, large flattened cells with disordered bundles of neurofilaments were occasionally observed in the cytoplasm (Fig. 3E, arrow) suggesting DGK-ζ-induced expression of neurofilaments does not always result in productive process outgrowth. Nonetheless, these results suggest DGK-ζ induces the formation of cellular processes with neurite-like properties.

**DGK-ζ mutants that do not interact with syntrophin inhibit outgrowth.** Since C-terminally FLAG-tagged versions of DGK-ζ failed to stimulate outgrowth in serum-containing medium, we tested their ability to inhibit neurite outgrowth induced by serum deprivation of N1E-115 cells. Expression of either DGK-ζ^{FLAG} or DGK-ζ^{M1-FLAG} dramatically inhibited

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**FIG. 3.** DGK-ζ-induced cellular processes contain neurofilaments. N1E-115 cells were grown in the presence of serum (A and C to E') or were differentiated for 48 h by serum deprivation (B). Uninfected cells (A and B) or cells infected with an adenoviral vector engineered to express HA-tagged DGK-ζ^{M1} (see Materials and Methods) were fixed and stained with anti-phospho-neurofilament (NF) and anti-HA (DGK-ζ) antibodies. The arrows indicate bundles of neurofilaments. (E) Note the large neurofilament bundle in a cell with no neurites. Scale bars, 20 μm.

neurite outgrowth (Fig. 4A, E, and F). Interestingly, these constructs occasionally induced the formation of large intracellular vesicles (Fig. 4E and F, arrows). Dominant-negative Rac1 (Rac1^{N17}; see below) inhibited neurite outgrowth to a
similar extent (Fig. 4A), but vesicles were not observed (Fig. 4G). Vector alone, wild-type DGK-ζ/H9256, or DGK-ζ/H9256/H9004 ATP did not inhibit serum deprivation-induced neurite outgrowth (Fig. 4A to D). Collectively, these results suggest the DGK-ζ-syntrophin interaction is important for serum deprivation-induced neurite outgrowth in N1E-115 cells.

We next determined whether process outgrowth from cultured cortical neurons is affected by exogenous expression of DGK-ζ. Cortical neurons were infected 3 h after plating with adenoviral constructs encoding either wild-type DGK-ζ or DGK-ζ/FLAG. After 24 h, the cells were fixed and double labeled with anti-HA antibodies and monoclonal antibody 3A10 against neurofilament-associated proteins (23). Cells infected with DGK-ζ showed robust neurite outgrowth and often had several long, branched neurites (Fig. 5A to A’ and C). In contrast, cortical neurons infected with DGK-ζ/FLAG almost never extended neurites and were rounded, even though cells in the same field had neurites (Fig. 5B to B’ and D). In addition, cells infected with DGK-ζ/FLAG (D) but not wild-type DGK-ζ (C) had globular accumulations of actin in their cell bodies, suggesting a possible defect in actin organization. Thus, as in N1E-115 cells, DGK-ζ/FLAG prevents neurite extension in cortical neurons, supporting the idea that DGK-ζ-syntrophin interactions play an important role in neurite outgrowth.

DGK-ζ-induced morphological changes are Rac1 dependent. Studies using a dominant-negative mutant (Rac1N17) have established a key role for Rac1 in lamellipodium formation and neurite outgrowth (38). Rac1N17 is mainly GDP bound and blocks endogenous Rac1 function by sequestering regulatory molecules required to activate the wild-type protein. In N1E-115 cells, coexpression with Rac1N17 completely blocked process outgrowth induced by either wild-type DGK-ζ (Fig. 2G and H) or DGK-ζ/FLAG (data not shown). These results suggest the induction of neurites by DGK-ζ is dependent on Rac1 and tentatively place DGK-ζ upstream of Rac1 activation.

To further investigate the relationship of DGK-ζ to Rac1 in neurite outgrowth, N1E-115 cells grown in the presence of serum were cotransfected with DGK-ζ and a constitutively

FIG. 4. DGK-ζ mutants that do not interact with syntrophins inhibit serum starvation-induced neurite outgrowth. N1E-115 cells transfected with the indicated DGK-ζ constructs were induced to differentiate by serum withdrawal for 48 h. The cells were fixed, stained, and assessed for neurite outgrowth as in Fig. 2. (A) The graph shows the percentage of cells bearing neurites for each construct indicated. The error bars represent the standard error of the mean for at least three independent experiments (n = 300 cells per condition). An asterisk indicates a statistically significant difference from the vector control (P < 0.05, two-tailed t test). (B to G) Representative photomicrographs showing the morphology of N1E-115 cells expressing the indicated constructs with either an N-terminal HA (C and D), C-terminal FLAG (E and F), or Myc (G) epitope tag. The cell in panel B is expressing green fluorescent protein. The arrows in panels E and F indicate large cytoplasmic vesicles or vacuoles. Scale bars, 20 μm.
active Rac1 mutant (Rac1V12). Previous studies have shown that Rac1V12, which persists in a GTP-bound state and activates downstream Rac1 effectors, has dramatic effects on the morphology of neuronal cells but does not induce neurite outgrowth. In agreement with these studies, we found that cells expressing Rac1V12 had a characteristic round, flattened appearance but did not form neurite-like extensions (Fig. 6A, A’ left, and C). Thus, Rac1 activity alone appears to be insufficient for process outgrowth, at least in N1E-115 cells. In contrast, cells expressing both wild-type DGK-ζ and Rac1V12 extended long processes within 24 h and had large lamellipodia at their leading edges (Fig. 6A, A’ right, and C). Furthermore, in cells expressing HA-tagged DGK-ζ alone, endogenous Rac1 closely colocalized with DGK-ζ in membrane ruffles at the leading edge of cellular extensions (Fig. 6B and B’, arrows). Together, these
data raise the possibility that DGK-ζ and Rac1 act in a coordinate manner to regulate process outgrowth.

**DGK-ζ interacts with Rac1 and Rho.** We next determined whether the observed effects of DGK-ζ on neurite outgrowth are the result of a cooperative interaction between DGK-ζ and Rac1. We first performed affinity precipitation experiments using N1E-115 cell lysates that had been infected with an adenoviral construct encoding wild-type HA-tagged DGK-ζ. Beads charged with GST or GST fusion proteins with Rac1V12 and Rac1N17 were incubated with the lysates. Bound DGK-ζ was detected by immunoblotting with an anti-HA antibody. We included GST-RhoA as a control, expecting that DGK-ζ would not interact with it. Surprisingly, we found that Rac1V12, Rac1N17 and RhoA all captured DGK-ζ from the lysate, while none was captured by control GST beads (Fig. 7A).

Since DGK-ζ was closely colocalized with Rac1 and not RhoA in cortical neurons (discussed below), we further characterized its interaction with Rac1. To determine if the DGK-ζ that is bound to Rac1 is also able to bind syntrophin, beads charged with a GST fusion protein of the α1-syntrophin PDZ domain (α1-PDZ) were used to capture HA-DGK-ζ from N1E-115 cell lysates (Fig. 7B, top panel). Endogenous Rac1 was coprecipitated with DGK-ζ (Fig. 7B, bottom panel). Since PDZ domains generally only bind a single ligand at a time, the most likely interpretation of these results is that the captured Rac1 was bound to DGK-ζ, which itself bound to α1-PDZ through its C-terminal PDZ-binding motif.

**FIG. 6.** Morphological characteristics of N1E-115 cells expressing DGK-ζ and constitutively active Rac1. (A to A') Cells grown in the presence of serum were cotransfected with Myc-Rac1V12 and HA-DGK-ζ. After 24 h, the cells were fixed and double-labeled with anti-Myc (A) and anti-HA (A') antibodies. Cells expressing Rac1V12 had a characteristic round, flattened appearance (A, left) while those expressing both Rac1V12 and DGK-ζ had neurite-like extensions and had prominent lamellipodia (A and A', right). (B and B') Cells expressing HA-DGK-ζ were double-labeled with anti-HA and anti-Rac1 antibodies. The arrows indicate close colocalization of endogenous Rac with DGK-ζ in membrane ruffles at the leading edge of a process. Scale bars, 50 μm (A and A'), 20 μm (B and B'). (C) The graph shows the percentage of cells bearing neurite-like extensions for each construct indicated. The error bars represent the standard error of the mean for at least three independent experiments.
To test whether DGK-ζ binds directly to Rac1, soluble His6 fusion proteins of Rac1V12 and Rac1N17 (containing S-tag epitopes) were used to overlay various GST fusion proteins of DGK-ζ, shown schematically in Fig. 7C. As shown in Fig. 7D, both Rac1V12 and Rac1N17 bound specifically to a DGK-ζ fusion protein containing amino acids 97 to 233, which includes two cysteine-rich regions homologous to the C1A and C1B motifs of PKCs (30). That both Rac1 mutants displayed comparable binding to DGK-ζ suggests the interaction is not significantly dependent on the activation state of the GTPase. As expected, only the Rac1V12 mutant bound to a GST fusion protein corresponding to the p21-binding domain of PAK1 (PAK1 PBD), indicating that the two mutants retained their binding specificity for this particular Rac1 effector. Neither Rac1 mutant bound to GST or to other DGK-ζ domains, further demonstrating the specificity of the interaction.

**Phosphorylation of the MARCKS domain causes DGK-ζ and Rac1 to dissociate.** The increased activity of DGK-ζM1 in neurite outgrowth assays prompted us to determine whether phosphorylation of the MARCKS domain affects the interaction with Rac1. To begin to address this possibility, we compared the ability of wild-type DGK-ζ and DGK-ζM1 to bind to Rac1 in pull-down assays as described for Fig. 7A. As before, Rac1V12, Rac1N17 and RhoA but not GST efficiently bound wild-type DGK-ζ (Fig. 8A, top panel). In contrast, DGK-ζM1 did not bind to Rac1V12 and weakly bound to Rac1N17, whereas binding to RhoA was unaffected (Fig. 8A, bottom panel).

We repeated this experiment using lysates of transiently transfected HeLa cells. In this cell type as well, GST-Rac1V12 captured wild-type DGK-ζ but not DGK-ζM1 from the offered extracts (Fig. 8B). Moreover, endogenous syntrophins were captured in the presence of wild-type DGK-ζ but not in the presence of DGK-ζM1 (Fig. 8B). These data suggest phosphorylation of the MARCKS domain attenuates the interaction of DGK-ζ and syntrophins with Rac1.

To further test this possibility, we examined whether phorbol esters, potent activators of PKC, affected the interaction of DGK-ζ with Rac1. We monitored the binding of DGK-ζ to Rac1V12 in lysates of N1E-115 cells infected with wild-type DGK-ζ and observed that treating the cells with PMA completely inhibited their interaction (Fig. 8C). A PKC inhibitor abolished this inhibition, indicating that PKC activity was required to attenuate the binding. Collectively, these results suggest PKC-mediated phosphorylation of the MARCKS domain causes DGK-ζ and syntrophin to dissociate from active Rac1.

To provide additional evidence that DGK-ζ and Rac1 specifically associate in neurons, cultures of mouse cortical neurons double labeled for DGK-ζ and Rac1 were visualized by confocal microscopy. Single optical sections from a deconvolved Z-series of images showed that DGK-ζ and Rac1 were closely colocalized in neurites (Fig. 9A to A*). In contrast, DGK-ζ immunoreactivity did not overlap with labeling for RhoA. Moreover, punctate RhoA labeling was mainly present in areas depleted of DGK-ζ labeling (Fig. 9B to B*). Thus, these results support the idea that DGK-ζ and Rac1 physically interact in neurons.

**DISCUSSION**

The results presented here establish a key role for DGK-ζ in the process of neurite outgrowth in N1E-115 cells. Expression of DGK-ζ induced outgrowth on laminin (and other substrates) in the presence of serum, which normally prevents neurite outgrowth. Surprisingly, the ability of DGK-ζ to stimulate neurite formation was largely independent of its lipid kinase activity, since a catalytically inactive DGK-ζ mutant (DGK-ζCATP) (54) induced outgrowth almost to the same extent as the wild-type protein. This result, combined with the finding that DGK-ζCATP did not inhibit serum deprivation-induced neurite outgrowth, suggests DGK-ζ activity is not required for neurite formation. It is possible, though, that DGK-ζ catalytic activity impinges on an aspect of neurite morphology, such as branching, that was not quantified in our studies.

In previous studies, we demonstrated that DGK-ζ mutants with a blocked C terminus do not interact with syntrophins and are associated with the plasma membrane less often (1, 26). In the present study, these mutants (DGK-ζFLAG and DGK-ζM1FLAG) were unable to induce neurite outgrowth in N1E-115 cells, despite the fact that they were expressed at levels equivalent to the wild-type construct (H. Abramovic and...
S. Gee, unpublished observations). They also inhibited neurite outgrowth of cultured cortical neurons and of serum-deprived N1E-115 cells. These findings demonstrate that the C-terminal PDZ-binding motif of DGK-ζ/H9256 is necessary for neurite outgrowth.

While we cannot exclude the possibility that the effect of these mutants is due in part to disruption of interactions with PDZ-containing proteins other than syntrophins, our previous studies showing that the C terminus of DGK-ζ/H9256 specifically binds to syntrophin PDZ domains and not to other closely related PDZ domains (26) strongly suggest it is the interaction with syntrophins that is required for neurite outgrowth. Indeed, accumulating evidence suggests syntrophins are necessary for recruiting and organizing their protein partners into specialized complexes at the plasma membrane (2, 5, 33, 35, 46).

How do DGK-ζ mutants that do not bind to syntrophins exert a dominant-negative effect on neurite outgrowth? In principle, they should not block the interaction of endogenous DGK-ζ with PDZ domains of other syntrophins, our previous studies showing that the C terminus of DGK-ζ specifically binds to syntrophin PDZ domains and not to other closely related PDZ domains (26) strongly suggest it is the interaction with syntrophins that is required for neurite outgrowth. Indeed, accumulating evidence suggests syntrophins are necessary for recruiting and organizing their protein partners into specialized complexes at the plasma membrane (2, 5, 33, 35, 46).

How do DGK-ζ mutants that do not bind to syntrophins exert a dominant-negative effect on neurite outgrowth? In principle, they should not block the interaction of endogenous DGK-ζ with syntrophins because they do not bind them. The mutants might interfere with endogenous DGK-ζ function by sequestering effector proteins such as Rac1 (see below). If signaling complexes formed by DGK-ζFLAG are not directed to specialized domains of the plasma membrane by syntrophins, then they might be nonproductive and, consequently, no morphological changes would ensue. Alternatively, indiscriminate targeting of DGK-ζFLAG complexes to the plasma membrane could result in spatially unrestricted activity, leading to gross, unpolarized changes in cell morphology.

At first glance, our finding that dominant-negative Rac1 completely blocked DGK-ζ-induced neurite outgrowth argues that DGK-ζ functions upstream of Rac1. However, this interpretation may be overly simplistic because constitutively active Rac1 was unable to induce neurite outgrowth by itself. Instead, expression of Rac1V12 produced highly flattened and spread cells, often with edge ruffles, similar to its effects on other cell types. These findings agree with two previous reports which showed that Rac1 induces dramatic morphological changes but does not result in polarized outgrowth of neuron-like cells (11, 51).

We favor the idea that Rac1 functions cooperatively with DGK-ζ to induce neurite outgrowth. Several lines of evidence support this interpretation. First, DGK-ζ and Rac1 together induced ~5-fold more neurite-like processes in N1E-115 cells than Rac1 alone and both proteins were colocalized at the leading edge of cellular extensions in these cells. Second, biochemical experiments demonstrated that Rac1 directly interacts with DGK-ζ. Both the constitutively active and the dominant-negative mutants displayed comparable interaction with DGK-ζ, suggesting the interaction is not significantly dependent on the activation state of Rac1. Finally, DGK-ζ and Rac1 were closely colocalized in neurites of cultured cortical neurons. Evidence for the specificity of the interaction comes from our finding that a DGK-ζ mutant that mimics constitutive phosphorylation of the MARCKS domain (DGK-ζM1) did not bind to Rac1V12 and that PMA-induced activation of PKC inhibited the interaction of wild-type DGK-ζ with Rac1V12. Collectively, these results strongly indicate that DGK-ζ and Rac1 exist in a regulated signaling complex that controls neurite outgrowth.

Additionally, we provide evidence that DGK-ζ and syntrophin form a tertiary complex with Rac1 in N1E-115 cells. We
favor the idea that DGK-ζ mediates the interaction between syntrophin and Rac1, because DGK-ζ binds directly to both proteins and because syntrophin was not captured by Rac1V12 in the presence of DGK-ζ, which itself does not interact with Rac1V12. The association with DGK-ζ and syntrophins provides a potential mechanism for targeting Rac1 to specialized membrane domains within neurons.

Syntrophins bind directly to members of the dystrophin family of cytoskeletal proteins (14). Dystrophin, in turn, associates with a complex of intracellular, transmembrane, and extracellular proteins collectively referred to as the dystrophin glycoprotein complex, which forms a transmembrane axis that tightly links the extracellular matrix to the actin cytoskeleton. Together, these interactions provide a connection between DGK-ζ/Rac1 complexes, the actin cytoskeleton, and the extracellular matrix. In this way, Rac1 activity can be spatially regulated and changes in actin organization mediated by Rac1 may be transmitted to the extracellular matrix to coordinate regulation of intracellular and extracellular structural changes during neurite outgrowth.

Receptor-induced recruitment of effector proteins to the plasma membrane is a major theme in signaling (29) and is particularly important for enzymes that act upon lipid second messengers, because it brings them into close proximity to their substrates. Membrane translocation appears to be a common paradigm for regulating DGK activity, but the molecular mechanisms underlying this process are not well understood (31, 49). Recently, Santos et al. (50) showed DGK-ζ rapidly translocates to the plasma membrane of T cells in response to activation of an exogenously expressed muscarinic receptor. They demonstrated that the kinase activity of DGK-ζ is not necessary for this translocation, but that PKC-driven phosphorylation of the MARCKS domain is required.

In agreement with their findings, we recently found that phosphorylation-mimicking DGK-ζ (DGK-ζM1) exhibits increased association with the plasma membrane of C2C12 muscle cells compared to the wild-type protein (1). The present study demonstrates that DGK-ζM1 is ∼2-fold more effective than the wild-type protein at eliciting neurite outgrowth from N1E-115 cells. This effect is not due to enhanced kinase activity because DGK-ζM1 is ∼50% less active than the wild-type enzyme (42). In addition, catalytically inactive DGK-ζM1 (DGK-ζM1-ΔATTT) was as effective as the wild-type protein at inducing neurite outgrowth, confirming that kinase activity is not essential for this process. Taken together, these findings suggest membrane localization is an important aspect of DGK-ζ’s ability to induce neurite outgrowth.

How might translocation of DGK-ζ from the cytosol to the plasma membrane drive neurite outgrowth? Our finding that phosphorylation of the MARCKS domain dissociates DGK-ζ from Rac1 (especially Rac1V12) offers a possible mechanistic basis for this effect. We propose a model where, in the basal state, DGK-ζ and syntrophin associate with Rac1-GDP in the cytoplasm (Fig. 10). Signals that stimulate neurite outgrowth cause PKC-mediated phosphorylation of the MARCKS domain, which induces the complex to translocate to the plasma membrane. This allows nucleotide exchange on Rac1 (GTP for GDP) and its subsequent dissociation from DGK-ζ and syntrophin. In this scenario, Rac1 is delivered to the plasma membrane, activated, and then released, which may account for the lack of complete overlap between DGK-ζ and Rac1 in cortical neurons.

This model implies that the MARCKS domain of DGK-ζ is a physiologic target of PKC. Indeed, it is the predominant nuclear localization signal of DGK-ζ and its phosphorylation by PKC reduces nuclear accumulation of DGK-ζ, which alters nuclear diacylglycerol levels (54). In addition, a recent study showed that DGK-ζ directly associates with PKCα and negatively regulates its protein kinase activity (41). This association was abolished when the MARCKS domain of DGK-ζ was phosphorylated by PKCα, relieving the inhibition of PKCα activity. Thus, it appears that PKCα-mediated phosphorylation of the MARCKS domain causes DGK-ζ to dissociate from both PKCα and Rac1. Presumably, phosphorylation of the MARCKS domain causes a conformational change in DGK-ζ that alters its binding to different effector proteins. In support of this idea, we found that MARCKS domain phosphorylation increases the binding of DGK-ζ to syntrophin PDZ domains (E. Daher and S. Gee, unpublished observations).

We have yet to find evidence for DGK-ζ-induced activation of Rac1, as measured by the binding of GTP-bound Rac1 to the PBD of PAK1 (H. Abramovici and S. Gee, unpublished observations). How can this result be reconciled with our finding that dominant-negative Rac1 inhibits DGK-ζ-induced neurite outgrowth? One possibility is that DGK-ζ activates Rac1, but the GTP-bound Rac1 remains bound to the cytoskeleton and is “overlooked” by the PAK1 PBD assay, which only measures soluble GTP-bound Rac1 (57). Consistent with this possibility, a significant fraction of both DGK-ζ and syntrophins is
associated with the insoluble cytoskeleton (Fig. 2A). Alternatively, DGK-ζ might not directly activate Rac1, but a basal level of Rac1 activity may be necessary to induce the actin cytoskeletal changes that underlie DGK-ζ-induced neurite outgrowth.

Several studies have shown that inhibition or sequestration of Rho induces neurite outgrowth (22). Interestingly, Houssa et al. (27) showed that activated RhoA binds to DGK-θ in N1E-115 cells and inhibits its catalytic activity, although it was not determined what effect this inhibition had on neurite outgrowth. Because we found that DGK-ζ bound to RhoA in pull-down assays, it is possible that it induces neurite outgrowth by inhibiting RhoA. However, this does not seem to be a likely mechanism because it does not easily account for the results obtained with the different DGK-ζ constructs (Fig. 2).

In addition, DGK-ζ and Rho immunoreactivity did not overlap in cortical neurons (Fig. 9B to B'). Thus, further studies are needed to more thoroughly characterize the association of DGK-ζ with RhoA and to determine whether this interaction has any functional consequences.

The region of DGK-ζ that binds to Rac1 (amino acids 97 to 233) comprises two tandem C1 domains, C1A and C1B. C1 domains were initially identified as the targets of diacylglycerol and phorbol ester binding in conventional PKC isozymes. Indeed, many C1 domain-containing proteins bind phorbol esters, but intriguingly, a large number do not (30). The C1 domains in DGK-ζ differ substantially in sequence from “typical” C1 domains and do not fit the profile for phorbol ester binding. Interestingly, among the C1 domains that do not bind phorbol esters, several have been shown to bind to small G-proteins (30). Moreover, there are several reports describing interactions of DGKs with Rho GTPases (27, 53, 55), and thus it is interesting to speculate that atypical C1 domains in DGKs may represent a common motif for interaction with this subfamily of small G-proteins.

A recent study by Tsushima et al. (55) showed that DGK-γ functions as an upstream suppressor of Rac1 in NIH 3T3 cells. In contrast to DGK-ζ, DGK-γ kinase activity was required to inhibit Rac1 activation. A kinase-dead DGK-γ acted as a dominant-negative mutant, inhibiting endogenous DGK-γ activity and inducing lamellipodia and membrane ruffling in the absence of growth factor stimulation. Although it was not determined whether DGK-γ binds directly to Rac1, it is interesting to speculate that the C1 domains in this isoform function in an analogous manner to those in DGK-ζ. Since DGK-γ is abundantly expressed in neurons (20, 34), one possibility is that the two DGK isoforms compete for binding to Rac1 and provide fine tuning of growth and retraction signals that regulate process formation.

In conclusion, our results suggest syntrophins provide localization cues that direct DGK-ζ-containing complexes to specialized plasma membrane domains and that this activity is required for neurite formation. We propose that DGK-ζ is an integral part of signal transduction pathways in neurons that translate receptor activation by extracellular ligands into cytoskeletal rearrangements and local growth. In the absence of localization cues, DGK-ζ may fail to properly regulate actin cytoskeleton rearrangements that are required for neurite formation.

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