Overcoming Amino-Nogo-induced Inhibition of Cell Spreading and Neurite Outgrowth by 12-O-Tetradecanoylphorbol-13-acetate-type Tumor Promoters

Kangwen Deng, Ying Gao, Zixuan Cao, Edmund I. Graziani, Andrew Wood, Patrick Doherty, and Frank S. Walsh

From Neuroscience Research, Pfizer Global Research and Development, Princeton, New Jersey 08543; Pfizer Global Research and Development, Pearl River, New York 10965; and the Wolfson Centre for Age-related Diseases, King’s College, London SE1 9RT, United Kingdom

The N-terminal domain of NogoA, called amino-Nogo, inhibits axonal outgrowth and cell spreading via a largely unknown mechanism. In the present study, we show that amino-Nogo decreases Rac1 activity and inhibits fibroblast spreading. 12-O-Tetradecanoylphorbol-13-acetate-type tumor promoters, such as phorbol 12-myristate 13-acetate (PMA) and teleocidin, increase Rac1 activity and overcome the amino-Nogo-induced inhibition of cell spreading. The stimulating effect of tumor promoters on cell spreading requires activation of protein kinase D and the subsequent activation of Akt1. Furthermore, we identified Akt1 as a new signaling component of the amino-Nogo pathway. Akt1 phosphorylation is decreased by amino-Nogo. Activation of Akt1 with a cell-permeable peptide, TAT-TCL1, blocks the amino-Nogo inhibition. Finally, we provide evidence that these signaling pathways operate in neurons in addition to fibroblasts. Our results suggest that activation of protein kinase D and Akt1 are approaches to promote axonal regeneration after injury.

Injured neurons in the adult mammalian central nervous system fail to regenerate, in part due to the existence of myelin-associated inhibitors (MAIs), such as NogoA, myelin-associated glycoprotein, and oligodendrocyte-myelin glycoprotein. Two inhibitory regions of NogoA, a C-terminal 66-amino acid region (Nogo-66) and a lengthy N-terminal region (amino-Nogo), have been characterized based on their inhibitory function on cell spreading and neurite outgrowth. Nogo-66 and two other major MAIs, myelin-associated glycoprotein and oligodendrocyte-myelin glycoprotein, exert their inhibitory effects by interacting with a receptor complex that contains the Nogo receptor 1 (NgR1), Lingo-1, p75NTR, and higher order gangliosides (1) or a newly identified receptor, PirB (paired immunoglobulin-like receptor-B) (3). However, amino-Nogo restricts non-neuronal cell spreading and axonal outgrowth through an unknown mechanism independent of the above NgR complex (5, 6). Despite evidence supporting the existence of a specific receptor on neurons and some non-neuronal cells, the identity of the amino-Nogo receptor(s) remains obscure. A recent study has provided evidence that amino-Nogo can bind to and selectively block signal transduction initiated by certain integrins (7).

The intracellular signaling mechanisms responsible for amino-Nogo-induced inhibition of cell spreading and regeneration are not well understood. So far, Rho GTPases, mainly RhoA and Rac1, remain the most established mediators. Amino-Nogo activates RhoA and suppresses Rac1 in primary neurons (6, 8). Activation of RhoA by MAIs has been extensively studied and is generally viewed as perhaps the key convergence point at which various MAIs exert similar functional outcomes (6, 8). Blockade of RhoA signaling improves axonal regeneration in both in vitro and in vivo models (13). Rac1 activity, on the other hand, is required for neurite formation and neurite outgrowth on permissive substrates (14–17). Although one of the consequences of MAI treatment is the decrease of Rac1 activity (6, 8), this has not been studied in detail.

In the present study, using fibroblasts and fibroblast spreading as a simple model, we show that TPA-type tumor promoters can effectively increase Rac1 activity and overcome amino-Nogo-induced inhibition of cell spreading through a protein kinase D (PKD; also known as PKC_\(\mu\))- and Akt1-dependent mechanism. Moreover, we identify Akt1 as a novel signaling component of the amino-Nogo pathway and demonstrate that activation of Akt1 blocks the inhibitory effects of amino-Nogo. Finally, we provide evidence that the same pathway or a similar pathway operates in neurons.

**Experimental Procedures**

**Materials**—A human Nogo construct (amino acids 567–748), equivalent to rat Nogo-A (amino acids 544–725), was synthesized as a codon-optimized cDNA using a PCR-directed gene synthesis method and was cloned into a mammalian expression vector to express as a human Fc fusion protein. The recombinant NogoA-Fc protein (hereafter called Nogo) was purified from transient transfected HEK293 cells by a protein A affinity column to more than 99% purity (Pfizer Research). Myelin was purified from adult rat CNS medulla (18). Teleocidi...
din was from an internal natural product collection at Pfizer. The teleocidin we used is a mixture of teleocidin B1, B2, B3, and B4, confirmed by HPLC analysis (Melissa Wagenaar, Pfizer Research). Phorbol 12-myristate 13-acetate (PMA) and 4α-PA were purchased from Sigma. NSC23763, Gö-6983, Gö-6976, and Akt inhibitor VII (TAT-Akt-in) were purchased from Calbiochem. Synthetic peptides TAT-TCL-1 and TAT-TCL-1G (19) were synthesized by NeoMPS, Inc. with purity of >96% by HPLC. Anti-Rac1 clone 23A8, anti-Akt1, and anti-phospho-Akt (Ser473) clone 11E6 were purchased from Cell Signaling Technology.

**Cell Culture**—NIH/3T3 cells were obtained from ATCC (CRL-1658). Cells were grown in Dulbecco’s modified Eagle’s medium supplemented with 10% bovine calf serum (Invitrogen), 100 units/ml penicillin/streptomycin (Invitrogen), and 200 mM l-glutamine in an incubator maintained at 37 °C with 5% CO₂.

Cerebella from postnatal day 3–5 rat pups were dissociated into single cell suspension following the protocol in a papain dissociation kit (Worthington). The pellets of cells were resuspended in Dulbecco’s modified Eagle’s medium supplemented with SATO (200 nM progesterone, 224 nM selenium, 4 µg/ml insulin, 0.35 mg/ml bovine serum albumin, 0.4 µg/ml l-thyroxine, 0.34 µg/ml tri-iodothyronine, 100 µM putrescine) for neurite outgrowth or in Neurobasal-A medium supplemented with B27 (Invitrogen) for Western blotting.

**Rac1 Activation Assay**—An Rac1 activation assay was performed following the protocol in a Rac1 activation assay kit (Millipore). Water (control) or Nogo (8 µg/well) was added to a well of 6-well poly-D-lysine (PDL)-coated plates (BD Bioscience) and air-dried overnight in a fume hood. Samples were prepared as follows. NIH/3T3 cells were seeded at 1.5 × 10⁶ cells/well for 6 wells/sample (total 9 × 10⁶ cells) on either control or Nogo substrate in the presence or absence of indicated treatments for 10 min. Cells were then washed once with ice-cold Tris-buffered saline and lysed in 1× Mg²⁺ lysis/wash buffer supplemented with protease inhibitors and phosphatase inhibitors (Calbiochem). Genomic DNAs were eliminated by passing the lysates through Qia-shredders (Qiagen). Protein concentrations of the flow-through were determined by Bradford assay (Bio-Rad). 1.5 mg of protein was aliquoted to each tube, PAK-1 protein-binding domain-agarose was added immediately, and samples were rotated overnight at 4 °C. Agarose beads were collected by centrifugation for 5 s at 14,000 rpm, followed by removal and discarding of the supernatant. The beads were washed three times with 0.5 ml of Mg²⁺ lysis/wash buffer and then resuspended in 40 µl of 2× Laemmli reducing sample buffer (Bio-Rad) and boiled for 5 min. Western blot and Rac1 detection were performed as above. The change of Rac1 activity was expressed as GTP-bound Rac1 over total Rac1 content in each sample.

**Western Blotting**—NIH/3T3 cells were seeded at 40,000 cells/cm² and incubated for 1 h on control substrate or Nogo substrate. Cells were stimulated with either teleocidin, PMA, Gö-6983, Gö-6976, or TAT-Akt-in. Cells were rinsed once with ice-cold Tris-buffered saline and then lysed with ice-cold radio-immune precipitation buffer (Sigma) supplemented with protease inhibitors and phosphatase inhibitors (Calbiochem). Samples were spun at 14,000 rpm for 20 min at 4 °C, and supernatants were transferred to a new tube. Protein concentration was measured with a Bradford assay (Bio-Rad). Electrophoresis was performed on 10% or 4–12% NuPAGE® Novex® Bis-Tris gels (Invitrogen) with equal amounts of protein loaded onto each lane. Proteins were then transferred to nitrocellulose membrane (Bio-Rad) using the Mini Trans-Blot® electrophoretic transfer cell system (Bio-Rad) at 100 V for 1 h. Membranes were blocked with Odyssey blocking buffer (Li-COR Biosciences) at room temperature for 1 h and then incubated with primary antibodies diluted in Odyssey blocking buffer with 0.05% Tween 20 overnight at 4 °C. The membranes were washed with PBS, 0.1% Tween 20 four times at room temperature and stained with secondary goat anti-rabbit IR 680 and goat anti-mouse IR 800 (Li-COR Biosciences) in Odyssey blocking buffer with 0.05% Tween 20 at room temperature for 1 h. After four more rinses, membranes were scanned with an Odyssey infrared imaging system. Visualization and quantification of bands were performed with the Odyssey 2.1 software.

**Cell-spreading Assay**—NIH/3T3 cells were cultured to 70–80% confluence before being trypsinized and plated at a subconfluent density (2.67 × 10⁶ cells/well) on either control or Nogo substrate. Nogo (2–4 µg/ml) or myelin (2–4 µg/ml in H₂O, 50 µl/well) or control (50 µl of H₂O) was air-dried on a PDL-coated 96-well plate overnight in a tissue culture hood. Cells were detached by 0.05% trypsin/EDTA (Invitrogen). The reaction was neutralized with Dulbecco’s modified Eagle’s medium with 10% bovine calf serum. Then NIH/3T3 cells were added to wells at 2500 cells/well and grown for 1 h at 37 °C in a 5% CO₂ incubator. Cells were fixed with 4% paraformaldehyde, permeabilized with 0.3% Triton X-100 in PBS for 5 min, and then stained with Hoechst 33342 (Invitrogen) and Alexa Fluor 488-conjugated phalloidin (Invitrogen) in PBS for 20 min. Cell images were scanned by MetaXpress (Molecular Devices). Cell spreading was quantified by the MetaXpress cell scoring module, which measures the spreading area of all Hoechst-positive cells. All data were exported as mean cell area. Data from each experiment were normalized to the cell spreading area on PDL (100%) in the same plate. Results from several independent experiments were combined and presented as “relative cell area” ± S.E. Statistical significance was determined using a two-tailed unpaired Student’s t test.

**Neurite Outgrowth Assay**—Either Nogo or myelin was coated on wells of a 96-well plate by a nitrocellulose-based coating method. In brief, nitrocellulose was air-dried at room temperature before 50 µl of Nogo (2–4 µg/ml) or myelin (2–4 µg/ml) in PBS was added to wells and incubated overnight at 4 °C. Medium was then aspirated, and PDL (Sigma) at 17 µg/ml in PBS was coated onto each well, followed by blocking with 10% FBS for an additional 1 h at 37 °C.

Cerebellar neurons were isolated and resuspended in Sato medium as described above. A neurite outgrowth assay was carried out by adding 1 × 10⁶ cerebellar neurons to Nogo or...
myelin-coated wells in triplicate with or without teleocidin. After 18–20 h of incubation, the neurons were fixed with 4% paraformaldehyde for 30 min and permeabilized with 0.3% Triton X-100 in PBS for 2 min. The cells were then blocked for 30 min with blocking buffer (0.1% Triton X-100, 3% normal goat serum in PBS) and incubated overnight with a rabbit polyclonal antibody against β-III tubulin (TuJ1, Covance). Cells were washed three times with PBS and then incubated for 1 h at room temperature with goat anti-rabbit Alexa Fluor 488 (Invitrogen).

The total neurite length for each neuron for more than 200 neurons was captured automatically for each well, and the mean total neurite length per neuron was quantified by a neurite outgrowth module (MetaXpress, Molecular Devices). All data were presented as a percentage of control neurite length of neurons grown on PDL (100%) ± S.E. Statistical significance was determined using a two-tailed unpaired Student’s t test.

RESULTS

Amino-Nogo Decreases the Activity of Rac1 in Fibroblasts—Molecular characterization of amino-Nogo has demonstrated that a central region, called NiG-Δ20, is responsible for its inhibitory activity (5). We have generated a recombinant Nogo protein, connecting two central regions (amino acids 567–748) of amino-Nogo with a human IgG Fc fragment, as a dimerized human equivalent of NiG-Δ20. This recombinant version of amino-Nogo (referred to hereafter as Nogo) has been tested in a cell-spreading assay and shown to exhibit a strong inhibitory function (Fig. 1A). In contrast, myelin-associated glycoprotein or a human Fc control protein had no effect on cell spreading in this assay (data not shown).

In addition to inhibiting cell spreading, staining cells with phalloidin revealed different actin filament arrangements in cells grown on control versus the Nogo substrate (Fig. 1A). Small GTPases, including RhoA, Rac, and Cdc42, are key regulators of cytoskeletal dynamics (20), suggesting the possible involvement of small Rho GTPase in Nogo signaling. We performed a Rac1 activity assay to investigate whether Nogo could change the level of GTP-bound Rac1 in cells. GTP-bound Rac1 was isolated from cell lysates with GST-PAK1 protein-binding domain, a probe to specifically isolate active (GTP-bound) forms of Rac1. When cells were plated on Nogo for 10 min, levels of GTP-bound Rac1 increased by about 48% compared with cells plated on control substrate (Fig. 1B). In addition, there was no detectable difference in the amount of total Rac1 and actin from cells grown on control versus Nogo substrate. Our data suggest that decreasing Rac1 activity is an acute response to Nogo treatment, and this may account for the rearrangement of actin when cells are grown on the Nogo substrate.

TPA-type Tumor Promoters Activate Rac1 and Promote Fibroblast Spreading on Nogo—The observation that Nogo inhibited Rac1 activity prompted us to determine if activation of Rac1 was sufficient to overcome Nogo inhibitory activity in the cell-spreading assay. Benard et al. (21) showed that levels of Rac1-GTP can be dramatically increased by TPA-type tumor promoters, such as PMA. We found that two structurally different TPA-type tumor promoters, PMA and teleocidin (22), activated Rac1 in fibroblast cells (Fig. 2A). Teleocidin treatment resulted in a more robust increase of Rac1 activity in fibroblasts on both the control and Nogo substrate at the same concentration as PMA (10 nm).

We then investigated whether teleocidin or PMA can overcome Nogo inhibition in a cell-spreading assay. The results showed that both teleocidin and PMA promoted cell spreading on the Nogo substrate (Fig. 2, B and C). Teleocidin significantly increased cell area on the Nogo substrate at 3.7 nm with a plateau reached at 10–35 nm. At the same concentrations, teleocidin little effect on cell area on the control substrate (Fig. 2B). Similar results were obtained with PMA (Fig. 2C), yet 4α-PMA, an inactive analog of PMA, did not promote cell spreading on the Nogo substrate. Also, the effects of teleocidin on cell spreading were effectively blocked in the presence of the Rac1 inhibitor, NSC23766 (Fig. 2D). These results suggest that activation of Rac1 by TPA-type tumor promoters overcomes Nogo inhibition of cell spreading.

PKD Acts as the Major Target for Teleocidin in Overcoming Nogo Inhibition—PKC and PKD are two groups of well characterized high affinity receptors for TPA-type tumor promoters (23–26). Among all of the tested PKC isoforms, none showed significant increase of their phosphorylation levels by teleocidin on either control or Nogo substrates (Fig. 3A). Thus, PKCs may not be involved in teleocidin action in NIH/3T3 cells. In contrast, there was a robust and persistent increase of phospho-PKD by teleocidin at both serine 744/748 and serine 916 sites in
Tumor Promoters Overcome Amino-Nogo Inhibition

FIGURE 2. TPA-type tumor promoters activated Rac1 and promoted fibroblast spreading on Nogo in a Rac1-dependent manner. NIH/3T3 cells were seeded on control (ctrl) or Nogo substrate, A, cells were incubated without (NT) or with PMA (10 nM) or teleocidin (Tele) (10 nM) for 10 min. Cell lysates were subjected to a Rac1 activation assay. Change of Rac1 activity was expressed as Rac1-GTP over total Rac1. Shown is fibroblast spreading on control or Nogo substrate in the presence of different concentrations of teleocidin (B), PMA (C), or 1 μM 4α-PMA (D) or in the presence of 10 nM teleocidin with or without 100 μM NSC23766 (E) for 1 h. Cells were fixed and stained for F-actin. The cell area of each Hoechst-positive cell was measured. Triplicate wells per treatment were measured in each experiment. Relative mean cell area combined from at least three independent experiments is presented. *, p < 0.05; **, p < 0.01.

cells plated on both the control and Nogo substrates (Fig. 3B). Activation of PKD by PMA or teleocidin was markedly diminished in the presence of Gö-6983, a pharmacological inhibitor of PKD, in cell-based assays (27) (Fig. 3C), but not Gö-6976, an inhibitor of PKCs. In order to test the importance of PKD activation in overcoming Nogo inhibition, a fibroblast-spreading assay was performed with various concentrations of Gö-6983 with or without teleocidin. Gö-6983 itself had little effect on cell spreading even at the highest dose tested (1 μM). However, if added along with teleocidin, Gö-6983 attenuated the promoting effect of teleocidin in a dose-dependent manner (Fig. 3E). On control substrates, teleocidin treatment induced actin polymerization at the plasma membrane, leading to the formation of lamellipodia and membrane ruffles. In the presence of Gö-6983, the cell morphology appeared more like the cell spreading on non-treated control (Fig. 3D). On Nogo substrates, most of the cells were non-spreading. Teleocidin treatment induced the cells to spread well regardless of the non-permissive substrate, and this spreading effect was reversed by Gö-6983 on either cell area or cell morphology (Fig. 3D). Together, our data indicate that PKD is a mediator for TPA-type tumor promoters in overcoming Nogo inhibition.

Teleocidin Activates Akt1 to Overcome Inhibition of Fibroblast Spreading by Nogo—Despite evidence suggesting that activation of PKD can lead to Rac1 activation (28), the link between PKD and Rac1 activation remains unclear. To obtain more insight into teleocidin signaling on Nogo substrate, we adapted the Kinetworks® signal transduction phosphoprotein profiling strategy. In detail, NIH/3T3 fibroblast cells were treated for various times (2, 5, 15, and 30 min) with teleocidin on inhibitory Nogo substrate before cell lysates were harvested. Protein samples were processed for the Kinexus phosphorylation sites version 1.3 broad signaling pathway screen (KPSS-1.3, Kinexus Bioinformatics Corp.). This screen examines 38 phosphorylation sites in 32 proteins by antibodies that recognize specific phosphorylated epitopes. Screening results were summarized in Table 1. Phosphorylation of Akt1 at both serine 473 and threonine 308 sites was increased by teleocidin with time (Table 1), which was confirmed in repeated experiments (Fig. 4A). In addition, phosphorylation of GSK3β (glycogen synthase kinase 3β), a substrate for Akt1, was also increased by teleocidin. These results indicate that the Akt1 pathway is the most prominent responder to teleocidin treatment under our assay conditions.

Next, we investigate whether PKD is involved in the activation of Akt1. As shown in Fig. 4B, phosphorylation of Akt1 by teleocidin was blocked by the presence of Gö-6983. In addition, the importance of Akt1 for teleocidin function was tested by applying a cell-permeable Akt-inhibitory peptide, called TAT-Akt-in (29), in the cell-spreading assay. TAT-Akt-in at 50 μM, the effective dose used to block Akt1 phosphorylation in HEK293 (29), attenuated the effect of teleocidin in overcoming Nogo inhibition (Fig. 4C). Thus, Akt1 serves as an essential component of teleocidin signaling, and Akt1 is a downstream effector of PKD.

Nogo Decreases Akt1 Phosphorylation and Activation of Akt1 Is Sufficient to Reverse Nogo Inhibition of Cell Spreading—We have shown that not only can Akt1 be activated by teleocidin, but also its activity is regulated by Nogo. We found that the levels of phospho-Akt1 decreased on the Nogo substrate, suggesting that Akt1 is involved in Nogo signaling (Fig. 5A).
Tumor Promoters Overcome Amino-Nogo Inhibition

FIGURE 3. Teleocidin activated PKD to overcome Nogo inhibition. A and B, NIH/3T3 cells were seeded on either control (ctrl) or Nogo substrate and incubated for 1 h prior to stimulation with 10 nM teleocidin (Tele) for 10 min or 30 min. Cell lysates were analyzed by SDS-PAGE and Western blot using antibodies against different phosphorylation sites of PKC isozymes and pan-PKC (A) and phospho-PKD and actin (B) as indicated. C, NIH/3T3 cells were treated with either 10 nM teleocidin or 10 nM PMA in the presence (+) or in the absence (−) of either Gó-6983 (1 μM) or Gó-6976 (1 μM) for 30 min on control substrate. Levels of phospho-PKD and pan-PKC in cell lysates were detected. D, representative images of cells grown on either control or Nogo substrate without treatment (NT) or with either 10 nM teleocidin or 1 μM Gó-6983. Cells were incubated for 1 h and then fixed and stained with Hoechst 33342 (purple) and phalloidin (green). E, quantification of NIH/3T3 cell spreading with different concentrations of Gó-6983 (1 μM) or Gó-6976 (1 μM) along with 10 nM teleocidin. The relative cell area of each treatment on Nogo substrate is presented in comparison with cell area on control substrate. n.s., not significant; **, p < 0.01; ***, p < 0.001.

TABLE 1
Akt1 pathway was activated by teleocidin

|                      | Without teleocidin | With teleocidin |
|----------------------|--------------------|-----------------|
|                      | 2 min             | 5 min           | 15 min          | 30 min          |
| Akt1 (Ser473)        | 1.74              | 3.57            | 6.00            | 6.03            |
| Akt1 (Thr308)        | 1.97              | 3.51            | 2.66            | 3.96            |
| GSK3β (Ser9)         | 1.06              | 2.15            | 1.97            | 1.31            |
| MEK1/2 (MAP2K1/2) (Ser218/222) | 1.24 | 2.57 | 3.36 | 3.46 |
| p38α MAPK (Thr180 + Tyr182) | 1.68 | 1.32 | 3.64 | 2.17 |

NIH/3T3 cells were seeded on Nogo substrate and incubated for 1 h prior to stimulation with 10 nM teleocidin for the indicated durations. Cell lysates were processed for Kinexus phosphorylation sites version 1.3 broad signaling pathway screen, which tracks for phosphorylation levels of 38 phosphorylation sites in 32 phosphoproteins with antibodies against specific epitopes. Band intensity was quantitated and is presented as counts/min (cpm) (Kinexus Corp.). The cpm in each lane was measured to confirm equivalent loading of samples. Hence, deactivation of Akt1 is a consequence of a Nogo-induced inhibitory signal. Activation of Akt1 can partially overcome Nogo-induced inhibition of cell spreading.

**Figure 3 continued.**

Next, a cell-permeable peptide that contains the Akt-binding domain of the endogenous Akt coactivator, TCL-1 (T-cell leukemia 1) (19), was applied to determine whether activation of Akt1 would be sufficient to overcome the inhibitory effects of Nogo. Fig. 5B showed that TAT-TCL-1 strongly increased levels of phospho-Akt1 compared with the control peptide, TAT-TCL-1G, an inactive peptide that recapitulated the D16G point mutant of TCL1. In addition, the inhibition by Nogo on cell

spreading was significantly reversed by TAT-TCL-1 but not by TAT-TCL-1G (Fig. 5C). Therefore, deactivation of Akt1 is a consequence of a Nogo-induced inhibitory signal. Activation of Akt1 can partially overcome Nogo-induced inhibition of cell spreading.

Rac1 Stimulation by Teleocidin Is Dependent on PKD and Akt1 Activation—We have also assessed the impact of PKD or Akt1 activation on Rac1 activity. NIH/3T3 fibroblast cells were seeded on a Nogo substrate with teleocidin in the presence of either Gó-6983 or Gó-6976. Gó-6983 blocked the elevation of Rac1-GTP by teleocidin. The Rac1-GTP level remained at 54% compared with teleocidin-treated control, whereas Gó-6976 failed to affect Rac1 activity (Fig. 6A). In Fig. 6B, the increase of Rac1-GTP by teleocidin was reversed when an inhibitor of Akt, called TAT-Akt-in, was added along with teleocidin.

Based on these results, we can propose a potential mechanism as follows. Nogo inhibits non-neuronal cell spreading via a decrease of Akt1 and Rac1 activity, whereas TPA-type tumor promoters, such as teleocidin and PMA, activate PKD, which in turn increases phosphorylation of Akt1 and lead to activation of Rac1. As a result, the signals induced by TPA-type tumor promoters probably counteract the inhibitory effect of Nogo and promote cell spreading on Nogo substrate (Fig. 7).

Teleocidin Reverses Inhibition of Neurite Outgrowth by Nogo and Myelin—In addition to inhibition of cell spreading, it has also been shown that NiG-Δ20 inhibited neurite outgrowth of cultured primary neurons (5). To address whether the signaling mechanism that we established in non-neuronal cells exists in neurons and whether activation of this pathway by teleocidin can overcome the inhibition of neurite outgrowth by Nogo, the response of PKD to teleocidin in neurons was examined. We showed (Fig. 8A) that teleocidin induced a rapid increase of phospho-PKD, resembling the results from NIH/3T3 fibroblast cells (Fig. 3A). The activation of PKD by teleocidin in neurons was also blocked by Gó-6983. Expression levels of tubulin were measured to confirm equivalent loading of samples. Hence, teleocidin-induced phosphorylation of PKD appears very similar between neurons and fibroblasts.

In neurite outgrowth assays, Nogo inhibits neurite extension of cerebellar granule neurons. Most neurons did not bear neurites or extend short neurite on Nogo substrate (Fig. 8B). Quantification of results from repeated experiments showed that neurites of neurons grown on Nogo substrates were about 50%
shorter than those of neurons grown on control (Fig. 8C), which was consistent with previous studies (5). Teleocidin overcame the inhibition of neurite extension by Nogo; neurite length in the presence of teleocidin was reversed to 80% of the neurite length of non-treated control (Fig. 8B and C).

We then examined whether teleocidin treatment can overcome the overall myelin-induced inhibition. Fig. 8D demonstrated that teleocidin-treated neurons grew significantly longer neurites than cells without teleocidin at concentration as low as 3.7 nM. The effect of teleocidin on neurite outgrowth on the myelin substrate plateaued at 10 nM. The extent of growth promotion was comparable with that with inhibition of Rho kinase by Y-27632 at 10 μM; the average total neurite length on the myelin substrate in repeated experiments with teleocidin and Y-27632 was about 70 and 65% of control neurite outgrowth without myelin, respectively (result not shown). Thus, teleocidin not only can improve fibroblast spreading but also can promote neurite outgrowth on inhibitory substrates.

**DISCUSSION**

Regrowth of axons in the injured nervous system is hampered by MAIs in the myelin debris or expressed by surrounding glial cells. Although amino-Nogo is characterized as a major MAI in constraining neurite outgrowth, the molecular mechanism of amino-Nogo action remains largely unknown at the level of both its receptor and intracellular signaling mechanisms. In the present study, we confirmed and extended a role for Rac1 in amino-Nogo-induced inhibitory signaling. In addition, we identified TPA-type tumor promoters as a group of molecules that promote cell spreading and neurite outgrowth on inhibitory MAI substrates. TPA-type tumor promoters might be structurally different; however, they bind to the same group of receptors to exert similar functions. We demonstrated that TPA-type tumor promoters, both PMA and teleocidin,
activated Rac1 and promoted fibroblast cell spreading on a Nogo substrate in a dose-dependent manner. Moreover, the promoting effect of TPA-type tumor promoters on cell spreading is Rac1-dependent. Decrease of Rac1 activity is an acute response to amino-Nogo. In addition, the activation of Rac1 by TPA-type tumor promoters shifts the balance of Rac1 to a GTP-bound active state, counteracts the inactivation of Rac1 by Nogo, and switches the cell spreading back to a normal growth state. Furthermore, our results indicate that teleocidin is a potent neurite outgrowth promoter on inhibitory substrates, extending a previous finding that the addition of low concentrations of TPA promoted neurite outgrowth from explants of chick dorsal root ganglia on a permissive substrate (30).

Receptors for TPA-type tumor promoters were divided into kinase type, including PKCs and PKD, and non-kinase type, including chimaerins, Unc-13/Munc-13, and RasGRPs (31). In the present study, we investigated the response of PKCs and PKD to TPA-type tumor promoters. Our results suggest that PKD is probably the major responder to TPA-type tumor promoters in both fibroblast cells and neurons. TPA-type tumor promoters regulate the intracellular localization of PKD, and they activate PKD through PKC by phosphorylation (32). PKD is activated via PKC-dependent trans-phosphorylation of the activation loop at Ser744 and Ser748, followed by PKD autophosphorylation at Ser916 (32). Here, PKD was phosphorylated at both serine 744/748 and serine 916 sites in response to either PMA or teleocidin. This increase of phosphorylation was blocked by Go¨-6983. Go¨-6983 has been developed as a specific inhibitor for novel PKCs (nPKC$^\text{ a}$, -$^\text{ b}$, -$^\text{ c}$, and -$^\text{ d}$), and it has low affinity for PKD in a cell-free system (33). Recently,
Tumor Promoters Overcome Amino-Nogo Inhibition

Gö-6983 has been used as a pharmacological inhibitor for PKD in several cell-based assays (27). Gö-6983 operates by blocking the activities of PKD-activating nPKCs. Among the nPKCs we explored, there was little change of phospho-PKCδ and phospho-PKCθ, and phospho-PKCε, by teleocidin in fibroblast cells. One explanation could be that the basal level of phospho-PKCδ in fibroblast cells masked any further increase in phospho-PKCδ by teleocidin. In neurons that have a much lower basal level of phospho-PKCδ, an increase of phospho-PKCδ was observed (supplemental Fig. S1), suggesting that PKCδ might be the corresponding PKD activator in neurons. Another possibility could be that TPA-type tumor promoters evoke a change of intracellular localization instead of overall activity of PKCs. It has been documented that TPA-type tumor promoters bind to the DAG site in conventional and novel PKC and act as a “glue” that fixes the PKC protein to the membrane (34). Thus, a plausible mechanism of the function of TPA-type tumor promoters in the activation of PKD might be that TPA-type tumor promoters recruit both PKD and its activating PKCs to close proximity on the membrane, so that PKD can be rapidly and persistently activated. Then PKD accumulates the activation signal from PKC and serves as a mediator to convey the signal. Theoretically, a small interfering RNA approach to knock down PKD could unequivocally demonstrate the key role of PKD in cell spreading and neurite outgrowth on inhibitory substrates. However, the spreading feature of fibroblast cells is affected by transfection, and primary neurons are difficult to transfect. Therefore, definitive evidence for a role of PKD in promoting cell spreading and neurite outgrowth awaits studies from PKD null mice. Based on our results, the activity of PKD, but not nPKCs, is highly correlated with Rac1 activity and cell spreading results, suggesting that PKD is a key player in the cell spreading promotion function of TPA-type tumor promoters.

It has been reported that classical PKCs (cPKCa, -βI, -βII, and -γ) were activated by MAIs (35, 36). Blockades of cPKCs, such as Gö-6976, promoted neurite outgrowth on inhibitory substrates (35, 36). However, in the current study, little change at the level of phospho-cPKCa and -β by Nogo was observed. In addition, there was no significant promotion of cell spreading on a Nogo substrate by Gö-6976 (Fig. 3E). These seemingly contradictory observations might be due to the different nature of primary neurons and fibroblast cells that may have distinct expression and activation profiles of PKCs. Also, our study focused on amino-Nogo that activates an NgR1-independent pathway, whereas these authors studied NgR1-related inhibitory signals. In both fibroblast cells and neurons, the basal activities of cPKC are high and are not altered by teleocidin. Furthermore, in the presence of Gö-6976, the cell spreading-promoting effect of teleocidin remains. In summary, these data imply that the activities of cPKCs are dispensable for either amino-Nogo or TPA-type tumor promoter signaling in this circumstance.

In addition to the kinase type receptors for TPA-type tumor promoter, the non-kinase type receptors, namely chimaerins (a family of Rac-GTPase-activating proteins), Unc-13/Munc-13 (a family of proteins involved in exocytosis), and RasGRPs (a Ras exchange factor), that have not been investigated here, might contribute to the observed signaling and functional outcomes of TPA-type tumor promoters as well. The signaling of these receptors is linked to Rac1 GTPase activity at different levels (37–43). Therefore, it is likely that some tumor promoter-induced effects are mediated via one or several of these proteins in parallel pathways. Determination of how they contribute to blocking amino-Nogo signaling will require further investigations. In this study, our results suggest that PKD is a key mediator in teleocidin-induced Rac1 activation, and activation of PKD is required to bring about its cell spreading-promoting function.

Although multiple downstream effectors of PKD exist, based on our results, the Akt1 pathway is the most prominent candidate for mediating the cell spreading-promoting function of teleocidin. Teleocidin increased phosphorylation of Akt1 at both Thr308 and Ser473 sites (Table 1 and Fig. 4A). In addition, the phosphorylation of GSK3β, an established downstream effector of Akt1, was elevated simultaneously. Akt1 has been characterized as a key component in the phosphatidylinositol 3-kinase/phosphatidylinositol 3,4,5-triphosphate signaling. However, our results indicated that inhibition of the phosphatidylinositol 3-kinase pathway by LY294002 could not block the effect of teleocidin on Rac1 activation and cell spreading promotion on an inhibitory substrate (supplemental Fig. S2). In addition, platelet-derived growth factor, an activator of Akt1 acting via phosphatidylinositol 3-kinase/phosphatidylinositol 3,4,5-triphosphate, could not mimic the promoting effect of teleocidin on cell spreading (supplemental Fig. S2). The association of Akt1 with distinct signaling pathways may have different functional outcomes (44). A more in depth explanation of this phenomenon awaits further study. Here, we showed that activation of Akt1 by teleocidin was dependent on PKD activity, implying that Akt1 is a downstream effector of PKD. Blocking Akt1 activity with TAT-Akt-in, a cell-permeable Akt1 inhibitor, attenuated the cell spreading-promoting effect of teleocidin. Furthermore, we demonstrated that Akt1 activity was crucial for teleocidin-induced Rac1 activation. Similarly, Somanath (45) reported that activation of Akt1 resulted in translocation of Rac1 to membrane and enhanced Rac1 activity.

We also found that the level of phospho-Akt1 was decreased by Nogo, a phenomenon similar to that reported as a response of neurons to chondroitin sulfate proteoglycans, another neurite outgrowth inhibitor highly expressed after injury (46). These authors further indicated that inactivation of GSK3 promoted axonal growth and recovery (46). Importantly, we showed that activation of Akt1 by a cell-permeable TCL1 peptide (19), which facilitated Akt oligomerization and led to its activation, significantly reversed amino-Nogo inhibition of cell spreading. Also, the inhibition of neurite outgrowth by myelin was reversed in the presence of TCL1 peptide.3 Our studies point out that Akt1 is a key component of a Nogo-induced inhibitory signaling pathway.

In conclusion, our findings show first that TPA-type tumor promoters are a group of molecules that can stimulate cell spreading and neurite outgrowth on inhibitory MAIs substrates; second, that TPA-type tumor promoters activate their receptor PKD and the downstream effectors Akt1 and Rac1 to

3 Y. Gao, unpublished results.
bring about these functions, and third, that Akt1 is a novel signaling component in the amino-Nogo-induced inhibitory pathway. Our results suggest that activation of PKD or Akt1 may be alternative means to promote axonal regeneration on inhibitory substrates.

Acknowledgments—We thank Dr. Pranab Chanda for human Nogo/Fc construct design; Dr. Richard Zoller, Grace Yan, and Angela Robak for the production and purification of the Nogo protein; and Dr. Yan Liu for helpful discussions.

REFERENCES

1. Cao, Z., Gao, Y., Deng, K., Williams, G., Doherty, P., and Walsh, F. S. (2010) Mol. Cell Neurosci. 43, 1–14
2. Fournier, A. E., GrandPre, T., and Strittmatter, S. M. (2001) Nature 409, 341–346
3. Atwal, J. K., Pinkston-Gosse, J., Syken, J., Stawicki, S., Wu, Y., Shatz, C., and Tessier-Lavigne, M. (2002) Science 298, 967–970
4. Chen, M. S., Huber, A. B., van der Haar, M. E., Frank, M., Schnell, L., Spillmann, A., Christ, F., and Schwab, M. E. (2000) Nature 403, 434–439
5. Oertle, T., van der Haar, M. E., Bandlow, C. E., Robeva, A., Burfeind, P., Buss, A., Huber, A. B., Simonen, M., Schnell, L., Brösamle, C., Kaupmann, K., Vallon, R., and Schwab, M. E. (2003) J. Neurosci. 23, 5393–5406
6. Schweigreiter, R., Walmsley, A. R., Niederoest, B., Zimmermann, D. R., Oertle, T., Casademunt, E., Frontzel, S., Dechant, G., Mir, A., and Bandtlow, C. E. (2004) Mol. Cell Neurosci. 27, 163–174
7. Hu, F., and Strittmatter, S. M. (2008) J. Neurosci. 28, 1262–1269
8. Niederoest, B., Oertle, T., Fritsche, J., McKinney, R. A., and Bandtlow, C. E. (2004) J. Neurosci. 24, 10368–10376
9. Lehmann, M., Fournier, A., Selles-Navarro, I., Dergham, P., Sebok, A., Leclerc, N., Tigyi, G., and McKerracher, L. (1999) J. Neurosci. 19, 7537–7547
10. Dergham, P., Ellezam, B., Essadiq, C., Avedissian, H., Avedissian, H., Avedissian, N., Leclerc, N., Tigh, G., and McKerracher, L. (2002) J. Neurosci. 22, 6570–6577
11. Monnier, P. P., Sierra, A., Schwab, J. M., Henke-Fahle, S., and Mueller, B. K. (2003) Mol. Cell Neurosci. 23, 319–330
12. Hiromura, M., Okada, F., Obata, T., Auguin, D., Shibata, R., Roumestand, C., and Noguchi, M. (2004) J. Biol. Chem. 279, 53407–53418
13. Hsu, C., Nativ, D., and Trupin, G. L. (1984) Muscle Nerve 7, 211–217
14. Kazanietz, M. G., Caloca, M. I., Eroles, P., Fujii, T., Garcia-Bernejo, M. L., Reilly, M., and Wang, H. (2000) Biochem. Pharmacol. 60, 1417–1424
15. Rybin, V. O., Guo, J., and Steinberg, S. F. (2009) J. Biol. Chem. 284, 2332–2343
16. Gschwendt, M., Dieterich, S., Rennicke, R., Kettstein, W., Mueller, H. J., and Johannes, F. J. (1996) FEBS Lett. 392, 77–80
17. Zhang, G., Kazanietz, M. G., Blumberg, P. M., and Hurley, J. H. (1995) Cell 81, 917–924
18. Sivasankaran, R., Pei, J., Wang, K. C., Zhang, Y. P., Shields, C. B., Xu, X. M., and He, Z. (2004) Nat. Neurosci. 7, 261–268
19. Hasegawa, Y., Fujitani, M., Hata, K., Tohyama, M., Yamagishi, S., and Yamashita, T. (2004) J. Neurosci. 24, 6826–6832
20. Ahmed, S., Kozma, R., Monfries, C., Hall, C., Lim, H. H., Smith, P., and Lim, L. (1990) Biochem. J. 272, 767–773
21. Ahmed, S., Kozma, R., Monfries, C., Hall, C., Lim, H. H., Smith, P., and Lim, L. (1999) Mol. Cell Biol. 19, 5069–5080
22. Leung, T., Chen, X. Q., Tan, I., Manner, E., and Lim, L. (1998) Mol. Cell Biol. 18, 130–140
23. Tan, I., Seow, K. T., Lim, L., and Leung, T. (2001) Mol. Cell Biol. 21, 2767–2778
24. Wilkinson, S., Paterson, H. F., and Marshall, C. J. (2005) Nat. Cell Biol. 7, 255–261
25. Ebini, J. O., Bottriff, D. A., Chan, E. Y., Stang, S. L., Dunn, R. J., and Stone, I. C. (1998) Science 280, 1082–1086
26. Tognon, C. E., Kirk, H. E., Passmore, L. A., Whitehead, I. P., Der, C. J., and Kay, R. J. (1998) Mol. Cell Biol. 18, 6995–7018
27. Kane, L. P., Shapiro, V. S., Stokoe, D., and Weiss, A. (1999) Curr. Biol. 9, 601–604
28. Somanath, P. R., and Byzova, T. V. (2009) J. Cell Physiol. 218, 394–404
29. Dill, J., Wang, H., Zhou, F., and Li, S. (2008) J. Neurosci. 28, 8914–8928