

Promotion of Axon Regeneration by Myelin-Associated Glycoprotein and Nogo through Divergent Signals Downstream of G_i/G

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Several myelin-derived proteins have been identified as components of the CNS myelin that prevents axonal regeneration in the adult vertebrate CNS. Activation of RhoA has been shown to be an essential part of the signaling mechanism of these proteins. Here we report an additional signal, which determines whether these proteins promote or inhibit axon outgrowth. Myelin-associated glycoprotein (MAG) and Nogo trigger the intracellular elevation of Ca²⁺ as well as the activation of PKC, presumably mediated by G_i/G. Neurite outgrowth inhibition and growth cone collapse by MAG or Nogo can be converted to neurite extension and growth cone spreading by inhibiting conventional PKC, but not by inhibiting inositol 1,4,5-triphosphate (IP₃). Conversely, neurite growth of immature neurons promoted by MAG is abolished by inhibiting IP₃. Activation of RhoA is independent of PKC. Thus, a balance between PKC and IP₃ is important for bidirectional regulation of axon regeneration by the myelin-derived proteins.

Key words: p75; myelin; myelin-derived proteins; G-protein; PKC; spinal

Introduction

Three distinct myelin proteins, myelin-associated glycoprotein (MAG), Nogo, and oligodendrocyte myelin glycoprotein, inhibit axon growth by binding to a common receptor, the Nogo receptor (McKerracher and Winton, 2002). Because the Nogo receptor is glycosylphosphatidylinositol-linked to the cell surface and does not have an intracellular signaling domain, it plays a role as a binding partner for the myelin proteins. Recently, p75^{NTR} in complex with the Nogo receptor has been shown to be a signal-transducing element for these proteins (Wang et al., 2002; Wong et al., 2002; Yamashita et al., 2002). One potential clue to understanding the signal transduction mechanism involved is found through observations demonstrating that the small GTPase Rho is a key intracellular effector for growth inhibitory signaling by myelin. In its active GTP-bound form, Rho rigidifies the actin cytoskeleton, thereby inhibiting axon elongation and mediating growth cone collapse (Davies, 2000; Schmidt and Hall, 2002). RhoA, a member of the Rho GTPases family, is activated by MAG, Nogo, and oligodendrocyte myelin glycoprotein through a p75^{NTR}-dependent mechanism, thus inhibiting neurite outgrowth from postnatal sensory neurons and cerebellar neurons

(Wang et al., 2002; Yamashita et al., 2002). Regulation of RhoA activity by MAG and Nogo through p75^{NTR} is mediated by the release of RhoA from Rho GDP dissociation inhibitor (GDI), which suppresses the activity of RhoA (Yamashita and Tohyama, 2003).

Although RhoA seems to play a key role in regulating axon growth, we were interested in the possibility that some other signals also may influence the effects of the myelin-derived inhibitors. An intriguing observation is that MAG promotes axon growth from dorsal root ganglion (DRG) neurons of rats up to postnatal day 4 (P4) (Johnson et al., 1989; Mukhopadhyay et al., 1994). This finding led to the possibility that the myelin-derived proteins are bifunctional molecules inhibiting or promoting axon regeneration. To test this hypothesis, we examined other signals that may be regulated by these proteins.

Here we report a new signal, which determines whether these proteins promote or inhibit axon outgrowth or growth cone spreading. MAG and Nogo trigger the intracellular elevation of Ca²⁺ as well as the activation of PKC. Neurite outgrowth inhibition and growth cone collapse by MAG or Nogo can be converted to neurite extension and growth cone spreading by inhibiting conventional PKC. Conversely, neurite growth of immature neurons promoted by MAG is abolished by inhibiting inositol 1,4,5-triphosphate (IP₃). A balance between PKC and IP₃ is suggested to be a key factor for bidirectional regulation of axon regeneration by the myelin-derived proteins.

Materials and Methods

Calcium imaging. Cultured cells were coloaded with the cell permeant 4 mM Fura Red AM and 4 mM Oregon Green 488 BAPTA-1 (Molecular

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Probes, Eugene, OR) for 1 hr at 37°C and imaged with the Leica confocal imaging system. Hank's MEM was used to prevent pH changes during experiments. The antibody against the extracellular domain of p75^{NTR} was added 2 hr before imaging, and U73122 (50 nM), U73343 (50 nM), or pertussis toxin (PTX) (20 ng/ml) was added 30 min before imaging. The cells were illuminated with 488 nm light from an argon laser. Fluorescence images for the entire cell body were used for ratiometric calcium measurements. Fura Red and Oregon Green emission signals were collected at 605–700 and 500–560 nm, respectively, and analyzed at 10 sec intervals. The Oregon Green/Fura Red ratio was calculated by dividing pixel values at 530 nm by those at 640 nm. MAG–Fc chimera (R & D Systems, Minneapolis, MN) or human Siglec-3-Fc chimera (R & D Systems) was used at the concentration of 25 µg/ml. The number of the cells examined is 70–120 for each experimental group (five to seven independent experiments were done for each).

Assessment of PKC activities. PKC assays were performed using the PepTag assay kit for nonradioactive detection of protein kinase C system (Promega, Madison, WI). Serum-starved cultured cerebellar cells were stimulated by MAG–Fc (25 µg/ml) and the Nogo peptide (4 µM; Alpha Diagnostic, San Antonio, TX) in the presence or absence of PTX (20 ng/ml). The inhibitors at the indicated concentration have been shown previously to be effective in enhancing the Rho activity as well as neurite inhibition (Tang et al., 1997b; Yamashita et al., 2002; Yamashita and Tohyama, 2003). Each sample was incubated with the PKC substrate PepTag C1 peptide (2 µg) at 30°C for 30 min. The samples were separated on a 0.8% agarose gel at 100 V for 15 min. Phosphorylated peptide substrate migrated toward the anode (+), whereas nonphosphorylated peptide substrate migrated toward the cathode (–). The gel was photographed on a transilluminator.

For assessment of phosphorylated PKC, phosphospecific antibodies were used. The cells were lysed with 0.1% NP-40 containing 10 mM Tris-HCl, pH 7.5, 150 mM NaCl, 10% glycerol, 100 mM sodium orthovanadate, 50 mM sodium fluoride, 5 mM sodium pyrophosphate, 1 mM EGTA, and protease inhibitor mixture (Roche Biochemicals). The lysates were subjected to SDS-PAGE, followed by immunoblot analysis. Polyclonal anti-phospho PKC-α (Ser 657; Upstate Biotechnology, Charlottesville, VA), phospho-PKC-ζ/λ (Cell Signaling Technology, Beverly, MA), and PKCα (Santa Cruz Biotechnology, Santa Cruz, CA) antibodies were used. In other sets of experiments, the lysates were incubated with anti-PKC-α, -PKC-β1, or -PKC-β2 (all from Santa Cruz Biotechnology), followed by incubation with protein G–Sepharose (Amersham Biosciences, Arlington Heights, IL) for 2 hr at 4°C. The beads were washed three times and subjected to SDS-PAGE, followed by immunoblot analysis. Western blotting was done using the antibody against phosphorylated pan-PKC (Cell Signaling Technology). Specificity of antibodies against PKC-β1 or PKC-β2 has been described in multiple references (Miranti et al., 1999). Anti-PKC-α antibody reacts with PKC-α and very weakly with PKC-β1 and -β2. Anti-PKC-β1 and -PKC-β2 are specific to corresponding isoform.

Neurite outgrowth assay. Dorsal root ganglia were removed from P1 rats and dissociated into single cells by incubation with 0.025% trypsin (Sigma, St. Louis, MO) for 15 min at 37°C. For cerebellar neurons, the cerebella from two animals (both P7 rats) were combined in 5 ml of 0.025% trypsin, triturated, and incubated for 10 min at 37°C. DMEM containing 10% FCS was added, and the cells were centrifuged at 800 rpm. Neurons were plated in Sato media (Doherty et al., 1990) on poly-L-lysine-coated chamber slides. For outgrowth assays, plated cells were incubated for 24 hr, fixed in 4% (weight per volume) paraformaldehyde, and immunostained with a monoclonal antibody (TuJ1) for the purpose of recognizing the neuron-specific β-tubulin III protein. The length of the longest neurite or the total process outgrowth for each β-tubulin III-positive neuron was then determined. For each experiment, we measured at least 100 neurites length and repeated the same experiments three times. Where indicated, MAG–Fc (25 µg/ml), the Nogo peptide (4 µM), PTX (2 ng/ml; Sigma), U73122 (20 nM; Sigma), Xestspingon C (XestC) (1 µM; Sigma), the cell-permeable PKC inhibitor 20–28 (2 µM; Calbiochem, La Jolla, CA), G₀6976 (200 nM; Sigma), or myristoylated PKCθ pseudosubstrate (2 µM; Sigma) was added to the medium after plating.

Growth cone collapse assay. Explants of embryonic day 12 (E12) chick dorsal root ganglion were incubated for 24 hr on plastic slides precoated with 100 µg/ml poly-L-lysine and treated for 30 min with soluble CNS myelin extracts (Sigma) at the indicated concentrations, MAG–Fc (25 µg/ml) or the Nogo peptide (4 µM). Explants were fixed in 4% (w/v) paraformaldehyde and stained with fluorescence-labeled phalloidin (Sigma). For each experiment, we assessed at least 100 growth cones, and we repeated the same experiments three times.

Affinity precipitation of GTP–RhoA. Cells were lysed in 50 mM Tris, pH 7.5, containing 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS, 500 mM NaCl, and 10 mM MgCl₂, with leupeptin and aprotinin, each at 10 µg/ml (Ren et al., 1999). Cell lysates were clarified by centrifugation at 13,000 × g at 4°C for 10 min, and the supernatants were incubated with 20 µg of GST–Rho binding domain of Rhotekin beads (Upstate Biotechnology) at 4°C for 45 min. The beads were washed four times with a washing buffer (50 mM Tris, pH 7.5, containing 1% Triton X-100, 150 mM NaCl, 10 mM MgCl₂, and 10 µg/ml each of leupeptin and aprotinin). Bound Rho proteins were detected by Western blotting using a monoclonal antibody against RhoA (Santa Cruz Biotechnology).

Results

Phospholipase C–PKC/IP₃ pathways are activated by MAG and Nogo

It has been shown previously that MAG induces a rise in intracellular Ca²⁺ concentration in cultured *Xenopus* spinal neurons (Wong et al., 2002) and that MAG-dependent repulsion of axonal growth cones requires Ca²⁺ signaling (Song et al., 1998). We confirmed these findings through a series of experiments using cerebellar neurons from P7 rats. Fluorescence imaging using the Ca²⁺-sensitive dyes Oregon Green 488 BAPTA-1 and Fura Red showed that the cytosolic Ca²⁺ was significantly elevated in the soma of the cells within 1 min after the addition of MAG–Fc to the medium (Fig. 1a–c). We were unable to monitor Ca²⁺ signals on the neurites because of the limited amount of fluorescent dyes loaded into these small cerebellar cell neurites (Xiang et al., 2002). The rapid and massive Ca²⁺ elevation we observed in the cerebellar neurons is different from the previous observation using *Xenopus* spinal neurons, for which the Ca²⁺ increase was mild (Wong et al., 2002). This may be attributable to the fact that our imaging was performed in the cell bodies, whereas Wong et al. (2002) observed the growth cones. No increase in the intracellular Ca²⁺ was observed by human Siglec-3-Fc, one of the members of the Ig superfamily (Fig. 1c). Results showed that the Ca²⁺ elevation was blocked by U73122, an inhibitor of phospholipase C (PLC), but not by U73343 (Fig. 1c). Because PLC is a major downstream effector of G_β, a heterotrimeric GTP-binding protein, in neurons, intracellular Ca²⁺ elevation may be dependent on the activation of G_β–PLC. Involvement of the G_i pathway is suggested by the observation that MAG blocks neurotrophin-induced cAMP accumulation (Cai et al., 1999), which is attenuated by PTX, an inhibitor of the G_i and G₀ proteins. These results suggest that the G-proteins activated by MAG inhibit accumulation of cAMP. Indeed, the increase in the Ca²⁺ concentration by MAG–Fc was attenuated by PTX as well (Fig. 1c). As reported previously (Wong et al., 2002), elevation of Ca²⁺ by MAG–Fc was inhibited by the antibody against the extracellular domain of p75^{NTR} (data not shown), thereby demonstrating that p75^{NTR} participates in the Ca²⁺ signal. These findings not only confirm the previous results but also suggest that G_i–PLC is activated by MAG.

Activation of PLC leads to the hydrolysis of phosphatidylinositol 4,5-bisphosphate (PIP₂) and produces two cytoplasmic second messengers (Berridge, 1998): diacylglycerol (DAG) and IP₃. PKC activation results from the production of DAG together with the elevation of Ca²⁺ because of IP₃-sensitive Ca²⁺ release

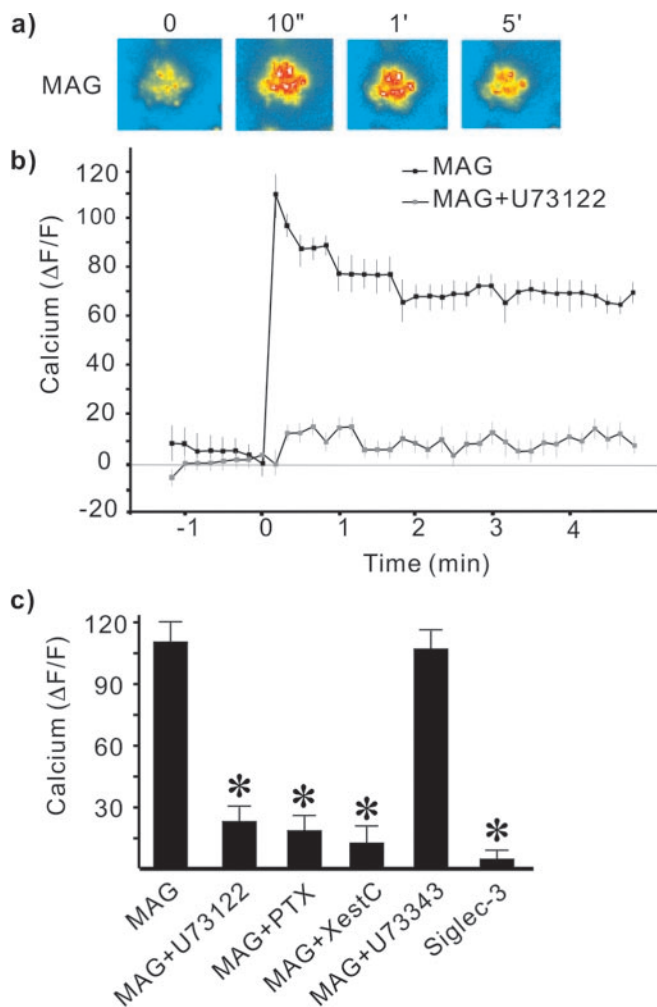


Figure 1. PLC-IP₃ pathways are activated by MAG and Nogo. *a*, Fluorescence images of the cell bodies of a cultured cerebellar neuron, which was loaded with the Ca²⁺-sensitive fluorescence indicators Fura Red and Oregon Green 488 BAPTA-1. *b*, Ca²⁺ signaling triggered by MAG is dependent on G_i-PLC activation. The percentage change in the fluorescence ratio (F_{530}/F_{640}) at 530 and 640 nm at the soma of the cerebellar neurons before and after application of MAG-Fc (25 μg/ml) in normal medium and in medium supplemented with U73122 (50 nM) is shown. Data are mean ± SE. *c*, Summary of the percentage change (± SE) in the fluorescence ratio (F_{530}/F_{640}) 0–4 min after MAG application with or without U73122, PTX (20 ng/ml), or XestC pretreatment. Data are mean ± SE ($n = 5$). Asterisks indicate statistical significance. * $p < 0.01$ (Student's *t* test).

from internal stores. These results prompted us to examine whether PKC is involved in MAG or Nogo signaling in cerebellar neurons. When cultured cells were treated for 5 min with 25 μg/ml MAG or a 4 μM concentration of the Nogo peptide, the PKC activity was significantly increased (Fig. 2*a*). Activation of PKC by MAG-Fc or the Nogo peptide was prevented by 20 ng/ml PTX. These results suggest the activation of the MAG- or Nogo-mediated G_i pathways, which in turn trigger PKC activation as well as IP₃ receptor activation. Our results are consistent with data published very recently (Sivasankaran et al., 2004) showing the activation of PKC by anti-phospho-PAN-PKC antibody. PKC-α, -β1, -β2, and -γ are expressed as conventional PKC in postnatal cerebellar neurons (data not shown). Western blot analysis revealed a significant increase in phosphorylation of PKC-α at Ser 657 by MAG-Fc, whereas protein levels of PKC-α were not significantly changed (Fig. 2*b*). However, experiments using phosphospecific antibodies showed that some of atypical

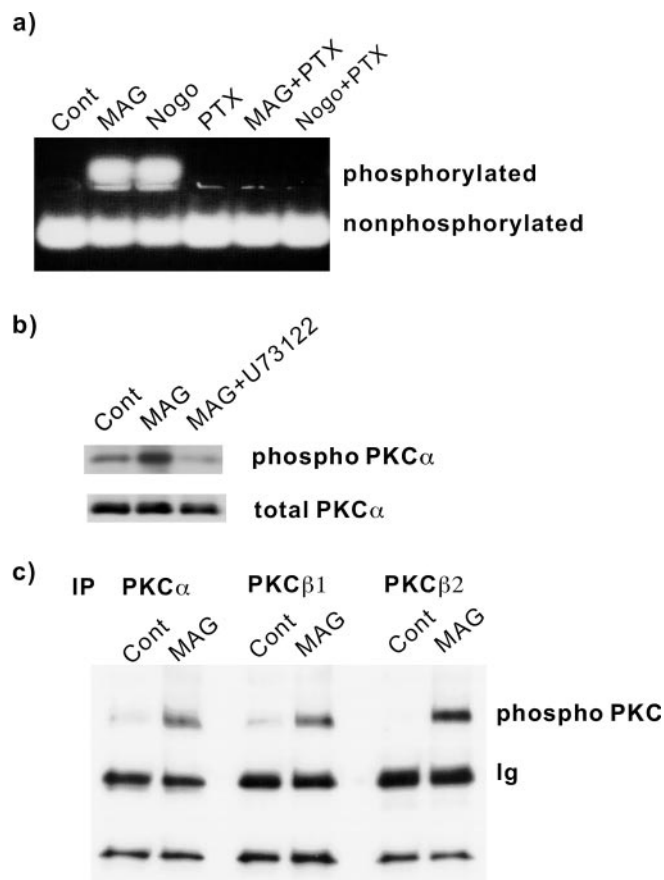


Figure 2. Conventional PKCs are activated by MAG and Nogo. *a*, Representative ($n = 4$) image that shows activation of PKC by MAG and Nogo in cultured cerebellar neurons. Note that the activation of PKC (phosphorylated) is abolished by pretreatment with PTX. MAG, MAG-Fc (25 μg/ml); Nogo, the Nogo peptide (4 μM). *b*, Representative image ($n = 3$) that shows phosphorylation of PKC-α by MAG-Fc in cultured cerebellar neurons. The bottom panel shows total PKC-α. *c*, Representative image ($n = 3$) that shows phosphorylation of PKC-α, -β1, or -β2 by MAG-Fc in cultured cerebellar neurons. Each isoform of conventional PKCs is immunoprecipitated (IP) with specific antibody and blotted with anti-phosphorylated pan-PKC antibody. Cont, Control.

PKCs, PKC-ζ/λ, were not phosphorylated by MAG (data not shown). To explore further isoform specific activation of conventional PKC, we immunoprecipitated each isoform of conventional PKC with the specific antibodies and immunoblotted with pan-phospho PKC antibody. Results showed that PKC-α, -β1, and -β2 were activated by MAG-Fc (Fig. 2*c*). These results demonstrate that conventional PKCs are activated by MAG-Fc.

Promotion of neurite outgrowth by MAG and Nogo when PKC is inhibited

We next examined whether the G_i pathway is associated with the effects of MAG or Nogo on the neurite outgrowth. It was shown that soluble MAG, released in abundance from myelin and found *in vivo*, and MAG-Fc could inhibit axon growth (Tang et al., 1997*a,b*). MAG-Fc at 25 μg/ml inhibited neurite outgrowth of cerebellar neurons from P7 rats (Fig. 3*a*). Fc had no effect on the neurons (data not shown). The exact same results were obtained regardless of whether total process outgrowth or length of the longest neurite was measured (data not shown). The Nogo peptide (4 μM) also significantly inhibited the neurite outgrowth (Fig. 3*a*). However, neither PTX nor U73122 modulated the action of MAG-Fc or the Nogo peptide (Fig. 3*a*). These results

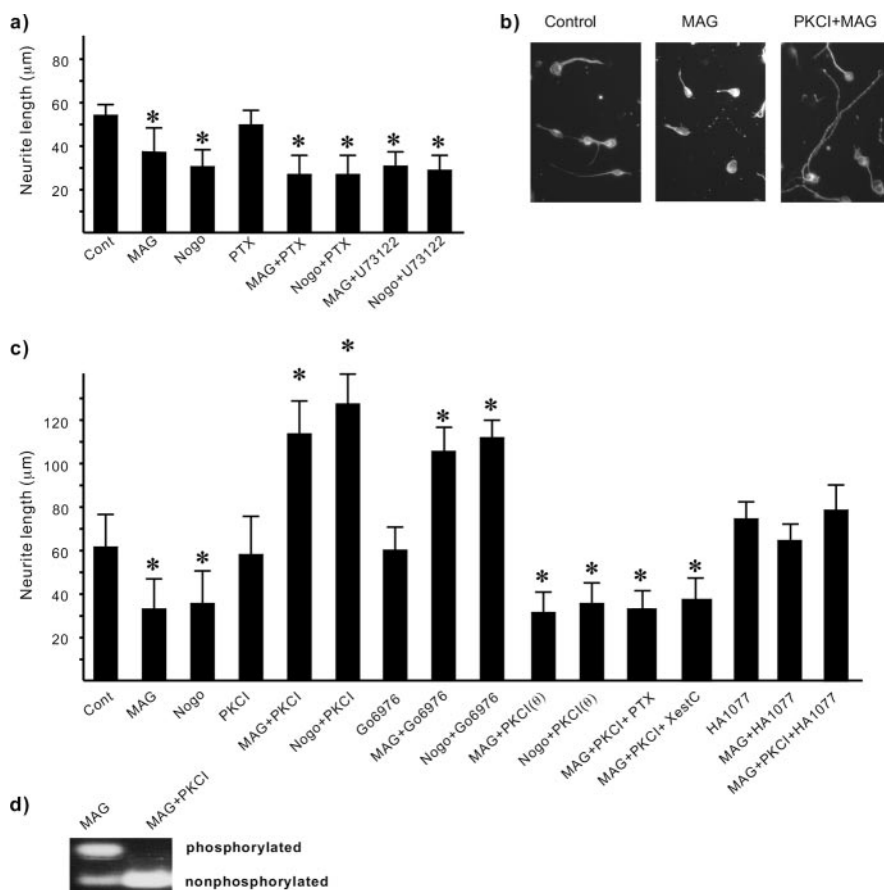


Figure 3. MAG and Nogo stimulate neurite outgrowth when PKC is inhibited. *a*, Neurite outgrowth of cerebellar neurons. MAG, MAG-Fc (25 µg/ml); Nogo, the Nogo peptide (4 µM); PTX, PTX (2 ng/ml); U73122, U73122 (20 nM). Data are mean ± SE. *b*, Dissociated cerebellar neurons were incubated for 24 hr with or without MAG-Fc and the PKC inhibitor peptide. Neurons were then immunostained with monoclonal antibody (TuJ1) for the purpose of recognizing the neuron-specific β-tubulin III protein. MAG, MAG-Fc (25 µg/ml); PKCI, the PKC inhibitor (2 µM). *c*, Neurite outgrowth of cerebellar neurons. MAG-Fc as well as the Nogo peptide stimulates neurite outgrowth in the presence of the PKC inhibitor. MAG, MAG-Fc (25 µg/ml); Nogo, the Nogo peptide (4 µM); PKCI, the PKC inhibitor (2 µM); HA1077, a Rho-kinase inhibitor (10 mM); PKCI(θ), myristoylated PKCθ pseudosubstrate (2 µM). Data are mean ± SE. Asterisks indicate statistical significance. **p* < 0.01 (Student's *t* test). *d*, Representative figure showing that activation of PKC (phosphorylated) by MAG-Fc is abolished by pretreatment with the PKC inhibitor (2 µM). Cont, Control.

suggest that neither G_i nor PLC is associated with the inhibitory effects of MAG or Nogo in regard to regulation of neurite elongation.

There are two divergent signaling cascades downstream of PLC activation: the PKC and IP_3 pathways. Therefore, we tested the hypothesis that a balance of the two signals may have an effect on these inhibitors. The involvement of PKC in the function of MAG and Nogo was assessed first. Surprisingly, MAG-Fc and the Nogo peptide dramatically stimulated neurite outgrowth in the presence of a specific membrane-permeable inhibitor of PKC- α and - β , although the PKC inhibitor itself had no effect on the growth (Fig. 3*b,c*). PKC activity was blocked by the inhibitor (Fig. 3*d*). The extent of neurite outgrowth induced by MAG-Fc or the Nogo peptide was found to be approximately twice as much as that in the control condition. Myristoylated PKC θ pseudosubstrate did not modulate the effect of MAG-Fc or the Nogo peptide (Fig. 3*c*). The neurite length induced by MAG-Fc and the PKC inhibitor returned to that with MAG-Fc alone, when pertussis toxin or XestC was added together with the PKC inhibitor (Fig. 3*c*). The same results as the PKC inhibitor were obtained when G₀6976, another inhibitor of PKC- α , - β , and - γ , was used (Fig. 3*c*). Although our data show that G₀6976 promoted neurite

outgrowth in the presence of soluble MAG-Fc, Sivasankaran et al. (2004) demonstrated that G₀6976 abolished the neurite inhibitory effect of the MAG-Fc substrate. Different observations of the effects of G₀6976, which should be elucidated in the future, might be attributable to the culture conditions: soluble MAG-Fc in our assay and MAG as a substrate in their assay. These data show bidirectional regulation of neurite elongation by MAG and Nogo, which is dependent on the activity of conventional PKC.

Growth cone spreading by myelin-derived inhibitors

To confirm the neurite growth assay data, we adopted another assay to test the function of MAG or Nogo-66. Chick E12 DRG neurons are known to respond to MAG or Nogo-66 (Fournier et al., 2001). We used chick E12 DRG explants to monitor the effects of MAG-Fc and the Nogo peptide on neuronal growth cones. Bath applications of MAG-Fc (25 µg/ml) or the Nogo peptide (4 µM) exhibited significant growth cone-collapsing activity (Fig. 4*a,b*). Consistent with the data obtained by the neurite outgrowth assays, MAG-Fc and the Nogo peptide enhanced the spreading of growth cones in the presence of the PKC inhibitor compared with the control. Although purified myelin from bovine white matter elicited growth cone collapse at 0.1–10 ng/µl, the PKC inhibitor completely reversed the effects mediated by myelin (Fig. 4*b*). These findings suggest that MAG, Nogo, and myelin inhibit neurite outgrowth and elicit growth cone collapse by activating PKC, whereas promotion of neurite outgrowth and spreading of

growth cones by these inhibitors are mediated by a mechanism that is independent of PKC. Considering that the inhibition of G_i or PLC did not result in the modulation of the effects mediated by MAG or Nogo, a balancing mechanism of two pathways, diverging at a point downstream of heterotrimeric G_i and PLC, may determine whether these inhibitors promote or inhibit neurite outgrowth as well as growth cone spreading.

Dependence on PLC–PKC/ IP_3 pathways

Because our data demonstrate that conventional PKC is involved in the effects of myelin-derived inhibitors, we next focused on IP_3 , another signal downstream of G_i and PLC. To test whether the IP_3 pathway mediates the effect of MAG and Nogo, we bath-applied XestC, an inhibitor of the IP_3 receptor. The increase in the Ca^{2+} concentration by MAG-Fc was attenuated by XestC (Fig. 1*c*). In contrast to the PKC inhibitor, neurite outgrowth inhibition by MAG-Fc or the Nogo peptide in cerebellar neurons was enhanced further by XestC (Fig. 5*a*). In these neurons, therefore, the PKC pathway may dominate over the IP_3 pathway, leading to inhibition of neurite outgrowth in response to MAG and Nogo.

A possible mechanism of the conversion from inhibition to

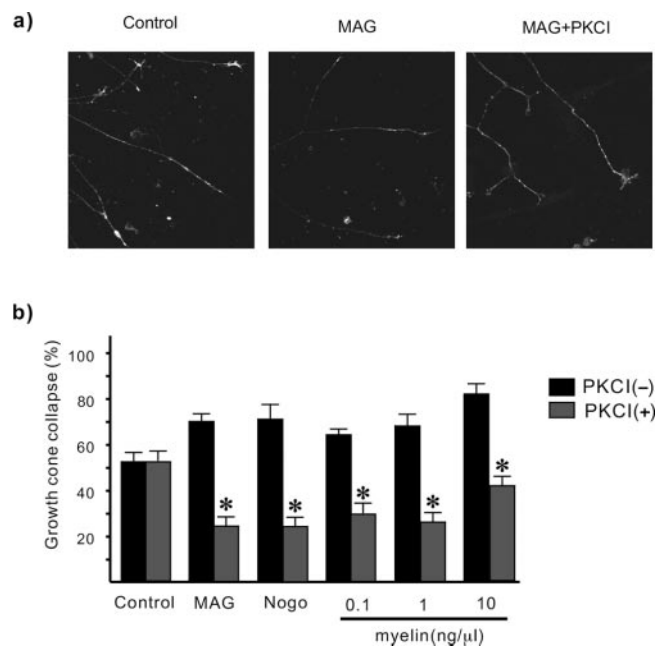


Figure 4. PKC regulates myelin-elicited growth cone collapse. *a*, Growth cone collapse assays. E12 chick DRG explants were treated with MAG-Fc (25 μ g/ml) in the presence or absence of the PKC inhibitor (PKCI; 2 μ M). Note the prominent spreading of growth cones induced by MAG-Fc in the explant pretreated with the PKC inhibitor. *b*, Results of growth cone collapse assays. We used 0.1–10 ng/ μ l CNS myelin for the treatment. MAG, MAG-Fc (25 μ g/ml); Nogo, the Nogo peptide (4 μ M); PKCI, the PKC inhibitor peptide (2 μ M). Data are mean \pm SE. Asterisks indicate statistical significance. * p < 0.01 (Student's *t* test).

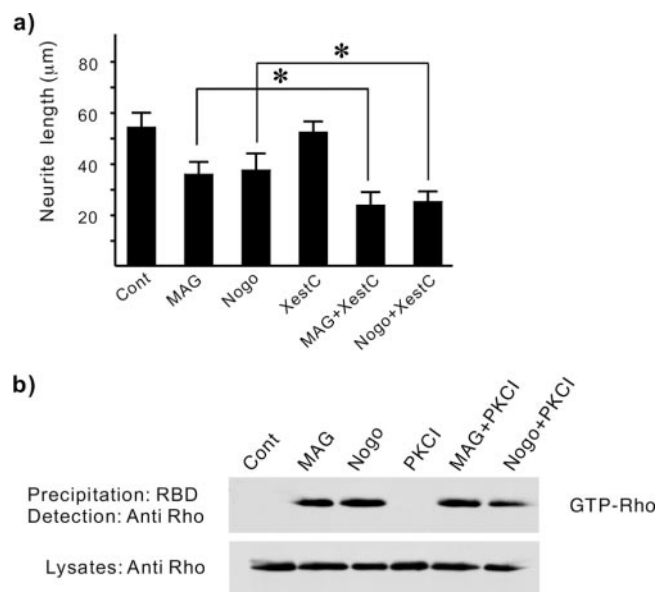


Figure 5. PKC is independent of Rho activation. *a*, Neurite outgrowth of cerebellar neurons. MAG, MAG-Fc (25 μ g/ml); Nogo, the Nogo peptide (4 μ M); XestC, XestC (1 μ M). XestC enhanced the effect of MAG-Fc or the Nogo peptide. Data are mean \pm SE. Asterisks indicate statistical significance. * p < 0.01 (Student's *t* test). *b*, Affinity precipitation of RhoA in cerebellar neurons. RBD, Rho binding domain. MAG-Fc and the Nogo peptide activate RhoA in the presence or absence of the PKC inhibitor. MAG, MAG-Fc (25 μ g/ml); Nogo, the Nogo peptide (4 μ M); PKCI, the PKC inhibitor peptide (2 μ M). These results were reproducible (n = 3). Cont, Control.

promotion of the axon regeneration induced by MAG or Nogo is that PKC modulates Rho activity, because Rho has been shown to be a key signaling molecule in inhibiting neurite elongation (Wang et al., 2002; Yamashita et al., 2002). To address this, we

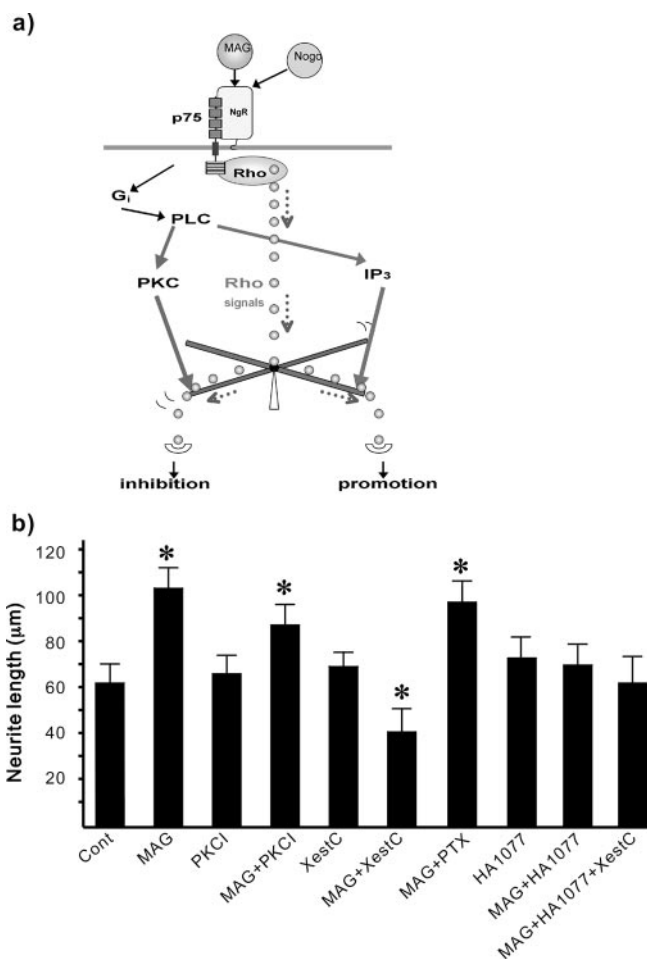


Figure 6. Balancing mechanism is important for the regulation of neurite outgrowth. *a*, MAG and Nogo activate RhoA as well as the G_i -PLC pathway. When PKC dominates, MAG and Nogo inhibit neurite outgrowth as well as growth cone spreading, as opposed to when IP_3 dominates. *b*, Promotion of neurite outgrowth of P1 DRG neurons by MAG is dependent on IP_3 but not on PKC. Neurite outgrowth of DRG neurons from P1 rats is shown. MAG, MAG-Fc (25 μ g/ml); PKCI, the PKC inhibitor peptide (2 μ M); XestC, XestC (1 μ M); HA1077 (10 mM), a Rho-kinase inhibitor. Data are mean \pm SE. Asterisks indicate statistical significance. * p < 0.01 (Student's *t* test). Cont, Control.

measured RhoA activity in the neurons. Using the RhoA-binding domain of the effector protein Rhotekin (Ren et al., 1999), the GTP-bound form of RhoA can be affinity-precipitated. The assay revealed that RhoA was activated 30 min after the addition of MAG-Fc or the Nogo peptide to the P7 rat cerebellar neurons (Fig. 5*b*). The PKC inhibitor had no effect on the Rho activity induced by MAG-Fc or the Nogo peptide. Thus, the promotion of neurite outgrowth of the cerebellar neurons by the inhibiting influence of PKC was not mediated by blocking RhoA activation, showing that conventional PKC is not upstream of RhoA.

MAG promotes axon growth from DRG neurons of rats up to postnatal day 4 (Johnson et al., 1989; Mukhopadhyay et al., 1994), suggesting that responses to these inhibitory cues are dependent on cell type and cell context and not simply on age. Because inhibition of PKC leads to the promotion of axon outgrowth by MAG in postnatal cerebellar neurons, it is postulated that the IP_3 pathway dominates over the PKC pathway when G_i -PLC is activated in these DRG neurons (Fig. 6*a*). To assess this, the neurite outgrowth from dissociated DRG neurons from P1 rats was measured. As before, MAG-Fc (25 μ M) promoted neurite outgrowth from the DRG neurons (Fig. 6*b*). Results showed

that MAG–Fc significantly inhibited neurite outgrowth if treated with XestC, whereas the PKC inhibitor or PTX had no modulating effect on the growth. These findings clearly show that MAG promotes neurite outgrowth, depending on the activity of IP₃.

Finally, to assess whether activation of RhoA is necessary for these effects mediated by MAG–Fc or Nogo, a Rho-kinase inhibitor, HA1077, was used at 10 μM. HA1077, which efficiently blocked the effects of MAG–Fc on the neurite outgrowth from cerebellar neurons, abolished also the effects of MAG–Fc plus the PKC inhibitor (Fig. 3c). In DRG neurons from P1 rats, the neurite-promoting effect of MAG–Fc was abolished by HA1077, although HA1077 itself had no effect (Fig. 6b). In addition, HA1077 abolished the effect of XestC in these neurons.

Discussion

A new signal was identified with regard to the important effects mediated by MAG, Nogo, and myelin. These inhibitors efficiently promoted neurite outgrowth and elicited growth cone spreading of the neurons when conventional PKC was inhibited, compared with the controls. Our data are consistent with those of Sivasankaran et al. (2004) and extend them. Promotion of neurite outgrowth of the immature neurons by MAG was converted to inhibition if IP₃ was inhibited. Because inhibition of G_i or PLC did not result in the modulation of the effects mediated by MAG or Nogo, it is suggested that a balance of the divergent signals is the determinant of these effects. Because PTX did not modulate the effects mediated by MAG on both types of neurons, basal activities of PKC and IP₃ may be important for the regulation of neurite outgrowth in the presence of PTX. Future study will focus on the molecular mechanism of how these divergent signals are regulated.

Because the elevation of intracellular Ca²⁺ concentration induced by MAG is abolished when treated with the antibody against p75^{NTR}, p75^{NTR} may be required for the signal transduction. Therefore, some G-protein-coupled receptors may be functionally associated with p75^{NTR} to transduce the conventional PKC/IP₃ signals. It has long been known that p75^{NTR} is a receptor for neurotrophins that promote survival and differentiation. Consistent with its function in controlling the survival and neurite formation of neurons, p75^{NTR} is expressed during the developmental stages of the nervous system. In contrast, p75^{NTR} is re-expressed in various pathological conditions in the adult and may even act as an inhibitor of axon regeneration. Our data provide conceptual evidence that the myelin-derived proteins are bifunctional regulators of axon growth. Diverse effects mediated by p75^{NTR} are, in part, the consequence of the interaction of p75^{NTR} with other membrane-associated proteins, such as Trk tyrosine kinases, the Nogo receptor and the ganglioside GT1b, and multiple intracellular signaling molecules (Dechant and Barde, 2002). The precise molecular mechanism of G_i–PLC signals related to p75^{NTR} should be explored, perhaps by searching for interactors with p75^{NTR}.

Previous studies suggest that Rho plays a central role in integrating myelin-derived growth inhibitory signals. Rho is activated by myelin, MAG, and NogoA (McKerracher and Winton, 2002). We note that Rho signal is a prerequisite for the regulation of neurite elongation, because inhibition of Rho results in disappearance of the effects (Yamashita et al., 2002). Rho activity was not affected by modulating the activity of conventional PKC. Activation of RhoA by soluble MAG was slow (5–10 min after exposure) (Niederost et al., 2002; Yamashita et al., 2002), whereas the elevation of the intracellular Ca²⁺ concentration by MAG–Fc is very rapid. Therefore, it is suggested that there is some cross

talk between PKC and the downstream signals of Rho, eliciting bidirectional effects on actin cytoskeleton reorganization.

There are some reports exploring the role of Ca²⁺ with regard to neurite outgrowth. Takei et al. (1998) demonstrated that IP₃ receptor in the neurons has an important role in neurite extension and that local loss of IP₃ receptor function results in growth arrest and neurite retraction, although the neurons undergo Ca²⁺ influx across the plasma membrane. Considering these results and our data indicating that XestC inhibits the neurite extension induced by MAG–Fc, IP₃ receptor function, but not Ca²⁺ itself, might be important for the regulation of neurite elongation. The precise molecular events should be explored in the future.

It is known that inactivation of Rho or one of its intracellular targets, Rho kinase, actually abolishes the effects of myelin, MAG, and NogoA, thereby providing potential therapeutic agents against CNS injuries (McKerracher and Winton, 2002). Another promising agent is the silencing peptide that associates with the intracellular domain of p75^{NTR} (Yamashita and Tohyama, 2003). p75^{NTR}, which transduces the signal from every myelin-derived inhibitor found to date, facilitates the release of Rho GDI from RhoA, thus enabling RhoA to be activated by guanine nucleotide exchange factors. Therefore, the peptide inhibits the association of Rho GDI with p75^{NTR} and the signal transduction. In addition, the peptide antagonist of the Nogo receptor and the IN-1 antibody that was generated against a fraction of myelin are shown to be effective in CNS axon regeneration (McKerracher and Winton, 2002). Previous work implicates the cAMP–PKA pathway in the neurite outgrowth regulation (Cai et al., 1999, 2001; Neumann et al., 2002; Qiu et al., 2002), showing that previous activation of this pathway counteracts the effects of the inhibitors *in vitro* as well as *in vivo*. A consequence of activation of cAMP–PKA was shown to be the synthesis of polyamines, resulting from an upregulation of Arginase I, a key enzyme in their synthesis (Cai et al., 2002). Inhibition of polyamine synthesis blocked the cAMP effect on regeneration. Many of the proposed strategies either block inhibitory proteins or block signaling by inhibitory proteins. In contrast, our data demonstrate that PKC has the effect of reversing the function of these inhibitors and instead promoting neurite outgrowth or growth cone spreading, providing a potent molecular target against CNS injuries. Myelin-derived inhibitors may act as trophic factors for axotomized neurons under certain conditions. However, it should be noted that none of the neurons used in this study are relevant ones for spinal cord injury. This is a general problem, because much work investigating the *in vitro* function of the axon growth inhibitors does not use relevant neurons because of the technical difficulty. Because different neurons have different responses to the inhibitors, it is important to focus on the relevant target population to permit identification of relevant pathways in future studies.

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