Neogenin-RGMa Signaling at the Growth Cone Is Bone Morphogenetic Protein-independent and Involves RhoA, ROCK, and PKC*§

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The repulsive guidance molecule RGMa has been shown to induce outgrowth inhibition of neurites by interacting with the transmembrane receptor neogenin. Here we show that RGMa-induced growth cone collapse is mediated by activation of the small GTPase RhoA, its downstream effector Rho kinase and PKC. In contrast to DRG cultures from neogenin−/− mice, in which no RGMa-mediated growth cone collapse and activation of RhoA occurred, treatment of wild type DRG neurites with soluble RGMa led to a marked activation of RhoA within 3 min followed by collapse, but left Rac1 and Cdc42 unaffected. Furthermore, preincubation of DRG axons with the bone morphogenetic protein (BMP) antagonist noggin had no effect on RGMa-mediated growth cone collapse, implying that the role of RGM in axonal guidance is neogenin- and not BMP-receptor-dependent. Pretreatment with 1) C3-transferase, a specific inhibitor of the Rho GTPase; 2) Y-27632, a specific inhibitor of Rho kinase; and 3) Gö6976, the general PKC inhibitor, strongly inhibited the collapse rate of PC12 neurites. Growth cone collapse induced by RGMa was abolished by the expression of dominant negative RhoA, but not by dominant negative Rac1. In contrast to RGMa, netrin-1 induced no growth cone retraction but instead reduced RGMa-mediated growth cone collapse. These results suggest that activation of RhoA, Rho kinase, and PKC are physiologically relevant and important elements of the RGM-mediated neogenin signal transduction pathway involved in axonal guidance.

During the development of the central and peripheral nervous system, target-derived axon extension is guided by attractive and repulsive diffusible or membrane-bound factors acting over short and long distances (1). During axonal chemorepulsion, repulsive guidance cues hinder neurite outgrowth, turn growth cones away from a guidance cue, and in the case of growth cone collapse induce a dramatic retraction of the growth cone. Although the repulsive guidance molecules (RGM)² and their receptor neogenin have been identified as guidance factors (2–6), the cytoplasmic signaling mechanisms responsible for triggering neogenin-mediated -directed growth cone collapse remain to be clarified. Recently Hata et al. (7) demonstrated that neurite extension of cerebellar granule neurons was inhibited when these cells were plated on a monolayer of RGMa overexpressing cells and that this effect can be blocked with RhoA and Rho kinase inhibitors. Furthermore, increased amounts of GTP-RhoA were observed in these primary neurons after the addition of soluble RGMa.

Neogenin, which is expressed by growing nerve cells in the developing vertebrate brain, consists of four immunoglobulin-like domains followed by six fibronectin type III domains, a transmembrane domain, and an intracellular domain (4, 8–10). Neogenin is closely related to the netrin-1 receptor deleted in colorectal carcinoma (DCC); it binds RGMa and, to a lesser extent, netrin-1 directly (4, 11). Although its functional role in neuronal development has not been elucidated in detail, high levels of neogenin expression correlate with axon guidance and neuronal survival in in vitro models (6, 12–14). Recently Watanabe et al. (15) reported finding no effect of netrin-1 on either axon attraction or repulsion of dorsal root ganglia (DRG) neurites, but did see a general suppression of axon outgrowth from DRG by netrin-1 after 24–48 h in co-culture experiments.

In addition to the ligand-receptor interaction of RGMa and neogenin it has been shown that RGMa, RGMb (dragon), and RGMc (hemojevelin) act as BMP co-receptors (16–18). Although no functional data were provided it was hypothesized that RGM family members might increase the sensitivity of cells in which they are expressed to BMP stimulation, thereby allowing these cells to respond earlier or more robustly to low levels of BMP ligand (18). It remains open whether BMPs could be involved in mediating the RGMa role in axonal guidance and growth cone collapse.

Each guidance molecule has to activate a cascade of intracellular effectors that eventually results in a cytoskeletal rearrangement underlying the guidance of axon extension or repulsion (19, 20). There is a great deal of evidence indicating that

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2 The abbreviations used are: RGM, repulsive guidance molecule; DCC, deleted in colorectal carcinoma; RasGAP, Ras GTPase-activating protein; DRG, dorsal root ganglia; PKC, protein kinase C; NGF, nerve growth factor; BMP, bone morphogenetic protein; GST, glutathione S-transferase; GFP, green fluorescent protein.
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axon guidance cue signaling involves the action of proteins belonging to the Rho family of small GTP-binding proteins, key regulators of actin cytoskeletal dynamics. Rho family GTPases orchestrate actin filament assembly and disassembly by controlling actin polymerization, branching, and depolymerization. These GTPases are thus likely to function as key mediators linking the guidance signal to cytoskeletal dynamics and controlling the organization of actin filaments.

Indeed, marked changes in the morphology, motility, and pathfinding of axons have been observed after perturbation of the Rho family GTPases in vitro and in vivo (21, 22). In general, these studies suggest that Rac and Cdc42 are positive regulators that promote neurite extension, whereas Rho is a negative regulator that causes the inhibition or collapse of growth cones. Several known guidance factors, including netrins, slits, semaphorins, ephrins, receptor tyrosine phosphatases, neurotrophins, cell adhesion receptors, myelin-associated receptors, and also neogenin have been shown to regulate intracellular Rho GTPase activity (4, 7, 22).

We therefore theorized that different Rho GTPases might have distinct roles in the RGMa guidance signal’s mediation of growth cone collapse. To analyze the signal transduction machinery in primary sensory neurons and PC12 cells underlying RGMa-neogenin-induced collapse, we focused on PKC, the small GTPase Rho and its downstream effectors, and serine/threonine Rho kinase.

In several cell systems, such as fibroblasts, neuroblastoma cells, neuron-like PC12 cells, and primary neurons, activation of Rho leads to rapid growth cone collapse, neurite retraction, or neurite growth inhibition. These responses are prevented by a specific inhibitor of Rho and the bacterial exoenzyme C3-transferase; C3-transferase ADP ribosylates RhoA, -B, and -C (but not Cdc42 and Rac) at Asn-41, thereby inhibiting these GTPases (23, 24). One of the downstream targets of active GTP-bound Rho is the Rho-associated kinase ROCK (25), which is specifically inhibited by the pyridine derivative Y-27632 (26).

In this study, we analyzed the signal transduction of RGMa-mediated growth cone collapse. Using neogenin knock-out mice we found strong evidence that the interaction of RGMa and neogenin is specific and important in axon repulsion. We could exclude BMP signaling in this context. We used primary sensory neurons and differentiated PC12 cells to examine the effects of expressing mutant forms of Rho and Rac1 in growth cone collapse induced by RGMa. Pull-down assays showed that RhoA mediates chemorepulsion, but not Rac1 or Cdc42. We found that PKC is involved in addition to the RhoA pathway and that regulation of each of these can trigger repulsive behavior mediated by the neogenin receptor. Experiments with netrin-1 indicated that the repulsive effect of RGMa could be silenced by preincubation with netrin-1.

**Experimental Procedures**

**Neogenin Mutants**—Mice homozygous for neogenin were kindly provided by Dr. M. Tessier-Lavigne (Genentech).

**In Situ Hybridization**—For in situ hybridization experiments, E14.5 Bl6 mice embryos were frozen over liquid nitrogen and embedded in OCT. Hybridization was performed with digoxygenin-labeled antisense riboprobes, corresponding to coding frames of mouse RGMa and neogenin cDNA (27, 28), on sagittal cryostat sections (20 μm).

**Dorsal Root Ganglia and PC12 Cell Culture**—For differentiation and neurite outgrowth a single cell culture of dorsal root cells was prepared from E12 wild type or neogenin−/− mutant embryos and cultured for 48 h on laminin-coated 12-well plates in Neurobasal medium supplemented with 5% fetal calf serum, B27, glutamine, mitose inhibitors, and 50 ng/ml NGF. To analyze RGMa-induced growth cone collapse in more detail, we also used PC12 cells seeded in 12-well tissue culture plates coated with laminin (20 μg/ml) and cultivated in 500 μl of RPMI 1640 media supplemented with 1% fetal calf serum, 1% l-glutamine, and 1% penicillin/streptomycin for 12 h at 37 °C in a CO₂ incubator. 50 ng/ml of NGF was then added to the culture medium and the cells were incubated for an additional 48 h.

**PCR of DRG, PC12 Cell cDNA, and Embryonic Mouse Brain cDNA**—The following primer combinations were used to amplify RGMa, RGMb, BMP-2, and BMP-4: RGMa, 5′-tgcaaaatcctcaagtg, 5′-ggcggcgccgcccagcgtct; RGMb, 5′-ggattggcacaagcgc, 5′-ctatccctcatcccccagctctg; BMP-2, 5′-aagaagcctggtaggagaaactt, 5′-tgagcgctttctctgtgttggt; BMP-4, 5′-ttctcaacctcgacagcatcc, 5′-caattaatcagcagcagcag.

**Coimmunoprecipitation of Clustered RGMa and the Extracellular Domain of Neogenin**—HEK293 cell supernatant containing neogenin-AP (neogenin in AP-tag vector) or supernatant containing RGMa-Fc (RGMa in pigtail plus vector) was concentrated with Nanosep 10K columns. Samples containing equal amounts of control supernatants (AP-tag alone) or supernatants containing neogenin-AP were mixed with samples containing clustered RGMa-Fc. These mixtures were incubated at room temperature for 2 h. Then an equal volume of an immunoprecipitation buffer (20 mM Tris, pH 8.0, 140 mM NaCl, 0.5 mM EDTA, and 2% Nonidet P-40) was added to each mixture, and the samples were centrifuged at 15,000 × g for 15 min at 4 °C. Supernatants were recovered and mixed with monoclonal AP-Sepharose beads (GenHunter), and the samples were incubated under stirring at 4 °C for 2 h. The samples were centrifuged again at 5,000 × g for 1 min, and the immune complexes were washed twice with immunoprecipitation buffer and once with phosphate-buffered saline. The beads were mixed with SDS sample buffer and boiled for 2 min at 100 °C. Immunoprecipitated proteins were separated using SDS-PAGE, transferred to nitrocellulose membranes, and subjected to immunoblot analysis with antibodies to AP-TAG and RGM protein.

**Immunohistochemistry**—After fixation with 4% paraformaldehyde, the PC12 cells were stained with monoclonal antibody specific for RhoA and a polyclonal antibody for neogenin (both from Santa Cruz, Biotechnology). As negative control we used a specific blocking peptide for the neogenin antibody (Santa Cruz, Biotechnology); for RhoA the primary antibody was omitted in the control. An alkaline phosphatase-conjugated antibody (Dianova) was used as a secondary antibody, and nitro blue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate as substrate. PC12 cells were also stained with Alexa 488-phalloidin (Molecular Probes). The In Situ Cell Death Detection Kit
(Roche) and caspase-3 immunostaining (Cell Signaling Technology, Roche) were used for the analysis of cell death.

**Collapse Assay**—RGMa-Fc was generated in a pigtail plus vector (Novagen) by inserting the mouse RGMa coding sequence (27, 28) without the signal peptide or glycosylphosphatidylinositol anchor and amplifying by PCR with the HindIII and XbaI sites. RGMa-Fc was stably transfected into HEK293 cells, and the supernatants with the fusion protein were further processed for collapse experiments. The proteins were clustered with an anti-human IgG1-Fc antibody (Calbiochem) to induce a stronger collapsing activity of the RGMa fusion protein. 500 μl of medium containing 60 μl of RGMa-pigtail protein clustered (RGMa-pl + IgG) were used for the collapse experiments; pigtail protein alone (pl + IgG) served as the control. In all experiments, a baseline was set to check the vitality of the differentiated DRGs or PC12 cells. RGMa-pl + IgG or pl + IgG was added 5 min after the beginning of the collapse assay, and every experiment was observed and recorded for 30 min with a Live Cell Imaging System (Zeiss) at a magnification of ×20. The collapsed and non-collapsed growth cones (n = 100) were counted in each culture, and the data were analyzed with Microsoft Excel. Data are expressed as mean ± S.E. For toxin A experiments, the cells were treated with 5–10 nM toxin A and incubated for 2 h before start here as mean ± S.E. For toxin A experiments, the cells were treated with 5–10 nM toxin A and incubated for 2 h before start.

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**RESULTS**

**RGMa Induces Growth Cone Collapse in Neogenin-expressing Primary Dorsal Root Ganglion Cells**—Nonradioactive in situ hybridization showed that neogenin is expressed by dorsal root

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**FIGURE 1.** Neogenin and RGMa interact specifically during axonal guidance. **A**, neogenin, but not RGMa, is expressed in dorsal root ganglia. In situ hybridization of sagittal paramedian sections of an E14.5 embryo hybridized with neogenin and RGMa DIG-labeled cDNA probes. Arrows show neogenin signals in dorsal root ganglia of the thoracic level. Note that no staining is observed at this location with RGMa. **B**, to monitor the integrity of the neogenin protein, anti-AP was detected on Neo-AP and AP-tag alone. **B’**, the specificity of the antibody was confirmed when anti-RGM stained a lane of the size expected for the RGM protein. C, from the different coimmunoprecipitations conducted (neogenin-AP/AP-tag alone, RGMa-Fc/AP-tag alone, and neogenin-AP/RGMa-Fc) it was evident that only RGMa interacts directly with neogenin.

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For the quantification of Rho and PKC kinase activities, we used non-radioactive enzyme-linked immunosorbent assay kits for Rho (Cycllex Co., Ltd.) and PKC (Stressgene). Kinase activities were inhibited with 100 nM of the specific inhibitor for Rho and 2 μM of the specific inhibitor for PKC. The kinase activities were measured at a wavelength of 450 nm.

**Statistical Analysis of the Different Groups**—Statistics were derived by one-way analysis of variance with at least 3 independent experiments performed in each experimental setting. In all analyses, differences were considered statistically significant at p = 0.01. The criteria for collapsed DRG and PC12 neurites were a total loss of growth cones and retraction of the neurites. Images were collected with the Zeiss Live Cell Imaging System and AxioVision analysis software.

**RESULTS**

**RGMa Induces Growth Cone Collapse in Neogenin-expressing Primary Dorsal Root Ganglion Cells**—Nonradioactive in situ hybridization showed that neogenin is expressed by dorsal root...
ganglia at E14.5, but RGMa is not (Fig. 1A). Furthermore, coimmunoprecipitation assays confirmed that neogenin and RGMa interact directly (Fig. 1B and C).

In a first step, we analyzed the growth cone collapse activity of RGMa with primary dorsal root ganglion cells from wild-type and neogenin knock-out mutants (Fig. 2, A–D). In the in vitro assay, the growth cones of NGF-stimulated dorsal root ganglion cells started to collapse 3–9 min after the addition of RGMa-Fc to the culture medium (Fig. 2, A and D). No effect was observed after the application of Fc-control protein (Fig. 2, B and D). Furthermore, no RGMa-induced growth cone collapse was observed in DRG cells prepared from neogenin \( ^{-/-} \) mutants (Fig. 2C, D). The RGMa protein was clustered with a specific antibody against the anti-human IgG1-Fc domain. In comparison to unclustered RGMa, which also induced growth cone retraction in PC12 cells (data not shown), the clustered form induced a faster and stronger effect. To determine whether neogenin directly regulates Rho GTPase activity, we carried out biochemical assays with the DRG cultures and observed a marked activation of RhoA in wild-type but not in neogenin \( ^{-/-} \) mutants within 3 min after the application of clustered RGMa (Fig. 2E). Furthermore, no activation of Rac1 and Cdc42 was observed in wild-type DRGs (data not shown).

**FIGURE 2.** Wild-type but not neogenin \( ^{-/-} \) DRG growth cones collapse when confronted with soluble clustered RGMa. A, growth cone collapse in neurites of NGF-stimulated DRG cells occurred 3–9 min after addition of clustered RGMa. This did not happen with control protein (B) or with DRG neurites treated with RGMa and (C) obtained from neogenin \( ^{-/-} \) mutants. D, quantification of the growth cone collapse assay shown in B–D. \( p < 0.001 \) RGMa-Fc + IgG on wild-type \( ^{+/+} \) compared with Fc-control on wild-type \( ^{+/+} \) and RGMa-Fc + IgG on neogenin \( ^{-/-} \) mutants. E, 3–6 min after stimulation much higher levels of RhoA were detected in RGMa-treated wild-type \( ^{+/+} \) DRG cell cultures than in controls and neogenin \( ^{-/-} \) cultures. No difference is observed in the amount of total RhoA in lysates of control-treated DRG cultures.

**FIGURE 3.** BMPs are not involved in RGMa-induced growth cone collapse. A, PCR analysis revealed that BMP-2, but not BMP-4, is expressed in E14.5 DRGs and PC12 cells. B, quantification of the treatment with noggin before the addition of RGMa. Noggin did not influence the RGMa-induced collapse response. RGMa and noggin + RGMa \( p < 0.001 \) compared with Fc-control.
to the cellular pathway to initiate the collapse response in the primary neurons. We believe that the results are strong evidence that neogenin plays a role in RGMa-induced signaling events.

**Expression of Neogenin and Rho GTPases but Not RGMs in PC12 Neurons**—We selected PC12 cells to study growth cone collapse induced by RGMa because, as confirmed by PCR analysis, these cells constitutively express neogenin but not RGMa or RGMb (Fig. 4A).

To analyze the role of the Rho GTPase in RGMa-induced collapse of PC12 cells, we next used immunohistochemistry to determine whether RhoA and neogenin proteins are expressed by these cells (Fig. 4, B and C). Differentiated PC12 cells growing on a laminin substrate were fixed after 48 h of NGF treatment and stained with neogenin- and RhoA-specific antibodies. Neogenin and RhoA were strongly detected in the cell bodies, the growth cones, and to a lesser extent in the neurites of PC12 cells as well (Fig. 4, B, B/1, C, and C/1). The growth cones of the PC12 cells also stained positive with Alexa 488-phalloidin (Fig. 4D).

**RGMa Induces Growth Cone Collapse in PC12 Cells**—We used differentiated PC12 cells as a cell culture model to analyze RGMa-induced growth cone collapse in more detail. During live-cell imaging, clustered RGMa induced a 85–95% collapse of PC12 cells (Fig. 5, B and C), and retraction of the growth cones occurred ~3–6 min after the addition of 4.8 μg/ml of RGMa to the culture medium. We observed no unspecific, spontaneously collapsed PC12 cells in time-lapse experiments after the addition of the RGMa-Fc protein, and the onset of growth cone retraction was stable in the same time frame. In contrast, the addition of the clustered control protein did not induce collapse (Fig. 5, A and C).

**Toxin A, C2–C3, Rho Inhibitor, and Neogenin Antibody Block RGMa-induced Growth Cone Collapse**—To test whether Rho GTPases are involved in RGMa-induced growth cone collapse via the neogenin receptor, we first examined the effect of toxin A, an inhibitor of Rho GTPases (Fig. 5C; supplementary Fig. S1A). Toxin A completely abolished the repulsive effect of clustered RGMa on PC12 growth cones. The neurites did not show retraction, leaving growth cones with long, hair-like protrusions.

The collapse-inducing activity of clustered RGMa was also abolished after PC12 cells were incubated with C2–C3 (Fig. 5C; supplementary Fig. 1B), whereas individual components of the fusion toxin (C2I or C3) did not block the RGMa-induced collapse of PC12 cells (data not shown). The fraction of collapse-resistant PC12 cells increased from 5 to 10% in control cultures to 80% in C2–C3-treated cultures, and PC12 cells advanced despite the presence of RGMa.

Similar results were obtained after inhibition of ROCK by the Rho inhibitor Y-27632. Two hours before the addition of RGMa, PC12 cells were treated with 10 mM Y-27632 (Fig. 5C; supplementary Fig. 1C). Inhibition of ROCK reduced the collapse-inducing activity of RGMa significantly, and the number of non-collapsed PC12 cells increased from 5–10 to 85–90%.

To investigate the functional interaction between RGMa and neogenin, we further treated the PC12 culture with an antibody directed against the ectodomain of neogenin. This pre-treat-
sponse studies (10 nM to 10 μM) were carried out with Gö6976 (data not shown), and the strongest effects were seen with 100 nM. Treatment with Gö6976 significantly reduced neurite growth cone collapse after the addition of clustered RGMa (Fig. 5C; supplementary Fig. 1E). Quantification of neurite retraction outgrowth suggested that the effect of this PKC inhibitor was comparable with that of Y27632, a ROCK inhibitor that also blocks RGMa-induced neurite retraction.

Expression of Dominant Negative RhoA, but Not Rac1 Inhibits RGMa-induced Growth Cone Collapse—To further examine the role of Rho family GTPases in mediating the growth cone collapse induced by RGMa, we expressed fusion proteins of normal active and dominant negative or constitutively active Rho GTPase, RhoA, and Rac1 green fluorescent protein (GFP). In cultures of PC12 cells, GFP fluorescence provided a reliable marker for identifying transfected cells (Fig. 5D; supplementary Fig. S2). The effects of normal active, dominant negative, and constitutively active RhoA and Rac1 on neurite retraction were quantitatively examined in cultures 72 h after transfection. The expression of dominant negative RhoA caused a robust and significant reduction in the average length of neurites and the average number of collapsing growth cones, whereas expression of active RhoA did not reduce the RGMa-induced neurite retraction response. In contrast, expression of dominant negative Rac1 caused no reduction in the amount of neurite retraction (supplementary Fig. S3A). These findings are consistent with the concept that RhoA initiates neogenin-mediated growth cone collapse.

Regulation of RhoA by RGMa—To determine whether neogenin directly regulates Rho GTPase activity, we carried out biochemical assays on PC12 cultures. We observed a marked activation of RhoA within 3 min after the application of clustered RGMa (Fig. 6A), whereas neither Cdc42 nor Rac1 activity was significantly affected (Fig. 6B).

FIGURE 5. RhoA and PKC are involved in RGMa-induced growth cone collapse, but Rac1 is not. A, in contrast to treatment with clustered Fc-control protein; B, the addition of clustered RGMa-Fc induced collapse of PC12 growth cones (circled in red) after 0–12 min. Magnification, ×40. C, incubation of PC12 cells with toxin A, C2–C3, Rho-inhibitor Y-27632, neogenin antibody, and PKC inhibitor reduced the RGMa-induced growth cone collapse. p < 0.001 RGMa-Fc compared with all other groups. D, the RGMa-induced collapse was significantly reduced after transfection of PC12 cells with dominant negative RhoA (N19), p < 0.001 control (pl + IgG) and N19 compared with normal active WT and constitutively active Val-14. E, quantification of growth cone collapse after transfection with dominant negative Rac1, which did not reduce the RGMa-induced growth cone collapse. p < 0.001 control compared with normal active Rac1 WT, dominant negative Rac1 T17N, and constitutively active Rac1 G12V.
Pull-down assays with GST fusion proteins were performed to measure activation of RhoA, Cdc42, and Rac1 in RGMα-treated PC12 cell cultures. To this end, PC12 cells were treated with either clustered RGMα or control protein. Cells were subsequently lysed, and the level of GTP-bound RhoA was determined using GST-C21 (Rhotekin Rho-binding domain), a downstream target of Rho, which binds specifically to GTP-bound Rho. RGMα induced an increase in GTP-bound RhoA, whereas no RhoA activation was detectable in control cultures (Fig. 6A). C2–C3 treatment and/or active neogenin-antibody blocking eliminated this effect (Fig. 6A). In contrast to RGMα-induced activation of RhoA, affinity precipitation of GTP-bound Rac or Cdc42 with GST-Pak-CD (Pak CRIB-domain) showed no alteration of GTP-Rac and Cdc42 in RGMα-treated cultures compared with the control cultures (Fig. 5B). These results suggest that treatment of PC12 cells with RGMα results in activation of Rho, but does not influence Rac or Cdc42. In control-treated PC12 cultures, no differences were observed in the levels of total RhoA, Cdc42, and Rac (Fig. 6, A–C).

Rho and PKC kinase activities were increased in PC12 cells 3 min after stimulation with RGMα in comparison to control conditions and kinase inhibition (Fig. 6, D and E). The maximal increase in Rho kinase activity was observed after 6 min, dropping to baseline level after 15 min, whereas PKC kinase activity maximum was reached 9 min after stimulation and remained elevated after 15 min.

Effect of RhoA in Combination with PKC—Different concentrations of RhoA and PKC inhibitors were tested to define half-maximal collapse activity of these inhibitors. As Fig. 6F shows, Rho kinase and PKC inhibitors additively blocked RGMα-induced growth cone collapse more than single half-maximal applications of these substances. This implies that RhoA and PKC may be involved parallel to one another in the same signaling pathway induced by RGMα (7, 22).

Netrin-1 Silences RGMα Collapse—We performed experiments with netrin-1 to determine whether netrin-1 induces growth cone collapse in PC12 cells and to analyze the activation of RhoA. After the addition of 300 ng/ml netrin-1 to the culture medium no retracting growth cones could be observed over a period of 30 min (Fig. 7). Furthermore, no activation of RhoA was observed (data not shown). This is consistent with Watanabe et al. (15), who did not find a specific effect of netrin-1 on either axon attraction or repulsion of DRG neurites, but did observe a general suppression of axon outgrowth from DRG by netrin-1 after 24–48 h in co-culture experiments. In addition we could observe that preincubation of PC12 neurites with netrin-1 reduces the RGMα-mediated collapse response (Fig. 7).

Neogenin and RGMα Are NotInvolved in Cell Death of DRGs and PC12 Cells—Next we analyzed the influence of RGMα on the viability of DRGs and PC12 cells. In both cases, no increase in cell death was observed 24 h after addition of unclustered or clustered RGMα. TUNEL staining and caspase-3 cell death detection showed no difference after the addition of RGMα (data not shown). The collapsing activity therefore occurred independently and did not involve a cell death pathway.

DISCUSSION

Both in vitro and in vivo, neogenin and its ligand RGMα have been implicated in chemorepulsive mechanisms during axonal guidance (4). In axonal chemorepulsion growth cones are pushed away from a guidance cue and in the case of growth cone collapse a dramatic retraction of the growth cone occurs. To understand the signal transduction underlying the repulsive guidance of RGMα by its receptor neogenin, we performed growth cone collapse experiments with primary DRG neurites and with differentiated PC12 cells as a more simplified model system for transfection experiments and pull-down assays.

To provide direct evidence for a role of neogenin in RGMα-induced signaling events we closely analyzed the interaction of the ligand RGMα with its receptor neogenin in DRGs from wild-type and neogenin knock-out mice. DRG neurites from the knock-out mice did not respond to RGMα and showed no activation of RhoA after stimulation with RGMα. These experiments with neogenin knock-out mice provide evidence that RGMα does not induce growth cone collapse in primary dorsal root ganglia cells without its receptor neogenin. Whereas the growth cones of wild-type DRG neurites collapsed by nearly 100%, growth cone retraction was not observed in the DRG neurites of the neogenin mutants. Furthermore, activation of the RhoA signal transduction pathway was not induced in the mutants’ DRG cells after the addition of RGMα to the culture medium.

Another point concerned the involvement of the BMP pathway in the RGMα-induced collapse response, independently of the described interaction with the receptor neogenin. Because morphogens are active in early developmental stages (dorsal-ventral patterning) that precede axon guidance in the neuronal tube, RGMα and RGMβ may be implicated in both morphogen-instructed patterning and repulsive axon guidance. Recently it was shown that RGMα, RGMβ (dragon), and RGMc (hemojevelin) act as BMP co-receptors under certain conditions (16–18). Therefore we tested whether BMPs could be involved in mediating RGMα-mediated growth cone collapse. The experiments with the neogenin−/− mutants provide clear evidence that neogenin, not a BMP receptor, is necessary to mediate the RGMα signal to the cellular pathway to initiate the collapse response in the primary neurons. In addition we tested if the BMP antagonist noggin might influence RGMα-induced growth cone collapse. Again, we found no instance in which the RGMα-induced collapse response could be influenced by the BMP antagonist noggin. In the field of axonal guidance the only BMP found to play a role in axonal guidance so far is BMP-7, which was shown to induce the collapse of commissural axon growth cones, providing evidence that it can act directly on growth cones to elicit a rapid change in cytoskeletal organization (29, 30). The molecular mechanisms underlying the effect of BMP-7 on growth cones is not known. Although BMPs typically activate signaling through type I and II receptors, it is not clear whether these receptors also transduce the BMP-7 chemorepellent activity of commissural axons of the spinal cord. Recently Babitt et al. (18) demonstrated that RGMα is a BMP-2 and -4 co-receptor, but they did not provide functional data. It was hypothesized that RGM family members might increase the sensitivity of cells in
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A

B

C

D

E

F

Rho Kinase Assay

PKC Kinase Assay

number of collapsed neurites (n=60)

additive effect

Rho+PKC

5nM 10nM 50nM 100nM Rho Kinase inhibitor

100nM 200nM 1µM 2µM PKC inhibitor
which they are expressed to BMP stimulation, thereby allowing these cells to respond earlier or more robustly to low levels of BMP ligand. Because the collapse response of DRG growth cones induced by the addition of RGMa to the culture medium did not occur without the neogenin receptor, and because BMP-2 or -4 do not induce growth cone collapse in DRGs, we conclude that the RGMa/BMP-2 and -4 co-receptor model is not applicable to the growth cone collapse response of DRG neurites. We also performed experiments with the BMP antagonist noggin, which did not block RGMa-induced growth cone collapse. The in the amount of RhoA in lysates of control-treated PC12 cultures. In contrast, no difference was observed in the same time course after

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protein. Moreover, RGMa-induced neurite retraction was blocked by transfection of the PC12 cells with dominant-nega-
tive RhoA rather than with Rac1, suggesting that activation of Rho and ROCK transmits an unknown signal to the cytoskele-
ton, such as coflin or LIM kinase (31, 32). Furthermore, pull-
down assays revealed that neither Rac1 nor Cdc42 is involved in the collapse of growth cones after contact with RGMa. In general, these results indicate that RGMa can specifically trigger the activation of RhoA in PC12 cells. This is supported by the results of Hata et al. (7), who showed that outgrowth inhibition of cerebellar granule neurons induced by RGMa overexpressing cells can be blocked with RhoA and Rho kinase inhibitors. We also demonstrated that PKC is involved parallel to RhoA in neogenin-mediated growth cone collapse behavior.

Vielmetter et al. (9) originally isolated neogenin as a deleted homologue in colorectal cancer (DCC). Based on our present knowledge about the structure of proteins belonging to the Ig superfamily, the amino acid sequence of neogenin shows four types of major structural characteristics. The extracellular part of the molecule is comprised of four Ig domains or loops of the C2 subcategory of Ig domains, followed by six fibronectin-type III (FNIII) repeats. The extracellular domains are followed by the strongly hydrophobic putative transmembrane region (resi-
dues 1089 to 1111). The remaining 331 amino acid residues constitute the cytoplasmatic domain, which contains a site for potential alternative splicing and three conserved domains referred to as P1, P2, and P3. The cytoplasmatic domain contains 14 potential serine and threonine phosphorylation sites, a histidine cluster that may chelate a divalent metal ion like some DNA-binding proteins and metalloproteases, and a PXXP motif that may be involved in the binding of Src homology type 3 (SH3) domains. Such motifs are also found in the intracellular domain of the netrin receptor DCC and will be of interest in future investigations.

Thus far, no binding partners in the axonal guidance mech-
anism have been identified for the intracellular domain of neo-
genin after interaction with the RGMa ligand. In contrast, sev-
eral proteins, such as phosphatidylinositol transfer protein α3 or focal adhesion kinase, interact with the P3 domain of neoge-
nin and are involved in netrin binding to neogenin (33, 34).

Neogenin is involved in epithelial morphogenesis in the mammary gland, in neural tube closure, angiogenesis, myogen-
esis, and during somite formation (35). DCC is a netrin-1 recep-
tor that mediates growth cone attraction by netrin-1 (11). In contrast to DCC, there is still no evidence that neogenin medi-

FIGURE 7. Neogenin Signaling during Growth Cone Repulsion. Quantification of the treatment with netrin-1 before the addition of RGMa. Netrin-1 did not induce a growth cone collapse by itself, but completely abolished the RGMa-induced collapse response. RGMa, p < 0.001, compared with netrin-1, netrin-1 + RGMa, and Fc-control.

FIGURE 6. RGMa activates RhoA in PC12 cells after 3–6 min but does not influence Rac1 or Cdc42. A, time course of affinity precipitation of GTP-RhoA with a GST fusion protein of the rhotekin Rho-binding domain. Much higher levels of RhoA were detectable in RGMa-treated PC12 cell cultures (right lanes 0, 3, 6, and 9) than in control cultures (left lanes 0, 3, 6, and 9). The highest level of RhoA was observed 3 min after stimulation. In comparison, no difference was observed in the amount of RhoA in lysates of control-treated PC12 cultures. In contrast, no difference in RhoA expression was observed in the same time course after treatment with C2–C3 or neogenin antibody followed by RGMa collapse. The lower panel of A gives the total amount of RhoA in the lysates. B, time course of affinity precipitation of Cdc42 and GTP-Rac using the GST Pak1-CRIB domain. No difference was detected between levels of Cdc42 in RGMa-treated PC12 cell cultures (right lanes 0, 3, 6, and 9) and those in control cultures (left lanes 0, 3, 6, and 9). Moreover, no difference in the amount of Rac1 was observed between lysates of control-treated and RGMa-treated PC12 cell cultures. C, quantification of Rho GTPase activities after stimulation with 2.4 μM of clustered RGMa. All experiments were repeated at least three times. Rho (D) and PKC kinase (E) activity assay. Kinase activities (OD at 450 nm) in PC12 cells were measured by enzymelinked immunosorbsent assay 0–15 min after stimulation with FC-Control, FC-RGMa, and FC-RGMa + inhibitor. The tables show the kinase activity of Rho in picograms and PKC in nanograms ± S.D., respectively. * = p < 0.01 compared with control and inhibition. P, PKC and RhoA are additively involved in neogenin signaling. Dose-response curves of the Rho inhibitor (red) and the PKC inhibitor (black) ranging from 5 to 100 nM (Rho kinase inhibitor) and 100 nM to 2 μM (PKC inhibitor). The ordinate shows the number of collapsed PC12 neurites (n = 60 per group) after treatment with clustered RGMa-Fc for 30 min. The upper two dots on the gray line indicate the concentrations at which a 50% reduction in growth cone collapse activity of RGMa was induced by the inhibitors alone. In comparison, the lower red and black dots on the gray line mark the additive blocking of a combination of both inhibitors.
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ates netrin-1 action. Chicken RGM affinity for neogenin is higher than for netrin-1 (4). Moreover, neither DCC nor Unc5Hs (another netrin-1 receptor (36, 37) interact with RGM, indicating that of the netrin-1-binding proteins only neogenin is a receptor for RGMs.

We performed experiments with netrin-1 to determine whether netrin-1 induces growth cone collapse in PC12 cells, and analyzed activation of RhoA. No retracting growth cones were observed and no activation of RhoA was induced after the addition of netrin-1. Our data are consistent with those of Watanabe et al. (15), who did not find a specific effect of netrin-1 on either axon attraction or repulsion of DRG neurites, but did observe a general suppression of axon outgrowth from DRGs by netrin-1 in long-term co-culture experiments. In addition we found that preincubation of DRG neurites with netrin-1 silenced the RGMa-mediated collapse response. This could indicate that netrin-1 pre-occupies the binding site for RGMa at the neogenin receptor (which is unlikely) or that it initiates a DCC-mediated induction of Rac1/Cdc42, counteracting the RGMa collapsing response. Further experiments would have to be conducted to analyze the mechanism behind this interaction.

At present little is known about the way in which ligand-induced activation of the neogenin receptor regulates the RhoA and PKC pathways. As with the netrin receptor DCC, neogenin may also interact via autoprophosphorylated juxtamembrane tyrosine residues with RasGAP (Ras GTPase-activating protein), which is constitutively associated with RhoGAP (22). RhoGAP is a negative regulator of Rho, and the strong activation of RhoA by RGMa in our experiments would seem to require inactivation of RhoGAP activity. It remains to be seen whether additional elements of the RasGAP-RhoGAP complex are responsible for such an inhibition.

It has been demonstrated that RGMa binds to neogenin during axonal guidance. On the other hand, neogenin expression in the absence of its ligand RGM leads to neuronal cell death, consistent with the recently formulated dependence receptor concept (12). Matsunaga et al. (6) have postulated that neogenin is a dependence receptor that induces cell death in the absence of RGM, whereas the presence of RGM inhibits this effect. The pro-apoptotic activity of neogenin in the neuronal tube in immortalized neuroblasts was associated with the cleavage of its cytoplasmic domain by caspase-3. This is certainly not the case with PC12 cells, which express neogenin but not RGMs and do not die under these conditions.

Schwab et al. (38, 39) observed spinal cord injury-induced lesional expression of the RGM. Lesional RGM expression was frequently confined to hypertrophic β-APP+ and RhoA+ neurites/retraction bulbs. In addition it has been observed that neogenin expression is increased at the lesion site.3 Both the presence of RGM in neogenin in a glial barrier and its inhibitory activity in vitro suggest that it would exert inhibitory effects on regenerating axons in the glial scar. Furthermore, Hata et al. (7) demonstrated the importance of RGMa signaling in spinal cord lesions. This observation and the findings of this study that PKC and RhoA are involved in neogenin-mediated growth cone collapse may represent a further step toward understanding the molecular inhibition of axonal regeneration lesions in the central nervous system.

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