We previously reported that the activation of p160 RhoA-binding kinase ROKα induces neurite retraction via small GTPase Rho in the EP3B receptor-expressing PC12 cells (Katoh, H., Negishi, M., and Ichikawa, A. (1996) J. Biol. Chem. 271, 29780–29784). However, a potential downstream effector of Rho that induces neurite retraction was not identified. Here we examined the morphological effect of p160 RhoA-binding kinase ROKα, a target for RhoA recently identified, on the nerve growth factor-differentiated PC12 cells. Microinjection of the catalytic domain of ROKα rapidly induced neurite retraction similar to that induced by microinjection of a constitutively active Rho, RhoV14A37, whereas microinjection of the kinase-deficient catalytic domain of ROKα did not induce neurite retraction. This morphological change was observed even though C3 exoenzyme, which was known to inactivate Rho, had been preinjected. On the other hand, microinjection of the Rho-binding domain or the pleckstrin homology domain of ROKα inhibited the EP3 receptor-induced neurite retraction. These results demonstrate that ROKα induces neurite retraction acting downstream of Rho in neuronal cells.

*p160 RhoA-binding Kinase ROKα Induces Neurite Retraction*

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Rho, a member of the Ras superfamily of small GTPases, is implicated in various cellular morphological functions, such as formation of stress fibers and focal adhesion (1), cell motility (2), cytokinesis (3), cell aggregation (4), and smooth muscle contraction (5). When cells are activated by extracellular stimuli, inactive GDP-bound Rho is converted to active GTP-bound Rho. Once activated, Rho probably interacts with its specific targets, leading to a variety of biological functions (6). Recently, several target proteins that interact only with GTP-bound Rho have been identified, including p128 protein kinase N (7, 8), p160 RhoA-binding kinase ROKα (9) also known as its bovine counterpart Rho kinase (10) or its mouse counterpart ROCK-II (11), rhophilin (7), rhotekin (12), and p140mDia (13). Among them, ROKα has been reported to be involved in several functions of Rho: the regulation of myosin phosphorylation (14, 15), the formation of stress fibers and focal adhesions (16, 17), and probably the regulation of cytokinesis (18).

Rho has also been implicated in the control of neuronal cell morphologies. The activation of a certain heterotrimeric GTP-binding protein (G-protein)-coupled receptor, such as lysophosphatic acid and thrombin receptors, caused the rapid retraction of extended neurites in several neuronal cell lines (19–21). Clostridium botulinum C3 exoenzyme, which specifically ADP-ribosylates Rho and suppresses the actions of Rho (22, 23), inhibits the receptor-mediated neurite retraction (24, 25), indicating that Rho activity is required for this morphological change. Although this effect appears to be induced by the contractility of the actin-based cytoskeleton (24, 26), a downstream effector of Rho that induces neurite retraction has not yet been identified.

We previously reported that the activation of prostaglandin EP3 receptor caused Rho-dependent neurite retraction in the NGF-differentiated PC12 cells expressing the EP3B receptor (27), one of the EP3 receptor isoforms isolated from bovine adrenal medulla (28). In non-neuronal cells, the activation of EP3 receptor stimulates the Rho-mediated formation of stress fibers (29), indicating that EP3 receptor is a potent activator of Rho in various cell types. In this report, we have examined the putative role of ROKα in the EP3 receptor-mediated neurite retraction in the NGF-differentiated PC12 cells. We show that ROKα is involved in the EP3 receptor-mediated neurite retraction and that the activation of ROKα is sufficient for inducing neurite retraction.

**EXPERIMENTAL PROCEDURES**

Materials—M&B28767 was a generous gift from Dr. M. P. L. Caton of Rhone-Poulenc Ltd. NGF 2.5S was purchased from Promega Corporation, and C. botulinum C3 exoenzyme was from Seikagaku Kogyo (Tokyo, Japan). The sources of the other materials are shown in the text.

Expression and Purification of Recombinant Proteins—The coding region of human RhoA was generated by reverse transcription-polymerase chain reaction (PCR) from HeLa cells using primers 5′-CTGGACTCAATTCGTTGCGAGAATGG-3′ and 5′-GACAGGATATTCTGTATGATCTAGG-3′. The PCR product was digested with EcoRI, cloned into the pBluescript KS(+), and completely sequenced. cDNAs for RhoA V14 (30) and RhoA V14A37 were generated by PCR-mediated mutagenesis (30), subcloned into the BamHI/EcoRI sites of pGEX-4T-2 vector, and sequenced. Recombinant RhoA V14 and RhoA V14A37 were expressed as glutathione S-transferase (GST) fusion proteins in Escherichia coli, and purified on glutathione-Sepharose beads according to the method of Self and Hall (31).

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The sequence encoding the putative C. botulinum C3 exoenzyme-sensitive site of RhoA was PCR amplified from mouse genomic DNA using primers 5′-ATGACCGGCTGCTCGCGGCTCGGCTAGCTGAG-3′ and 5′-GACAGGATATTCTGTATGATCTAGG-3′. The PCR product was digested with EcoRI, cloned into the pBluescript KS(+), and completely sequenced. cDNAs for RhoA V14 (30) and RhoA V14A37 were generated by PCR-mediated mutagenesis (30), subcloned into the BamHI/EcoRI sites of pGEX-4T-2 vector, and sequenced. Recombinant RhoA V14 and RhoA V14A37 were expressed as glutathione S-transferase (GST) fusion proteins in Escherichia coli, and purified on glutathione-Sepharose beads according to the method of Self and Hall (31).
retraction of the neurites within 30 min. More than 70% of the NGF-differentiated PC12 cells (data not shown). Therefore, we cultured in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum, 5% horse serum, 4 mM glutamine, 100 units/ml penicillin, and 0.2 mg/ml streptomycin under humidified conditions in 95% air and 5% CO2 at 37 °C. Cells were photographed at × 200 magnification under a phase contrast microscope. For the quantitative examination, shown in Fig. 3, neurite-retracted cells were defined as the cells that retracted by more than 10% of their original length within 30 min of the addition of the agonist or of the microinjection of recombinant proteins. The percentages of neurite-retracted cells were calculated by counting at least 30 cells in the same field. Data were obtained from triplicate experiments.

RESULTS AND DISCUSSION

In our previous study, we obtained evidence that M&B28767, an EP3 agonist, induced neurite retraction in the EP3B receptor-expressing PC12 cells and that this morphological change was completely inhibited when the cells were microinjected with C3 exoenzyme, which ADP-ribosylates and inactivates Rho (22, 23), indicating that the EP3B receptor induced neurite retraction through the activation of Rho (27). To determine whether activation of Rho is sufficient for inducing neurite retraction in the PC12 cells, we microinjected a constitutively activated recombinant RhoA, RhoAV14 into the NGF-differentiated PC12 cells and examined its effect. As shown in Fig. 1 (C and D), microinjection of RhoAV14 into the cytoplasm caused retraction of the neurites within 30 min. More than 70% of the injected cells retracted their neurites (Fig. 3). This morphological change was similar to that stimulated by M&B28767 (Fig. 1, A and B). The neurite-retracted cells by microinjection of RhoAV14 were not stained with trypan blue (data not shown), indicating that they did not undergo cell death. On the other hand, RhoAV14 containing a T37A substitution in the effector region, RhoAV14AT, had no effect on the differentiated cells after microinjection (Fig. 1, E and F, and Fig. 3), suggesting that this mutation blocked the interaction of Rho with its target to induce neurite retraction. This result also indicated that there were not any nonspecific effects due to the microinjection itself.

Previous studies suggested that the generation of actin-based contractile forces was required for neurite retraction (24, 26). Among several targets of Rho, ROKα appears to participate in Rho-dependent contractile events, such as the formation of stress fibers (16, 17) and the regulation of cytokinesis (18). By Northern blot analysis, ROKα was expressed in the NGF-differentiated PC12 cells (data not shown). Therefore, we examined whether ROKα was involved in the Rho-mediated neurite retraction in the NGF-differentiated PC12 cells. ROKα contains the catalytic domain in its amino terminus, the coiled-coil domain, the Rho-binding domain, and the PH domain in its carboxyl terminus (9). It was recently shown that the truncation mutant of ROKα containing the catalytic domain displayed constitutive kinase activity without the addition of active form of Rho, whereas the Rho-binding domain and the PH domain of ROKα served as dominant negative forms of the kinase (16, 17). Based on these characters, we generated recombinant proteins containing these domains: the catalytic domain of ROKα (CD-ROKα), amino acids 1–543, the kinase-deficient mutant of CD-ROKα (CD-ROKαK112D), the Rho-binding domain of ROKα (RBD-ROKα, amino acids 932–1065), and the PH domain of ROKα (PHD-ROKα, amino acids 1116–1379). To examine the effects of these domains of ROKα on neurite retraction, we microinjected these recombinant proteins into the NGF-differentiated cells and analyzed their morphologies.

After the cells had been microinjected with CD-ROKα, they rapidly retracted their neurites within 30 min (Fig. 2, A and B, and Fig. 3). This morphological change was similar to that induced by microinjection of RhoAV14. The neurite-retracted cells by microinjection of CD-ROKα were not stained with trypan blue (data not shown), indicating that they did not undergo cell death. On the other hand, microinjection of the kinase-deficient mutant of CD-ROKα mutant CD-ROKαK112D had no effect (Fig. 2, C and D, and Fig. 3), indicating that the kinase activity of CD-ROKα was required for inducing neurite retraction. When the cells were microinjected with C3 exoenzyme, the M&B28767-induced neurite retraction was completely inhibited (Fig. 2, E and F). However, the CD-ROKα-induced neurite retraction was not inhibited after the cells had been microinjected with C3 exoenzyme (Fig. 2, G and H), indicating that CD-ROKα acted downstream of Rho.

Next we microinjected RBD-ROKα or PHD-ROKα, which served as dominant negative forms of ROKα, into the differentiated cells and examined each effect on the M&B28767-induced neurite retraction. When the cells had been microinjected with RBD-ROKα or PHD-ROKα, the M&B28767-induced neurite retraction was inhibited (Fig. 4). All the cells...
microinjected with RBD-ROKα or PHD-ROKα had no response to M&B28767. These results suggest that ROKα is involved in the EP3 receptor-mediated neurite retraction in the PC12 cells. Taken together, our results suggest that ROKα induces neurite retraction acting downstream of Rho in the NGF-differentiated PC12 cells.

Recently, ROKα was shown to be involved in Rho-induced formation of stress fibers and focal adhesion in other cell types such as fibroblasts. However, the organization of stress fibers induced by constitutively active ROKα was apparently different from that induced by lysophosphatidic acid or constitutively active Rho (16, 17), suggesting that additional signals were required for Rho-induced stress fiber formation. In this study, however, microinjection of CD-ROKα sufficiently induced neurite retraction similar to that induced by RhoV14 even though C3 exoenzyme had been preinjected, whereas CD-ROKαK112G failed to induce neurite retraction (Figs. 2 and 3), suggesting that the increase in the kinase activity of ROKα by Rho appears to be sufficient for inducing neurite retraction. Because myosin-binding subunits of myosin phosphatase and myosin light chain are known to be substrates for ROKα and activation of ROKα leads to phosphorylation and activation of myosin (14, 15), neurite retraction may be induced by ROKα-mediated regulation of myosin phosphorylation. In addition, it was recently reported that glial fibrillary acidic protein, an intermediate filament protein expressed in the cytoplasm of astroglia, was identified as another substrate for ROKα (18). Therefore, we will consider substrate(s) of this kinase for neurite retraction in future studies. Until now, we have obtained evidence that the activation of EP3B receptor, coupling to Rho activation, did not affect the NGF-induced mitogen-activated protein kinase activation in the PC12 cells (data not shown), suggesting that the activation of Rho or ROKα did not inhibit the NGF-induced signaling to the Ras-mitogen-activated protein kinase pathway. To examine the direct effect of Rho or ROKα on the NGF-induced differentiation, we are currently establishing PC12 cell lines that express RhoAV14 or CD-ROKα under the control of an inducible promoter.

As shown in Fig. 4, two fragments of ROKα, the Rho-binding domain and the PH domain of ROKα on M&B28767-induced neurite retraction. The NGF-differentiated cells were microinjected with 2 mg/ml of RBD-ROKα (A and B) or 1 mg/ml of PHD-ROKα (C and D) and photographed before (A and C) and 30 min after (B and D) addition of 1 μM M&B28767. The arrows indicate injected cells. The results shown are representative of three independent experiments. At least 20 cells were microinjected in each experiment, and all cells microinjected gave the described response. 

The bar represents 50 μm.
reported for the formation of stress fibers and focal adhesion (16, 17). ROKα has been shown to be translocated to peripheral membranes upon transfection with RhoV14 (9). Because PH domains are supposed to play a key role in localization of molecules to the specific target regions in the membranes, the PH domain of ROKα may localize this kinase at the specified region in response to the EP3 receptor-induced activation of Rho, and this translocation of ROKα to its target region seems to be essential for inducing neurite retraction. On the other hand, RBD-ROKα may block the interaction between endogenous Rho and ROKα. We also showed that RhoAV14A37, a mutant at the effector region, lost the ability to induce neurite retraction (Fig. 3). Indeed, RhoAV14 bound to the RBD-ROKα, but RhoAV14A37 did not (data not shown). This defect in binding to ROKα seems to be the reason for the inability of RhoAV14A37 to induce neurite retraction.

In conclusion, we have here shown that ROKα is an essential component of Rho-mediated neurite retraction in neuronal cells. Considering that ROKα is enriched in the brain (16), ROKα may play a critical role in the regulation of neuronal cell morphology in the brain. However, many questions have not yet been elucidated in this field, for example how the G-protein coupled receptor activates Rho. Further investigations are necessary to understand Rho-mediated signal transduction in neuronal cells.

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