RESEARCH COMMUNICATION

Signal transduction pathway regulating prostaglandin EP3 receptor-induced neurite retraction: requirement for two different tyrosine kinases

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We reported previously that activation of the prostaglandin E receptor EP3 subtype triggered neurite retraction through the small GTPase Rho-, and its target, RhoA-binding kinase α (ROKα)-, dependent pathway in EP3 receptor-expressing PC12 cells. Here we examined the involvement of tyrosine kinases in this pathway in nerve growth factor-differentiated PC12 cells. Tyrphostin A25, a tyrosine kinase inhibitor, blocked neurite retraction and cell rounding induced by activation of the EP3 receptor, however, it failed to block neurite retraction and cell rounding induced by microinjection of constitutively active RhoA, RhoA^{V14}, indicating that a tyrphostin-sensitive tyrosine kinase was involved in the pathway from the EP3 receptor to Rho activation. On the other hand, genistein, another tyrosine kinase inhibitor, blocked neurite retraction and cell rounding induced by both activation of the EP3 receptor and microinjection of RhoA^{V14}. However, genistein did not block neuronal morphological changes induced by microinjection of a constitutively active mutant of ROKα. These results indicate that two different tyrosine kinases, tyrphostin A25-sensitive and genistein-sensitive kinases, are involved in the EP3 receptor-mediated neurite retraction acting upstream and downstream of Rho, respectively.

Key words: prostaglandin, Rho, tyrosine kinase, neurite retraction.

INTRODUCTION

The small G-protein, Rho, mediates various morphological functions in response to extracellular stimuli in various cell types [1]. In neuronal cell lines including rat pheochromocytoma PC12 cells, the activation of a certain heterotrimeric G-protein-coupled receptor, such as the lysophosphatidic acid receptor, triggered the rapid retraction of extended neurites and the rounding of the cell body [2–4]. *Clostridium botulinum* C3 exoenzyme, which specifically ADP-ribosylates and suppresses the action of Rho [5,6], inhibited receptor-mediated neurite retraction and cell rounding [7–10], and microinjection of a constitutively active mutant of Rho caused morphological changes [11,12], indicating that Rho is involved in this action. Recently, our laboratory and others revealed that a Rho-associated serine/threonine kinase, RhoA-binding kinase α (ROKα)/Rho-kinase and p160ROCK, a recently identified direct target for RhoA, mediated the agonist-induced neurite retraction acting downstream of Rho [12–14]. It was also suggested that the kinase-mediated elevation of myosin light chain phosphorylation and the resultant activation of the actomyosin-based contractility appeared to be involved in the neurite retraction [13,14]. Furthermore, we examined upstream signal pathways for Rho activation of neurite retraction, and recently demonstrated that expression of constitutively active G proteins, $G\alpha_{12}$, $G\alpha_{13}$, and $G\alpha_q$, induced Rho-dependent neurite retraction in nerve growth factor (NGF)-differentiated PC12 cells, but that these G proteins used different signalling pathways for Rho activation; the $G\alpha_{13}$-induced retraction was sensitive to a tyrosine kinase inhibitor, tyrphostin A25, while the effect of $G\alpha_{12}$ was insensitive to this inhibitor, and that of $G\alpha_q$ was mediated by protein kinase C [15]. These findings suggest that there are at least three different signalling pathways for heterotrimeric G-proteins to relay signals from cell surface receptors to Rho in neuronal cells.

Prostaglandin (PG) E_2 is one of the major PGs synthesized in the nervous system [16]. PGE_2 has several important functions in the nervous system, such as the generation of fever [17], pain modulation [18], and the regulation of neurotransmitter release [19]. Furthermore, cyclo-oxygenase products, including PGE_2, have been suggested to be involved in the regulation of memory consolidation [20], but the molecular mechanisms of the synaptic functions of PGE_2 are not yet understood. PGE_2 acts on cell surface receptors to exert its actions [21]. PGE receptors are pharmacologically divided into four subtypes, EP1, EP2, EP3 and EP4, on the basis of their responses to various agonists and antagonists [22]. We have cloned the four subtypes of mouse PGE receptors and have demonstrated that they are heterotrimeric GTP-binding protein (G-protein)-coupled rhodopsin-type receptors [23]. Among these subtypes, the EP3 receptor was most abundant in the brain and was specifically localized to the neurons [24]. We reported previously that the activation of the EP3 receptor caused neurite retraction through a pertussis toxin-insensitive pathway in NGF-differentiated PC12 cells stably expressing the EP3B receptor, one of the EP3 receptor isoforms isolated from bovine adrenal medulla [25]. This morphological effect was inhibited by C3 exoenzyme and the dominant negative form of ROKα [9,12], indicating that the EP3 receptor induced neurite retraction through activation of the Rho–ROKα path-

Abbreviations used: CD, catalytic domain; NGF, nerve growth factor; PG, prostaglandin; ROKα, RhoA-binding kinase α.

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way. In this study, we further investigated the signal transduction pathway involved in EP3 receptor-mediated neurite retraction. We showed that at least two different tyrosine kinases were involved in EP3 receptor-mediated neurite retraction acting upstream and downstream of Rho respectively.

MATERIALS AND METHODS

Materials

M&B28767 and Y-27632 were generous gifts from Dr M. P. L. Caton of Rhone-Poulenc Ltd. (Dagenham, Essex, U.K.) and Yoshitomi Pharmaceutical Industries (Saitama, Japan) respectively. NGF 2.5S was obtained from Promega Corporation, genistein was from Research Biochemical International (Natick, MA, U.S.A.), tyrphostin A25 was from Calbiochem, mouse monoclonal antibody against phosphotyrosine (4G10) was from Upstate Biotechnology Incorporated (Lake Placid, NY, U.S.A.), and mouse monoclonal antibodies against chick paxillin (clone 349) were from Transduction Laboratories (Lexington, KY, U.S.A.).

Expression and purification of recombinant proteins

Recombinant RhoA^{V14} was expressed in *Escherichia coli* as glutathione S-transferase fusion protein, purified on glutathione-Sepharose beads and cleaved with thrombin. The catalytic domain of ROKα (CD-ROKα, amino acids 1–543) was expressed in Sf9 cells as glutathione S-transferase fusion protein and purified on glutathione-Sepharose beads, as described previously [12]. Recombinant proteins were dialysed with an injection buffer (10 mM Tris/HCl, pH 7.6/150 mM NaCl/2 mM $MgCl_2$/0.1 mM dithiothreitol) at 4 °C overnight for microinjection. The protein concentration was determined by comparison with BSA standards after SDS/PAGE and staining with Coomassie Brilliant Blue. Purified proteins showed a single band on a Coomassie-stained gel.

Cell culture and microinjection

PC12 cells stably expressing the EP3B receptor [9] were cultured in Dulbecco's modified Eagle's medium containing 10 % (v/v) fetal bovine serum, 5 % (v/v) horse serum, 4 mM glutamine, 100 units/ml penicillin and 0.2 mg/ml streptomycin under humidified conditions in 95 % air and 5 % CO_2 at 37 °C. For studies of neurite retraction, the cells were seeded on to poly-D-lysine-coated (Sigma) 35-mm dishes at a density of 2×10^4 cells/dish or 24-well plates at a density of 5×10^3 cells/well in serum-containing Dulbecco's modified Eagle's medium, and cultured for 12 h. Then they were differentiated in serum-free Dulbecco's modified Eagle's medium containing 50 ng/ml NGF and 20 μM indomethacin for 3 or 4 days. For microinjection, dishes were marked with a cross to facilitate the localization of microinjected cells. During microinjection, cells were maintained in Hepes-buffered Dulbecco's modified Eagle's medium (pH 7.4). Microinjection was performed using an IMM-188 microinjection apparatus (Narishige, Tokyo, Japan). Recombinant proteins (2 mg/ml) were microinjected into the cytoplasm. Cells were photographed at ×400 magnification under a phase-contrast microscope. Injected cells were identified by co-microinjection with Texas Red-coupled dextran. A neurite was identified as a process greater than 1 cell body diameter in length. To establish quantitative examinations, 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 microinjection of the recombinant proteins. The percentages of neurite-retracted cells were calculated by counting at least 30 cells in the same field. All data were obtained from triplicate experiments.

Western blotting and immunoprecipitation

PC12 cells were lysed in ice-cold lysis buffer containing 20 mM Tris/HCl, pH 7.5, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 10 % (v/v) glycerol, 1 % (v/v) Nonidet P-40, 1 mM sodium orthovanadate, 2 mg/ml NaF, 2 mg/ml β-glycerophosphate, 10 μg/ml leupeptin, 10 μg/ml aprotinin, 4.5 mM sodium pyrophosphate and 1 mM PMSF. After cell lysates had been clarified by centrifugation at 18000 ***g*** for 20 min at 4 °C, the supernatants were subjected to SDS/PAGE [7.5 % (w/v) gel], and the proteins were transferred to poly(vinylidene difluoride) (PVDF; Immobilon) membranes. Immunoreactive bands were visualized with anti-phosphotyrosine antibody (4G10) using an enhanced chemiluminescence (ECL) kit (Amersham), as described previously [26]. For the immunoprecipitation of paxillin, cell lysates clarified by centrifugation at 18000 ***g*** for 20 min at 4 °C were immunoprecipitated at 4 °C for 4 h by anti-paxillin antibody (clone 349), as described previously [27]. Immunoprecipitates were subjected to SDS/PAGE (10 % gel), and tyrosine-phosphorylated paxillin and total paxillin were visualized with anti-phosphotyrosine antibody (4G10) and anti-paxillin antibody (clone 349) respectively.

RESULTS

Effect of tyrphostin A25 on M&B28767- or RhoA-induced neurite retraction

We demonstrated previously that M&B28767, an EP3 receptor agonist, induced Rho-dependent neurite retraction in NGF-differentiated PC12 cells stably expressing the EP3B receptor [9]. In this study, we further examined the signal transduction pathway of EP3 receptor-mediated neurite retraction. We first examined the effect of a tyrosine kinase inhibitor, tyrphostin A25, on M&B28767-induced neurite retraction in NGF-differentiated PC12 cells. The vehicle-treated cells retracted their extended neurites and showed rounding of the cell body within 30 min, in response to 1 μM M&B28767 (Figures 1A and 1B). In contrast, pretreatment of the cells with 300 μM tyrphostin A25 for 15 min blocked M&B28767-induced neurite retraction (Figures 1C and 1D). Table 1 shows the quantitative examination of the effect of tyrphostin A25. This inhibitor markedly decreased the percentage of neurite-retracted cells, in response to M&B28767, from 97.0 to 13.7 %. The treatment with tyrphostin A25 caused no morphological change in the cells for at least 60 min (results not shown). These results indicated that a tyrphostin A25-sensitive tyrosine kinase was involved in EP3 receptor-mediated neurite retraction.

To determine whether this tyrphostin A25-sensitive tyrosine kinase functioned upstream or downstream of Rho, we examined the effect of tyrphostin A25 on neurite retraction induced by constitutively active RhoA, RhoA^{V14}, microinjected into the cytoplasm of differentiated PC12 cells. As shown in Figures 2(A) and 2(B), and Table 1, more than 80 % of the cells microinjected with 2 mg/ml RhoA^{V14} showed neurite retraction and cell rounding within 30 min. However, pretreatment of the cells with 300 μM tyrphostin A25 for 15 min did not block the neurite retraction induced by microinjection of RhoA^{V14} (Figures 2C and 2D, and Table 1). These results indicated that a tyrphostin A25-sensitive tyrosine kinase was not involved in the neurite retraction induced by Rho.

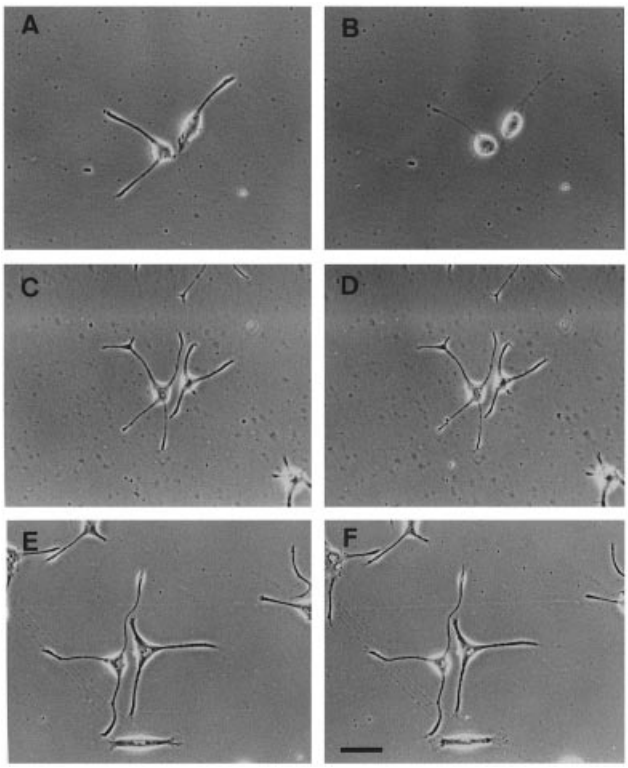

Figure 1 Effect of tyrphostin A25 or genistein on neurite retraction induced by M&B28767

Cells differentiated with NGF for 3 days were pretreated with vehicle (**A** and **B**), 300 μM tyrphostin A25 (**C** and **D**) or 100 μM genistein (**E** and **F**) for 15 min. The cells were then photographed before (**A**, **C** and **E**) and 30 min after (**B**, **D** and **F**) the addition of 1 μM M&B28767. The results shown are representative of three independent experiments. The bar represents 50 μm.

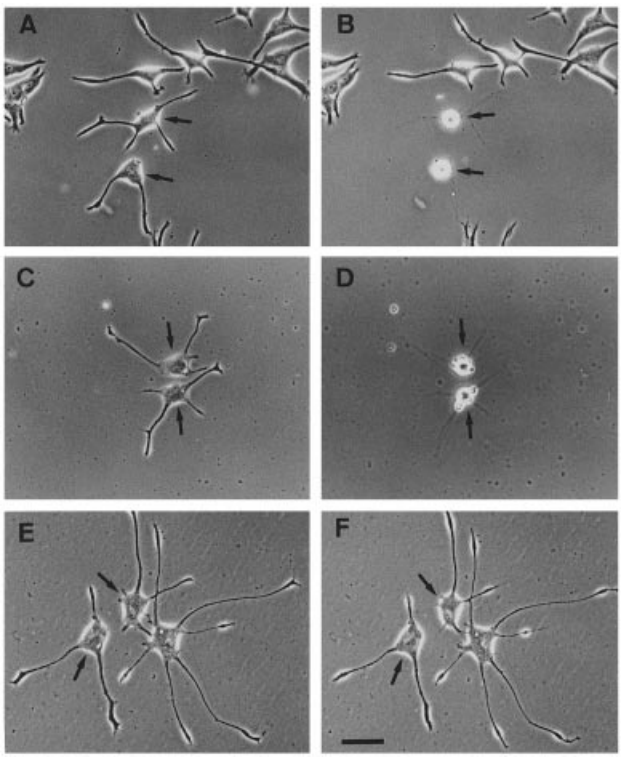

Figure 2 Effect of tyrphostin A25 or genistein on neurite retraction induced by RhoA^{V14}

Cells differentiated with NGF for 3 days were pretreated with vehicle (**A** and **B**), 300 μM tyrphostin A25 (**C** and **D**) or 100 μM genistein (**E** and **F**) for 15 min. The cells were then photographed before (**A**, **C** and **E**) and 30 min after (**B**, **D** and **F**) microinjection of 2 mg/ml RhoA^{V14}. The arrows indicate injected cells. The results shown are representative of three independent experiments. The bar represents 50 μm.

Table 1 Quantification of the effect of tyrphostin A25 or genistein on neurite retraction induced by M&B28767, RhoA^{V14} or CD-ROKα

After cells differentiated with NGF for 3 days had been pretreated with vehicle, 300 μM tyrphostin A25 or 100 μM genistein for 15 min, they were exposed to 1 μM M&B28767 or microinjected with 2 mg/ml of RhoA^{V14} or CD-ROKα. The percentages of neurite-retracted cells were determined 30 min after the addition of the agonist or microinjection of the recombinant proteins, as described in the Materials and methods section. Data are the means $\pm$ S.E.M. of triplicate experiments.

	Neurite-retracted cells (%)		
Stimulants ...	None	+ tyrphostin A25	+ genistein
M&B28767	97.0 $\pm$ 3.0	13.7 $\pm$ 3.0	11.4 $\pm$ 2.8
RhoA^{V14}	86.0 $\pm$ 5.6	86.5 $\pm$ 5.3	16.0 $\pm$ 3.6
CD-ROKα	96.9 $\pm$ 3.1	–	95.8 $\pm$ 4.2

Effect of genistein on M&B28767-, RhoA- or ROKα-induced neurite retraction

Previous studies have shown that another tyrosine kinase inhibitor, genistein, blocked lysophosphatidic acid-induced neurite retraction in the N1E115 neuronal cell line [3,28]. We also examined the effect of genistein on the EP3 receptor- or active RhoA-induced morphological changes in NGF-differentiated PC12 cells. Pretreatment of the cells with 100 μM genistein for 15 min blocked M&B28767-induced neurite retraction and cell rounding (Figures 1E and 1F). Furthermore, genistein also prevented the morphological changes induced by microinjection of RhoA^{V14} (Figures 2E and 2F). The treatment with genistein decreased the percentage of neurite-retracted cells in response to M&B28767 from 97.0 to 11.4%, and that of neurite-retracted cells induced by the microinjection of RhoA^{V14} from 86.0 to 16.0% (Table 1). The treatment with genistein caused no morphological change in the cells for at least 60 min (results not shown). These results indicated that a genistein-sensitive tyrosine kinase was involved in the pathway of EP3 receptor-mediated neurite retraction at the downstream step of Rho.

We demonstrated previously that ROKα, a target for RhoA, was involved in EP3 receptor-mediated neurite retraction [12]. In fact, a specific inhibitor of ROKα, Y-27632 [29], completely suppressed EP3B receptor-induced neurite retraction and cell rounding (results not shown). Microinjection of the catalytic domain of ROKα (CD-ROKα, amino acids 1–543), which exerts constitutive kinase activity without the addition of active form of Rho [30,31], induced neurite retraction and cell rounding in differentiated PC12 cells (Figures 3A and 3B). We then examined the effect of genistein on the morphological changes induced by CD-ROKα. As shown in Figures 3(C) and 3(D), and Table 1, pretreatment of the cells with 100 μM genistein for 15 min failed to suppress the neurite retraction and cell rounding induced by

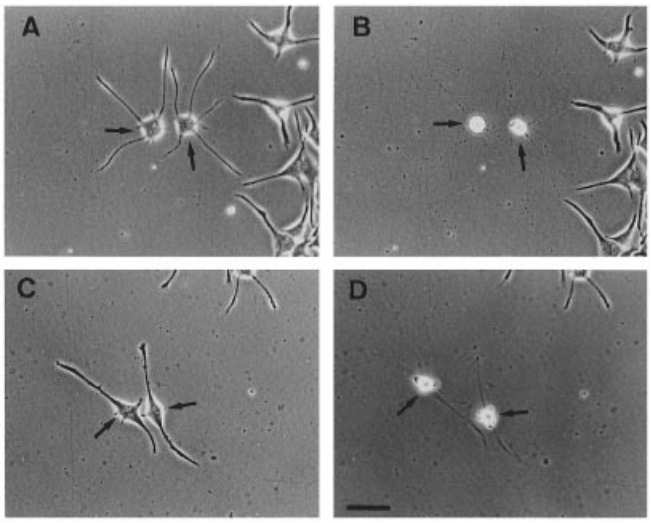

Figure 3 Effect of genistein on neurite retraction induced by CD-ROKα

Cells differentiated with NGF for 3 days were pretreated with vehicle (**A** and **B**) or 100 μM genistein (**C** and **D**) for 15 min. The cells were then photographed before (**A** and **C**) and 30 min after (**B** and **D**) microinjection of 2 mg/ml CD-ROKα. The arrows indicate injected cells. The results shown are representative of three independent experiments. The bar represents 50 μm.

CD-ROKα. These results indicated that a genistein-sensitive tyrosine kinase did not act downstream of ROKα for neurite retraction.

Effect of genistein and ROKα inhibitor on tyrosine phosphorylation of paxillin

Activation of Rho has been shown to result in tyrosine phosphorylation of multiple proteins, including paxillin and focal adhesion kinase [32]. We examined tyrosine phosphorylation induced by the EP3 receptor. As shown in Figure 4(A), M&B28767 most prominently stimulated tyrosine phosphorylation of a protein of approx. 68 kDa, and this phosphorylation was strongly inhibited by genistein but not by a specific ROKα inhibitor, Y-27632. The molecular mass of the tyrosine-phosphorylated protein prompted us to speculate that the protein was paxillin. To test whether the tyrosine-phosphorylated 68-kDa protein represented paxillin, we immunoprecipitated paxillin, and anti-phosphotyrosine immunoblots of paxillin immunoprecipitates demonstrated increased tyrosine phosphorylation of paxillin in response to M&B28767 in a genistein-sensitive manner, while this phosphorylation was insensitive to Y-27632 (Figure 4B). These data indicated that tyrosine phosphorylation of paxillin and Rho-induced ROKα activation were separable events.

DISCUSSION

In our previous studies, activation of the EP3 receptor caused neurite retraction in EP3B receptor-expressing PC12 cells through the Rho-ROKα pathway [9,12]. In this study, we examined the requirement for tyrosine kinase activity in EP3 receptor-mediated morphological changes in PC12 cells, and we demonstrated that two different tyrosine kinases were involved in EP3 receptor-mediated neurite retraction and cell rounding in PC12 cells: tyrphostin A25-sensitive and genistein-sensitive tyrosine kinases acting upstream and downstream of Rho respectively.

We demonstrated previously that the EP3 receptor was coupled to a pertussis toxin-sensitive heterotrimeric G-protein, Gi, which inhibited forskolin-stimulated adenylate cyclase, and to a pertussis toxin-insensitive G-protein, which induced neurite retraction through the Rho-activation pathway in PC12 cells [9]. In our recent study, the expression of constitutively active mutants of $G\alpha_{12}$, $G\alpha_{13}$, and $G\alpha_q$, but not $G\alpha_{i2}$, induced Rho-dependent neurite retraction and cell rounding through different signalling pathways in NGF-differentiated PC12 cells [15]. Among these $G\alpha$ subunits inducing neurite retraction, tyrphostin A25 blocked the morphological changes induced by $G\alpha_{13}$ and $G\alpha_q$, but not by $G\alpha_{12}$, and the inhibition of protein kinase C or the elimination of extracellular Ca^{2+} suppressed the changes induced by $G\alpha_q$, but not by $G\alpha_{13}$ and $G\alpha_{12}$. Therefore, there are three different pathways for heterotrimeric G-proteins transmitting the receptor signals to Rho. Here we showed that tyrphostin A25 blocked EP3 receptor-mediated neurite retraction and cell rounding. We found previously that activation of the EP3 receptor did not increase the intracellular Ca^{2+} concentration in PC12 cells, and the neuronal morphological changes induced by the EP3 receptor were not blocked by the inhibition of protein kinase C activity [9]. In addition, the elimination of extracellular Ca^{2+} failed to

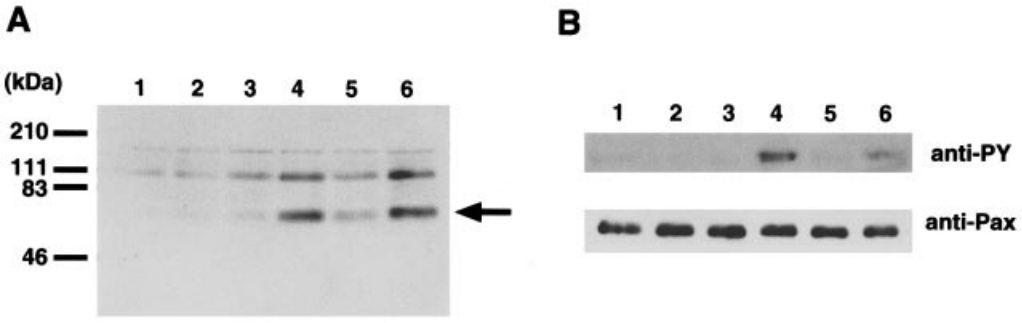

Figure 4 Effect of genistein or ROKα inhibitor on EP3 receptor-induced tyrosine phosphorylation of paxillin

After cells differentiated with NGF for 3 days had been pretreated with vehicle (lanes 1 and 4), 100 μM genistein (lanes 2 and 5) or 10 μM Y-27632 (lanes 3 and 6) for 15 min, they were stimulated for 3 min with (lanes 4–6) or without (lanes 1–3) 1 μM M&B28767. (**A**) Equal aliquots of the cell lysates were separated by SDS/PAGE and blotted with anti-phosphotyrosine antibody (4G10), as described in the Materials and methods section. (**B**) After the cell lysates had been immunoprecipitated with the anti-paxillin antibody (clone 349), the immunoprecipitates were analysed by immunoblotting with anti-phosphotyrosine antibody (4G10) for the phosphorylated paxillin (upper panel), and anti-paxillin antibody (clone 349) for total paxillin content (lower panel), as described in the Materials and methods section. The results shown are representative of three independent experiments.

suppress EP3B receptor-mediated neurite retraction (J. Aoki, H. Katoh and M. Negishi, unpublished work). Considering these results, we speculate that the EP3 receptor may be coupled to the $G\alpha_{13}$-tyrphostin A25-sensitive tyrosine kinase pathway leading to neurite retraction in differentiated PC12 cells.

Recently, our laboratory and others have shown that ROKα/Rho-kinase and p160ROCK, a Rho-associated serine/threonine kinase, mediated neurite retraction and cell rounding acting downstream of Rho, and that activation of this kinase was sufficient to induce these morphological changes [12–14]. Rho-kinase phosphorylates the myosin-binding subunit of myosin phosphatase and inactivates its activity [33], and also directly phosphorylates the myosin light chain [34], leading to the activation of myosin ATPase. Therefore, neurite retraction and cell rounding induced by ROKα/Rho-kinase and p160ROCK appeared to be mediated by the activation of myosin-based contractility. In our present study, however, a tyrosine kinase inhibitor, genistein, blocked constitutively active RhoA-induced neurite retraction and cell rounding (Figure 2), indicating that RhoA-induced activation of the contractility in neuronal cells required a genistein-sensitive tyrosine kinase. On the other hand, genistein did not block the neurite retraction and cell rounding induced by the active kinase domain of ROKα (Figure 3), indicating that the genistein-sensitive tyrosine kinase involved in the Rho-mediated neuronal morphological changes did not act downstream of ROKα. Activation of Rho has been shown to induce tyrosine phosphorylation of multiple proteins, such as paxillin and focal adhesion kinase, known to be implicated in the regulation of cell migration and cell attachment [32]. Here we showed activation of the EP3 receptor-induced tyrosine phosphorylation of paxillin, a putative adaptor protein, and that this phosphorylation was inhibited by genistein but not by the ROKα inhibitor, Y-27632 (Figure 4). Therefore, tyrosine phosphorylation of paxillin was mediated by a genistein-sensitive tyrosine kinase, but that this phosphorylation was not a downstream effect of ROKα, suggesting that stimulation of the tyrosine kinase by Rho was separable from Rho-induced ROKα activation. It was reported that *in vitro* stimulation of ROKα by GTP-bound Rho was only 1.5–2-fold, and that this level was much lower than the activity of the catalytic domain of the kinase (5–6-fold), suggesting that there was an additional signalling component downstream of Rho for full activation of ROKα [35]. Considering that CD-ROKα is apparently sufficient for inducing neurite retraction, Rho-mediated stimulation of a genistein-sensitive tyrosine kinase might be involved in the activation of ROKα. At present, a tyrosine kinase which acts downstream of Rho to induce neurite retraction and cell rounding has not yet been identified. Therefore, we will consider identification and characterization of this tyrosine kinase in future studies.

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