Prostaglandin E Receptor EP3 Subtype Induces Neurite Retraction via Small GTPase Rho*

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Prostaglandin E receptor EP3 subtype is widely distributed in the nervous system and is specifically localized to neurons, suggesting that the EP3 receptor plays important roles in the nervous system. We established a PC12 cell line that stably expresses the EP3B receptor isoform isolated from bovine adrenal chromaffin cells and examined the effect of agonist stimulation on the morphological changes of the PC12 cells. In the differentiated cells, M&B28767, an EP3 agonist, caused neurite retraction in a pertussis toxin-insensitive manner. 12-O-Tetradecanoylphorbol-13-acetate (TPA) also induced neurite retraction. However, when protein kinase C was down-regulated by long term exposure to TPA, TPA failed to induce neurite retraction, while the EP3B receptor-mediated retraction occurred normally. Clostridium botulinum C3 exoenzyme completely inhibited both EP3 agonist- and TPA-induced neurite retraction when microinjected into the cells, indicating that the morphological effect of the EP3B receptor is dependent on Rho activity. Thus, the activation of the EP3B receptor induced neurite retraction through a protein kinase C-independent Rho-activation pathway.

The function of the nervous system depends on the highly specific pattern of connections formed between neurons during development. The specificity of these connections requires neurite extension toward the correct targets guided by the growth cone and refinement and remodeling of the initial pattern of connections, referred to as synaptic plasticity, that are dependent on patterns of synaptic activity (1). The growth cone receives several kinds of environmental signals, such as diffusible chemorepellents and chemorepellents, extracellular matrix components, and cell adhesion molecules (2), and then it interprets them and changes shape and motility that results in the advance, turning, or collapse of the growth cone. The Rho family of small GTPases, Rac, CDC42, and Rho, has been shown to be involved in morphological changes of a variety of cells (3). In neuronal cells, Rac or CDC42 appears to be required for the outgrowth of neurites while Rho is required for their retraction (4).

Prostaglandin (PG)1 E2 is one of the major PGs synthesized in the nervous system (5). PGE2 has several important functions in the nervous system, such as generation of fever (6, 7), regulation of luteinizing hormone-releasing hormone secretion (8), pain modulation (9), and regulation of neurotransmitter release (10, 11). Although cyclooxygenase products including PGE2 have been suggested to be involved in regulation of memory consolidation (12), the biological significance of PGE2 in synaptic plasticity is not yet understood. PGE2 acts on cell surface receptors to exert its actions (13). PGE receptors are pharmacologically divided into four subtypes, EP1, EP2, EP3, and EP4, on the basis of their responses to various agonists and antagonists (14, 15). We have recently cloned the four subtypes of mouse PGE receptors and demonstrated that they are heterotrimeric GTP-binding protein (G protein)-coupled rhodopsin-type receptors (16–19). Among these subtypes, the EP3 receptor was most abundant in the brain and was specifically localized to the neurons (20).

PC12 cells were derived from rat pheochromocytomas, expressing a chromaffin-like phenotype, and serve as a useful model system for the study of neuronal differentiation. When PC12 cells are exposed to NGF for several days, they acquire many features of sympathetic neurons, such as an outgrowth of neurites. We recently isolated four EP3 receptor isoforms (EP3A, EP3B, EP3C, and EP3D) from bovine adrenal chromaffin cells that are produced through alternative splicing (21). To assess the role of the EP3 receptors in the nervous system, we introduced the cDNA for the EP3B receptor isoform, the most abundant isoform, into PC12 cells. In this report, we show that the activation of the EP3B receptor causes neurite retraction via small GTPase Rho.

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. Pertussis toxin (PT) and Clostridium botulinum C3 exoenzyme were obtained from Seikagaku Kogyo (Tokyo, Japan), dibutyril cyclic AMP Bt2cAMP was from Sigma, and 12-O-tetradecanoylphorbol-13-acetate (TPA) and 4α-phorbol 12,13-di decanoate were obtained from Funakoshi Pharmaceuticals (Tokyo, Japan). The sources of the other materials are shown in the text.

Cell Culture—PC12 cells were 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. DNA encoding the EP3B receptor was inserted into the expression vector pcDNA3 (Invitrogen), and the plasmid constructed was transfected into PC12 cells by lipofection (22). Stable transformants were cloned by selection with G418 (Life Technologies, Inc.).

Neurite Retraction Assay—After the cells had been seeded on poly-L-lysine (Sigma)-coated 24-well plates at a density of 104 cells/well in serum-containing Dulbecco’s modified Eagle’s medium, and cultured for 24 h, they were differentiated in serum-free Dulbecco’s modified Eagle’s medium containing 50 ng/ml NGF and 20 μM indomethacin for 5 days. In the neurite retraction experiments, the differentiated cells were acid; NGF, nerve growth factor.
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RESULTS

Effect of M&B28767 on Morphology of the EP3B Receptor-expressing PC12 Cells Differentiated with NGF—To assess the role of EP3 receptors in neuronal morphology we established PC12 cells that stably expressed the EP3B receptor (151 fmol/mg protein). We initially examined the coupling of the EP3B receptor to the classical signal transduction pathways, the adenylate cyclase and Ca²⁺ mobilization pathways. In these cells, the EP3B receptor inhibited adenylate cyclase activity through a PT-sensitive heterotrimeric GTP-binding protein, but the receptor could not stimulate adenylate cyclase, phosphatidylinositol hydrolysis, or Ca²⁺ mobilization (data not shown), suggesting that the EP3B receptor is coupled to Gᵢ but not to Gₛ or Gq. We next examined the effect of M&B28767, a specific EP3 agonist, on the neuronal morphology of the EP3B receptor-expressing PC12 cells. As shown in Fig. 1, treatment with NGF induced neurite outgrowth. The addition of M&B28767 caused a dramatic morphological change in the NGF-differentiated PC12 cells. Within 10 min of the addition of the agonist neurites began to retract, and within 30 min most neurites had retracted completely. Fig. 2A shows the time course of the effect of the agonist on neurite length. The neurite length became half of the original length within 20 min. The rate of neurite retraction was not dependent on the concentration of the agonist (data not shown). M&B28767 concentration-dependently increased the population of neurite-retracted cells, and more than 90% of the total cells responded to 1 μM M&B28767 (Fig. 2B). However, a few cells resisted even high concentrations of the agonist. M&B28767-induced neurite retraction was not seen for more than 60 min in the untransfected cells (data not shown).

The EP3B receptor inhibited adenylate cyclase via a PT-sensitive heterotrimeric G protein. Therefore, we examined whether M&B28767-induced neurite retraction was mediated through a PT-sensitive G protein. As shown in Fig. 3 and Table I, pretreating the EP3B receptor-expressing cells with PT for 12 h did not affect agonist-induced neurite retraction. We further examined the effect of protein kinase A activation on EP3B receptor-mediated neurite retraction. As shown in Fig. 4, whereas B_{t2}cAMP did not affect the morphology of the differentiated cells, it strongly prevented M&B28767-induced neurite retraction. Table I shows the quantitative examination of the effect of B_{t2}cAMP. It decreased the percentage of neurite-retracted cells in response to M&B28767 from 93.9 to 13.4%. These results indicate that the activation of protein kinase A suppresses the EP3B receptor-mediated neurite retraction.

Effect of TPA on Neuronal Cell Morphology—Protein kinase C activation has been reported to induce membrane ruffling...
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The percentage of neurite-retracted cells was determined as described under "Experimental Procedures." Data are means ± S.E. of triplicate experiments.

| Treatment | Neurite-retracted cells |
|-----------|-------------------------|
| None      | 93.9 ± 1.0              |
| PT        | 92.2 ± 2.3              |
| Bt2cAMP   | 13.4 ± 1.9              |

**Fig. 4.** Effect of Bt2cAMP on M&B28767-induced neurite retraction. A, cells were differentiated with NGF for 5 days. B, cells in A were exposed to 1 μM M&B28767 for 60 min. C, differentiated cells were pretreated with 500 μM Bt2cAMP for 15 min. D, cells in C were exposed to 1 μM M&B28767 for 60 min. The results shown are representative of three independent experiments that yielded similar results. The bar represents 50 μm.

**Fig. 5.** TPA-induced retraction of neurites. A, cells were differentiated with NGF for 5 days. B, cells in A were exposed to 1 μM M&B28767 for 60 min. C, cells were differentiated with NGF for 5 days. D, cells in C were exposed to 100 nM TPA for 60 min. E, cells were differentiated with NGF for 5 days in the presence of 1 μM TPA. F, cells in E were exposed to 1 μM M&B28767 for 60 min. G, cells were differentiated with NGF in the presence of 1 μM TPA. H, cells in G were exposed to 100 nM TPA for 60 min. The results shown are representative of three independent experiments that yielded similar results. The bar represents 50 μm.

**Table I**

| Treatment | Neurite-retracted cells |
|-----------|-------------------------|
| None      | 93.0 ± 1.5              |
| TPA       | 87.5 ± 2.5              |

**Table II**

The percentage of neurite-retracted cells was determined as described under "Experimental Procedures." Data are means ± S.E. of triplicate experiments.

| Treatment | Neurite-retracted cells |
|-----------|-------------------------|
| None      | 93.9 ± 1.0              |
| TPA       | 87.5 ± 2.5              |

**DISCUSSION**

Information on the function of the EP3 receptors in the nervous system is scarce, although several findings have been reported, for example, they inhibit neurotransmitter release and have hyperalgesic effects (28, 29). In this study, we described a new possible function of the EP3 receptors, modulation of a neuronal morphological change through a novel pathway distinct from adenylyl cyclase inhibition or protein kinase C activation. In the EP3B receptor-expressing PC12 cells dif-
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The average neurite length of differentiated cells that responded to 1 μM M&B28767 (●) or 100 nM TPA (□) was determined at the times indicated, as described under “Experimental Procedures,” and expressed as a percentage of the length before retraction. Data are means ± S.E. of triplicate experiments.

FIG. 6. The time course of M&B28767- or TPA-induced neurite retraction. The average neurite length of differentiated cells that responded to 1 μM M&B28767 (●) or 100 nM TPA (□) was determined at the times indicated, as described under “Experimental Procedures,” and expressed as a percentage of the length before retraction. Data are means ± S.E. of triplicate experiments.

Fig. 7. Effect of the C3 exoenzyme on M&B28767-induced neurite retraction. Differentiated cells were microinjected with 100 μg/ml C3 (A and C) and then stimulated with 1 μM M&B28767 (B) or 100 nM TPA (D) for 60 min. M&B28767 or TPA was added 30 min after injection. The arrows indicate injected cells. The results shown are representative of three independent experiments. At least 20 cells were microinjected in each experiment for C3 exoenzyme and the control buffer, and all cells microinjected gave the described response. The bar represents 50 μm.

Among classical second messenger pathways, the EP3B receptor is coupled to adenylate cyclase inhibition through G12 and G13 (30), which inhibits adenylyl cyclase, and to a PT-insensitive heterotrimeric G protein other than G12 and G13 that induces neurite retraction dependent on Rho activity. It has recently been reported (30) that GTPase-deficient constitutively activated Gα12 and Gα13 stimulate Rho-dependent stress fiber formation and focal adhesion assembly in Swiss 3T3 cells. As Gα12 and Gα13 are PT-insensitive G proteins, we suggest that the EP3B receptor may regulate neuronal morphology through G12 or G13.

Protein kinase C is also thought to be involved in the regulation of cell shape, contraction, and motility (31), and it has recently been reported (32) that protein kinase C activation induced membrane ruffling and translocation of activated Rho to the membrane ruffling area. We have shown here that protein kinase C activation also induced neurite retraction within 60 min in EP3B receptor-expressing PC12 cells (Fig. 5, C and D, and Table II). Long-term exposure to TPA suppressed the TPA-induced neurite retraction by down-regulating protein kinase C (Fig. 5, G and H, and Table II). In contrast, long-term exposure to TPA did not prevent M&B28767-induced neurite retraction (Fig. 5, E and F, and Table II), indicating that EP3 receptor inhibition induces neurite retraction through a mechanism not involving the protein kinase C pathway. Both M&B28767- and TPA-induced neurite retractions were sensitive to the C3 exoenzyme (Fig. 7), indicating that these effects are mediated by Rho activation. Thus, there are two different signaling pathways, protein kinase C-dependent and -independent pathways, involved in Rho-mediated neurite retraction. TPA-induced neurite retraction was preceded by a lag period (5–10 min), while the EP3B receptor activation induced neurite retraction without a lag period (Fig. 6). Thus, the EP3B receptor-mediated retraction is faster than that mediated by the protein kinase C pathway. This finding shows that the intermediate pathways from the EP3B receptor to Rho and from the protein kinase C to Rho differ, and the latter requires a longer time to activate Rho. It has recently been reported (4) that lysophosphatidic acid (LPA) caused neurite retraction dependent on Rho activity in N1E-115 cells as well as in PC12 cells and that the LPA-induced neurite retraction was mediated by Gα-coupled phospholipase C stimulation and subsequent protein kinase C activation (33). Furthermore, the time course of LPA-induced neurite retraction in PC12 cells appears to be slower than the EP3B receptor-mediated retraction but similar to that of TPA-induced retraction (34). These findings indicate that LPA induces neurite retraction through a protein kinase C-dependent pathway, while the EP3B receptor induces neurite retraction through a protein kinase C-independent pathway. Thus, there are two different types of heterotrimeric G protein-coupled receptors that induce Rho-dependent neuronal morphological change. One type is Gα-phospholipase C-coupled receptors, and the other is coupled to a heterotrimeric G protein other than Gα, leading to Rho-dependent action.

Our examination of the regulation of the EP3B receptor-mediated neurite retraction by protein kinase A revealed that the activation of protein kinase A inhibited the EP3B receptor-mediated neurite retraction and indicated that protein kinase A is a negative regulator. As the potential protein kinase A phosphorylation site is located in the first cytoplasmic loop of the EP3B receptor, it is possible that protein kinase A phosphorylates the EP3B receptor. The phosphorylation of this site by protein kinase A may stop the EP3B receptor-mediated signaling. The second possible site of the action of protein kinase A is on a downstream component. LPA-induced neurite retraction was recently reported (35) to be prevented by pre-
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treatment with Bt-cAMP in PC12 cells. Furthermore, it was recently reported (36) that protein kinase A directly phosphorylated Rho, and this phosphorylation resulted in termination of Rho signaling. As LPA and the EP3B receptors use different pathways to activate Rho, protein kinase A may block Rho-mediated morphological regulation by phosphorylating Rho in PC12 cells.

From this study, we propose new possible functions of PGE2 acting through the EP3 receptors in the nervous system. We previously showed that EP3 receptors were expressed in a variety of neurons in the brain (20), and neurons in the dorsal root ganglion (37). PGE2 is normally produced in the brain, and its production is dramatically stimulated by brain injuries such as concussion, trauma, and asphyxia (38–41). When the brain is injured, newly synthesized PGE2 may retract the neurites of the EP3 receptor-expressing neurons and reorganize damaged neuronal connections. In addition, the levels of PGE2 are also increased in the brain by synaptic activity or during development (42). Furthermore, cyclooxygenase, a rate-limiting enzyme in PG synthesis, has been reported to be markedly increased in the brain by synaptic activity or during development (43). We previously showed that EP3 receptors were expressed in a neuronal PGE2 function but will also help to elucidate the molecular mechanisms of signal transduction pathways between heterotrimeric G protein-coupled receptors and Rho.

REFERENCES
1. Goodman, C. S., and Shatz, C. J. (1993) Cell 10, (suppl.) 77–98
2. Keynes, R., and Cook, G. M. W. (1995) Cell 83, 161–169
3. Hall, A. (1994) Annu. Rev. Cell Biol. 10, 31–54
4. Jinlin, K., Cornes, E. J., Hengeveld, T., Mori, N., Narumiya, S., and Moolenaar, W. H. (1994) J. Cell Biol. 128, 801–810
5. Wolfe, L. S. (1982) J. Neurochem. 38, 1–14
6. Milton, A. S., and Wendlandt, S. (1970) J. Physiol. (Lond.) 207, 76–77
7. Stitt, J. T. (1986) Yale J. Biol. Med. 59, 137–149
8. Ojeda, S. R., and Campbel, W. B. (1982) Endocrinology 111, 1031–1037
9. Ferreira, S. H. (1972) Nat. Neurol. 240, 200–203
10. Roberts, P. J., and Hillier, K. (1976) Brain Res. 112, 425–428
11. Reimann, W., Steinhauser, H. B., Hedler, L., Stark, K., and Hertting, G. (1981) Eur. J. Pharmacol. 69, 421–427
12. Holscher, C. (1995) Eur. J. Pharmacol. 294, 253–259
13. Negishi, M., Sugimoto, Y., and Ichikawa, A. (1995) Biochim. Biophys. Acta 1259, 109–120
14. Coleman, R. A., Kennedy, I., Hamphey, P. A., Bunce, K., and Lumley, P. (1990) in Comprehensive Medicinal Chemistry (Hansch, C., Sammes, P. G., Taylor, J. B., and Emmett, J. C., eds) Vol. 3, pp. 643–714, Pergamon Press, Oxford.
15. Coleman, R. A., Grix, S. P., Head, S. A., Louttit, J. L., Mallett, A., and Sheldrick, R. L. (1994) Prostaglandins 47, 151–168
16. Watabe, A., Sugimoto, Y., Honda, A., Irie, A., Namba, T., Negishi, M., Narumiya, S., and Ichikawa, A. (1993) J. Biol. Chem. 268, 20170–20176
17. Katsuyma, M., Nishigaki, N., Sugimoto, Y., Morimoto, K., Negishi, M., Narumiya, S., and Ichikawa, A. (1995) FEBS Lett. 372, 151–156
18. Sugimoto, Y., Namba, T., Honda, A., Hayashi, Y., Negishi, M., Ichikawa, A., and Narumiya, S. (1992) J. Biol. Chem. 267, 6463–6466
19. Honda, A., Sugimoto, Y., Namba, T., Watabe, A., Irie, A., Negishi, M., Narumiya, S., and Ichikawa, A. (1993) J. Biol. Chem. 268, 7759–7762
20. Sugimoto, Y., Shigemoto, R., Namba, T., Negishi, M., Mizuno, N., Narumiya, S., and Ichikawa, A. (1994) Neuroscience 62, 919–928
21. Namba, T., Sugimoto, Y., Negishi, M., Irie, A., Ushikubi, F., Kakizuka, A., Ito, S., Ichikawa, A., and Narumiya, S. (1993) Nature 363, 166–170
22. Felgen, P. L., Gadek, T. R., Holm, M., Roman, R., Chan, H. W., Wenz, M., Northrop, J. P., Ringold, G. M., and Danielson, M. (1987) Proc. Natl. Acad. Sci. U. S. A. 84, 7413–7417
23. Ridley, A. J., and Hall, A. (1992) Cell 70, 389–399
24. Nishiyama, T., Sasaki, T., Takashi, K., Kato, M., Yaku, H., Araki, K., Matsumura, Y., and Takai, Y. (1994) Mol. Cell. Biol. 14, 2447–2456
25. Matthews, J. H. G., Palfrey, H. C., Hirning, L. D., and Miller, R. J. (1987) J. Neurosci. 7, 1198–1206
26. Aktories, K., Weller, U., and Chhatwal, G. S. (1987) FEBS Lett. 212, 109–113
27. Sekine, A., Fujimura, M., and Narumiya, S. (1989) J. Biol. Chem. 264, 8602–8605
28. Ensner, H. J., and Schlicker, E. (1995) Naunyn-Schmiedeberg's Arch. Pharmacol. 351, 46–52
29. Kumazawa, T., Mizumura, K., and Koda, H. (1993) Brain Res. 632, 321–324
30. Buhl, A. M., Johnson, N. L., Dhanasekaran, N., and Johnson, G. L. (1995) J. Biol. Chem. 270, 24631–24634
31. Takai, Y., Sasaki, T., Tanaka, K., and Nakanishi, H. (1995) Trends Biochem. Sci. 20, 227–231
32. Takai, K., Sasaki, T., Kameyama, T., Tsukita, S., Tsukita, S., and Takai, Y. (1995) Oncogene 11, 39–48
33. Tigi, G., Fischer, D. J., Sekob, A., Yang, C., Dyer, D. L., and Mileedi, R. (1996) J. Neurochem. 66, 537–548
34. Dyer, D., Tigi, G., and Mileedi, R. (1992) Mol. Brain Res. 14, 293–301
35. Tigi, G., Fischer, D. J., Sekob, A., Yang, C., Dyer, D. L., and Mileedi, R. (1996) J. Neurochem. 66, 549–558
36. Lang, P., Greber, F., Carmagnat, M. D., Stancou, R., Pouchelet, M., and Bertoglio, J. (1996) EMBO J. 15, 510–519
37. Oida, H., Namba, T., Sugimoto, Y., Ushikubi, P., Ohishi, H., Ichikawa, A., and Narumiya, S. (1993) Br. J. Pharmacol. 116, 2828–2837
38. Ment, L. R., Stewart, W. B., Duncan, C. C., Pitt, B. R., and Cole, J. (1987) J. Neurosurg. 67, 278–283
39. Shohami, E., Shapira, Y., Sidki, A., and Cotev, S. (1987) J. Cereb. Blood Flow Metab. 7, 58–63
40. Dewitt, D. S., Kong, D. L., Lyeth, B. G., Jenkins, L. W., Hayes, R. L., Wooten, E. D., and Prough, D. S. (1988) J. Neurotrauma 5, 303–313
41. Ellis, E. F., Fulcher, B. J., Rice, Y. L., Grabee, M., and Holt, S. (1989) J. Neurotrauma 6, 31–37
42. Hertting, G., and Seregi, A. (1989) Ann. N. Y. Acad. Sci. 559, 84–99
43. Yamagata, K., Andreassen, K. I., Kaufmann, W. E., Barnes, C. A., and Worley, P. F. (1993) Neuron 11, 571–586