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Short communication

The prostaglandin E_2/EP4 receptor/cyclic AMP/T-type Ca^{2+} channel pathway mediates neuritogenesis in sensory neuron-like ND7/23 cells

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ABSTRACT

We investigated mechanisms for the neuritogenesis caused by prostaglandin E_2 (PGE_2) or intracellular cyclic AMP (cAMP) in sensory neuron-like ND7/23 cells. PGE_2 caused neuritogenesis, an effect abolished by an EP4 receptor antagonist or inhibitors of adenylyl cyclase (AC) or protein kinase A (PKA) and mimicked by the AC activator forskolin, dibutyryl cAMP (db-cAMP), and selective activators of PKA or Epac. ND7/23 cells expressed both $Ca_v3.1$ and $Ca_v3.2$ T-type Ca^{2+} channels (T-channels). The neuritogenesis induced by db-cAMP or PGE_2 was abolished by T-channel blockers. T-channels were functionally upregulated by db-cAMP. The PGE_2/EP4/cAMP/T-channel pathway thus appears to mediate neuritogenesis in sensory neurons.

Injury to the peripheral axons of the primary sensory neurons triggers a variety of cell signals in the neurons and related cells, which stimulates axonal regeneration and may eventually contribute to the later development of neuropathic pain (1). One of the most important intracellular molecules for the axonal regeneration is cyclic AMP (cAMP) in the neuronal cytoplasm, since dibutyryl cAMP (db-cAMP), a membrane-permeable cAMP analogue capable of inhibiting phosphodiesterase (PDE), a cAMP-degrading enzyme, enhances neurite outgrowth in cultured dorsal root ganglion (DRG) neurons (2). There is also *in vivo* evidence that microinjection of db-cAMP into the DRG promotes the regrowth of injured axons (3), although the surrounding cells such as Schwann cells and macrophages also participate in the regenerative process (1,4). On the other hand, prostaglandin E_2 (PGE_2) causes cAMP-dependent neuritogenesis in cultured DRG neurons (2). We have shown that PGE_2 enhances protein kinase A (PKA)-dependent enhancement of T-type Ca^{2+} channel (T-channel) activity via EP4 receptors, contributing to the development of mechanical hyperalgesia (5), and that the enhanced activity of T-channels by hydrogen sulfide, a gasotransmitter, causes neuritogenesis in the neuron-like NG108-15 cells (6,7). To clarify the cell signals underlying the axonal regeneration in injured primary afferents, in the present study, we analyzed possible involvement of PGE_2, cAMP and T-channels in the neuritogenesis in ND7/23 cells, created by cell fusion of N18Tg2 mouse neuroblastoma and DRG neurons from neonatal rats, which express substance P and have nociceptive sensory neuron-like properties (8,9).

ND7/23 cells were cultured in a low glucose-containing Dulbecco's modified Eagle's medium (Wako Pure Chemical Industries, Osaka, Japan) supplemented with 100 U/mL penicillin, 100 μg/mL streptomycin (Gibco, Carlsbad, CA, USA) and 10% fetal bovine serum (FBS) (Thermo Electron Corporation, Waltham, MA, USA). The cells were seeded at a density of 1×10^4/mL in a culture dish (35 mm in diameter) coated with poly-L-ornithine and filled with 1 mL of the above medium containing 1% FBS, and stimulated with distinct chemicals including PGE_2 and db-cAMP for 24, 48, 72 or 96 h at 37 °C. Neurite outgrowth was evaluated by counting cells with neurites that were longer than the cell body diameter (two fields from each dish) under the microscope, as described elsewhere (6,7). T-channel-dependent membrane currents (T-currents) were determined by whole-cell patch-clamp recordings in ND7/23 cells stimulated with db-cAMP, as reported previously (5–7). Briefly, Ba^{2+} currents were recorded from randomly chosen cells at room temperature (22–24 °C) using a computer-controlled

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microelectrode amplifier (MultiClamp 700B, Axon Instruments, Foster City, CA, USA). The resistance of patch pipettes ranged from 3 to 5 MΩ, and series-resistance was compensated by 80%. Data were acquired and digitized with a Digidata interface (1322A, Axon Instruments), and analyzed by a personal computer using pClamp8 software (Axon Instruments). Current-voltage relations were analyzed by applying 200-ms test-pulse potentials ranging in 10-mV steps from −120 to +30 mV, from a holding potential of −90 mV. T-currents were evaluated by depolarizing the cells to −20 mV from the same holding potential. Protein expression was analyzed by Western blotting, essentially as reported previously (5). The primary antibodies used (dilution) were: a goat anti-Epac1 (C-17) antibody (1:200), goat anti-Epac2 (M-18) antibody (1:200), rabbit anti-GAPDH antibody (1:3000) (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA), rabbit anti-$Ca_v3.1$ antibody (1:500) (Alomone Labs, Jerusalem, Israel), and rabbit anti-$Ca_v3.2$ antibody (1:500) (Sigma-Genosis/Sigma–Aldrich, St. Louis, MO, USA). The secondary antibodies used (dilution) were: a horseradish peroxidase (HRP)-conjugated anti-rabbit IgG antibody (1:2000) (Chemicon International Inc., Billerica, MA, USA) or anti-goat IgG antibody (1:3000) (Millipore Corporation, Billerica, MA, USA). Expression of mRNA for Epac1 and Epac2 was confirmed by the conventional RT-PCR method, as described elsewhere (10). The PCR primers used were: 5′-GCT CTC CCC TCC TGT CAT CC-3′ (forward) and 5′-GTT CCC GCT GGT TGT CAA TG-3′ (reverse) for Epac1 (PCR product size: 323 bp); 5′-CCT TCC TGG AGG AGT TCT AT-3′ (forward) and 5′-GTG ACA ACC CAA AAC TGA AT-3′ (reverse) for Epac2 (PCR product size: 688 bp). According to the procedures approved by the Committee for the Care and Use of Laboratory Animals at Kindai University, the cerebellum and DRG were excised from male Wistar rats (9–12 weeks old) (Japan SLC Inc., Shizuoka, Japan) that had been sacrificed by exsanguination under anesthesia with i.p. urethane at 1.5 g/kg. The homogenates were used as positive controls that express mRNA and/or protein of Epac1 or Epac2. PGE_2, db-cAMP, 3-isobutyl-1-methylxanthine (IBMX), forskolin, KT5720, SC19220, NNC55-0396 (NNC), mibefradil, AH6809, sulprostone, SH23848, SQ22536, N^6-benzoyl-cAMP (6-Bnz-cAMP) and ascorbic acid were purchased from Sigma–Aldrich (St. Louis, MO, USA). $ZnCl_2$ was obtained from Kishida Chemical (Osaka, Japan), and 8-(4-chlorophoenylthio)-2′-*O*-methyl-cAMP (8-CPT-2′-O-Me-cAMP) was from BIOLOG Life Science Institute (Bremen, Germany). Data are shown as the mean ± SEM. Statistical analysis was performed by Student's t-test for two group data and Tukey's test for multiple comparisons. Significance was set at a $p < 0.05$ level.

In ND7/23 cells, stimulation with PGE_2 at 2–10 μM for 24 h caused significant neurite outgrowth in the presence of IBMX, a PDE inhibitor, at 50 μM, a subeffective concentration (Fig. 1A–C). The neurite outgrowth caused by PGE_2 at 10 μM plus IBMX at 50 μM was significantly inhibited by SH23848, an EP4 receptor antagonist, at 10 μM, but not SC19220, an EP1 receptor antagonist, or AH6809, an EP2 receptor antagonist, at 10 μM (Fig. 1D–F). Sulprostone, an EP3/EP1 agonist, in a range of 1–100 μM failed to mimic the PGE_2-induced neuritogenesis in the presence of IBMX (Fig. 1G). The PGE_2/IBMX-induced neurite outgrowth was also significantly suppressed by SQ22536 at 500 μM and KT5720 at 1–10 μM, inhibitors of adenylyl cyclase and PKA, respectively, known as downstream signals of the trimeric G_s protein-coupled receptor EP4 (Fig. 1H, I). Collectively, PGE_2 is considered to cause neuritogenesis via activation of EP4 receptors, adenylyl cyclase and PKA in ND7/23 cells.

Stimulation with db-cAMP, a membrane-permeable cAMP analogue capable of inhibiting PDE, at 1 mM for 24, 48 or 72 h produced neurite outgrowth (Fig. 2A, B), an effect reduced by the PKA inhibitor KT5720 (Fig. 2E). Like PGE_2, forskolin, an adenylyl cyclase activator, at 3 μM also elicited neuritogenesis in the presence, but not absence, of IBMX (Fig. 2C, D). 6-Bnz-cAMP, a selective PKA activator, at 500 μM caused KT5720-sensitive neurite outgrowth (Fig. 2F, G). 8-CPT-2′-O-Me-cAMP, a selective activator of Epac, another target for cAMP, at 100 μM mimicked the db-cAMP-induced neuritogenesis (Fig. 2H). Combined application of 6-Bnz-cAMP and 8-CPT-2′-O-Me-cAMP at 100 and 20–50 μM, respectively, subeffective concentrations, synergistically produced neurite outgrowth (Fig. 2I). Proteins and mRNA for Epac1 and Epac2 were detected in ND7/23 cells (Fig. 2J, K).

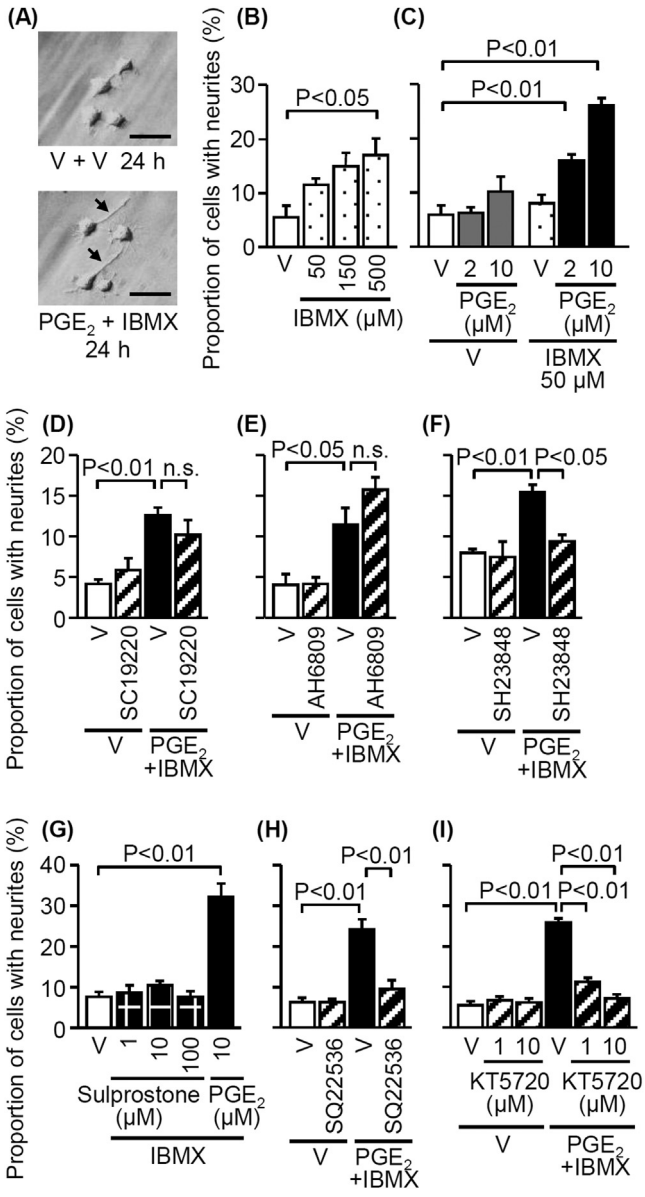

Fig. 1. PGE_2 causes neurite outgrowth via the EP4/adenylyl cyclase/PKA pathway in ND7/23 cells. (A) Typical photographs of neuritogenesis in ND7/23 cells stimulated with PGE_2 at 10 μM plus IBMX, a PDE inhibitor, at 50 μM or the vehicle (V) for 24 h. Arrows indicate neurites. (B, C) Neurite outgrowth after 24-h stimulation with IBMX (B) or PGE_2 plus IBMX (C). (D–F) Effects of SC19220, AH6809 and SH23848, EP1, EP2 and EP4 antagonists, respectively, at 10 μM on the neurite outgrowth caused by 24-h stimulation with PGE_2 at 10 μM plus IBMX at 50 μM. (G) Neurite outgrowth after 24-h stimulation with sulprostone, an EP3/EP1 agonist, or PGE_2 in the presence of 50 μM IBMX. (H, I) Effects of SQ22536, an adenylyl cyclase inhibitor, at 500 μM and KT5720, a PKA inhibitor, at 1–10 μM on the PGE_2/IBMX-induced neuritogenesis. Each inhibitor or antagonist was applied to the cells 30 min before the stimulation (D–F, H, I). Data show the mean with SEM for 4–8 (B, C), 8–12 (E–F) or 8 (G–I) different experiments. n.s., not significant.