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Full paper

Pituitary adenylate cyclase-activating polypeptide type 1 receptor signaling evokes long-lasting nociceptive behaviors through the activation of spinal astrocytes in mice

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ABSTRACT

Intrathecal (i.t.) administration of pituitary adenylate cyclase-activating polypeptide (PACAP) induces long-lasting nociceptive behaviors for more than 60 min in mice, while the involvement of PACAP type1 receptor (PAC_1-R) has not been clarified yet. The present study investigated signaling mechanisms of the PACAP-induced prolonged nociceptive behaviors. Single i.t. injection of a selective PAC_1-R agonist, maxadilan (Max), mimicked nociceptive behaviors in a dose-dependent manner similar to PACAP. Pre- or post-treatment of a selective PAC_1-R antagonist, max.d.4, significantly inhibited the nociceptive behaviors by PACAP or Max. Coadministration of a protein kinase A inhibitor, Rp-8-Br-cAMPS, a mitogen-activated protein kinase/extracellular signal-regulated kinase (ERK) kinase inhibitor, PD98059 or a c-Jun N-terminal kinase (JNK) inhibitor, SP600125, significantly inhibited the nociceptive behaviors by Max. Immunohistochemistry and immunoblotting analysis revealed that spinal administration of Max-induced ERK phosphorylation and JNK phosphorylation, and also augmented an astrocyte marker, glial fibrillary acidic protein in mouse spinal cord. Furthermore, an astroglial toxin, L-α-aminoadipate, significantly attenuated the development of the nociceptive behaviors and ERK phosphorylation by Max. These results suggest that the activation of spinal PAC_1-R induces long-lasting nociception through the interaction of neurons and astrocytes.

1. Introduction

Pituitary adenylate cyclase-activating polypeptide (PACAP), which shares 68% homology with vasoactive intestinal polypeptide (VIP), is a pleiotropic neuropeptide, belonging to the VIP/secretin/glucagon superfamily (1–3). PACAP receptor consists of three distinct G-protein-coupled receptors: the PACAP-specific receptor, PAC_1-R, which is mainly coupled to adenylate cyclase/protein kinase A (PKA) and phospholipase C (PLC)/protein kinase C (PKC) pathways, and the PACAP/VIP-indifferent receptors, $VPAC_1$- and $VPAC_2$-R, which are primarily coupled to adenylate cyclase (3,4). PAC_1-R is particularly abundant in central nervous system (CNS) including spinal dorsal horn (3,5,6), where PACAP-immunoreactive (IR) fibers are also considerably localized (7–10). These fibers are thought to be predominantly primary afferents in origin, since capsaicin treatment releases PACAP from rat spinal cord (11,12) and induces a significant decrease in the number of PACAP-IR nerve fibers within the spinal cord (7). Furthermore, PACAP mRNA/immunoreactivity is localized primarily in calcitonin-gene-related peptide- or substance P (SP)-IR containing neurons in rat dorsal root ganglion (DRG) (7,13) and is markedly upregulated in peripheral nerve injury or inflammation (14–18). Together with other lines of evidence, these suggest that PACAP-PAC_1-R system could play an important role in the modulation of spinal nociceptive transmission (19). However, the functional significance of PAC_1-R

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and its downstream signaling mechanism for spinal nociceptive transmission has not been completely understood yet.

Previously, we reported that intrathecal (i.t.) injection of PACAP-induced nociceptive behaviors, which gradually increased after relatively long latency (2–3 min), and reached to the maximum around 7–10 min, then lasting for more than 60 min. On the other hands, in the case of SP, nociceptive behaviors were induced immediately after the i.t. injection within 15 s, quickly reached to the maximum and then disappeared almost within 1 min (20).

To understand the nature of the PACAP-induced nociceptive behaviors, we attempted to clarify the role of PACAP receptor and its downstream signaling in the nociception using PAC_1 specific agonist, maxadilan (Max) and PAC_1 specific antagonist, max.d.4 (4,21–24).

2. Materials and methods

2.1. Animals

Male ddY mice (6–12 weeks old) were purchased from Kyudo Co. Ltd. (Kumamoto, Japan) and housed under controlled temperature (24 ± 1 °C) and humidity (55 ± 10%) with a 12-h light–dark cycle with food and water freely available. The animal experiments were approved by the Animal Care Committee of Kagoshima University (approval no. MD 12059), and were conducted in accordance with the ethical guidelines for the study of experimental pain in conscious animals of the International Association for the Study of Pain.

2.2. Intrathecal injection and behavioral observation

I.t. injection was given in a volume of 5 μl by percutaneous puncture through an intervertebral space at the level of the 5th or 6th lumbar vertebra, according to a previously reported procedure (25).

Before i.t. injection, mice were placed and habituated in a glass cylinder (φ14 × 18 cm) with a filter paper at the bottom over 20 min immediately after i.t. injection, the mice were placed again in the same glass cylinder and the number of nociceptive behaviors consisting of licking, biting and scratching directed toward the caudal part of the body was counted every 1 min. The cumulative number of events was pooled over 5 min bins or 30 min period of observation, and analyzed (20).

2.3. Drugs

Max and max.d.4 were kindly donated from Dr. Tajima (Shiseido, Japan) (21–24). PACAP (38-amino acid form) and VIP were purchased from Peptide Institute Inc. (Osaka, Japan). Rp-8-Br-cAMPS and L-α-aminoadipate were obtained from Sigma (St. Louis, MO, USA). PD98059 and SP600125 were from Millipore (Billerica, MA, USA). These drugs were administrated at the designated concentration in artificial cerebrospinal fluid (ACSF: NaCl 138 mM, KCl 3 mM, $CaCl_2$ 1.25 mM, $MgCl_2$ 1 mM, D-glucose 1 mM).

2.4. Immunohistochemistry

Immunohistochemistry was performed as previously described (26). Briefly, the animals were deeply anesthetized with sodium pentobarbital (60 mg/kg, i.p.) and perfused intracardially with heparinized saline followed by 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4). After laminectomy the spinal cord (L3–L5) was identified, excised and postfixed over night at 4 °C in the same fixative, and then replaced sequentially with 10, 15 and 20% sucrose in 0.1 M phosphate buffered saline (PBS) at 4 °C for cryoprotection. Transverse spinal sections (30 μm) were cut on a cryostat and collected in PBS at 4 °C to be processed immunohistochemically as free-floating sections.

Sections were blocked in PBS containing 1% normal donkey serum (ImmunoBioScience, Mukilteo, WA, USA) for 1 h at room temperature (RT), and incubated over night at 4 °C with the primary antibodies against phospho-p44/42 MAPK (ERK1/2) (Thr202/Tyr204) antibody (rabbit, 1:100, Cell Signaling Technology, Danvers, MA, USA) and glial fibrillary acid protein (GFAP) antibody (mouse monoclonal, 1:500, Millipore). The sections were then incubated for 1 h at RT with Alexa Fluor 568-labeled donkey anti-rabbit IgG antibody and Alexa Fluor 488-labeled donkey anti-mouse IgG antibody (Invitrogen, Carlsbad, CA). Stained sections were examined with a Zeiss LSM 700 confocal microscope (Carl Zeiss Microscopy, Jena, Germany).

2.5. Western blot analysis

Western blotting was performed as previously described (26). Briefly, after sacrifice of mice, the lumbar spinal cords (L3–L6) were quickly removed and homogenized in a lysis buffer [150 mM NaCl, 1 mM EDTA, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, and 50 mM Tris–HCl, pH 8.0] with a mixture of protease and phosphatase inhibitors (Roche Diagnostics, Mannheim, Germany). After the centrifugation at 13,000 ×g for 10 min at 4 °C, the protein concentrations of the supernatants were determined with a Bio-Rad protein assay kit (Bio-Rad, Hercules, CA). The proteins (12 μg) were separated by SDS-PAGE (12.5% gel) and then transferred onto polyvinylidene difluoride membrane (Millipore, Billerica, MA). Following antibodies were used. Anti-p44/42 MAPK (ERK1/2) (Thr202/Tyr204) (rabbit, 1:1000), anti-phospho-p44/42 MAPK (ERK1/2) (Thr202/Tyr204) (rabbit, 1:1000), anti-SAPK/JNK (rabbit, 1:1000), and anti-phospho-SAPK/JNK (Thr183/Tyr185) antibodies (rabbit, 1:1000) were obtained from Cell Signaling. Mouse anti-GFAP monoclonal antibody (1:10,000) is from Millipore. Immunoreactivity was detected by using an ECL prime kit (GE Healthcare, Buckinghamshire, UK). An anti-β-actin antibody (mouse monoclonal, 1:1000; Santa Cruz) was used to normalize protein loading. Relative intensities of the bands were quantified by using an image analysis system with ImageJ software, version 1.46 (National Institutes of Health, Bethesda, MD). At least two independent immunoblot experiments of two individual spinal cord samples were analyzed.

2.6. Statistical analysis

Experimental data are expressed as mean ± standard error of the mean (SEM). Single comparisons were made using Student's two-tailed unpaired *t*-test. One- or two-way analysis of variance followed by the Dunnett's test was used for multiple comparisons. $P < 0.05$ was considered statistically significant.

3. Results

3.1. Maxadilan, a selective PAC_1-R agonist, induces dose-dependent long-lasting nociceptive behaviors via PAC_1 receptor-PKA signaling

After single i.t. administration of Max, nociceptive behaviors such as licking and biting, which directed toward the caudal part of the body, gradually appeared at 3–5 min, reached to the plateau within 15–20 min, and maintained at least more than 30 min (Fig. 1A) as well as PACAP-induced nociceptive behaviors described previously (20). This aversive response subsided within 24 h after injection (data not shown). Max dose-dependently induced the nociception, although VIP did not (Fig. 1A and B).