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Sensitization of cutaneous neuronal purinergic receptors contributes to endothelin-1-induced mechanical hypersensitivity

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ABSTRACT

Endothelin (ET-1), an endogenous peptide with a prominent role in cutaneous pain, causes mechanical hypersensitivity in the rat hind paw, partly through mechanisms involving local release of algogenic molecules in the skin. The present study investigated involvement of cutaneous ATP, which contributes to pain in numerous animal models. Pre-exposure of ND7/104 immortalized sensory neurons to ET-1 (30 nM) for 10 min increased the proportion of cells responding to ATP (2 μM) with an increase in intracellular calcium, an effect prevented by the ET_A receptor–selective antagonist BQ-123. ET-1 (3 nM) pre-exposure also increased the proportion of isolated mouse dorsal root ganglion neurons responding to ATP (0.2–0.4 μM). Blocking ET-1-evoked increases in intracellular calcium with the IP_3 receptor antagonist 2-APB did not inhibit sensitization to ATP, indicating a mechanism independent of ET-1-mediated intracellular calcium increases. ET-1-sensitized ATP calcium responses were largely abolished in the absence of extracellular calcium, implicating ionotropic P2X receptors. Experiments using quantitative polymerase chain reaction and receptor-selective ligands in ND7/104 showed that ET-1-induced sensitization most likely involves the P2X4 receptor subtype. ET-1-sensitized calcium responses to ATP were strongly inhibited by broad-spectrum (TNP-ATP) and P2X4-selective (5-BDBD) antagonists, but not antagonists for other P2X subtypes. TNP-ATP and 5-BDBD also significantly inhibited ET-1-induced mechanical sensitization in the rat hind paw, supporting a role for purinergic receptor sensitization *in vivo*. These data provide evidence that mechanical hypersensitivity caused by cutaneous ET-1 involves an increase in the neuronal sensitivity to ATP in the skin, possibly due to sensitization of P2X4 receptors.

1. Introduction

Endothelin-1 (ET-1) is a potent vasoactive peptide that also plays a prominent role in peripheral pain, but its mechanisms are complex and incompletely understood. ET-1 acts through two G-protein-coupled receptors (ET_A and ET_B) on neuronal and non-neuronal cells in the skin to mediate both pro- and antinociceptive effects [3,17]. When injected into the rat plantar hind paw, ET-1 at high doses causes overt pain (as indicated by robust hind paw flinching) and sensitization to thermal and mechanical stimulation. Lower doses of ET-1 are also capable of producing tactile sensitization but do not cause overt pain [12]. The overt pain elicited by subcutaneous ET-1 injection into the paw has been almost exclusively attributed to the direct activation of ET_A receptors on nociceptive sensory neurons that innervate the skin because these nerves express ET_A receptors and increase their firing *in vivo* in response to ET-1 administration [12,28]. ET_A receptor activation also results in enhanced excitability in the soma of isolated nociceptive primary sensory neurons, through alterations in ionic currents and sensitization of excitatory receptors. Activation of ET_A receptors promotes TTX-sensitive sodium currents, reduces delayed rectifier potassium currents and sensitizes TRPV1 receptors, all processes that likely contribute to tactile sensitization after ET-1 administration [10,24,32,35].

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Recently, our laboratory has demonstrated that endogenous release of excitatory molecules in the skin appears to contribute to ET-1-induced mechanical sensitization. Pre-injection of antagonists for NMDA glutamate receptors reduced both the early and late phases of this sensitization, while an antagonist of the calcitonin gene–related peptide (CGRP) receptor reduced only the late phase [18]. ET-1 was found to increase the release of both of these molecules from cultured dorsal root ganglion (DRG) neurons through ET_A receptor activation, suggesting that ET-1 injection into the skin causes mechanical allodynia in part by enhancing the release of glutamate and CGRP from cutaneous nerve terminals.

In addition to causing increased release of algogenic substances in the skin, it is also possible that ET-1 sensitizes ligand-gated receptors on nociceptive nerve terminals. ET_A receptor–expressing nociceptive neurites terminate in the epidermis where they are surrounded by keratinocytes. Keratinocytes release a variety of pro-algesic molecules that activate excitatory receptors on nociceptive nerve endings, including glutamate, CGRP, and ATP [2,11,15,20,33]. In particular, cutaneous ATP release has been implicated in numerous types of acute and chronic pain [5,9]. Subcutaneously administered purinergic receptor antagonists are effective analgesics in a variety of animal pain models, including ones of inflammatory and neuropathic pain [6,22,30].

We hypothesized that ET-1-induced mechanical hypersensitivity is partly due to sensitization of purinergic receptors expressed by primary sensory neurons. We provide evidence that ET_A receptor activation enhances ATP responses in cultured sensory neurons independent of resulting intracellular calcium increases and that cutaneous ATP release in the skin contributes to ET-1-induced mechanical hypersensitivity in the rat hind paw.

2. Materials and methods

2.1. ND7/104 cell culture

ND7/104 model sensory neurons, a cell line derived from embryonic rat DRG neurons hybridized with mouse neuroblastoma N18GT2 cells, were generously donated in 2004 by Dr P. Hogan (Harvard Medical School, Boston, MA). These cells were cultured in Dulbecco modified Eagle medium (DMEM) supplemented with penicillin and streptomycin (100 μg) and 10% fetal bovine serum (Invitrogen, Carlsbad, CA) at 37°C and 5% CO_2. For calcium imaging, cells were plated onto poly-L-lysine-coated coverslips so that they were approximately 50% to 80% confluent when imaging was performed the next day.

2.2. Isolation and culture of mouse sensory neurons (mDRG neurons)

Male adult CD1 mice (Charles River, Wilmington, MA) were purchased and housed in the animal facilities of Children's Hospital Boston on a 12 h alternating light–dark cycle. Mice were experimentally treated and cared for using policies and procedures approved by the Harvard Committee on Animals and conformed to the guidelines of the Committee for Research and Ethical Issues of IASP. Animals for imaging were dissected after 7 weeks of age. After CO_2 asphyxiation and cervical translocation, and after spinal laminectomy, the left and right DRG from the whole spine were removed and placed in 4°C Hanks buffered saline solution (Life Technologies, Grand Island, NY). After the DRG were collected and centrifuged for 3 min at 1000 rpm (150 × *g*), they were placed in a collagenase/dispase solution (3 mg/mL dispase II and 1 mg/mL collagenase A, Roche Applied Science) and incubated at 37°C for 90 min. After incubation, the cells were washed in DMEM (Life Technologies), fortified with 4.5 g/L D-glucose, L-glutamine, 110 mg/L sodium pyruvate, 10% fetal bovine serum (Life Technologies), penicillin (500 U/mL, Cellgro, Manassas, VA), and streptomycin (500 μg/mL, Cellgro). DNAse (125 U/mL, Sigma) was then added, and the solution was triturated using successively smaller-caliber, flame-polished Pasteur pipettes. This solution was gently layered onto a bovine serum albumin gradient (10% albumin from bovine serum, Sigma in PBS, Life Technologies) and spun at 150 × *g* for 12 min to reduce the proportion of satellite cells. After removal of the supernatant, the cells were washed again in DMEM, suspended in neurobasal medium (Life Technologies) supplemented with L-glutamine (20 mM, Life Technologies), B-27 supplement (Life Technologies), penicillin (500 U/mL, Cellgro), streptomycin (500 μg/mL, Cellgro), nerve growth factor (50 ng/mL, Life Technologies), glial-derived nerve factor (2 ng/mL, Sigma), and arabinocytidine (10 μM, Sigma), plated onto poly-D-lysine (100 mg/mL, Sigma) and laminin-treated (1 mg/mL, Sigma) round glass coverslips (Fisher Scientific), and then maintained in an incubator at 37°C (5% CO_2). Media was completely replaced 2 days after dissection, and all imaging was performed 4 days after dissection and culture. Preliminary studies showed that both ET-1 and ATP responses were most robust 4 days after isolation (data not shown). Cultures of cells isolated from mouse DRG contained both primary sensory neurons and satellite cells. In calcium imaging experiments, neurons were distinguished on the basis of their increase in intracellular calcium in response to 40 mM potassium chloride (KCl).

2.3. Quantitative polymerase chain reaction

RNA was isolated from 3 separate passages of 80% confluent ND7/104 cultures with an RNeasy kit (Qiagen, Valencia, CA) according to the manufacturer's instructions. The concentration and quality of RNA were measured with a Nanodrop spectrophotometer (Thermo Scientific, Wilmington, DE), after which 1 μg of total RNA was synthesized to cDNA using an iScript kit (Biorad, Hercules, CA) according to the manufacturer's instructions. Samples were also prepared substituting reverse transcriptase with RNAse-free water to control for contamination. Quantitative polymerase chain reaction (qPCR) was conducted with Evagreen PCR supermix on a miniopticon thermocycler (Biorad, Hercules, CA) using the following protocol: 94°C for 3 min, 45 cycles of 94°C for 30 s, 60°C for 30 s, and 72°C for 1 min, and then a melting curve (65°C to 95°C in 0.5°C increments) to confirm primer specificity. Two separate qPCR experiments were each conducted in duplicate on the 3 ND7/104 samples for a total of 4 replicates per data point.

Sequences of the primers used to amplify P2X receptors have been previously published [21]. Data were analyzed using BioRad CFX manager. Expression of each target was normalized to the housekeeping gene Cyclo-A, adjusting for primer efficiencies calculated using REST 2009 software (Qiagen) from a serial dilution of each primer.

2.4. Calcium imaging of cells

Calcium imaging experiments were conducted largely as previously described [23]. Intracellular calcium was determined by excitation microfluorometry using the Ca^{2+}-sensitive fluorescent dye fura-2AM (Invitrogen). Coverslips were first rinsed with Ringer solution (NaCl 155 mM, KCl 4.5 mM, $CaCl_2$ 2 mM, $MgCl_2$ 1 mM, D-glucose 10 mM, HEPES 5 mM, pH 7.4) and incubated in fura-2AM (4 μM) in Ringer for 30 min in the dark at room temperature. The coverslip was then transferred to an imaging chamber and rinsed with Ringer solution to remove excess dye. With a pipette, 1 mL volumes of test solutions were added to an approximately 200 μL pool covering the cells, with the chamber's volume maintained by a vacuum removal line. Cells were monitored with an inverted IX17 microscope (Olympus America Inc, Center Valley, PA)