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Expression and function of purinergic $P2Y_{12}$ receptors in rat trigeminal ganglion neurons

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ABSTRACT

Purinergic receptors play key signaling roles in neuropathic pain in the orofacial region, which is innervated by trigeminal ganglion (TG) neurons. The neuropathology of purinergic $P2Y_{12}$ receptors is well characterized in glia; however, their physiological role in TG neurons remains to be fully elucidated. The present study investigated the expression and function of $P2Y_{12}$ receptors in rat TG neurons. $P2Y_{12}$ receptor immunoreactivity was intense in the soma, dendrites, and axons, and colocalized with a pan-neuronal marker, neurofilament H, isolectin B4, and substance P. In the presence of extracellular Ca^{2+}, 2-methylthio-ADP (an agonist of $P2Y_{1,12,13}$ receptors) transiently increased intracellular free Ca^{2+} concentrations ($[Ca^{2+}]_i$), an effect that was abolished by $P2Y_{12}$ receptor antagonists. In the absence of extracellular Ca^{2+}, ryanodine receptor/channel inhibitors diminished the 2-methylthio-ADP-induced increases in $[Ca^{2+}]_i$. A sarcoplasmic reticulum Ca^{2+}-ATPase (SERCA) inhibitor gradually increased $[Ca^{2+}]_i$, and after a plateau, application of 2-MeS-ADP induced a rapid and transient, but additive increase in $[Ca^{2+}]_i$. An adenylate cyclase inhibitor transiently increased $[Ca^{2+}]_i$, while a phosphodiesterase inhibitor prevented the 2-methylthio-ADP-induced increase in $[Ca^{2+}]_i$. Our study shows that $P2Y_{12}$ receptors are expressed in TG neurons, and act via a cAMP-dependent pathway to release intracellular Ca^{2+} from ryanodine-sensitive Ca^{2+} stores.

1. Introduction

Extracellular nucleotides, which are important for the transmission and/or integration of pain sensations (Burnstock, 2013), activate the P2 family of receptors, which includes ATP-gated ion channels (i.e., P2X purinergic receptors, subdivided in $P2X_1$ to $P2X_7$), and G protein-coupled receptors (i.e., P2Y receptors, subdivided in $P2Y_1$, $P2Y_2$, $P2Y_4$, $P2Y_6$, and $P2Y_{11}$ to $P2Y_{14}$). The $P2Y_{12}$ receptor has seven hydrophobic transmembrane regions linked by extracellular/intracellular loops and is activated by adenine and uracil nucleotides (ADP, UTP, UDP, and UDP-glucose). The $P2Y_{12}$ receptor generally couples to the Gi alpha subunit of the G protein, and reduces intracellular cAMP production (Abbracchio et al., 2006; Dussor et al., 2009; Moheimani and Jackson, 2012).

The relationship between $P2Y_{12}$ receptors and pain mechanisms has been mostly described in the spinal cord (Fried et al., 2001). After spinal nerve injury resulting in allodynia, the expression of $P2Y_{12}$ receptor mRNA increases in the dorsal horn of the spinal cord; however, its expression is highly restricted to microglia (Kobayashi et al., 2008; Tozaki-Saitoh et al., 2008). In the trigeminal ganglion (TG), an increased expression of $P2Y_{12}$ receptors in satellite glial cells (SGCs) has also been described following lingual nerve injury; this suggests that in the orofacial region, the activation of $P2Y_{12}$ receptors is involved in neuropathic pain induced by a peripheral nerve injury (i.e., hyperalgesia) (Katagiri et al., 2012). Thus, the functional role of $P2Y_{12}$ receptors is well described in glial cells. Although allodynia and hyperalgesia are associated with peripheral sensitization that originates from primary afferent neurons (Basbaum et al., 2009; Cervero and Laird, 1996; Ochoa, 2009; Scholz and Woolf, 2002), the expression and function of $P2Y_{12}$ receptors in the TG neurons remains to be fully elucidated.

In the present study, we analyzed the expression, localization, and physiological and pharmacological properties of $P2Y_{12}$ receptors in primary cultured TG neurons.

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2. Materials and methods

2.1. Ethical approval

All the animals were treated in accordance with the "Guiding Principles for the Care and Use of Animals in the Field of Physiological Sciences." approved by the Council of the Physiological Society of Japan, and by the American Physiological Society. This study also followed the guidelines established by the National Institutes of Health (USA) regarding the care and use of animals for experimental procedures. The "Animal Research Ethical Committee" of the Tokyo Dental College (approval No. 252502) approved all experimental procedures in this study.

2.2. Cell culture

Trigeminal ganglions (TGs) were rapidly excised from 7-day-old Wistar rats under sodium pentobarbital anesthesia (50 mg/kg) following administration of isoflurane (3.0 vol%). Cells in TGs were dissociated by enzymatic treatment with Hank's balanced salt solution (Invitrogen, Carlsbad, CA, USA) containing 20 U/mL papain (Worthington, Lakewood, NJ, USA), for 20 min at 37 °C, followed by dissociation by trituration. Dissociated TG cells were plated onto poly-L-lysine-coated 35 mm diameter culture dishes (Corning, Corning, NY, USA). The primary cultures were performed using Leibovitz's L-15 medium (Invitrogen) containing 10% fetal bovine serum (FBS), 1% penicillin-streptomycin (Invitrogen), 1% fungizone (Invitrogen), 26 mM $NaHCO_3$, and 30 mM glucose (pH 7.4). Cells were maintained in culture for 48 h at 37 °C in a humidified atmosphere containing 95% air and 5% CO_2 to allow cell attachment to the bottom of dishes. Recording solutions and drugs were applied to cells by superfusion using a pressurized (driven by N_2 gas) perfusion system (Automate Scientific, Berkeley, CA, USA) that allows a steady flow, thus avoiding unpredicted shear stress in the recording environment. Changes of solution were completed within 20 ms, using the pressurized perfusion system together with a multi-valve perfusion system (Warner Instruments, Hamden, CT, USA) and an aspirator (K.T. Labs, Saitama, Japan). When measuring $[Ca^{2+}]_i$ (see below), the temperature of solutions was maintained at 32 °C (Warner Instruments) to avoid unexpected thermal stimulation of the cells.

2.3. Immunocytochemistry

Primary TG cells were seeded and cultured on poly-L-lysine-coated coverslips (Matsunami, Osaka, Japan). TGs excised from 7-day-old Wistar rats were immersed in "optimal cutting temperature" (OCT) compound, and rapidly frozen in liquid nitrogen. These tissues were sectioned at 10-μm thickness and mounted on slides. After fixation with a mixture of 50% ethanol and 50% acetone at −20 °C for 30 min, cultured cells and cryosections were treated with 10% donkey serum at room temperature for 20 min, and then incubated overnight at 4 °C with primary antibodies (Kuroda et al., 2013). A cocktail of primary antibodies (Neuro-Chrom™ pan-neuronal marker, Millipore, Billerica, MA, USA; diluted 1:50), which contains mouse anti-neuronal nuclei (NeuN), anti-microtubule-associated protein 2 (MAP2), anti-βIII tubulin, and anti-neurofilament H (NF-H) antibodies, was used as a neuronal marker. TG cells were also incubated with either rabbit anti-NF-H antibody (Milllipore; 1:200 dilution) as an A-neuron marker, FITC-conjugated isolectin B4 antibody (IB4; 1:200 dilution) as a non-peptidergic C-neuron marker, mouse anti-substance P (SP; Alomone Labs; Jerusalem, Israel; 1:50 dilution) as a peptidergic C-neuron marker, and a rabbit anti-$P2Y_{12}$ receptor antibody (against amino acid residues 125–142 of the human $P2Y_{12}$-receptor) (Alomone Labs, Jerusalem, Israel; diluted 1:50) (Carrasquero et al., 2005; Giachini et al., 2014; Pinheiro et al., 2013). Cells and tissues were washed, and then incubated with a secondary antibody for 30 min at room temperature. The secondary antibodies included Alexa Fluor 488 donkey anti-rabbit IgG, Alexa Fluor 488 donkey anti-mouse IgG, Alexa Fluor 568 donkey anti-mouse IgG, and Alexa Fluor 568 donkey anti-rabbit IgG (Molecular Probes, Eugene, OR, USA; both diluted 1:50). In addition, 4′,6-diamino 2-phenylindole dihydrochloride (Invitrogen) was applied for 5 min at room temperature as a nuclear staining. Cells and tissues were examined under fluorescence microscopes (Zeiss, Jena, Germany).

2.4. Solutions and reagents

A standard solution containing 137 mM NaCl, 5.0 mM KCl, 2.0 mM $CaCl_2$, 0.5 mM $MgCl_2$, 0.44 mM KH_2PO_4, 0.34 mM Na_2HPO_4, 4.17 mM $NaHCO_3$, and 5.55 mM glucose (pH 7.4) was used as an extracellular solution. A high-K^+ solution (91 mM NaCl, 50 mM KCl, 2.0 mM $CaCl_2$, 0.5 mM $MgCl_2$, 0.44 mM KH_2PO_4, 0.34 mM Na_2HPO_4, 4.17 mM $NaHCO_3$, and 5.55 mM glucose, pH 7.4) was used to discern TG neurons from glial cells by the activation of depolarization-induced increases in the concentration of intracellular free Ca^{2+} in the neurons. The $P2Y_{1,12,13}$ receptor agonist 2-methylthioadenosine diphosphate trisodium salt (2-MeS-ADP), potent and selective $P2Y_{12}$ receptor antagonists AR-C66096 and PSB0739, sarcoplasmic reticulum Ca^{2+}-ATPase (SERCA) inhibitor cyclopiazonic acid (CPA), ryanodine receptor/channel inhibitor dantrolene (sodium salt), adenylate cyclase (AC) inhibitor 9-(Tetrahydro-2-furanyl)-9*H*-purin-6-amine (SQ22536), and phosphodiesterase inhibitor 3-isobutyl-1-methylxanthine (IBMX) were obtained from Tocris Bioscience (Bristol, UK). All the other reagents were purchased from Sigma Chemical Co. (St. Louis, MO, USA), except where indicated.

2.5. Measurement of intracellular free Ca^{2+} concentration ($[Ca^{2+}]_i$)

Primary cultured TG cells were loaded with 10 μM fura-2 acetoxymethyl ester (Dojindo, Kumamoto Japan) and 0.1% (w/v) pluronic acid F-127 (Invitrogen) in Hank's solution, for 90 min at 37 °C. Then, the cultured TG cells were rinsed with fresh Hank's solution, and mounted on a microscope stage (Olympus, Tokyo, Japan). The emission of the fura-2 fluorescence was measured at 510 nm in response to alternating excitation wavelengths of 340 (F340) and 380 (F380) nm using an Aquacosmos system and software (Hamamatsu Photonics, Shizuoka, Japan), which controls an excitation wavelength selector, and an intensified charge-coupled device camera system (Hamamatsu Photonics). The intracellular free Ca^{2+} concentration ($[Ca^{2+}]_i$) was measured as the fluorescence ratio of F340 and F380 ($R_{F340/F380}$), and expressed as F/F_0 units; the $R_{F340/F380}$ value (F) was normalized to the resting value (F_0).

2.6. Statistical and offline analysis

Data were expressed as the mean ± standard error (S.E.) or standard deviation (S.D.) of the mean of N observations, where N represents the number of independent experiments or cells, respectively. The data were analyzed using the following nonparametric tests: the Wilcoxon signed-rank test, the Kruskal–Wallis one-way analysis of variance followed by a Dunn's post hoc test, or the Mann–Whitney U-test. A P value of less than 0.05 was considered significant. The statistical analysis was performed using GraphPad Prism 5.0 (GraphPad Software, La Jolla, CA, USA).