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Expression and function of purinergic P2Y₁₂ 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+}]. 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.

**Keywords:** Purinergic receptor, orofacial, neuropathic pain, pain, trigeminal ganglion neuron, Ca$^{2+}$ signal
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 P2X1 to P2X7), and G protein-coupled receptors (i.e., P2Y receptors, subdivided in P2Y1, P2Y2, P2Y4, P2Y6, and P2Y11 to P2Y14). The P2Y12 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 P2Y12 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 P2Y12 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 P2Y12 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 P2Y12 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.
MATERIALS AND METHODS

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.

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 NaHCO₃, 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% CO₂ to allow cell attachment to the bottom of dishes. Recording solutions and drugs were applied to cells by superfusion using a pressurized (driven by N₂ 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 [Ca²⁺], see below), the temperature of solutions was maintained at 32°C (Warner Instruments) to avoid unexpected thermal stimulation of the cells.

**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 (Millipore; 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-P2Y₁₂ receptor antibody (against amino acid residues 125-142 of the human P2Y₁₂-receptor) (Alomone Labs, Jerusalem, Israel; diluted 1:50) (Carrasquero et al., 2005a; Giachini et al., 2014; Pinheiro et al.,
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).

**Solutions and reagents**

A standard solution containing 137 mM NaCl, 5.0 mM KCl, 2.0 mM CaCl₂, 0.5 mM MgCl₂, 0.44 mM KH₂PO₄, 0.34 mM Na₂HPO₄, 4.17 mM NaHCO₃, 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 CaCl₂, 0.5 mM MgCl₂, 0.44 mM KH₂PO₄, 0.34 mM Na₂HPO₄, 4.17 mM NaHCO₃, 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 Ca²⁺ in the neurons. The P2Y₁₂,₁₃ receptor agonist 2-methylthioadenosine
diphosphate trisodium salt (2-MeS-ADP), potent and selective P2Y\textsubscript{12} receptor antagonists AR-C66096 and PSB0739, sarcoplasmic reticulum Ca\textsuperscript{2+}-ATPase (SERCA) inhibitor cyclopiazonic acid (CPA), ryanodine receptor/channel inhibitor dantrolene (sodium salt), adenylate cyclase (AC) inhibitor 9-((Tetrahydro-2-furanyl)-9H-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, U.S.A.), except where indicated.

**Measurement of intracellular free Ca\textsuperscript{2+} concentration ([Ca\textsuperscript{2+}]\textsubscript{i})**

Primary cultured TG cells were loaded with 10 µM fura-2 acetoxyethyl 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\(^{2+}\)]) was measured as the fluorescence ratio of F340 and F380 (RF\(_{340/380}\)), and expressed as F/F\(_0\) units; the RF\(_{340/380}\) value (F) was normalized to the resting value (F\(_0\)).

**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).

The influence of each pharmacological agent on the changes in [Ca\(^{2+}\)]\(_i\) was analyzed using Origin 8.5 (OriginLab Corporation, Northampton, MA, USA) by fitting the data to the following function using a single-binding model.
\( F/F_0 = \left[ \left( F/F_{\text{int}} - F/F_{\text{fin}} \right) / (1 + ([x]_0/K) \right] + F/F_{\text{fin}} \)

where \( K \) is the equilibrium binding constant, \([x]_0\) indicates the applied concentration of pharmacological agents, and \( F/F_{\text{int}} \) and \( F/F_{\text{fin}} \) are the initial and final \( F/F_0 \) responses, respectively.
RESULTS

Characterization of cultured TG neurons

In brightfield image of the primary cultured rat TG cells (Fig. 1A), it was difficult to distinguish the neurons from the SGCs. In order to specifically reveal the neuronal responses, \([\text{Ca}^{2+}]_i\) in fura-2-loaded primary cultured TG cells was measured by the application of a solution containing a high concentration of \(K^+\) (50 mM \(K^+\)), which induces membrane depolarization. A series of applications of the high \(K^+\) solution induced transient increases in \([\text{Ca}^{2+}]_i\) in 41.0% of the tested cultured TG cells (red lines and bar), while the other 59.0% of the TG cells showed no response (orange lines and bar) (Figs. 1B, 1C and 1D). The cells showing \([\text{Ca}^{2+}]_i\) increases induced by depolarization were identified as TG neurons (red line in Fig. 1B). Application of 2-MeS-ADP produced a rise in \([\text{Ca}^{2+}]_i\), not only in neurons exhibiting depolarization-induced \([\text{Ca}^{2+}]_i\) increases (red in Fig. 1E), but also in other cell populations that showed no response to the high \(K^+\) solution (orange in Fig. 1E). The peak increase in \([\text{Ca}^{2+}]_i\), elicited by 2-MeS-ADP was \(1.5 \pm 0.02\ F/F_0\) units in TG neurons and \(2.2 \pm 0.7\ F/F_0\) units in other cell populations, i.e., SGCs. The \([\text{Ca}^{2+}]_i\) increases in satellite glial cells (SGCs) were significantly greater than those in neurons (Fig. 1F). Thus, in the present study, a
50-mM K⁺ extracellular solution was applied at the end of each experiment to confirm that results were obtained from a pure population of TG neurons.

**Immunolocalization of P2Y₁₂ receptors in the TG neurons**

The cultured TG neurons showed positive immunoreactivity to a neuronal marker cocktail (Neuro-Chrom™ pan-neuronal marker), which contained mouse anti-NeuN, anti-MAP2, and anti-βIII tubulin antibodies (Fig. 2A; see also Fig. 4A). Positive immunoreactivity was also observed to either substance P (SP; as a peptidergic C-neuron marker; Fig. 2D) or isolectin B4 (IB4; as a non-peptidergic C-neuron marker; Fig. 2G). Intense P2Y₁₂ receptor immunoreactivity was present in the soma, dendrites, and axons, as well as in the perinuclear region of primary cultured TG cells (Figs. 2B, 2E, 2H, 2K, and 4B). These P2Y₁₂ receptor immunoreactivities colocalized with neurons positive for the pan-neuronal marker (Fig. 2C), SP (Fig. 2F), and IB4 (Fig. 2I). In addition, positive immunoreactivity was observed to NF-H (an A-neuron marker; Fig. 2J), and to the P2Y₁₂ receptor in cells that morphologically resembled NF-H-positive TG neurons (Fig. 2K).

In cryosections, TG neurons showed positive immunoreactivity to the
pan-neuronal marker (Fig. 3A), SP (Fig. 3D), IB4 (Fig. 3G), and NF-H (Fig. 3J).

We also observed intense P2Y\textsubscript{12} receptor immunoreactivity in the TG cell bodies (Figs. 3B, 3E, 3H, and 3K). These P2Y\textsubscript{12} receptor immunoreactivities colocalized with neurons positive for the pan-neuronal marker (Fig. 3C), SP (Fig. 3F), and IB4 (Fig. 3I). Notably, NF-H and P2Y\textsubscript{12} receptor antibodies were produced from the same host. Both NF-H and P2Y\textsubscript{12} receptor immunoreactivity was identified in TG neurons (Figs. 3J and 3K).

In cultured cells (Figs. 4A to 4C) and cryosections (Figs. 4D to 4F), positive immunoreactivity to the P2Y\textsubscript{12} receptor was visible in pan-neuronal marker-positive neurons (Fig. 4) and pan-neuronal marker-negative non-neuronal cells (arrowheads in Figs. 4B, 4C, 4E, and 4F).

2-MeS-ADP increases $[\text{Ca}^{2+}]_i$ in cultured TG neurons

We analyzed the changes in $[\text{Ca}^{2+}]_i$ during the application of 2-MeS-ADP, which is a P2Y\textsubscript{1,12,13} receptor agonist. In the presence of extracellular $\text{Ca}^{2+}$ (2.0 mM), addition of five different concentrations of 2-MeS-ADP (1, 10, 50, 100, and 500 nM) induced rapid and transient increases in $[\text{Ca}^{2+}]_i$, in a concentration-dependent manner (Fig. 5A). A semilogarithmic plot (Fig. 5B)
illuminates $F/F_0$ values as a function of the applied concentration of 2-MeS-ADP, with an equilibrium-binding constant of 20 nM.

**P2Y_{12}-selective antagonists inhibit 2-MeS-ADP-induced increases in the $[\text{Ca}^{2+}]_i$ in the TG neurons**

In the presence of 2.0 mM extracellular $\text{Ca}^{2+}$, the increases in $[\text{Ca}^{2+}]_i$ induced by 2-MeS-ADP were inhibited by various concentrations of the selective P2Y_{12} receptor antagonists AR-C66096 (Fig. 6A) and PSB0739 (Fig. 6C), in a dose-dependent manner. The equilibrium binding constants, represented as the half maximal inhibitory concentrations (IC_{50}), were obtained at an AR-C66096 concentration of 6.0 nM (Fig. 6B) and a PSB0739 concentration of 1.0 nM (Fig. 6D).

**Effects of inhibitors of the ryanodine receptor/channel and SERCA on $[\text{Ca}^{2+}]_i$ in TG neurons**

In the presence of extracellular $\text{Ca}^{2+}$ (2.0 mM), the application of 2-MeS-ADP (50 nM) evoked transient increases in $[\text{Ca}^{2+}]_i$ (Figs. 7A to 7D) to peak values of $1.7 \pm 0.06$ $F/F_0$ units in the first application, and $1.6 \pm 0.06$ $F/F_0$
units in the second application (Fig. 7D). After completely removing Ca\(^{2+}\) from the extracellular solution, the repeated addition of 2-MeS-ADP (50 nM) also produced rapid and transient increases in [Ca\(^{2+}\)]\(_i\) (Fig. 7A), reaching a peak value of 1.5 ± 0.07 \(F/F_0\) units in the first application, while the peak value in the second application was significantly lower (Figs. 7A and 7D). Application of the SERCA inhibitor CPA (100 nM) gradually increased [Ca\(^{2+}\)]\(_i\) to a value of 1.6 ± 0.03 \(F/F_0\) units (Figs. 7B and 7D). After the CPA-induced [Ca\(^{2+}\)]\(_i\) increase had reached a plateau, subsequent application of 2-MeS-ADP resulted in a rapid and transient, but additive increase in [Ca\(^{2+}\)]\(_i\) (Figs. 7B and 7D). Preincubation of TG neurons with a ryanodine receptor/channel inhibitor (1.0 μM dantrolene sodium salt; dantrolene), in the absence of external Ca\(^{2+}\), significantly and almost completely abolished 2-MeS-ADP-induced [Ca\(^{2+}\)]\(_i\) increases to an \(F/F_0\) value of 1.1 ± 0.02 (Figs. 7C and 7D).

**The increase in [Ca\(^{2+}\)]\(_i\) induced by the activation of P2Y\(_{1,12,13}\) receptors is mediated by a decrease in intracellular cAMP**

To elucidate the intracellular signaling pathway mediated by the activation of the P2Y\(_{12}\) receptor in the TG neurons, we examined the effects of
the adenylate cyclase inhibitor SQ22536, and the phosphodiesterase inhibitor IBMX. Application of five different concentrations (0.01, 0.1, 1.0, 10, 100 μM) of SQ22536 elicited rapid and transient increases in [Ca\(^{2+}\)]\(_{i}\), in a concentration-dependent manner (Fig. 8A). A semilogarithmic plot (Fig. 8B) illustrates the \(F/F_0\) values as a function of the applied SQ22536 concentrations, with an EC\(_{50}\) of 0.08 μM. In the presence of extracellular Ca\(^{2+}\), 50 μM IBMX significantly and reversibly inhibited the increases in [Ca\(^{2+}\)]\(_{i}\) induced by 2-MeS-ADP (1.4 ± 0.05 \(F/F_0\) units) (Figs. 8C and 8D).
DISCUSSION

Previous work has shown that the mRNA of all the P2Y receptor subtypes (P2Y₁, P2Y₂, P2Y₄, P2Y₆, P2Y₁₁ to P2Y₁₄) is expressed in murine TG neurons (Villa et al., 2010b). However, in rat TG neurons, RT-PCR analysis revealed the mRNA expression of only P2Y₁, P2Y₂, P2Y₄, and P2Y₆ receptors (Li et al., 2014; Ruan and Burnstock, 2003). The present results clearly indicate that P2Y₁₂ receptors are functionally expressed in rat TG neurons, with localization to the soma, axons, and dendrites in A-neurons, non-peptidergic C-neurons, and peptidergic C-neurons. The activation of not only P2Y₁ and P2Y₁₃, but also P2Y₁₂ receptors, elicited mobilization of intracellular Ca²⁺ in the TG neurons. Although only 40% of the primary TG cells showed depolarization-induced increases in [Ca²⁺], all the Ca²⁺ responses in this study were analyzed using cells that exhibited [Ca²⁺] increases on depolarization, which indicates that the data were obtained from neurons, not glial cells.

To examine Ca²⁺ signaling following the activation of the P2Y₁₂ receptor, we utilized 2-MeS-ADP as an agonist. In general, there is a consensus on the lack of a selective agonist for the P2Y₁₂ receptor. As an agonist, 2-MeS-ADP has been shown to have a high affinity for P2Y₁,₁₂,₁₃ receptors (Kügelgen, 2008),
and has been commonly used to evaluate the functional properties of P2Y₁₂ receptors (Bodor et al., 2003; Pausch et al., 2004; Simon et al., 2002). Moreover, recent studies have reported that 2-MeS-ADP acts as a full agonist for the P2Y₁₂ receptor by binding to its pocket-like structure; a 2.5 Å structure of P2Y₁₂ receptor binds to the full agonist 2-MeS-ADP (J. Zhang et al., 2014a; K. Zhang et al., 2014b). The P2Y₁₂ receptor antagonist AR-C66096 displayed the same binding conformation as that of the P2Y₁₂ receptor/2-MeS-ADP complex. In the present study, application of 2-MeS-ADP increased [Ca²⁺]ᵢ, while the selective P2Y₁₂ receptor antagonists AR-C66096 and PSB0739 inhibited [Ca²⁺]ᵢ increases in a dose-dependent manner. In addition, selective antagonists of the P2Y₁₂ receptor almost completely abolished the increase in [Ca²⁺]ᵢ induced by 2-MeS-ADP, making it probable that a major component of the [Ca²⁺]ᵢ increase was mediated by P2Y₁₂ receptor activation.

We also observed that pan-neuronal marker-negative cells, which are located around TG neurons, were positive for P2Y₁₂ receptor immunoreactivity and displayed significantly larger 2-MeS-ADP-induced [Ca²⁺]ᵢ increases compared with neurons. These findings were in accordance with previous studies demonstrating that glial cells in the TG express the P2Y₁₂ receptor
(Ceruti et al., 2008; Katagiri et al., 2012; Villa et al., 2010a). Although the P2Y<sub>12</sub> receptor-expressing non-neuronal cells in this study were considered to be glial cells in the TG, Ca<sup>2+</sup> responses were not further analyzed.

In the present study, the increase in [Ca<sup>2+</sup>]<sub>i</sub> induced by 2-MeS-ADP in TG neurons in the absence of extracellular Ca<sup>2+</sup> was sensitive to ryanodine receptor/channel inhibitor. Application of the AC inhibitor SQ22536 increased the amplitude of the transient increases in [Ca<sup>2+</sup>]<sub>i</sub> in a concentration-dependent manner, while the phosphodiesterase inhibitor IBMX, significantly and reversibly inhibited the increases in [Ca<sup>2+</sup>]<sub>i</sub> induced by 2-MeS-ADP in TG neurons. These results indicate that the decrease in intracellular cAMP levels, produced by the suppression of AC following the activation of 2-MeS-ADP-sensitive P2Y receptors (including P2Y<sub>12</sub>), increases [Ca<sup>2+</sup>]<sub>i</sub>. The activation of P2Y<sub>12</sub> receptors reduces intracellular cAMP levels in both excitable and non-excitable cells (Kubista et al., 2003; Moskvina et al., 2003; Simon et al., 2002; Unterberger et al., 2002). Furthermore, the decrease in cAMP levels caused by P2Y<sub>12</sub> receptor activation, and the subsequent increases in [Ca<sup>2+</sup>]<sub>i</sub> are responsible for mediating platelet aggregation (Moheimani and Jackson, 2012).

However, it has been demonstrated that activation of cAMP-dependent
protein kinase (protein kinase A; PKA) positively modulates both ryanodine receptors/channels and SERCA on the Ca\(^{2+}\) store, to increase [Ca\(^{2+}\)]\(_i\) and accelerate Ca\(^{2+}\) reuptake, respectively (Lymperopoulos et al., 2013). In the present study, a SERCA inhibitor, which inhibits Ca\(^{2+}\) reuptake, also elicited gradual [Ca\(^{2+}\)]\(_i\) increases; however, subsequent application of 2-MeS-ADP produced an additional rapid and transient increase in [Ca\(^{2+}\)]\(_i\), as shown in Fig. 7. These results indicated that each 2-MeS-ADP-sensitive P2Y receptor activation and each SERCA inhibition acts independently to increase [Ca\(^{2+}\)]. Taken together, our results suggest that P2Y receptor activation in TG neurons decreases intracellular cAMP levels. The reduction in cAMP triggers an increase in [Ca\(^{2+}\)]\(_i\) without the contribution of SERCA inhibition. In addition, pre-treatment of TG neurons with dantrolene, a ryanodine receptor/channel inhibitor, suppressed 2-MeS-ADP-induced Ca\(^{2+}\) release in TG neurons. Notably, dantrolene also inhibits the ionotropic glutamate receptor (i.e., N-methyl-D-aspartate receptor) (Hayashi et al., 1997; Rossi et al., 2012; Salinska et al., 2008); however, the effect of dantrolene on the 2-MeS-ADP-induced Ca\(^{2+}\) release was examined in the absence of extracellular Ca\(^{2+}\), to avoid the contribution of Ca\(^{2+}\) influx. Thus, we suggest that the activation
of P2Y_{12} receptors induced Ca^{2+} release from intracellular stores via ryanodine receptors/channels, by a decrease in cAMP production via intracellular AC in the TG neurons. Further investigation will be needed to elucidate the detailed mechanism of cAMP-dependent Ca^{2+} release via ryanodine receptors/channels.

Each of the 2-MeS-ADP-sensitive P2Y receptors binds to a single heterotrimeric G protein. The Gq-coupled P2Y\textsubscript{1} receptor activates isoforms of the phospholipase C (PLC), leading to the formation of the second messengers 1,2-diacylglycerol and inositol 1,4,5-trisphosphate (IP\textsubscript{3}); this mobilizes Ca^{2+} via an IP\textsubscript{3}-dependent pathway (Abbracchio et al., 2006). On the other hand, the Gi/o-coupled P2Y\textsubscript{12} and P2Y\textsubscript{13} receptors inhibit the AC and activate the phosphoinositide 3-kinase (Gachet et al., 1997; Murugappa and Kunapuli, 2006). P2Y\textsubscript{13} receptor has also been reported to couple to G alpha\textsubscript{16} and stimulate PLC (Kim et al., 2005). Although the increase in [Ca^{2+}]_i induced by the Gi protein was associated with AC and cAMP, Gi protein subunits, including the Gi_{\beta\gamma} protein, also activate PLC\textsubscript{p} subtypes and [Ca^{2+}]_i-increasing pathways via the IP\textsubscript{3} receptor (Bugrim, 1999; Smrcka and Sternweis, 1993). Recently, it has been reported that Gi/o-coupled receptors activate the PLC pathway in dystrophin-deficient mouse muscle cells (Balghi et al., 2006), Chinese hamster ovary cells (Langer and
Robberecht, 2005), and human airway smooth muscle cells (Mizuta et al., 2011).
A P2Y<sub>13</sub>-like receptor was coupled to PLC stimulation and AC inhibition in rat cerebellar astrocytes (Carrasquero et al., 2005b). In the present study, although pre-application of dantrolene almost completely inhibited 2-MeS-ADP-induced Ca<sup>2+</sup> release in TG neurons, a small residual component of [Ca<sup>2+</sup>]<sub>i</sub> increase was detected during dantrolene application. Thus, the contribution of the PLC signaling cascade to the 2-MeS-ADP-induced Ca<sup>2+</sup> mobilization in TG neurons could not be excluded; however, Ca<sup>2+</sup> mobilization by 2-MeS-ADP may be mediated by a cAMP-dependent Gi/o pathway rather than a PLC-coupling Gq pathway.

In conclusion, our results showed that the TG neurons express P2Y<sub>12</sub> receptors. An agonist for the P2Y<sub>12</sub> receptor, 2-MeS-ADP, activates [Ca<sup>2+</sup>]<sub>i</sub> increase via activation of intracellular Ca<sup>2+</sup> releasing pathway through the ryanodine receptors/channels located in the Ca<sup>2+</sup> store, in cooperation with P2Y<sub>1</sub>, P2Y<sub>12</sub> and P2Y<sub>13</sub> receptors. These pathways that increase [Ca<sup>2+</sup>]<sub>i</sub> are activated by inhibition of intracellular cAMP production, and we suggest that the P2Y<sub>12</sub> receptor in TG neurons couples with a cAMP-dependent pathway. Gi-coupled P2Y receptors play an important regulatory role in the inhibition of nociceptive
signaling (Malin and Molliver, 2010); thus, P2Y_{12} receptors in TG neurons could also be involved in pain signaling in the oral and maxillofacial regions.
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FIGURE LEGENDS

Figure 1: Characterization of primary cultured TG cells from neonatal Wistar rats.

(A) Phase-contrast image shows primary cultures of TG cells. Cells were cultured for 48 h and contained neurons, glial cells, and fibroblasts; Scale bar: 50 μm. (B) Image of high magnification shows neurons (red) eliciting depolarization-induced \([\text{Ca}^{2+}]_{i}\) increases and other cell populations (orange). Scale bar: 20 μm. (C) In the presence of 2.0 mM extracellular \(\text{Ca}^{2+}\) (lower white box), application of 50 mM KCl solution (upper gray boxes) increased \([\text{Ca}^{2+}]_{i}\), indicating that the recorded cells were TG neurons (red solid line in B and C). In contrast, depolarization-induced \([\text{Ca}^{2+}]_{i}\) increases were not observed in some cell populations (orange solid line in B and C). (D) Summary bar graph displays the normalized number of cells tested and the presence or absence of depolarization-induced \([\text{Ca}^{2+}]_{i}\) increases. The percentage of cells responding to the application of a 50-mM KCl solution was 41.0% (red column; neurons), and these cells formed a neuronal cell population. Other cell populations (orange column; 59.0%) did not respond to 50 mM K\(^+\). Data represent the mean ± S.D. of the indicated number of independent experiments (in parentheses). (E) In the
presence of 2.0 mM extracellular Ca\textsuperscript{2+} (lower white box), application of 50 nM 2-MeS-ADP (upper black boxes) increased [Ca\textsuperscript{2+}]\textsubscript{i} in TG neurons that elicited depolarization-induced [Ca\textsuperscript{2+}]\textsubscript{i} increases (red solid line), and other cell populations (orange solid line). Gray box indicates the application of the 50 mM KCl solution. (F) Summary bar graph of [Ca\textsuperscript{2+}]\textsubscript{i} increases following the application of 50 nM 2-MeS-ADP in neurons (red column) and other cell populations (orange column). Each column denotes the mean ± S.E. of the indicated number of independent experiments (in parentheses). The statistical significance between columns (shown by solid lines) is indicated by asterisks: * \( p < 0.05 \).

**Figure 2**: Immunolocalization of P2Y\textsubscript{12} receptors in primary cultured TG neurons.

(A) Primary cultured TG neurons positive for the pan-neuronal marker. (B, E, H and K) P2Y\textsubscript{12} receptor immunoreactivity. (C) Triple immunofluorescence staining with antibodies against P2Y\textsubscript{12} receptors (green) and pan-neuronal marker (red). Nuclei are shown in blue. (D) Positive immunoreactivity to SP as a peptidergic C-neuron marker in primary cultured TG neurons. (F) Triple staining
with antibodies against P2Y$_{12}$ receptors (green) and SP (red). Nuclei are shown in blue. (G) Positive immunoreactivity to IB4 as a non-peptidergic C-neuron marker in primary cultured TG neurons. (I) Triple staining with antibodies against P2Y$_{12}$ receptors (red) and IB4 (green). Nuclei are shown in blue. (J) Positive immunoreactivity to NF-H as an A-neuron marker in primary cultured TG neurons. NF-H positive cells exhibited large soma with axons spreading in multiple directions. (K) Neurons of the morphology shown in (J) were also positive for P2Y$_{12}$ receptor immunoreactivity (green). No fluorescence was detected in the negative control (data not shown). Scale bars: 50 μm.

**Figure 3**: Immunolocalization of P2Y$_{12}$ receptors in the soma of TG neuron cryosections.

(A) TG neurons positive for the pan-neuronal marker. (B, E, H and K) P2Y$_{12}$ receptor immunoreactivity. (C) Triple immunofluorescence staining with antibodies against P2Y$_{12}$ receptors (green) and pan-neuronal marker (red). Nuclei are shown in blue. (D) Positive immunoreactivity to SP as a peptidergic C-neuron marker in TG neurons. (F) Triple staining with antibodies against P2Y$_{12}$ receptors (green) and SP (red). Nuclei are shown in blue. (G) Positive
immunoreactivity to IB4 as a non-peptidergic C-neuron marker in TG neurons. (I) Triple staining with antibodies against P2Y$_{12}$ receptors (red) and IB4 (green). Nuclei are shown in blue. (J) Positive immunoreactivity to NF-H as an A-neuron marker in TG neurons. Colocalization of P2Y$_{12}$ receptor and NF-H immunoreactivity could not be examined because both proteins were produced from the same host. No fluorescence was detected in the negative control (data not shown). Scale bars: 20 μm.

**Figure 4:** Neuron marker-negative TG cells exhibited P2Y$_{12}$ receptor immunoreactivity.

(A) Primary cultured TG neurons positive for the pan-neuronal marker. (B) P2Y$_{12}$ receptor immunoreactivity was observed in both pan-neuronal marker-positive and pan-neuronal marker-negative cells (arrow heads). (C) Triple immunofluorescence staining with antibodies against P2Y$_{12}$ receptors (green) and pan-neuronal marker (red). Nuclei are shown in blue. (D) Neurons in a TG cryosection positive for the pan-neuronal marker. (E) P2Y$_{12}$ receptor immunoreactivity was observed in both pan-neuronal marker-positive and pan-neuronal marker-negative cells (arrow heads). (F) Triple
immunofluorescence staining with antibodies against P2Y_{12} receptors (green) and the pan-neuronal marker (red). Nuclei are shown in blue. Scale bars: 20 μm. Notably, the existence of P2Y_{12} receptor-expressing neuron marker-negative TG cells confirms the specificity of the P2Y_{12} receptor antibody, as a positive control for the results shown in Figs. 2 and 3, because expression of the P2Y_{12} receptor in glial cells has been widely reported (see text).

**Figure 5:** 2-MeS-ADP induces changes in [Ca^{2+}]_{i} in the TG neurons.

(A) Example of transient increases in [Ca^{2+}]_{i} following the application of a series of concentrations of 2-MeS-ADP (1–500 nM; upper white boxes) in the presence of extracellular Ca^{2+} (2.0 mM). Application of a 50 mM KCl solution is shown by a gray box. (B) The data points illustrate the $F/F_{0}$ values as a function of the applied concentration of 2-MeS-ADP. Each data point represents mean ± S.E. of eight independent experiments (numbers in parentheses represent the number of cells tested). The curve on the semilogarithmic scale was fitted according to *Equation 1* described in the text. The equilibrium binding constant for 2-MeS-ADP was 20 nM. Notably, the dose-dependent relationship between 2-MeS-ADP and [Ca^{2+}]_{i} increases displayed a good fit to the function when using
the single-binding model (Equation 1).

**Figure 6:** Pharmacological identification of P2Y$_{12}$ receptors in the TG neurons.

(A and C) Dose-dependent inhibitory effect of the selective P2Y$_{12}$ receptors antagonists AR-C66096 (A) and PSB0739 (C), on increases in [Ca$^{2+}$]$_i$ induced by 2-MeS-ADP in the TG neurons. Example of transient increases in [Ca$^{2+}$]$_i$ by 50 nM 2-MeS-ADP (upper white boxes), with (black boxes) or without various concentrations of AR-C66096 (1–1000 nM; A) and PSB0739 (1–1000 nM; C), in the presence of extracellular Ca$^{2+}$ (2.0 mM). Times of application of the 50 mM KCl solution are shown in gray boxes. (B and D) Dose-response relations for the inhibitory effects of AR-C66096 (B) and PSB0739 (D) on the increases in [Ca$^{2+}$]$_i$ induced by 2-MeS-ADP. The data points in each figure illustrate $F/F_0$ values as a function of the applied concentration of the inhibitors, and represent the mean ± S.E. of eight experiments (numbers in parentheses represent the number of cells tested). The curve on the semilogarithmic scale was fitted according to Equation 1 described in the text. Notably, the relationship between the dose of P2Y$_{12}$ receptor antagonists (AR-C66096 and PSB0739) and 2-MeS-ADP-induced [Ca$^{2+}$]$_i$ increases was a good fit to the function using a
single-binding model (Equation 1).

Figure 7: Effects of ryanodine receptor/channel and sarcoplasmic reticulum Ca\(^{2+}\)-ATPase inhibitors on [Ca\(^{2+}\)].

(A) Example of transient increases in [Ca\(^{2+}\)], following the application of 50 nM 2-MeS-ADP, with (lower gray boxes) or without (lower white box), extracellular Ca\(^{2+}\) (2.0 mM). (B) In the presence of external Ca\(^{2+}\), additions of 50-nM 2-MeS-ADP increased [Ca\(^{2+}\)]. Application of 100 nM of a sarcoplasmic reticulum Ca\(^{2+}\)-ATPase (SERCA) inhibitor (cyclopiazonic acid; CPA) gradually elicited an increase in [Ca\(^{2+}\)], and subsequent application of 2-MeS-ADP (50 nM) induced a further transient [Ca\(^{2+}\)] increase. (C) The increase in [Ca\(^{2+}\)] induced by 50 nM 2-MeS-ADP was inhibited by the application of a 1.0 μM dantrolene in the absence of external Ca\(^{2+}\). (A to C) Upper white and black boxes indicate the timing of application of 2-MeS-ADP and the other inhibitors (CPA and dantrolene), respectively. Upper gray boxes indicate the application of the 50 mM KCl solution. (D) Summary bar graph shows increases in [Ca\(^{2+}\)], following the first (upper column) and second (second column from upper) application of 50 nM 2-MeS-ADP, as well as the application of 100 nM CPA.
without (third column from upper) and with (fourth column from upper) 50 nM 2-MeS-ADP in the presence of external Ca^{2+} (2.0 mM) (gray boxes on the right side). $F/F_0$ values after first (fifth column from top) and second (sixth column from top) application of 50 nM 2-MeS-ADP, as well as application of 50 nM 2-MeS-ADP with 1.0 μM dantrolene (seventh column from top) in the absence of external Ca^{2+} (white box on the right side) are shown. After-recovery effect is shown in the eighth column from the top. Each column denotes the mean ± S.E. of the indicated number of independent experiments (in parentheses). Statistical significance between columns (shown by solid lines) is indicated by asterisks: * $p < 0.05$.

**Figure 8:** Intracellular cAMP levels modulate the increases in [Ca^{2+}]\textsubscript{i} induced by 2-MeS-ADP in the TG neurons.

(A) Example of transient increases in [Ca^{2+}]\textsubscript{i} following the application of SQ22536 (0.01-100 μM) (upper white boxes) with external Ca^{2+} (2.0 mM). (B) The data points illustrate $F/F_0$ values as a function of the applied SQ22536 concentration, and represent the mean ± S.E. of five independent experiments (numbers in parentheses represent the number of cells tested). The curve on the
semilogarithmic scale was fitted according to Equation 1 described in the text, and the equilibrium binding constant of SQ22536 was 0.08 μM. (C) Example of transient increases in [Ca^{2+}]_{i} during the application of 50 nM 2-MeS-ADP (upper white boxes), with (upper black box), or without 50 μM IBMX, in the presence of external Ca^{2+} (2.0 mM). (D) Summary bar graph of the increases in [Ca^{2+}]_{i} following the application of 50 nM 2-MeS-ADP, with (bottom black column), or without IBMX (top white column). Each column denotes the mean ± S.E. of the indicated number of independent experiments (in parentheses). Gray boxes indicate the application of the 50 mM KCl solution (A and C). The statistical significance between columns (shown by solid lines), is indicated by asterisks: * \( p < 0.05 \).
Figure 4

Pan neuronal marker

P2Y12 receptor

Pan neuronal marker + P2Y12 receptor

Pan neuronal marker

P2Y12 receptor

Pan neuronal marker + P2Y12 receptor
Figure 5

(A) 

(F/F₀) vs. Time (s)

- 2.0 mM [Ca²⁺]₀

- 1, 10, 50, 100, 500 nM [2-MeS-ADP]₀

- 50 mM [K⁺]₀

(B) 

(F/F₀) vs. [2-MeS-ADP]₀ (nM)

EC₅₀ = 20 nM

n = 8
Figure 6

A

\[ \frac{F}{F_0} \]

Time (s)

2.0 mM [Ca^{2+}]_o

1 10 100 1000 nM [AR-C66096]_o

50 nM [2-MeS-ADP]_o

50 mM [K^+]_o

B

\[ \frac{F}{F_0} \]

[AR-C66096]_o (nM)

IC_{50} = 6.0 nM

n = 8

C

\[ \frac{F}{F_0} \]

Time (s)

2.0 mM [Ca^{2+}]_o

1 10 100 1000 nM [PSB0739]_o

50 nM [2-MeS-ADP]_o

50 mM [K^+]_o

D

\[ \frac{F}{F_0} \]

[PSB0739]_o (nM)

IC_{50} = 1.0 nM

n = 8
Figure 7

A

B

C

D

Application of
1st 50 nM 2-MeS-ADP
2nd 50 nM 2-MeS-ADP
100 nM CPA
100 nM CPA + 50 nM 2-MeS-ADP
1st 50 nM 2-MeS-ADP
2nd 50 nM 2-MeS-ADP
50 nM 2-MeS-ADP + 1.0 μM Dantrolene
After recovery

[Ca^{2+}]_o (mM)

F/F_0

0 400 800 1200 1600 2000

Time (s)
Figure 8

(A) Graph showing the effect of different concentrations of SQ22536 on calcium influx over time. The x-axis represents time (s), and the y-axis represents F/F₀.

(B) Graph showing the EC₅₀ value of SQ22536. The x-axis represents the concentration of SQ22536 (μM), and the y-axis represents F/F₀. The EC₅₀ is 0.08 μM, with n = 5.

(C) Graph showing the effect of 50 nM 2-MeS-ADP, 50 μM IBMX, and 50 mM K⁺ on calcium influx. The x-axis represents time (s), and the y-axis represents F/F₀.

(D) Graph showing the effect of 50 nM 2-MeS-ADP with 50 μM IBMX on calcium influx. The x-axis represents F/F₀, and the y-axis represents different concentrations. A symbol indicating a p-values less than 0.05 is present.