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$P2Y_{12}$ and bradykinin B_2 receptor activation attenuates cAMP-mediated inhibitory effects on intracellular Ca^{2+} release via ryanodine receptor channels in trigeminal ganglion neurons

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Abstract

Receptor expression for ATP and bradykinin (BK), which are inflammatory substances, remains to be fully elucidated in trigeminal ganglion (TG) neurons. We investigated functional expression of purinergic $P2Y_{12}$ receptors and BK, B_1 and B_2, receptors in primary cultured rat TG neurons. We observed intense $P2Y_{12}$ receptor immunoreactivity in somata, dendrites, and axons, which colocalized with a pan-neuronal marker, neurofilament H, isolectin B4, and substance P. B_1 receptors were also expressed in somata, while B_2 receptors were present in somata, dendrites, axons, and in the perinuclear region. In the presence and absence of extracellular Ca^{2+} ($[Ca^{2+}]_o$), a $P2Y_{12}$ receptor agonist 2-methylthio-ADP (2-MeS-ADP) transiently increased intracellular free Ca^{2+} concentration ($[Ca^{2+}]_i$), which was sensitive to $P2Y_{12}$ receptor antagonists. Sarcoplasmic reticulum Ca^{2+}-ATPase and ryanodine receptor channel inhibitors reduced 2-MeS-ADP-induced $[Ca^{2+}]_i$ increases in the absence of $[Ca^{2+}]_o$. BK also increased $[Ca^{2+}]_i$ in the presence and absence of $[Ca^{2+}]_o$, which was sensitive to a B_2 receptor antagonist but not to a B_1 receptor antagonist. BK-induced $[Ca^{2+}]_i$ increases were facilitated by 2-MeS-ADP and suppressed by $P2Y_{12}$ receptor antagonists. A B_2 receptor antagonist also inhibited 2-MeS-ADP-induced $[Ca^{2+}]_i$ increases. An adenylate cyclase inhibitor

induced transient $[Ca^{2+}]_i$ increases, while a phosphodiesterase inhibitor inhibited both 2-MeS-ADP-induced and BK-induced $[Ca^{2+}]_i$ increases. Thus, $P2Y_{12}$ receptor activation induced Ca^{2+} release from ryanodine-sensitive Ca^{2+} stores. B_2 receptor activation mobilized $[Ca^{2+}]_i$ from intracellular stores with partial Ca^{2+} influx from the extracellular medium. Function of $P2Y_{12}$ and B_2 receptors interaction attenuates cAMP-mediated inhibitory effects on Ca^{2+} release from ryanodine-sensitive stores in TG neurons.

Keywords: Purinergic receptor, bradykinin receptor, neuropathic pain, pain, trigeminal ganglion neuron

Introduction

Nucleotide receptors are important for the transmission and/or integration of pain sensations (Burnstock, 2013). Extracellular nucleotides activate the P2 family of receptors, which include ATP-gated ionic channels (P2X purinergic receptors: subdivided into $P2X_1$ to $P2X_7$) and guanosine nucleotide-binding protein (G protein) coupled receptors (P2Y receptors: subdivided into $P2Y_1$, $P2Y_2$, $P2Y_4$, $P2Y_6$, and $P2Y_{11}$ to $P2Y_{14}$). The $P2Y_{12}$ receptor is activated by adenine and uracil nucleotides (ADP, UTP, UDP, and UDP-glucose), and consists of seven hydrophobic transmembrane regions linked with extracellular/intracellular loops. The $P2Y_{12}$ receptor generally couples to G protein alpha subunit of Giα 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 nervous system (Fried et al.,2001). After spinal nerve injury, showing allodynia behavior, $P2Y_{12}$ receptor mRNA expression increases in the spinal dorsal horn; however, expression is highly restricted to microglia (Kobayashi et al., 2008; Tozaki-Saitoh et al.,2008). In the

trigeminal ganglion (TG), increased $P2Y_{12}$ receptor expression in satellite glial cells (SGCs), but not in neurons, has also been described following lingual nerve injury, suggesting that $P2Y_{12}$ receptor activation is involved in peripheral nerve injury-induced neuropathic pain, as hyperalgesia, in the orofacial region (Katagiri et al.,2012).

Neuropathic pain, including hyperalgesia and allodynia, is associated with peripheral and central sensitization and developing and increasing chronic pain (Cervero and Laird, 1996; Ochoa, 2009; Scholz and Woolf, 2002). Inflammation-associated changes in the chemical environment surrounding axons causes peripheral sensitization (Basbaum et al., 2009). Tissue damage results in an accumulation of endogenous chemical substances, as an “inflammatory soup,” released by nociceptive afferents and/or non-neural cells in the tissue-injured area (Basbaum et al., 2009). This “inflammatory soup” includes signaling factors, such as nucleotides, bradykinin (BK), calcitonin gene-related peptide (CGRP), substance P, prostaglandins, serotonin, and neurotrophins (Julius and Basbaum, 2001). BK, contributes to the acute inflammatory responses, and its BK receptors divided into B_1 and B_2 receptors (Hall, 1992). Recently, it has been reported that BK facilitates P2Y receptor

activation in trigeminal SGCs (Ceruti et al., 2008). Furthermore, application of BK to cells in TG cultures evoked CGRP release from neurons, and potentiated P2Y-mediated responses in neighboring SGCs, promoting the release of pro- and anti-inflammatory cytokines (Ceruti et al., 2011). Thus, the functional role of $P2Y_{12}$ receptors and their inter-receptor interactions via intracellular signal transduction during pathological pain conditions is well described in glial cells. Although these pathological pain mechanisms involve in primary nerve fibers, knowledge of the functional expression of $P2Y_{12}$ receptors in TG neurons remains to be fully elucidated. Moreover, knowledge of BK receptors in TG neurons also has been limited. TG neurons, as a primary afferent neuron, sense and relay not only innocuous but also nociceptive sensations including acute and chronic conditions (Fried et al., 2001).

In the present study, we aimed to investigate the expression, localization, and physiological and pharmacological properties of $P2Y_{12}$ receptors, as well as their interactions with BK receptors in primary cultured TG neurons.

Materials and methods

Ethical approval

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

Cell culture

Under pentobarbital sodium anesthesia (50 mg/kg), following administration of isoflurane (3.0 Vol%) TGs were rapidly excised from neonatal Wistar rats (7 days old). Cells were dissociated by enzymatic treatment with Hank's balanced salt solution (Invitrogen, NY, 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 dishes (Corning, NY, USA). Primary culture was performed with Leibovitz's L-15 medium (Invitrogen) containing 10% FBS, 1%

penicillin-streptomycin (Invitrogen), 1% fungizone (Invitrogen), 26 mM $NaHCO_3$, and 30 mM glucose (pH 7.4). Cells were maintained in culture at 37°C in a humidified atmosphere of 95% air and 5% CO_2 for 48 h.

Immunohistocytochemistry

Primary TG culture cells were seeded and cultured on poly-L-lysine-covered glass slides (Matsunami, Osaka, Japan). Excised TGs were fixed in optimal cutting temperature (OCT) compound and rapidly frozen in liquid nitrogen. Frozen tissues were cut at a thickness of 10 μm and placed on slides. After fixation with 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 neuronal marker cocktail (Neuro-Chrom™ Pan Neuronal Marker, Millipore, Billerica, MA, USA; 1:50 dilution) including mouse anti- Neuronal nuclei (NeuN), anti-microtubule-associated protein 2 (MAP2), anti-βIII tubulin, and anti- neurofilament H (NF-H) antibodies was included with the primary antibodies. 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, rabbit anti-B_1 bradykinin receptor (Alomone Labs; 1:50 dilution), rabbit anti-B_2 bradykinin receptor (Alomone Labs; 1:50 dilution) and rabbit anti-$P2Y_{12}$ receptor (Alomone Labs; 1:50 dilution). Cells and tissues were then washed and incubated with a secondary antibody at room temperature for 30 min. Secondary antibodies were Alexa Fluor 488 donkey anti-rabbit IgG, Alexa Fluor 568 donkey anti-mouse IgG, or Alexa Fluor 568 donkey anti-rabbit IgG (Molecular Probes, Eugene, OR, USA; 1:50 dilution) for fluorescence staining and 4′,6-diamino 2-phenylindole dihydrochloride (Invitrogen) for nuclear staining (at room temperature for 5 min). Cells (Zeiss, Jena, Germany) and tissues (Keyence, Osaka, Japan) were examined under fluorescence microscopes.

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 containing 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 activation of depolarization-induced increases in intracellular free Ca^{2+} concentration in neurons. High potency $P2Y_{12}$ agonist, 2-methylthioadenosine diphosphate trisodium salt (2-MeS-ADP) (Zhang et al., 2014a), bradykinin (BK), potent and selective $P2Y_{12}$ antagonists (AR-C66096 and PSB0739), sarcoplasmic reticulum Ca^{2+}-ATPase inhibitor (cyclopiazonic acid), ryanodine receptor channel inhibitor (dantrolene sodium salt: dantrolene), adenylate cyclase inhibitor (9-(Tetrahydro-2-furanyl)-9*H*-purine-6-amine; SQ22536), phosphodiesterase inhibitor (3-isobutyl-1-methylxanthine; IBMX), selective bradykinin B_2 receptor antagonist (HOE140), selective bradykinin B_1 receptor antagonist (R715), and highly selective bradykinin B_1 receptor agonist (Lys-[Des-Arg^9] bradykinin) were obtained from Tocris Bioscience (Bristol, UK). All other reagents were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.), except where indicated.

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

Primary cultured TG cells were loaded for 90 min at 37°C in Hank’s

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

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 Wilcoxon t-test, Kruskal–Wallis test, Dunn's post hoc test, or Mann–Whitney *U*-test were used to determine nonparametric statistical significance. A *P* value of less than 0.05 was considered significant. Statistical analysis was performed using GraphPad Prism

5.0 (GraphPad Software, La Jolla, CA, USA).

The dependence of changes in $[Ca^{2+}]_i$ on each pharmacological agent was obtained by fitting data to the following function using Origin 8.5 (OriginLab Corporation, Northampton, USA):

$$F/F_0 = [(F/F_{0int} - F/F_{0fin}) / (1 + ([x]_o / K)) + F/F_{0fin} \qquad \textit{Equation 1}$$

where K is the equilibrium binding constant, $[x]_o$ indicates the applied concentration of pharmacological agents, and F/F_{0int} and F/F_{0fin} are the initial and final F/F_0 responses, respectively.

Results

Characterization of primary cultured TG cells

In bright-field images of rat trigeminal primary cell cultures (Fig. 1A), we could not visually or independently distinguish neurons and glial cells. To reveal neuronal responses alone, not those of glial cells, $[Ca^{2+}]_i$ in fura-2-loaded primary cultured TG cells was measured by application of an extracellular solution containing high extracellular K^+ (50 mM K^+), which induces membrane depolarization. A series of applications of 50 mM K^+ induced transient increases in $[Ca^{2+}]_i$ in 41.0% of tested primary cultured TG cells, while others (59.0%) showed no response (Figs. 1B and 1C). Cells showing the depolarization-induced $[Ca^{2+}]_i$ increase elicited by high K^+ were identified as TG neurons. 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 $P2Y_{12}$ receptors in TG neurons

Primary cultured TG cells all showed positive immunoreactivity to a neuronal marker cocktail (Neuro-Chrom^{TM} Pan Neuronal Marker), which included mouse anti-NeuN, anti-MAP2, anti-βIII tubulin (Fig. 2A). They also

showed positive immunoreactivity 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). We also observed intense $P2Y_{12}$ receptor immunoreactivity in somata, dendrites, axons, and in the perinuclear region of primary cultured TG cells (Figs. 2B, 2E, 2H, and 2K). This $P2Y_{12}$ receptor immunoreactivity colocalized with Pan Neuronal Marker- (Fig. 2C), SP- (Fig. 2F), and IB4- (Fig. 2I) positive neurons. Additionally, we observed positive immunoreactivity to NF-H (as an A-neuron marker; Fig. 2J) and identified $P2Y_{12}$ receptor immunoreactivity in cells that morphologically resembled NF-H-positive TG neurons (Fig. 2K).

In cryosections, intense $P2Y_{12}$ receptor immunoreactivity was observed in the cell bodies of TG neurons (Figs. 3B, 3E, 3H, and 3K) which were positive for the Pan Neuronal Marker (Fig. 3C), SP (Fig. 3F), and IB4 (Fig. 3I) immunoreactivity. Note that both NF-H and $P2Y_{12}$ receptor antibodies are produced from the same host. Each NF-H and $P2Y_{12}$ receptor immunoreactivity was identified in TG cells (Figs. 3J and 3K).

Treatment with 2-MeS-ADP increases $[Ca^{2+}]_i$ in TG neurons

We investigated $[Ca^{2+}]_i$ increases during application of 2-MeS-ADP,

which is a $P2Y_{12}$ receptor agonist. Increases in $[Ca^{2+}]_i$ in TG neurons were induced by addition of five different concentrations of 2-MeS-ADP (1, 10, 50, 100, and 500 nM). In the presence of extracellular Ca^{2+} (2.0 mM), TG neurons showed rapid and transient increases in $[Ca^{2+}]_i$ following addition of 2-MeS-ADP in a concentration-dependent manner (Fig. 4A). A semilogarithmic plot (Fig. 4B) illustrates 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 $[Ca^{2+}]_i$ increases in TG neurons

In the presence of 2.0 mM extracellular Ca^{2+}, 2-MeS-ADP-induced $[Ca^{2+}]_i$ increases were inhibited by various concentrations of selective $P2Y_{12}$ receptor antagonists, AR-C66096 (Fig. 5A) and PSB0739 (Fig. 5C), in a dose-dependent manner. Equilibrium binding constants, as half-maximal 50% inhibitory concentrations (IC_{50}), were obtained at an AR-C66096 concentration of 6.0 nM (Fig. 5B) and a PSB0739 concentration of 1.0 nM (Fig. 5D).

Release of Ca^{2+} from intracellular Ca^{2+} stores mediates 2-MeS-ADP-induced

$[Ca^{2+}]_i$ mobilization

In the presence of extracellular Ca^{2+} (2.0 mM), 2-MeS-ADP (50 nM) evoked transient increases in $[Ca^{2+}]_i$ (Figs. 6A, 6B, and 6C) to peak values of 1.7 ± 0.06 F/F_0 units in the first application, and 1.6 ± 0.06 F/F_0 units in the second application (Fig. 6D). After removing extracellular Ca^{2+} (0 mM Ca^{2+} in the extracellular solution), repeated addition of 2-MeS-ADP (50 nM) also produced a rapid increase in $[Ca^{2+}]_i$ (Fig. 6A) to a peak value of 1.5 ± 0.07 F/F_0 units in the first application, while the peak value in the second application of 2-MeS-ADP was significantly lower than that of the first application (Figs. 6A and 6D). These increases in $[Ca^{2+}]_i$ were significantly lower in the presence of a sarcoplasmic reticulum Ca^{2+}-ATPase inhibitor (100 nM cyclopiazonic acid; CPA) with a peak value of 1.1 ± 0.01 F/F_0 units (Fig. 6B). Note that CPA was administered simultaneously with 2-MeS-ADP (Figs. 6B and 6D). 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. 6C and 6D).

Immunolocalization of bradykinin (BK) receptors in TG neurons

BK receptors are classified into B_1 and B_2 receptors. Intense B_1 receptor immunoreactivity was observed in both TG primary cultured cells (Fig. 7B) and in cryosections (Fig. 7H) with intense staining in nuclei and slight fluorescence in somata, although dendrites and axons were not stained. B_1 receptor-positive cells exhibited immunoreactivity to the Pan Neuronal Marker (Figs. 7C and 7I). Intense B_2 receptor immunoreactivity was observed not only in primary cultured TG neurons (Fig. 7E) but also in cryosections (Fig. 7K), which all showed positive immunoreactivity to the Pan Neuronal Marker (Figs. 7F and 7L) in somata, dendrites, axons, and in perinuclear regions.

HOE140, a B_2 receptor antagonist, inhibits BK-induced $[Ca^{2+}]_i$ increases in TG neurons

We observed rapid and transient concentration-dependent $[Ca^{2+}]_i$ increases in TG neurons following addition of five different concentrations of BK (0.01, 0.1, 1.0, 10, 100 nM) in the presence of external Ca^{2+} (2.0 mM; Fig. 8A). A semilogarithmic plot (Fig. 8B) illustrates F/F_0 values as a function of applied BK concentrations, with an equilibrium binding constant, as a half maximal 50%

effective concentration (EC_{50}), of 1.0 nM. We also examined BK-induced $[Ca^{2+}]_i$ responses in both the presence and absence of external Ca^{2+}. Application of BK (1.0 nM) produced rapid increases in $[Ca^{2+}]_i$ to a peak F/F_0 value of 1.7 ± 0.03 F/F_0 units in the presence (2.0 mM) of external Ca^{2+} and 1.4 ± 0.03 F/F_0 units in the absence (0 mM) of external Ca^{2+} (Figs. 8C and 8F). There were significant differences in the amplitudes of BK-induced $[Ca^{2+}]_i$ increases between those determined in the presence and absence of extracellular Ca^{2+}. In the absence of extracellular Ca^{2+}, BK (1.0 nM)-induced $[Ca^{2+}]_i$ increases were significantly inhibited by a B_2 receptor antagonist (100 nM HOE140) (Figs. 8E and 8F) but were not inhibited by a B_1 receptor antagonist (1.0 μM R715) (Figs. 8D and 8F).

Signal coupling between $P2Y_{12}$ and BK receptors in TG neurons

We further examined $P2Y_{12}$ and BK receptor crosstalk in TG neurons by simultaneous application of agonists for both receptors. In the presence of extracellular Ca^{2+} (2.0 mM), BK (1.0 nM) evoked transient increases in $[Ca^{2+}]_i$ to a peak value of 1.6 ± 0.06 F/F_0 units. BK-induced $[Ca^{2+}]_i$ increases were significantly augmented by application of 2-MeS-ADP (50 nM) (2.3 ± 0.17 F/F_0 units) (Figs. 9A and 9B).

In addition, a B_2 receptor inhibitor (100 nM HOE140) significantly and reversibly inhibited the 50 nM 2-MeS-ADP-induced $[Ca^{2+}]_i$ increase to a F/F_0 value of 1.3 ± 0.02 (Figs. 10C and 10D), while a B_1 receptor inhibitor (1.0 μM R715) did not (Figs. 10A and 10B). Both $P2Y_{12}$ antagonists also significantly and reversibly inhibited 100 nM BK-induced $[Ca^{2+}]_i$ increases (2.01 ± 0.06 F/F_0 units for the control) to 1.3 ± 0.04 F/F_0 units (Figs. 10E and 10F) with 100 nM AR-C66096 and 1.4 ± 0.10 F/F_0 units (Figs. 10G and 10H) with 1.0 μM PSB0739.

Release of Ca^{2+} from intracellular Ca^{2+} stores was driven by decreased intracellular cAMP levels following $P2Y_{12}$ receptor and B_2 receptor activation

To elucidate the intracellular Ca^{2+} signaling pathway mediated by $P2Y_{12}$ -B_2 receptor coupling in TG neurons, we examined effects of an adenylate cyclase (AC) inhibitor (SQ22536) and 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. 11A). A semilogarithmic plot (Fig. 11B) illustrates the F/F_0 values as a function of 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 not only 2-MeS-ADP-induced $[Ca^{2+}]_i$ increases (1.4 ± 0.05 F/F_0 units) (Figs. 11C and 11D) but also BK-induced increases (1.2 ± 0.03 F/F_0 units) (Figs. 11E and 11F).

Discussion

The present study demonstrated functional expression of $P2Y_{12}$ and BK receptors (B_1 and B_2) and their interaction in TG neurons. $P2Y_{12}$ receptors were localized either on soma, axons, or dendrites in A-neurons, non-peptidergic C-neurons, and peptidergic C-neurons. B_1 and B_2 receptors were also expressed in TG neurons; B_1 receptors were expressed on somata, while B_2 receptors were present on axons or dendrites. $P2Y_{12}$ receptor activation induced Ca^{2+} release from internal ryanodine-sensitive Ca^{2+} stores. BK activated B_2 receptors in TG neurons and mobilized $[Ca^{2+}]_i$ by releasing Ca^{2+} from internal Ca^{2+} stores with partial Ca^{2+} influx from the extracellular medium. Additionally, $P2Y_{12}$ and B_2 receptors interacted with each other by integrating intracellular signaling to release Ca^{2+} from intracellular stores. Decreased intracellular cAMP levels by suppression of AC following activation of both $P2Y_{12}$ and B_2 receptors elicited release of Ca^{2+} from internal Ca^{2+} stores, indicating that $P2Y_{12}$-B_2 receptor activation decreases intracellular cAMP to attenuate cAMP-mediated inhibitory effects on Ca^{2+} release from ryanodine receptor channels in TG neurons.

Primary TG cells were immunoreactive to either the Pan Neuronal Marker, NF-H (A-neuron marker), IB4 (non-peptidergic C-neuron marker), or SP

(peptidergic C-neuron marker). Although 40% of primary TG cells showed depolarization-induced $[Ca^{2+}]_i$ increases in an extracellular solution containing 50 mM K^+ (Gover et al., 2007), all Ca^{2+} responses in the present study were analyzed using cells showing depolarization-induced $[Ca^{2+}]_i$ increases, indicating that all results were obtained from neurons rather than glial cells.

To examine intracellular signaling following $P2Y_{12}$ receptor activation, we utilized 2-MeS-ADP as an agonist, since 2-MeS-ADP acts as a full agonist for the $P2Y_{12}$ receptor by binding to the extracellular pocket-like structure of the $P2Y_{12}$ receptor (Zhang et al., 2014a; Zhang et al., 2014b). Notably, 2-MeS-ADP increased $[Ca^{2+}]_i$, while selective $P2Y_{12}$ receptor antagonists, AR-C66096 and PSB0739 (Figs. 5), both inhibited this $[Ca^{2+}]_i$ increase in a dose-dependent manner. The 2-MeS-ADP-induced $[Ca^{2+}]_i$ increase in TG neurons in the absence of extracellular Ca^{2+} was sensitive to a sarcoplasmic reticulum Ca^{2+}-ATPase inhibitor (CPA) and ryanodine receptor channel inhibitor (dantrolene) (Figs. 6B and 6C). Therefore, $P2Y_{12}$ receptor activation in TG neurons mobilizes $[Ca^{2+}]_i$ by releasing Ca^{2+} from ryanodine receptor channels present on intracellular Ca^{2+} stores. All P2Y receptor subtypes ($P2Y_1$, $P2Y_2$, $P2Y_4$, $P2Y_6$, $P2Y_{11}$ to $P2Y_{14}$) are expressed (in terms of mRNA) in murine TG neurons (Villa et al., 2010).

However, in rat TG neurons, RT-PCR revealed mRNA expression of only $P2Y_1$, $P2Y_2$, $P2Y_4$, and $P2Y_6$ receptors (Li et al., 2014; Ruan and Burnstock, 2003). The present results clearly indicate that $P2Y_{12}$ receptors are localized to and functionally expressed in rat TG neurons.

BK receptors are classified into B_1 and B_2 receptors (Hall, 1992). Both B_1 and B_2 receptors were expressed on TG neurons; however, we observed differential localization patterns between these two receptors (Figs. 7C, 7F, 7I and 7L). We also observed that BK dose-dependently increased $[Ca^{2+}]_i$. Interestingly, the B_2 receptor antagonist (HOE140) had significant inhibitory effects on BK-induced $[Ca^{2+}]_i$ increases, while the B_1 receptor antagonist (R715) did not. These results are in line with the pharmacological properties of BK, which is a potent and endogenous agonist for B_2 receptors rather than B_1 receptors in sympathetic neurons of the rat superior cervical ganglion (Babbedge et al., 1995) and in CHO cells stably expressing recombinant human B_1 or B_2 receptors (Simpson et al., 2000). Furthermore, BK has a 500 times higher affinity for B_2 receptors than B_1 receptors (Simpson et al., 2000). Note that the B_1 receptor agonist, Lys-[Des-Arg^9] bradykinin, dose-dependently increased $[Ca^{2+}]_i$ in the presence of extracellular Ca^{2+}, and this increase was

suppressed by a B_1 receptor specific antagonist (supplemental figure 1), indicating that B_1 receptors are also functionally expressed in TG neurons. Note that the B_1 agonist is a metabolite of endogenous of BK produced by kininase in peripheral tissues (Regoli et al., 1998). Therefore, the present study indicates that TG neurons express B_1 and B_2 receptors, and endogenous BK preferentially activates B_2 receptors. Both B_1 and B_2 receptors mediate orofacial pain (Luiz et al., 2010; Chichorro et al., 2004). B_2 receptors are generally essential in the early stages of pain generation and are expressed ubiquitously and constitutively in healthy tissues, while B_1 receptors are involved in chronic inflammation or tissue injury (Hall, 1997). This implies, in addition to the immunohistochemical analysis showing B_2 receptor expression on dendrites and axons of TG neurons, that B_2 receptors respond to endogenous BK immediately after its production following peripheral tissue injury.

BK-induced $[Ca^{2+}]_i$ increases were observed in both the presence and absence of extracellular Ca^{2+}; however, the amplitude of the $[Ca^{2+}]_i$ increase in the absence of extracellular Ca^{2+} was significantly smaller (84.9 ± 11.3%, *N* = 161) than those in the presence of Ca^{2+} (as 100%; Figs. 8C and 8F), indicating that BK-induced $[Ca^{2+}]_i$ mobilization was mainly composed of Ca^{2+} release from

internal stores, with partial Ca^{2+} influx from the extracellular medium. Notably, BK activates voltage-dependent Ca^{2+} channels in rat submucosal plexus neurons (Avemary and Diener, 2010; Rehn et al., 2013), transient receptor potential cation channel, subfamily V, member 1 (TRPV1 channels) in rat dorsal root ganglion neurons (Ferreira et al., 2004; Mistry et al., 2014), and transient receptor potential cation channel, subfamily A, member 1 (TRPA1 channels) in murine sensory neurons (Bautista et al., 2006) or TRPA1 channel-expressing CHO cells (Bandell et al., 2004). However, it has been reported that BK-induced Ca^{2+} currents in TG neurons could not be recorded (Kitakoga and Kuba, 1993). Although further studies are required to clarify which Ca^{2+} influx pathways contributed to BK-induced Ca^{2+} influx in TG neurons, the present results clearly indicate that BK mobilizes $[Ca^{2+}]_i$ through both intracellular Ca^{2+} release and Ca^{2+} influx.

The most noteworthy result in the present study was the functional interaction of $P2Y_{12}$ and B_2 receptor activation. BK-induced Ca^{2+} responses were significantly increased by application of 2-MeS-ADP (Figs. 9A and 9B). Additionally, BK-induced increases in $[Ca^{2+}]_i$ were inhibited by the selective $P2Y_{12}$ receptor antagonists, AR-C66096 and PSB0739 (Figs. 10E to 10H).

Moreover, 2-MeS-ADP-induced $[Ca^{2+}]_i$ increases were inhibited by a B_2 receptor antagonist (Figs. 10C and 10D). When we applied a B_1 receptor antagonist during the 2-MeS-ADP-induced $[Ca^{2+}]_i$ increase in TG neurons, no changes were observed (Figs. 10A and 10B). Thus, $P2Y_{12}$ receptors were specifically and functionally coupled with B_2 receptor activity. Similarly, BK-induced increases in $[Ca^{2+}]_i$ were inhibited by selective $P2Y_{12}$ receptor antagonists in human subcutaneous fibroblasts (Pinheiro et al., 2013). Since BK and 2-MeS-ADP are specific for B_2 and $P2Y_{12}$ receptors, respectively (Zhang et al., 2014a; Zhang et al., 2014b; Simpson et al., 2000; Regoli et al., 1998), non-specific inhibition of $P2Y_{12}$ receptors by the B_2 receptor antagonist as well as that of B_2 receptors by the $P2Y_{12}$ receptor antagonists was unlikely. Additionally, while $P2Y_{12}$ receptor antagonists suppressed BK-induced $[Ca^{2+}]_i$ increases, the B_2 receptor antagonist, but not the B_1 receptor antagonist, inhibited 2-MeS-ADP-induced $[Ca^{2+}]_i$ increases. Therefore, although $P2Y_{12}$ and BK receptors were activated individually by their specific agonists, they likely activated a common intracellular signaling pathway. Additionally, activation of both $P2Y_{12}$ and B_2 receptors increased $[Ca^{2+}]_i$ via intracellular Ca^{2+} release, indicating that both receptors likely integrate signaling to ryanodine receptor channels on Ca^{2+} stores and

through intracellular signaling factors, including cAMP or inositol 1,4,5-trisphosphate, generated by activation of each receptor, to release Ca^{2+} from internal stores.

Application of SQ22536, an AC inhibitor, increased the amplitude of transient increases in $[Ca^{2+}]_i$ in a concentration-dependent manner (Fig. 11A and 11B), while IBMX, a phosphodiesterase inhibitor, significantly and reversibly inhibited 2-MeS-ADP-induced $[Ca^{2+}]_i$ increases (Figs. 11C and 11D) and BK-induced $[Ca^{2+}]_i$ increases (Figs. 11E and 11F) in TG neurons. These results indicated that decreased intracellular cAMP levels by suppression of AC following both $P2Y_{12}$ and B_2 receptor activation increased $[Ca^{2+}]_i$. $P2Y_{12}$ receptor activation 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, decreased cAMP levels and subsequent increases in $[Ca^{2+}]_i$ mediate platelet aggregation (Moheimani and Jackson, 2012). Thus, activation of $P2Y_{12}$ and B_2 receptors mobilizes $[Ca^{2+}]_i$ by attenuating cAMP-mediated inhibitory effects on intracellular Ca^{2+} release via ryanodine receptor channels; however, further studies are required to identify which signaling cascades (i.e., G-protein, AC, or others) mediate the decrease in

intracellular cAMP levels resulting from $P2Y_{12}$ and B_2 receptor activation.

Nucleotides (ATP and/or ADP) and BK, as component of the “inflammatory soup”, are involved in both acute pain and peripheral sensitization for pain. These factors are also responsible for enhancement of hyperalgesia and/or allodynia. We suggest that both ATP release and BK generation following/during tissue injury may enhance $P2Y_{12}$ and B_2 receptor activation independently, both of which cooperatively promote pain signals via augmentation of intracellular Ca^{2+} signaling in TG neurons. Thus, our ongoing experimental work aims to develop drugs to suppress pain caused by acute peripheral sensitization, and both $P2Y_{12}$ and B_2 receptors may be candidate drug targets.

In conclusion, $P2Y_{12}$ receptor activation in TG neurons elicited Ca^{2+} release from ryanodine-sensitive Ca^{2+} stores. B_2 receptor activation mobilized $[Ca^{2+}]_i$ by releasing Ca^{2+} from intracellular stores with partial Ca^{2+} influx. Interaction of $P2Y_{12}$ and B_2 receptors through intracellular cAMP signaling prevented cAMP-mediated inhibitory effects on Ca^{2+} release from ryanodine receptor channels in TG neurons. In TG neurons, this $P2Y_{12}$-B_2 receptor interaction may amplify nociceptive signaling resulting from extracellular

nucleotides and/or BK.

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Figure Legends

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

(A) Bright-field phase-contrast image showing primary cultured TG neurons. Cells were cultured for 48 h and contained neurons, glial cells, and fibroblasts. (B) In the presence of 2.0 mM extracellular Ca^{2+} (lower white box), application of 50 mM KCl solution (upper white boxes) increased $[Ca^{2+}]_i$ in TG neurons (black solid line). Increases in $[Ca^{2+}]_i$ were not observed in other cell populations (grey solid line). (C) Summary bar graph showing normalized cell numbers tested. The percentage of cells responding to application of 50 mM KCl solution was 41.0% (black column), indicating that these cells were a neuronal cell population. Data points represent mean ± S.D. of a number of independent experiments (numbers in parentheses represent the number of tested cells). Statistical significance between columns is indicated by asterisk: $*p<0.05$.

Figure 2: Immunolocalization of $P2Y_{12}$ receptors in primary cultured TG neurons.

(A) Primary cultured TG neurons positive to a Pan Neuronal Marker. (B, E, H, and K) $P2Y_{12}$ receptor immunoreactivity (B, E, K, (green) and H (red)) and nuclei (blue) in primary cultured TG neurons. (C) Double immunofluorescence

staining of $P2Y_{12}$ receptors (green) and the Pan Neuronal Marker (red). (D) Positive immunoreactivity to SP as a peptidergic C-neuron marker in primary cultured TG neurons. (F) Double staining of $P2Y_{12}$ receptors (green) and SP (red). (G) Positive immunoreactivity to IB4 as a non-peptidergic C-neuron marker in primary cultured TG neurons. (I) Double staining of $P2Y_{12}$ receptors (red) and IB4 (green). (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 many directions. (K) Neurons of this morphology 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 somata of TG neuron cryosections.

(A) TG neurons positive for the Pan Neuronal Marker. (B, E, H and K) $P2Y_{12}$ receptor immunoreactivity (B, E, K, (green) and H (red)) and nuclei (blue). (C) Double immunofluorescence staining of $P2Y_{12}$ receptors (green) and the Pan Neuronal Marker (red). (D) Positive immunoreactivity to SP as a peptidergic C-neuron marker in TG neurons. (F) Double staining of $P2Y_{12}$ receptors (green)

and SP (red). (G) Positive immunoreactivity to IB4 as a non-peptidergic C-neuron marker in TG neurons. (I) Double staining of $P2Y_{12}$ receptors (red) and IB4 (green). (J) Positive immunoreactivity to NF-H as an A-neuron marker in TG neurons. We could not examine colocalization of $P2Y_{12}$ receptors and NF-H since both are produced from the same host; however, neurons were also positive for $P2Y_{12}$ receptor immunoreactivity in the TG cryosections. No fluorescence was detected in the negative control (data not shown). Scale bars: 20 μm.

Figure 4: Ca^{2+} dependence of $P2Y_{12}$ receptors activated in TG neurons.

(A) Example of transient $[Ca^{2+}]_i$ increases following application of a series of concentrations of 2-MeS-ADP (1–500 nM; upper white boxes) in the presence of extracellular Ca^{2+} (2.0mM). Application of 50 mM KCl solution is shown by a gray box. (B) Data points illustrate F/F_0 values as function of the applied concentration of 2-MeS-ADP. Each data point represents mean ± S.E. of 8 independent experiments (numbers in parentheses represent the number of tested cells). 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.

Figure 5: Pharmacological identification of $P2Y_{12}$ receptors in TG neurons.

(A, B, C, and D) Dose-dependent inhibitory effect of AR-C66096 (A and B) and PSB0739 (C and D) on $P2Y_{12}$ receptors in TG neurons. Example of transient $[Ca^{2+}]_i$ increase with 50 nM 2-MeS-ADP (upper white boxes) and inhibition of this increases by 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). (A and C) Black boxes represent the timing of application of these antagonists. Application timings of the 50 mM KCl solution are shown in gray boxes. (B and D) Dose-response relationships for inhibitory effects of AR-C66096 (B) and PSB0739 (D) on 2-MeS-ADP-induced $[Ca^{2+}]_i$ increases. Data points in each figure illustrate F/F_0 values as function of the applied concentration of the inhibitors and represent mean ± S.E. of 8 experiments (numbers in parentheses represent the number of tested cells). The curve on the semilogarithmic scale was fitted according to *Equation 1* described in the text.

Figure 6: 2-MeS-ADP induces release of Ca^{2+} from intracellular Ca^{2+} stores via

the ryanodine receptor channel.

(A) Examples of transient $[Ca^{2+}]_i$ increases following application of 50 nM 2-MeS-ADP with (lower gray boxes) or without (lower white boxes) extracellular Ca^{2+} (2.0 mM). (B) The 2-MeS-ADP (50 nM)-induced $[Ca^{2+}]_i$ increase was inhibited by 100 nM of a sarcoplasmic reticulum Ca^{2+}-ATPase inhibitor (cyclopiazonic acid; CPA) in the absence of external Ca^{2+}. (C) The 2-MeS-ADP (50 nM)-induced $[Ca^{2+}]_i$ increase was inhibited by 1.0 μM dantrolene sodium salt (dantrolene) in the absence of external Ca^{2+}. (A, B and C) Upper white and black boxes indicate the timing of application of 2-MeS-ADP, CPA and dantrolene respectively. Upper gray boxes indicate applications of 50 mM KCl solution. (D) Summary bar graph shows increases in $[Ca^{2+}]_i$ following the first (upper column) and second (second column from upper) application of 50 nM 2-MeS-ADP in the presence of external Ca^{2+} (2.0 mM) (gray boxes on the right side), as well as the first (third column from upper) and second (fourth column from upper) application of 50 nM 2-MeS-ADP in the absence of external Ca^{2+}. F/F_0 values after application of 50 nM 2-MeS-ADP with 100 nM CPA (fifth column from top) and with 1.0 μM dantrolene (sixth column from top) in the absence of external Ca^{2+} (white box on the right side) are shown. Each column

denotes mean ± S.E. of the indicated (in parentheses) number of independent experiments. Statistical significance between columns (shown by solid lines) is indicated by asterisks: * $p < 0.05$.

Figure 7: Immunolocalization of bradykinin B_1 and B_2 receptors in primary cultured TG neurons and in the somata of TG neuron cryosections.

(A, D, G and J) Cells positive to the Pan Neuronal Marker in primary cultured TG neurons (A and D) and TG cryosections (G and J). (B and H) Positive immunoreactivity to B_1 receptors (green) in primary cultured TG neurons (B) and TG cryosections (H). (C and I) Double staining for the Pan Neuronal Marker (red) and B_1 receptors (green) in primary cultured TG neurons (C) and TG cryosections (I). (E and K) Positive immunoreactivity to B_2 receptors (green) in primary cultured TG neurons (E) and TG cryosections (K). (F and L) Double staining of B_2 receptors (green) and the Pan Neuronal Marker (red) in primary cultured TG neurons (F) and TG cryosections (L).

Figure 8: Pharmacological identification of BK receptors in TG neurons.

(A) Example of transient $[Ca^{2+}]_i$ increases following application of a

series of BK concentrations. In the presence of extracellular Ca^{2+} (2.0 mM; lower white boxes), application of BK induced transient $[Ca^{2+}]_i$ increases in a concentration-dependent manner. Applied concentrations of BK (0.01–100 nM) are shown in uppermost white boxes. (B) Data points illustrate F/F_0 values as function of the applied BK concentration. Each data point represents the mean ± S.E. of 7 experiments (numbers in parentheses represent the number of tested cells). The curve on the semilogarithmic scale was fitted according to *Equation 1* described in the text. The equilibrium binding constant of BK was 1.0 nM. (C) Example of transient $[Ca^{2+}]_i$ increases following application of 1.0 nM BK with (lower gray boxes) or without (lower white box) extracellular Ca^{2+} (2.0 mM). (D) Example of BK (1.0 nM)-induced $[Ca^{2+}]_i$ increases with (upper black box) or without (upper white boxes) R715 in the absence (lower white box) or presence (lower grey boxes) of external Ca^{2+}. (E) Example of BK (1.0 nM)-induced $[Ca^{2+}]_i$ increases with (upper black box) or without (upper white boxes) HOE140 in the absence (lower white box) or presence (lower grey boxes) of external Ca^{2+}. (A, C, D, and E) Upper grey boxes in indicate the timing of application of the 50 mM KCl solution. (F) Summary bar graph indicates $[Ca^{2+}]_i$ increases following the first (upper column) and second (second upper column) application of 1.0 nM BK

with external Ca^{2+} (2.0 mM) (gray boxes on the right side). Mean values for the increase in $[Ca^{2+}]_i$ following the first (third upper column) and second (fourth upper column) application of 1.0 nM BK in the absence of external Ca^{2+}, as well as application of 1.0 nM BK with 1.0 μM R715 (fifth upper column) and with 100 nM HOE140 (sixth upper column) in the absence of external Ca^{2+} (white boxes on the right side) are shown. Each column denotes the mean ± S.E. of the indicated (in parentheses) number of experiments. Statistical significance between columns (shown by solid lines) is indicated by asterisks: * $p < 0.05$.

Figure 9: BK-induced Ca^{2+} responses were significantly augmented by 2-MeS-ADP.

(A) Example of transient $[Ca^{2+}]_i$ increases following application of BK (1.0 nM; white boxes) with 2-MeS-ADP (50 nM; black boxes) in the presence of extracellular Ca^{2+} (2.0 mM). Application timings for the 50 mM KCl solution are indicated by the gray box. (B) Summary bar graph of $[Ca^{2+}]_i$ increases following 1.0 nM BK with (lower black column) or without (upper white column) 50 nM 2-MeS-ADP. Each column denotes the mean ± S.E. of 5 experiments. Statistical significance between columns (shown by solid lines) is indicated by asterisks: * *p*

< 0.05.

Figure 10: $P2Y_{12}$ receptors couple with bradykinin B_2 receptors in TG neurons.

(A and C) Examples of transient $[Ca^{2+}]_i$ increases following 50 nM 2-MeS-ADP with (upper black boxes) or without (upper white boxes) 1.0 μM R715 (A) or 100 nM HOE140 (C) in the presence of external Ca^{2+} (2.0 mM). (B and D) Summary bar graphs of 2-MeS-ADP (50 nM)-induced $[Ca^{2+}]_i$ increases with (black lower columns) or without (white upper columns) 1.0 μM R715 (B) or 100 nM HOE140 (D). (E and G) Examples of BK (100 nM)-induced $[Ca^{2+}]_i$ increases with (upper black boxes) or without (upper white boxes) 100 nM AR-C66096 (E) or 1.0 μM PSB0739 (G) in the presence of external Ca^{2+} (2.0 mM). (F and H) Summary bar graphs of increases in $[Ca^{2+}]_i$ following 100 nM BK with (black lower columns) or without (upper white columns) 1.0 nM AR-C66096 (F) or 1.0 μM PSB0739 (H). Each column denotes the mean ± S.E. of the indicated (in parentheses) number of independent experiments. Statistical significance between columns (shown by solid lines) is indicated by asterisks: * *p* < 0.05.

Figure 11: Intracellular cAMP levels modulate 2-MeS-ADP-induced $[Ca^{2+}]_i$ increases in TG neurons.

(A) Example of transient $[Ca^{2+}]_i$ increases following application of SQ22536 (0.01-100 μM) (upper white boxes) with external Ca^{2+} (2.0 mM). Application of a 50 mM KCl solution is shown by the gray box. (B) Data points illustrate F/F_0 values as function of the applied SQ22536 concentration and represent the mean ± S.E. of 5 independent experiments (numbers in parentheses represent the number of tested cells). The curve on 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 $[Ca^{2+}]_i$ increases following 50 nM 2-MeS-ADP with (upper black box) or without (upper white boxes) 50 μM IBMX in presence of external Ca^{2+} (2.0 mM). (D) Summary bar graph of increases in $[Ca^{2+}]_i$ following 50 nM 2-MeS-ADP with (lower black column) or without IBMX (upper white column). (E) Example of BK-induced (1.0 nM BK; upper white boxes) $[Ca^{2+}]_i$ increases that were significantly inhibited by 50 μM IBMX (upper black box) in the presence of external Ca^{2+} (2.0 mM). (F) Summary bar graph of $[Ca^{2+}]_i$ increases following 1.0 nM BK with (lower black column) or without (upper white column) 50 μM IBMX.

(D and F) Each column denotes the mean ± S.E. of the indicated number (in parentheses) of independent experiments. Gray boxes indicate applications of the 50 mM KCl solution (A, C and E). Statistical significance between columns (shown by solid lines) is indicated by asterisks: * $p < 0.05$.

Figure 12: Schematic representation of $P2Y_{12}$-B_2 receptor coupling to prevent cAMP-mediated inhibitory effects in TG neurons.

Schematic representation of the interaction between $P2Y_{12}$ and B_2 receptors in TG neurons. Both $P2Y_{12}$ and B_2 receptors share inhibitory signaling through intracellular AC, and prevent cAMP-mediated inhibitory effects on intracellular Ca^{2+} release from ryanodine receptors on intracellular Ca^{2+} stores by reducing cAMP levels in TG neurons. Gi, Gi protein alpha subunit; Gq, Gq protein alpha subunit (Abbracchio et al., 2006); SERCA, sarcoplasmic reticulum Ca^{2+}-ATPase.

Supplemental figure 1: Pharmacological identification of B_1 receptors in TG neurons.

(A) Example of BK (1.0 nM; upper white boxes) induced $[Ca^{2+}]_i$

increases showing insensitivity to R715 (1.0-1000 nM; upper black box) in the presence of external Ca^{2+} (2.0 mM). (B) Example of transient $[Ca^{2+}]_i$ increases following application of a series of concentrations of Lys-[Des-Arg^9]Bradykinin (0.01-100 nM; upper white boxes) in the presence of extracellular Ca^{2+} (2.0mM). (C) Data points illustrate F/F_0 values as function of the applied concentration of Lys-[Des-Arg^9]Bradykinin. Each data point represents mean ± S.E. of 6 independent experiments (numbers in parentheses represent the number of tested cells). The curve on the semilogarithmic scale was fitted according to *Equation 1* described in the text. The equilibrium binding constant for Lys-[Des-Arg^9]Bradykinin was 0.4 nM. (D) Example of Lys-[Des-Arg^9]Bradykinin-induced (1.0 nM; upper white boxes) $[Ca^{2+}]_i$ increases that were significantly inhibited by 1000 nM R715 (upper black box) in the presence of external Ca^{2+} (2.0 mM). Application of 50 mM KCl solution is shown by a gray box (A, B and D). (E) Summary bar graph of $[Ca^{2+}]_i$ increases following 1.0 nM Lys-[Des-Arg^9]Bradykinin with (lower black column) or without (upper white column) 1000 nM R715. Each column denotes the mean ± S.E. of the indicated number (in parentheses) of independent experiments. Statistical significance between columns (shown by solid lines) is indicated by asterisks: * *p*

< 0.05.