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Purinergic receptor expression and function in rat vagal sensory neurons innervating the stomach

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Abstract

The nodose ganglion (NG) is the main parasympathetic ganglion conveying sensory signals to the CNS from numerous visceral organs including digestive signals such as gastric distension or the release the gastrointestinal peptides. The response characteristics of NG neurons to ATP and ADP and pharmacological interrogation of purinergic receptor subtypes have been previously investigated but often in NG cells of undetermined visceral origin. In this study, we confirmed the presence of P2X3 and P2Y1 receptors and characterized P2X and P2Y responses in gastric-innervating NG neurons. Application of ATP-evoked large inward currents and cytosolic Ca^{2+} increases in gastric-innervating NG neurons. Despite the expression of P2Y1 receptors, ADP elicited only minor modulation of voltage-gated Ca^{2+} channels. Considering the sensitivity of NG neurons to comorbidities associated with disease or neural injury, purinergic modulation of gastric NG neurons in disease- or injury-states is worthy of further investigation.

Keywords

Nodose ganglion; vagal afferents; stomach; Ca^{2+} channels; P2X3 receptor; P2Y1 receptor

1. Introduction

The primary viscerosensory afferents of the vagus are comprised of a combination of unmyelinated C-type and lightly myelinated A δ -type axons, and the cell bodies for these afferents reside in the nodose ganglion (NG) [1]. Vagal afferents relay stimuli from multiple visceral tissues including the heart, lungs, vasculature, gastrointestinal (GI) tract and the accessory organs necessary for digestion. These primary visceral afferents must respond to organ specific stimuli in order to generate appropriate efferent responses. For example, vagal afferents innervating the aortic arch transduce mechanical stretch in response to arterial blood pressure [2] as opposed to vagal afferents innervating the GI lumen that relay chemical and mechanical stimuli to the brainstem as part of the parasympathetic control of

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nutrient homeostasis [3]. Therefore, the neurophysiology of the vagal circuitry may not be uniform across all organs systems.

Although neurons within sensory ganglia are devoid of synaptic contacts, numerous neurotransmitters and neuromodulators have been implicated in vagal afferent signaling through membrane-bound receptors [4]. In particular, adenosine 5'-triphosphate (ATP) is a major diffusible signaling molecule that is released by neurons, glia, and immune cells, and is responsible for the proper control of GI functions and sensory afferents [5]. Functionally, purinergic receptors are classified into three distinct types: the adenosine responsive P1 receptors (A1, A2a, A2b, A3); the ATP responsive P2X receptors (P2X1–7); and the ATP and adenosine 5'-diphosphate (ADP) responsive metabotropic P2Y receptors including P2Y1 [6]. The purinergic system is known to be present in NG [7–9] and involved in GI neural function [10]. However, previous research has not definitively characterized purinergic receptor expression in gastric-innervating vagal afferents: particularly, the P2X3 and P2Y1 subtypes.

There is strong evidence of sensory afferent neural plasticity that is mediated by purinergic receptors [11–17]. Specifically, the ionotropic P2X3 receptor is expressed in NG neurons and is sensitized following Ca^{2+} entry [18], thus causing neuroplastic changes within the NG neurons. In wild-type and P2X3^{-/-} mice, ATP-mediated inward currents were evoked in gastric-innervating NG neurons [19], however, no previous study has specifically examined the purinergic system in gastric-innervating NG neurons in rats. Additionally, recent evidence has shown that the P2Y1 receptor is expressed in peripheral afferent neurons and that activation of this G protein-coupled receptor may co-regulate P2X3 expression and signaling [12]. Furthermore, neurotransmitter release in autonomic neurons is regulated by Ca^{2+} entry through voltage-gated Ca^{2+} channels and P2 receptors: such as the P2X3 and P2Y1. The purinergic influence on Ca^{2+} channels and signaling within gastric-innervating NG neurons has yet to be explored.

The purpose of the present study was to begin to characterize purinergic expression and function in gastric-innervating NG neurons from rats. Neural tracing and immunofluorescence labeling was employed to identify of gastric-innervating P2X3 and P2Y1 positive neurons. Thereafter, Ca^{2+} imaging studies were conducted to visualize in real time the extracellular purinergic activation of gastric-innervating NG neurons. Whole-cell patch-clamp recordings and Ca^{2+} imaging were performed on dissociated gastric-innervating NG neurons in order to characterize the P2X-mediated responses and P2Y modulation of voltage-gated Ca^{2+} channels which are necessary for normal neuronal purinergic transmission.

2. Materials and methods

2.1. Animals

All procedures were approved by the Institutional Animal Care and Use Committee (IACUC) at the Penn State University College of Medicine. Male Wistar rats (Hsd:WI, Envigo, Indianapolis, IN) 8 wk of age, initially weighing 175–200 g, were used for all experimental procedures. Three days prior to cell isolation, animals were anesthetized with

isoflurane (3–5%, 1 l/min O₂), and a laparotomy was performed to expose the stomach. To label gastric-innervating NG neurons; 1,1'-dioctadecyl-3,3',3'-tetramethylindocarbocyanine perchlorate (DiI; Invitrogen/Molecular Probes, Waltham, MA) was dissolved in DMSO (3% solution) and injected into the superficial surface of the ventral and dorsal wall of the corpus of the stomach (10 µL/side). Following closure of the wound and recovery from anesthetic, rats were singly housed in a temperature-controlled room (23 °C) on a 12:12-h light-dark cycle with food and water ad libitum.

2.2. Nodose ganglion neuron isolation

Rats were anesthetized with CO₂ and then euthanized by decapitation. The NG were dissected in ice-cold Hank's balanced salt solution. Thereafter, the ganglia were enzymatically dissociated in Earle's balanced salt solution containing 0.6 mg/ml collagenase (Roche Diagnostics Corp. Indianapolis, IN), 0.4 mg/ml trypsin (Worthington Biochemical Corp. Lakewood, NJ), 0.1 mg/ml DNAase Type I (Sigma-Aldrich, St. Louis, MO) in a shaking water bath at 35°C for 65 min. Afterward, the neurons were centrifuged twice and the dispersed neurons were resuspended in minimal essential medium supplemented with 10% fetal bovine serum (VWR), 1% penicillin-streptomycin (all from Thermo-Fisher Scientific). The dissociated NG neurons were plated on poly-L-lysine coated dishes or coverslips and incubated (5% CO₂/95% air) at 37 °C overnight until experimentation. Power analysis and sample size calculations were performed with Sigmaplot 12.5 (Systat Software, San Jose, Ca). Based upon a 50% ± 10% expected difference with a power of 0.9 and alpha 0.05, a minimum of three animals were required for all experiments.

2.3. Fluorescence immunocytochemistry

All cells were labeled using a previously described indirect immunofluorescence technique [20]. Coverslips with dissociated NG neurons (n = 4 rats) were fixed with fresh 4% paraformaldehyde. Neurons were blocked with a solution of 10% normal donkey serum (NDS)/1% bovine serum albumin (BSA) (Jackson Laboratories, Bar Harbor, ME) in 1X PBS with 250 µg/ml digitonin (Sigma-Aldrich) prior to incubation with primary antibody (P2X3: 1:1000, GP10108, Neuromics, Edina, MN; or P2Y1: 1:1000, APR-009, Alomone Labs, Jerusalem, Israel) in 3% NDS/1% BSA/0.05% sodium azide (Acros, Thermo Fisher, NJ) in 1X PBS with 250 µg/ml digitonin (see [21]). After washing with 1X PBS, slides were then incubated with the secondary antibody (Donkey anti-Guinea Pig: 1:1000, CF488A, Biotium, Fremont, CA; or Donkey anti-Rabbit: 1:1000, Life Technologies, Carlsbad, CA) in 3% NDS/1% BSA in 1X PBS with 250 µg/ml digitonin for 30 min in the dark. Lastly, coverslips were mounted with Vecta-shield (Vector Laboratories Inc, Burlingame, CA). To verify specificity of the secondary antibody when the using the indirect immuno-fluorescence method, controls were prepared by omitting one primary antibody during the incubation. All antibodies were tested on rodent tissue preparations at least three times to confirm specificity and optimize concentration prior to performing any immunolabeling experiments and assure specific antibody labeling.

2.4. Confocal imaging

Images of isolated NG neurons were acquired using a Nikon C2+ confocal microscope (Nikon Instruments, Melville, NY) with Plan Apochromat 40X/1.30 Oil DIC objective with

Nikon Elements software (ver. 4.5). To identify fluorescent signals, specific band-pass filters were used to achieve proper separation of signals for each laser excitation (for double labeling 488 nm laser/505–530, 543 nm laser/560–590BP). Most confocal images were acquired at an approximate optical thickness of 0.5 μm or 1.0 Airy unit. Digital confocal images were saved as Nikon. ND2 format and final publication quality images were exported in the TIFF format as 300 dpi images. All images were processed and adjusted for brightness and contrast using Adobe Photoshop CC (Adobe Systems Inc., Mountain View, CA).

2.5. Solutions and drugs

For Ca^{2+} imaging: the superfusate consisted of (in mM): 140 NaCl, 5 KCl, 2 CaCl_2 , 1 MgCl_2 , 10 glucose, 10 HEPES, the pH was adjusted to 7.4 with NaOH. For elevated $[\text{K}^+]_o$ solutions, NaCl was replaced with an equal concentration of KCl. For recording P2X currents: the external solution consisted of (in mM): 145 NaCl, 2.5 KCl, 1 MgCl_2 , 2 CaCl_2 , and 20 HEPES. The pH was adjusted to pH 7.4 with NaOH. For recording Ca^{2+} currents: the external solution consisted of (in mM): 145 tetraethylammonium hydroxide (TEA-OH), 140 methanesulfonic acid, 10 HEPES, 15 glucose, 10 CaCl_2 , and 0.0003 TTX. The pH was adjusted to 7.40 with TEA-OH. The internal solution contained (in mM): 100 N-methyl-D-glucamine, 20 TEA-OH, 20 BAPTA, 10 HEPES, 4 MgATP, 0.3 Na_2GTP , and 14 creatine phosphate. The pH was adjusted to 7.20. Stimulus stock solutions: stock solutions of ATP, 2' (3')-O-(4-Benzoylbenzoyl)adenosine 5'-triphosphate (BzATP), ADP, and [D-Ala2-N-Me-Phe4-Glycol5]-enkephalin (DAMGO) were diluted in the external solution to their final concentrations on the day of the experiment.

2.6. Ca^{2+} imaging

Following isolation of NG neurons ($n = 7$ rats), cells were loaded with 2 μM Calbryte 520 (from a 1 mM stock in DMSO) (ATT Bioquest Sunnyvale, CA) for 30 min at 37 °C. Images were collected with a Nikon C2+ confocal mounted to an upright fixed stage microscope (Eclipse FN-1) equipped with a CFI Apo NIR 40 X(0.8 NA) water-immersion objective. To activate voltage-gated Ca^{2+} channels, cells were depolarized by increasing $[\text{K}^+]_o$ from 5 to 72 mM for 1 min. 12 bit images were acquired at 5–10 s intervals and controlled by Nikon Elements software. For analysis, a region of interest was drawn over the cell, and fluorescence changes were interpreted as changes in $[\text{Ca}^{2+}]_i$. Calbryte 520 was collected with a 488 nm excitation/505–530 nm BP emission filter. Fluorescence signals were expressed as $F/F = (F - F_o)/F_o$, where F_o denotes the resting fluorescence level at the beginning of the experiment, and $F/F \times 100$ to give a percentage. The baseline was established as the average of the first three measurements of fluorescence in every cell. The drug or stimulus (ATP, BzATP, or ADP) response was compared with the averaged baseline measurements. For measuring ADP effects on the K^+ -evoked depolarization of NG neurons, control conditions were determined by averaging the prior control and subsequent wash responses evoked by elevated $[\text{K}^+]_o$ with each drug application. These fractional responses were used to measure ADP's effect on the K^+ -evoked depolarization in NG neurons. Typically, 1 to 3 cells were analyzed on each coverslip and all experiments were performed on at least three different preparations. Data was excluded if there was shifting in the x, y, or z planes.

2.7. Electrophysiology and data analysis

Whole-cell Ca^{2+} currents were recorded employing the patch-clamp technique ($n = 5$ rats). The currents were acquired with the Axopatch 200B amplifier (Molecular Devices, Sunnyvale, CA), analog filtered at a frequency of 2 kHz (-3 dB, four-pole low-pass Bessel filter) and digitized with custom-designed F6 software (Stephen R. Ikeda, National Institute on Alcohol Abuse and Alcoholism, NIH). Agonist-mediated Ca^{2+} current inhibition was calculated as follows: $[(\text{peak } \text{Ca}^{2+} \text{ current before agonist}) - (\text{peak } \text{Ca}^{2+} \text{ current after agonist})] / [(\text{peak } \text{Ca}^{2+} \text{ current before positive NG neurons})]$. Microinjection of cDNA plasmids was performed with a 5246 microinjector and 5171 micromanipulator (Eppendorf) as described previously [22] 3 to 5 h after NG neurons were plated. The human P2Y₁₂ receptor, was injected at a final concentration of 100–150 ng/ μl . The enhanced green fluorescent protein (Clontech, Mountain View, CA) cDNA construct was co-injected at a concentration of 5 ng/ μl to allow for the identification of successfully injected neurons. P2X currents were also acquired with a holding potential of -80 mV. After compensating for membrane capacitance and series resistance, ATP was applied to the cell for 3 sec.

2.8. Statistical analysis

The electrophysiological recordings were analyzed with Igor Pro (Wavemetrics, Lake Oswego, OR). Statistical analysis for electro-physiological and Ca^{2+} imaging data was performed with Prism 7.5 software (GraphPad Software, San Diego, CA). The statistical significance between two groups was performed employing unpaired t-tests. All data are presented as mean $\pm$ standard error of the mean (SEM) with statistically significant differences defined as $p < 0.05$.

3. Results

3.1. Gastric-innervating NG neurons are immunopositive for P2X₃ and P2Y₁ receptors

Currently, it is unknown if gastric-innervating NG neurons express P2X₃ and P2Y₁ receptors. To confirm whether gastric-innervating NG neurons express both P2Y₁ and P2X₃ receptors, DiI labeled NG neurons from the stomach were dissociated and plated on poly-L-lysine coated coverslips. The P2X₃ labeling was punctate in DiI labeled NG neurons (Fig. 1A), as previously reported [8,9,23], and localized to different cellular compartments including the cell membrane. Similarly, P2Y₁ immunoreactivity (green) in DiI labeled NG neurons (red) revealed a punctate signal throughout the cell including the membrane (Fig. 1B). In contrast, DiI labeled NG neurons where the primary antibodies were omitted lacked any fluorescence signal (data not shown).

3.2. ATP evokes P2X inward currents and $[\text{Ca}^{2+}]_i$ increases in gastric-innervating NG neurons

Based upon the expression of P2X₃ in isolated gastric-innervating NG neurons (see Fig. 1A), we tested if gastric-innervating NG neurons functionally expressed P2X channels. We tested whether ATP (100 μM), BzATP (10 μM), a P2X selective agonist [24], and control K^+ -evoked depolarization would evoke $[\text{Ca}^{2+}]_i$ increases in gastric-innervating NG neurons. Fig. 2A. shows a DiI labeled gastric-innervating NG neuron loaded with Calbryte 520 (top

left panel), followed by pseudocolor images corresponding to numbered images taken at specific time points as indicated by arrows in Fig. 2B. Corresponding paired pseudocolor images of cellular fluorescence intensity (Fig. 2A, 1-7) are indicated by the arrows shown on the plot of the intensity (F/F) over time in Fig. 2B, 1-7. BzATP, elevated $[K^+]_o$, and ATP all evoked a $[Ca^{2+}]_i$ increase following bath application for 1 min in all cells tested (Fig. 2B). $p < 0.05$ increase over baseline $[Ca^{2+}]_i$ levels, elevated K^+ -evoked a $68\% \pm 13\%$ ($n = 13$, $p < 0.05$), and ATP evoked a $102\% \pm 19\%$ ($n = 16$, $p < 0.05$) increase over baseline $[Ca^{2+}]_i$ levels (See Fig. 2C). In the next set of experiments, DiI labeled NG neurons were exposed to ATP (10 μ M) to determine whether P2X currents would be activated. Fig. 2D shows that a 3 sec application of ATP evoked an inward P2X current that rapidly activated and then inactivated. The summary plot in Fig. 2E shows the peak P2X current density in the NG neurons tested.

3.3. Voltage-gated Ca^{2+} channels are not modulated by P2Y receptor activation in NG neurons

Based upon the expression of P2Y1 in isolated gastric-innervating NG neurons (see Fig. 1B), we tested if NG neurons innervating the stomach functionally expressed ADP-activated P2Y channels. Ca^{2+} to test whether ADP altered the depolarization-evoked $[Ca^{2+}]_i$ increases. Following isolation, Calbryte520/AM loaded NG cells were depolarized by elevating $[K^+]_o$ from 5 to 72 mM for 1 min. Fig. 3A shows a combined fluorescent DIC brightfield image of Calbryte 520 loaded NG neuron that has been labeled with DiI. Corresponding paired pseudocolor images of fluorescence from the same cells are displayed (Fig. 3A, 1-7), showing cells in both normal (5 mM) and elevated (72 mM) $[K^+]_o$ mammalian superfusate, ADP, ADP + elevated $[K^+]_o$ mammalian superfusate, and subsequent wash responses. Corresponding time-course of F/F levels for the same cell is illustrated in Fig. 3B. Fig. 3C further shows a summary plot of the $[Ca^{2+}]_i$ response measured in a neuron before, during, and after the application of ADP (10 μ M). ADP produced a small but reversible inhibition of the depolarization-evoked $[Ca^{2+}]_i$ increase. On average, ADP (10 μ M) caused a $13.7 \pm 3.5\%$ ($n = 14$, $p < 0.05$) reduction in the K^+ -evoked fluorescence change in DiI labeled NG neurons (Fig. 3C). In some cells (10/15), there was an ADP evoked Ca^{2+} increase upon application of ADP, however the effect of the K^+ -evoked Ca^{2+} was unaffected by this response (data not shown).

Fig. 4A shows the time course of peak Ca^{2+} current amplitude acquired before [Fig. 4A (trace 1) and Fig. 4A (trace 3)] and during [Fig. 4A (trace 2) and Fig. 4A (trace 4)] application of ADP and DAMGO, a high affinity mu-opioid receptor agonist that served as a positive control, (both at 10 μ M) in a control DiI labeled NG neuron. The Ca^{2+} currents were evoked every 10 sec with a test pulse (shown right side) to +10 mV from a holding potential of -80 mV. The superimposed Ca^{2+} currents shown in Fig. 4A correspond to those plotted. Application of ADP led to a ~10% inhibition [Fig. 4A (trace2)]. After a recovery period, neurons were treated with DAMGO. Since mu-opioid receptors are expressed by NG neurons, DAMGO elicited an 80% reduction in the Ca^{2+} currents. In the cells tested, ADP exerted weak Ca^{2+} current modulation. The time course shown in Fig. 4B was recorded from an NG neuron that was transfected with P2Y12 receptor cDNA (a positive control) and the corresponding numbered current traces are shown to the right. Initially, the neuron was exposed to ADP and the Ca^{2+} current was blocked by 29% [Fig. 4B (trace 2)]. Thereafter,

DAMGO was applied to the cell and the Ca^{2+} currents were blocked approximately 60% [Fig. 4B (trace 4)]. Fig. 4C is a summary scatter plot showing the ADP- and DAMGO-mediated Ca^{2+} current inhibition in control and in P2Y₁₂ receptor-expressing neurons. The plot indicates that the purinergic-mediated modulation of Ca^{2+} currents from P2Y₁₂ receptor-expressing NG neurons was significantly ($p < 0.05$) greater than that observed for control neurons.

4. Discussion

In the present study we observed large ATP induced $[\text{Ca}^{2+}]_i$ increases and inward ATP currents in gastric-innervating NG neurons which functionally support the immunopositive labeling of the P2X₃ receptor on NG neurons, and possibly expression of other P2X receptor channel subunits as previously reported [7,18,25]. Conversely, in spite of strong expression of the P2Y₁ receptors we observed only minor modulation of voltage-gated Ca^{2+} channels in the presence of the P2Y selective agonist ADP in gastric-innervating NG neurons. Therefore, ATP acting at both P2X and P2Y receptors may have a larger impact on ATP-mediated $[\text{Ca}^{2+}]_i$ increases in gastric-innervating NG neurons.

Previous electrophysiological and Ca^{2+} imaging studies have shown that the fast excitatory effects of ATP on NG neurons were mediated by pharmacologically identified P2X₂, P2X₃, and heteromeric P2X_{2/3} receptors [26]. In addition, P2Y receptors play an important role in the mobilization of $[\text{Ca}^{2+}]_i$ from intracellular stores and can be activated by released ATP [15,27,28]. Satellite glial cells are the predominant glial cell controlling the neuronal microenvironment of sensory ganglia, including the NG (see [29]). Although not shown, we did observe, ATP-induced $[\text{Ca}^{2+}]_i$ responses in the satellite glial cells retained with the isolated NG neurons, for both ATP and ADP, and $[\text{Ca}^{2+}]_i$ increases in some NG neurons following the application of ADP (data not shown), suggesting that functional P2Y receptors are present on both gastric-innervating NG neurons and satellite cells which is in agreement with previous studies [11,15,30]. Therefore, our immunohistochemical, electrophysiological and Ca^{2+} imaging findings are the first reported evidence supporting a role for ATP at both P2X and P2Y receptors on gastric-innervating NG neurons.

Previously, the P2X₃ purinergic receptor has been used to functionally characterize myelinated A-fiber vagal afferents while C-fibers are segregated by vanilloid (TRPV1) receptor sensitivity [31]. More recently, it has been shown that the P2X₃ receptor is widely dispersed throughout the nodose ganglia and expressed in both myelinated and unmyelinated vagal afferents [32]. Specifically, the functional dichotomy that is present in baroreflex afferents does not hold true for GI inputs at the level of the dorsal vagal complex [10]. In addition to central neural circuits, evidence is mounting that the function of neurons in peripheral sensory ganglia (e.g., nodose, dorsal root & trigeminal) is heavily modulated by the local interactions with adjacent satellite glial cells, and the responses of these sensory neurons are unique to their own microenvironment [33,34]. ATP is an important modulator within the local microenvironment of the NG, though the role of this local ATP in normal digestive afferent signaling remains obscure. Available data does suggest that the purinergic system is susceptible to alteration after insult and often leads to changes in excitability of sensory afferents [11,12]. In particular, ATP may initiate remodeling, excitotoxicity, and

dysregulation of sensory afferents, including those of the vagus. The vagal gastric neural circuitry could be substantially different from other vagal circuits, and understanding neuropathological changes in function require appropriate characterization of the cell bodies relevant to the system being studied.

The objective of this study was to specifically investigate gastric neurons within the NG. Since DiI was dissolved in DMSO and injected underneath the gastric serosa, it is likely that our procedure labeled both the mucosa and the smooth muscle. Therefore, our population of gastric NG neurons may include both muscular and mucosal afferents. While mechano- and chemosensitive afferent subtypes have been distinguished through selective application of tracers in ferrets [35], our present studies did not incorporate this differential tracing approach for the afferent subtypes of the rat.

5. Conclusions

We have shown that identified gastric vagal afferent neurons are under local purinergic modulation that includes the P2X3 receptor subtype. We further show that metabotropic P2Y receptors, though present on gastric-innervating NG neurons, modulate only a minor Ca^{2+} current. Our findings confirm and refine previous observations demonstrating differential purinergic-receptor expression and function of cultured NG neurons derived from the entire NG population [7]. Purinergic-mediated augmentation of NG signaling in the context of nociceptive neural transmission has received considerable attention [36]. While a subset of gastric NG neurons may be involved in the transmission of nociceptive stimuli, the majority of NG neurons relay the stimuli associated with digestive processes [1]. The purinergic modulation of digestion-relevant NG neurons in disease- or injury-states deserves further investigation.

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Abbreviations:

ATP	Adenosine 5'-Triphosphate
ADP	Adenosine 5'-Diphosphate
$[\text{Ca}^{2+}]_i$	Cytosolic Ca
BSA	Bovine Serum Albumin
BzATP	2'(3')-O-(4-Benzoylbenzoyl)adenosine 5'-triphosphate
DAMGO	[D-Ala2-N-Me-Phe4-Glycol5]-enkephalin
DiI	11'-dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine perchlorate

GI	Gastrointestinal
NDS	Normal Donkey Serum
NG	Nodose Ganglion
SEM	Standard Error of Mean

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Fig. 1. Immunohistochemical labeling of P2X3 and P2Y1 in isolated gastric-innervating NG neurons (n = 4 rats). (A) Confocal image of immunopositive P2X3 gastric-innervating NG neurons (15 cells analyzed). P2X3-immunopositive expression (480 nm excitation, *upper left*); DiI (530 nm excitation, *upper right*); Merged P2X3 and DiI channels (*lower left*); and merged channels with DIC to delineate the entire cell (*lower right*). (B) Confocal image of immunopositive P2Y1 gastric-innervating NG neurons (12 cells analyzed). P2Y1-immunopositive expression (480 nm excitation, *upper left*); DiI (530 nm excitation, *upper right*); Merged P2Y1 and DiI channels (*lower left*); and merged channels with DIC to delineate the entire cell (*lower right*). Scale bar = 50 μm.

Fig. 2.

ATP-stimulated Ca^{2+} responses and P2X currents (I_{ATP}) in DiI labeled NG neurons ($n = 7$ rats, cells for each analysis in parenthesis). (A) A brightfield DIC image of an isolated DiI labeled (red) NG neuron loaded with Calbryte 520 $[\text{Ca}^{2+}]$ -sensitive indicator dye (upper left panel). Corresponding pseudocolor images (1–7) from the same cell plotted in B. (B) A plot of the fluorescence intensity over time from an ROI drawn over the soma of the isolated DiI labeled NG neuron in A. The arrows correspond to the images shown in A: 1, control; 2, 10 μM BzATP containing superfusate; 3, wash; 4, elevated ($[\text{K}^+]_o = 72 \text{ mM}$) $[\text{K}^+]_o$ containing superfusate; 5, wash; 6, 10 μM ATP containing superfusate; and 7, wash. The numbers with arrows indicate the time point when the image was captured during the experiment. (C) A summary scatter plot of the stimulus-evoked Ca^{2+} increases in basal fluorescence. Percent increases over the basal fluorescence levels measured in DiI labeled NG neurons in response to a stimulus: 10 μM BzATP ($n = 9$, $*p = 0.0052$); 72 mM $[\text{K}^+]_o$ ($n = 13$, $*p = 0.0002$); 10 μM ATP ($n=16$, $*p < 0.0001$). Lines and error bars represent the mean $\pm$ SEM (D) in DiI labeled NG neurons. Sample trace of native P2X receptor currents stimulated by ATP (10 μM) exposure indicated by the bar above the current trace. The holding potential for the neurons was -80 mV . (E) Summary scatter plot showing the mean $\pm$ SEM. P2X current density mediated by ATP in NG neurons. Number in parenthesis indicates the number of neurons tested. Scale bar = 50 μm .

Fig. 3. ADP partially inhibits the K^+ -evoked-depolarization in DiI labeled NG neurons (n = 7 rats, yielding 14 cells for analysis). (A) A brightfield DIC image of an isolated DiI labeled (red) NG neuron loaded with Calbryte 520 [Ca^{2+} -sensitive indicator dye] (upper left panel). Corresponding pseudocolor images (1–7) from the same cell plotted in B. (B) A plot of the fluorescence intensity over time from an ROI drawn over the soma of an isolated DiI labeled NG neuron. The arrows correspond to the images shown in A: 1, control, normal ($[K^+]_o = 2.5$ mM) superfusate; 2, elevated ($[K^+]_o = 72$ mM) $[K^+]_o$ containing superfusate; 3, wash or normal superfusate; 4, ADP (10 μ M) and elevated $[K^+]_o$ superfusate; 5, wash, or normal $[K^+]_o$ superfusate; 6, elevated $[K^+]_o$ containing superfusate; and 7, wash, or normal $[K^+]_o$ superfusate. The numbers with arrows indicate the time point when the image was captured during the experiment. (C) A scatter plot of the fractional ADP-mediated Ca^{2+} response to a K^+ -evoked depolarization in DiI labeled NG neurons (n = 14, *p = 0.002). Line and error bars represent the mean $\pm$ SEM. Scale bar = 50 μ m.

Fig. 4. Time courses of peak Ca^{2+} current amplitude acquired from the application of ADP (10 μM) and DAMGO (10 μM) in control(A) and a P2Y12R-transfected NG neuron (B). Ca^{2+} currents were evoked every 10 sec with a depolarizing test pulse (right). The numbered Ca^{2+} current traces in A and B are shown to the right, where 1 and 3 represent control currents (before agonist exposure), and 2 and 4 represent currents in the presence of ADP or DAMGO, respectively. (C) Summary scatter plot showing the mean $\pm$ SEM. Ca^{2+} current inhibition mediated by ADP and DAMGO in control (circles) and P2Y12-expressing (triangles) NG neurons. *p < 0.05 compared to control, paired t-test. (n = 5, numbers in parenthesis indicate the number of neurons analyzed).