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Control of P2X3 channel function by metabotropic P2Y2 UTP receptors in primary sensory neurons

Gary Mo, Jennifer C. Peleshok, Chang-Qing Cao, Alfredo Ribeiro-da-Silva and Philippe Séguéla

Alan Edwards Research Centre on Pain *(G.M., J.C.P., A.R.-d-S., P.S.)*, Montreal, Canada; and Department of Bioscience *(C.-Q.C)*, AstraZeneca R&D Montreal, Ville-Saint-Laurent, Canada; and Montreal Neurological Institute, Department of Neurology & Neurosurgery *(G.M., P.S.),* Department of Pharmacology *(J.C.P., A.R.-d-S.)*, Department of Anatomy and Cell Biology *(A.R.-d-S.)*, McGill University, Montreal, Canada

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Running title: *P2Y2-P2X3 crosstalk in DRG neurons*

Corresponding author:

Dr. Philippe Séguéla

Montreal Neurological Institute

3801 University, Suite 778

Montreal (Quebec) Canada H3A 2B4

Phone: 1-514-398-5029

Fax 1-514-398-8106

philippe.seguela@mcgill.ca

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List of abbreviations and chemical structures:

DRG, dorsal root ganglion; MRS2279, [(1S,2R,4R)-4-[(2-chloro-6-methylaminopurin-9-yl)methyl]-2-(phosphonooxymethyl)cyclopentyl] dihydrogen phosphate; MRS2365, [(1R,2R,3S,5S)-4-[6-amino-2-(methylsulfanyl)-9H-purin-9-yl]-2,3-dihydroxybicyclo[3.1.0]hexan-1-yl]methyl sodium {[bis(sodiooxy)phosphoryl]oxy}phosphonate; PAR2, protease-activated receptor-2; PIP_2, phosphatidylinositol-4,5-bisphosphate; PKA, protein kinase A; PKC, protein kinase C; PLC,

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phospholipase C; TRPV1, transient receptor potential vanilloid 1 receptor; U73122, 1-[6-[[(17β)-3-methoxyestra-1,3,5(10)-trien-17-yl]amino]hexyl]-1H-pyrrole-2,5-dione; U73343, 1-[6-[[(17β)-3-methoxyestra-1,3,5(10)-trien-17-yl]amino]hexyl]-2,5-pyrollidinedione.

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Abstract

Purinergic signaling contributes significantly to pain mechanisms, and the nociceptor-specific P2X3 ATP receptor channel is considered a target in pain therapeutics. Recent findings suggesting the co-expression of metabotropic P2Y receptors with P2X3 implies that ATP release triggers the activation of both ionotropic and metabotropic purinoceptors, with strong potential for functional interaction. Modulation of native P2X3 function by P2Y receptor activation was investigated in rat dorsal root ganglia (DRG) neurons using whole-cell patch-clamp recordings. Application of the selective P2Y receptor agonist UTP decreased peak amplitudes of α,β-meATP-evoked homomeric P2X3-mediated currents, but had no effect on heteromeric P2X2/3-mediated currents. Treatment with phospholipase C (PLC) inhibitor U73122 significantly reversed P2X3 current inhibition induced by UTP-sensitive P2Y receptor activation. We previously reported the modulation of P2X receptors by phospholipids in DRG neurons and injection of exogenous phosphatidylinositol-4,5-bisphosphate (PIP_2) fully reverses UTP-mediated regulation of P2X3 channel activity. Pharmacological as well as functional screening of P2Y receptor subtypes indicates the predominant involvement of P2Y2 receptor in P2X3 inhibition and immunolocalization confirms a significant cellular co-expression of P2X3 and P2Y2 in rat DRG neurons. In summary, the function of P2X3 ATP receptor can be inhibited by P2Y2-mediated depletion of PIP_2. We propose that expression of P2Y2 purinoceptor in nociceptive sensory neurons provides an homeostatic mechanism to prevent excessive ATP signaling through P2X3 receptor channels.

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Introduction

The intrinsic complexity of purinergic signaling was predicted when several genes encoding P2 purinoceptors were identified (Abbracchio and Burnstock, 1994). ATP, activates different classes of P2 purinoceptors subdivided into ionotropic P2X and metabotropic P2Y gene families (Burnstock and Kennedy, 1985). Seven P2X receptor subunits (P2X1-7) have been cloned in rodents and humans, and their physiological function have been extensively investigated (Jarvis and Khakh, 2009). Initially isolated from the non-peptidergic subpopulation of peripheral nociceptors (Chen et al., 1995), the excitatory P2X3 receptor channel is a prominent pain transducer and it has been proposed as a target for pain therapeutics (Wirkner et al., 2007). However, its exact contribution to pathological pain remains unclear. While several reports describe decreased pain response in P2X3 deficient animals (Souslova et al., 2000; Honore et al., 2002) and increased P2X3 function after neuropathic injury (Chen et al., 2005), others document a decrease (Kage et al., 2002) or no significant change in P2X3 function in chronic pain states (Mo et al., 2011). It is possible that changes in P2X3 function under pathological conditions are more complex than simple up- or down-regulation of expression at the protein level. Understanding the regulatory mechanisms of P2X3 activity is critical in elucidating its function in pain and the modulation of P2X3 currents by intracellular pathways has recently gained attention (Giniatullin et al., 2003; Wang et al., 2007; Park et al., 2010). We previously reported that depletion of the plasma membrane-specific phospholipid phosphatidilinositol-4,5-bisphosphate (PIP_2) decreases P2X3 activity (Mo et al., 2009).

Although investigations of the contribution of purinergic signaling to nociception have largely focused on ionotropic P2X receptors, increasing evidence suggest that metabotropic P2Y receptors also play an important role in pain (for review see (Gerevich and Illes, 2004)). The 8 P2Y receptors (P2Y1, P2Y2, P2Y4, P2Y6, P2Y11-14) identified so far are ubiquitous G-protein

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coupled receptors that can activate a wide range of cellular processes (Abbracchio et al., 2006). For example, the P2Y2 receptor is considered a therapeutic target in the treatment of cystic fibrosis (Kellerman et al., 2002), but it has also been implicated in pain signaling (Moriyama et al., 2003; Stucky et al., 2004). P2Y1 and P2Y2 receptors, both Gq protein-coupled, have been reported to be the major P2Y subtypes expressed in peripheral small-diameter sensory neurons, including the non-peptidergic nociceptors that express P2X3 (Molliver et al., 2002; Kobayashi et al., 2006). Since P2Y receptors and P2X3 channels can be activated by the same endogenous agonist ATP, a functional cross-talk between co-expressed metabotropic and ionotropic purinoceptors would provide an homeostatic mechanism for controlling purinergic signaling.

The present study reports that activation of UTP-sensitive P2Y receptors reversibly inhibits inward currents mediated by the ionotropic ATP receptor P2X3 in rat DRG sensory neurons. The molecular mechanism of interaction between these two purinoceptors involves phospholipase C activation and depletion of PIP_2, which has a known modulatory effect on P2X3 receptor function (Mo et al., 2009). Furthermore, using pharmacological screening as well as immunocytochemistry, we propose that this inhibitory effect of UTP on P2X3 function is mediated through activation of P2Y2 receptors.

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Materials and Methods

Cell Culture

DRGs from lumbar segments were extracted from male Sprague-Dawley rats aged 4-6 weeks (Charles River Canada) under deep anaesthesia induced by halothane. The DRGs were placed in ice-cold DMEM (Gibco) for removal of connected tissue and dura matter. The isolated DRGs were then placed into DMEM containing 1 mg/mL of papain and 2 mg/mL collagenase type II (Sigma Aldrich) and incubated for 1 h at 37˚C and 100% humidity. After enzymatic digestion, the DRGs were transferred into DMEM containing 10% FBS and 1% L-glutamine, dissociated into single neurons by means of trituration using fire-polished pipettes. Dissociated neurons were then transferred into F-12 media (Gibco) containing 10% FBS, 1% L-glutamine, 100 U/mL penicillin and streptomycin and 30 ng/mL nerve growth factor (Sigma Aldrich), and plated onto 35 mm cell culture dishes (Sarstedt) coated with laminin (BD Bioscience) and poly-D-lysine (Sigma Aldrich), and cultured for 48 h at 37˚C and 100% humidity.

Patch-clamp recordings

Whole-cell patch-clamp recordings (V_h of −60 mV) on DRG neurons were conducted using pipettes filled with intracellular solution (ICS), pH 7.2, containing (in mM): 130 K-gluconate, 1 $MgCl_2$, 5 EGTA, 10 HEPES, and 0.4 GTP. Drug applications were performed using a fast microperfusion system at a rate of 1 ml/min (SF-77B, Warner Instruments, Morris Plains, NJ). The standard extracellular solution (ECS), pH 7.4, comprised (in mM): 152 NaCl, 5 KCl, 2 $CaCl_2$, 1 $MgCl_2$, 10 HEPES, and 10 glucose. Membrane currents were recorded using an Axopatch 200B amplifier, digitized with a Digidata 3200A interface (Axon Instruments, Foster City, CA.), and acquired at a frequency of 2 kHz using pClamp 9. Osmolarity of external solution was adjusted to 300 mOsm/l and that of pipette solutions to 280 mOsm/l. All

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experiments were carried out at room temperature (20–23°C). Recording electrodes were produced by pulling borosilicate glass tubes using a P-97 puller (both from Sutter Instrument, Novato, CA.), and fire-polishing with a MP-830 microforge (Narishige, Tokyo, Japan) to a tip resistance of 3-6 MΩ when filled with ICS. The culturing plates were also used as the recording chamber. Membrane capacitance (C_m) and series resistance (R_s) were measured through the peak amplitude and decay constant of transients induced by repetitive depolarizing pulses of 10 mV.

P2X receptor-mediated currents were induced by fast application of α,β-meATP. Successive currents were recorded to determine current stability. Multiple current responses were recorded 3 min apart, which was sufficient time for the P2X3 receptor to fully recover from desensitization. For every experiment, peak currents of four α,β-meATP-induced responses were recorded: 2 successive control responses of the same peak amplitude were recorded, then cells are treated with P2Y agonist and another response was recorded, and finally a response after a washout period. The amplitudes of currents recorded under the different conditions were compared to the first response and expressed as a percentage. Specifically, control ratios were determined by dividing the peak amplitude of the second control response by the first, P2Y agonist effects were determined by dividing the peak amplitude of the response after P2Y agonist treatment by that of the first control response, and washout ratio was determined by dividing peak amplitude of response after washout by the first control response.

Histological processing and immunolocalization

Rats were administered Equithesin (6.5 mg chloral hydrate and 3 mg sodium pentobarbital) in a volume of 0.3 mL, i.p., per 100 g body weight. Once animals were deeply anaesthetized, they were quickly transcardially perfused with vascular rinse (0.1% w/v sodium nitrite in a phosphate buffer) followed by histological fixatives (4% paraformaldehyde (PFA) in 0.1 M phosphate buffer pH 7.4) for 30 min. The entire vertebral column (containing the spinal cord and spinal

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nerves and ganglia) was removed and post-fixed overnight in the same fixative at 4°C, followed by 30% sucrose in 0.1 M phosphate buffer for cryoprotection. Dorsal root ganglia from segments (specify) were then dissected out and embedded in an optimum cutting temperature medium (OCT, TissueTek), sectioned using a cryostat (Leica) at a thickness of 14 μm and thaw-mounted onto gelatin coated slides. Dissociated DRG neurons were cultured on glass coverslips for 24 h prior to fixation with 4% PFA.

Slides were washed for 30 min with PBS followed by 1 h incubation with 10% blocking buffer. Slides were then incubated overnight at 4°C in humidified chamber with the following primary polyclonal antibodies: guinea-pig anti-P2X3 (1:7000, Neuromics, Edina MN) and rabbit anti-P2Y2 (1:300, Alomone, Israel). Sections were then washed and incubated with secondary antibodies for 2 h at room temperature: for P2Y2, goat anti-rabbit IgG conjugated to Alexa 488 (1:800, Molecular Probes, Eugene OR) and for P2X3 donkey anti-guinea pig IgG conjugated to rhodamine red-X (1:400, Jackson Immunoresearch, West Grove PA), then washed and coverslipped using Aquapolymount (Polysciences; Warrington, PA). Specificity of the anti-P2X3 antibody was previously validated (Ichikawa and Sugimoto, 2004; Dellarole and Grilli, 2008). Pre-adsorption of the primary antibody with the peptide immunogen abolished all selective P2X3 staining. We also performed control experiments for the P2Y2 antibody by pre-incubating the primary antibody with saturating amounts of the peptide immunogen (supplied by manufacturer) prior to incubation (Tung et al, 2004). The pre-adsorption abolished selective P2Y2 staining in DRG neurons.

Images in Figures 6A and 6B were taken using a fluorescent Zeiss Axioplan 2 microscope equipped with a 40× Plan-Fluotar oil-immersion objective. Images were acquired with a high-resolution color digital camera using the Zeiss AxioVision software. Images were exported directly in TIFF format and adjusted for brightness and contrast using Adobe Photoshop CS4.

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Statistical analysis

Data are presented as mean ± S.E.M. All statistical analyses for the difference in means were carried out using either Mann-Whitney or Wilcoxon matched pairs test depending on experimental conditions. Difference in data was considered statistically significant if P value is ≤ 0.05.

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Results

Whole-cell recordings were conducted on cultured small diameter DRG neurons. Neurons with processes were preferred for recording as process extension indicates greater cellular viability. Only neurons exhibiting a resting membrane potential of -35 mV or below, and able to respond to fast application of agonist with a current amplitude larger than 200 pA were used. The activity of P2X receptors were induced by 10 μM α,β-meATP, a selective agonist for P2X receptors without any reported activity on P2Y receptors (North, 2002a). Expression of P2X receptor subtypes was functionally determined by the kinetics of response to α,β-meATP as previously described in DRG neurons (Burgard et al., 1999). For experiments examining P2X3 receptors, only neurons that exhibited predominant fast activating and rapidly desensitizing P2X3 current profiles were retained for analysis (sample traces in Figure 1), and P2X2/3 experiments only utilized cells that contained a significant slowly-desensitizing P2X2/3 current component (sample traces in Figure 2). P2Y receptors were activated by UTP or the selective agonist MRS2365 with no known activity on P2X receptors.

UTP-mediated inhibition of P2X3 receptor activity

Application of α,β-meATP on homomeric P2X3 receptor-expressing neurons produced fast activating and fast desensitizing currents typical of the P2X3 subtype (Figure 1A). Given a 3 min washout period to allow full recovery, the cells were able to respond to the same degree as initial application of α,β-meATP. After two successive α,β-meATP-evoked P2X3-mediated responses with constant amplitude and current kinetics (to confirm the absence of rundown), the neuron was treated with the P2Y agonist UTP (100 μM) for 3 min. UTP treatment decreased the current amplitude of the subsequent response to α,β-meATP by ~50% (Control: 95.8 ± 5.3%, UTP: 47.4 ± 4.6%, all relative to initial response, Figure 1B). When the neurons were washed with control

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buffer for 3 min the current amplitude of response recovered to ~87% of control (Control: 95.8 ± 5.3%, Wash: 83.8 ± 7.1%, all relative to initial response, Figure 1B). Treatment with UTP did not cause any changes to current kinetics for activation nor desensitization (data not shown). These data indicate that activation of P2Y receptors by UTP causes an inhibition of P2X3 receptor activity.

Inhibition mediated by UTP is specific for the homomeric P2X3 subtype

Contrary to what we observed studying homomeric P2X3 receptor activity, UTP did not seem to affect the activity of the heteromeric P2X2/3 receptor subtype. When the same experiment outlined above was performed on neurons that significantly express the P2X2/3 receptor subtype, treatment with 100 μM UTP did not cause any inhibition to the sustained current typically mediated by P2X2/3 receptors (Figure 2A). Although the P2X2/3 receptor does not exhibit desensitization and run-down, for consistency we still waited 3 min between successive applications of α,β-meATP. The current amplitude of P2X2/3-mediated responses after 3 min treatment with UTP was not different compared to control responses (Control: 98.3 ± 4.4%, UTP: 85.8 ± 8.9%, all relative to initial response, Figure 2B) and UTP did not cause any changes in P2X2/3-mediated current kinetics. Most neurons that express the P2X2/3 receptor subtype also exhibit varying levels of homomeric P2X3-mediated currents, as indicated by a fast desensitizing current component (Figure 2A). The inhibition of the fast desensitizing component, while no change was observed in the sustained component, further demonstrates the P2X3 subtype specificity of this modulation by P2Y UTP receptors (Figure 2A). No analysis of the P2X3-mediated component was performed because peak amplitude cannot be accurately determined in presence of significant P2X2/3 generated currents.

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UTP-induced inhibition of P2X3 receptor activity involves PLC

Both predominant P2Y receptors subtypes in DRG neurons, P2Y1 and P2Y2, are linked to phospholipase C (PLC) activation via Gq coupling. To verify the involvement of PLC in the modulatory effect of UTP on P2X3 activity, we performed the same experiment as described above while intracellularly injecting the PLC blocker U73122. Although U73122 is membrane permeable, we could not treat DRG neurons extracellularly because the compound caused the cells to detach from the recording surface, therefore we applied it intracellularly through the patch pipet. In presence of 10 μM U73122, the inhibitory effect of UTP-induced P2Y receptor activation on P2X3 function was significantly attenuated (UTP: 47.0 ± 4.0%, UTP + U73122: 72.6 ± 4.9%, all relative to initial response, Figure 3B). Intracellular application of the control inactive enantiomer U73343 was not able to cause any change to UTP-induced inhibition of P2X3 receptor currents induced by α,β-meATP (UTP: 47.0 ± 4.0%, UTP + U73343: 43.4 ± 6.5%, all relative to initial response, Figure 3B). Treatment with U73122 did not cause any change in the control response or response after recovery from washout, nor did it induce any noticeable changes to current kinetics (Figure 3A). Evidence here confirms that UTP-mediated inhibition of P2X3 currents requires PLC activation.

Inhibition of P2X3 activity by UTP-sensitive P2Y receptors involves PIP_2

Previously we reported that P2X3 channel activity was sensitive to changes in intracellular levels of phosphoionositide PIP_2 (Mo et al., 2009), we tested if PIP_2 may be directly involved in the UTP-mediated PLC-dependent inhibition of the P2X3 receptor activity. When recordings were performed with 200 μM diC8-PIP_2 in the solution of the recording electrode, a 3 min application of UTP did not cause any significant inhibition of P2X3-mediated currents (Control + PIP_2: 84.8 ± 4.5%, UTP + PIP_2: 78.3 ± 5.8%, all relative to initial response, Figure 4B). Such intracellular

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injection of exogenous PIP_2 did not cause any significant change to the control P2X3 response or the response induced after a 3 min wash period in both amplitude and current kinetics (Figure 4A). These results indicates involvement of PIP_2 in the mechanism of UTP-induced inhibition of P2X3 activity.

Inhibitory effect of UTP does not involve the P2Y1 receptor subtype

The main candidates for Gq-coupled PLC-linked P2Y receptors expressed in DRG neurons are P2Y1 and P2Y2. Although the contribution of P2Y1 is less likely, since it is not sensitive to UTP, commercially available triphosphate nucleotides can be contaminated with di- or monophosphate nucleotides thus activation of P2Y1 remains a possibility. We checked that treatment of the DRG neurons with the selective P2Y1 agonist MRS2365 (5 nM) for 3 min was not able to produce any changes comparable to the inhibitory effect exerted by UTP (MRS2365: 78.8 ± 6.01%, UTP: 44.4 ± 4.0%, all relative to initial response, Figure 5B). In fact, MRS2365 was not able to produce any significant change to the P2X3 current amplitude (Control: 93.8 ± 4.2%, MRS2365 78.8 ± 6.0%, all relative to initial response, Figure 5A). To further confirm that P2Y1 is not involved, experiments were conducted with the P2Y1 selective antagonist MRS2279. Treatment with UTP for 3 min in constant presence of 500 nM MRS2279 did not cause any reversal of UTP-mediated inhibition of P2X3 receptor function (UTP: 49.8 ± 3.6%, UTP + MRS2279: 49.0 ± 2.8%, all relative to initial response, Figure 5B). These results strongly indicate that the inhibitory effect of UTP on P2X3 activity is mediated by activation of the metabotropic P2Y2 receptors, with no involvement of P2Y1 receptors.

Co-localization of P2Y2 and P2X3 receptors in DRG sensory neurons.

To confirm the co-expression of P2Y2 with P2X3 receptors in the same sensory neurons, the distribution of these two purinoceptors on primary afferents from dorsal root ganglia was

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investigated in double-labeling immunohistochemistry using well-characterized primary antibodies. We found that P2Y2 immunoreactivity in large diameter cell bodies was punctuate in appearance and, as expected, was not co-localized with P2X3 within this cell type. A significant subpopulation of small-diameter neurons immunoreactive for P2Y2 was also positive for P2X3 labeling in DRG sections (Figure 6A). Some sensory nerve fibers were found to be immunoreactive for both P2X3 and P2Y2 (Figure 6B), indicating that these two receptors are transported from the cell body to the peripheral terminals. In agreement with our electrophysiological data, P2Y2 and P2X3 receptors were found co-expressed in dissociated small-diameter DRG neurons in culture (data not shown). These results validate the fact that P2X3 and P2Y2 receptors are co-expressed in a subset of non-peptidergic DRG nociceptors.

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Discussion

Increasing evidence has implicated P2Y receptors in nociception, but detailed mechanisms of their involvement are largely unclear (Gerevich and Illes, 2004). Intrathecal application of P2Y agonist UTP has been reported to decrease mechanical sensitivity associated with neuropathic pain (Okada et al., 2002). Conversely, application of UTP has also been shown to induce action potentials in nociceptive afferents (Stucky et al., 2004). Interaction of P2Y receptors with nociceptive ion channels can underlie their involvement in pain. Activity of TRPV1 channels has been reported to be potentiated by activation of the P2Y2 receptor (Moriyama et al., 2003) and a recent report showed that both P2Y1 and P2Y2 receptors are able to increase excitability in DRG neurons by inhibiting M-type K channels (Yousuf et al., 2011). Here we provide evidence that the activity of the P2X3 ATP-gated channel is regulated by P2Y receptors as the P2Y receptor agonist UTP induced a significant inhibition of P2X3 function in DRG neurons. A P2Y-P2X crosstalk may provide a homeostatic mechanism to prevent excessive P2X3 activation and calcium overload in sensory neurons after injury. ATP released during tissue damage can activate both receptors, and although initial P2X3 channel activity will not be affected, as P2Y receptor transduction mechanisms are slower, subsequent P2X3 activity induced by additional ATP release will be much lower due to the inhibitory action of P2Y. This may explain some of the observed decrease of pain sensation in response to repeated ATP applications in human skin (Hamilton et al., 2000). Inhibition of P2X3 activity by an unidentified P2Y receptor in DRG was previously reported (Gerevich et al., 2005; Gerevich et al., 2007). In those studies the agonists ADP-β-S and 2-methylthio-ADP were used, both of which are more selective for the P2Y1, P2Y12, and P2Y13 receptor subtypes, whereas UTP is more selective for the P2Y2 and P2Y4 subtypes (Abbracchio et al., 2006).

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A subpopulation of small diameter sensory neurons expresses P2X3 and P2X2 subunits, resulting in the formation of heteromeric P2X2/3 receptors (Pankratov et al., 2001). The P2X2/3 receptor subtype is believed to be involved in ATP-induced nociceptive signaling as indicated by its upregulation in small diameter sensory neurons following neuropathic injury (Kage et al., 2002). The heteromeric P2X2/3 receptor subtype is also activated by α,β-meATP, but unlike the P2X3 subtype it does not exhibit rapid desensitization (North, 2002b). Interestingly, P2Y receptor activation by UTP did not cause any significant inhibition of sustained currents mediated by P2X2/3 receptors. In the same neurons, it was evident that while the fast currents generated by homomeric P2X3 channels were affected, the P2X2/3-mediated sustained currents were insensitive to UTP treatment. The functional properties of P2X2/3 and P2X3 suggest that the heteromeric receptor plays a specific role in ATP-induced pain signaling (Brederson and Jarvis, 2008). Due to lack of subtype-specific pharmacological tools, it is difficult to test this hypothesis *in vivo*, but the differential sensitivity of the two P2X receptor subtypes to modulation by P2Y receptors confirms that they have distinct regulatory mechanisms (Mo et al., 2009).

Activation of Gq-coupled P2Y receptors by UTP leads to activation of PLC and transient depletion of PIP_2, a process that has been demonstrated to modulate ion channels sensitive to phosphoinositides (Baukrowitz et al., 1998). Contribution of PLC to UTP-mediated inhibition of P2X3 activity was clearly demonstrated here using the PLC inhibitor U73122. Although PLC activation can trigger many intracellular signaling cascades, many of which depend on PLC-mediated hydrolysis of PIP_2, we reported in a previous work the sensitivity of P2X3 to depletion of intracellular PIP_2 (Mo et al., 2009). We confirmed the direct involvement of phosphoinositides in P2Y receptor-induced modulation of P2X3 activity by showing that injection of exogenous PIP_2 fully reverses the inhibition. The mechanism of interaction between P2X3 and PIP_2 remains unclear (Mo et al., 2009). Unlike P2X subunits that directly binds to PIP_2 through a dual

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polybasic motif, P2X3 does not display the consensus lipid-binding motif (Bernier et al., 2012). In agreement with previous findings, we also observed differences in sensitivity of native P2X3 and P2X2/3 receptors to UTP-evoked P2Y-mediated modulation in sensory neurons (Mo et al., 2009). It would be interesting to examine the sensitivity of homomeric P2X2 to P2Y activation since that subtype is sensitive to changes in cellular levels of phosphoinositides (Fujiwara and Kubo, 2006). Several other P2X receptor subtypes have also been shown to be regulated by phosphoinositides, such as P2X1 (Bernier et al., 2008b), P2X4 (Bernier et al., 2008a), and heteromeric P2X1/5 (Ase et al., 2010). However, since none of those receptors have been shown to be functionally expressed in rodent small-diameter sensory neurons, their potential modulation by P2Y receptors is beyond the scope of this study.

The molecular mechanism of P2Y-mediated inhibition of P2X3 presented here suggests that the function of P2X3 receptor may be modulated by other PLC-linked Gq-coupled receptors expressed in DRG sensory neurons. Interestingly, the activation of Gq-coupled protease-activated receptor-2 (PAR-2) was reported to increase P2X3 currents evoked by sub-saturating concentrations of α,β-meATP in rat sensory neurons (Wang et al., 2012). Wang and colleagues showed that the potentiation of P2X3 by PAR-2 involves PKC and PKA (Wang et al., 2012). Also, the potentiation of P2X3 activity by P2Y2 activation in mouse bladder sensory neurons was suggested to involve ecto-kinases (Chen et al., 2010). Notably, Chen and colleagues demonstrated that the crosstalk between P2Y2 with P2X3 in bladder afferents is G-protein independent. Based on these findings, the evidence presented here suggest that functional interaction of metabotropic receptors with P2X3 receptors may be negative or positive depending on the agonist concentration and the type of metabotropic receptors. It may involve either protein kinases for potentiation or phospholipid depletion for inhibition. We propose that the P2Y-mediated inhibition of P2X3 activity observed here represents a homeostatic mechanism to

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control ATP-evoked ionotropic signalling when ATP stimulation is strong or when ATP and UTP stimulation are redundant.

The diversity of metabotropic purinoceptors contributes to the complexity of their function in nociception. Identifying the P2Y receptor subtype involved in the interaction with P2X3 receptor on sensory neurons is required to enhance our understanding of the role of P2Y receptors and nucleotides in pain. The mRNA expression of various P2Y receptors on sensory neurons has been previously reported (Ruan and Burnstock, 2003). However, as in the case with P2X receptors, P2Y mRNA expression does not always coincide with physiological relevance (Kobayashi et al., 2005). Only the P2Y1 and P2Y2 receptors, both of which are Gq-coupled receptors (Abbracchio et al., 2006), have been convincingly shown to be functional in sensory neurons (Hussl and Boehm, 2006). We can exclude the involvement of P2Y1 because P2X3 channel activity was not affected by the P2Y1 selective agonist MRS2386 or P2Y1 selective antagonist MRS2279 in presence of UTP. Although the Gq-coupled P2Y6 receptor has not been functionally shown to be expressed in DRG neurons, its sensitivity to UTP and the fact that its mRNA was detected at low levels in sensory neurons (Ruan and Burnstock, 2003; Malin et al., 2008) make it a potential, albeit unlikely, candidate. In Ca^{2+} imaging experiments on DRG neurons, we were unable to induce any measurable intracellular Ca^{2+} release with the P2Y6 selective agonist UDP (data not shown). Expression of P2Y4, a Gq-coupled UTP receptor, has been observed in sensory ganglia (Ruan and Burnstock, 2003), but functional analysis shows low levels of P2Y4 receptor expression in DRG neurons (Sanada et al., 2002; Yousuf et al., 2011). Moreover, mRNA detection studies using RT-PCR or in situ hybridization confirm that P2Y4 is a minor purinoceptor subtype in small-diameter sensory neurons (Sanada et al., 2002) (Kobayashi et al., 2006). Logically, the only remaining candidate is the P2Y2 receptor.

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Further support for the involvement of P2Y2 receptor in UTP-mediated inhibition of P2X3 channel activity came from our immunolocalization results, which showed co-expression of P2Y2 and P2X3 proteins in sensory neurons. This is in agreement with studies reporting high expression of P2Y2 mRNA in rat DRG neurons (Molliver et al., 2002; Malin et al., 2008). The co-localization of P2Y2 and P2X3 receptors has been suggested, but was never directly demonstrated (Stucky et al., 2004; Chen et al., 2010). The two receptors were found to be co-localized both in cell bodies and nerve fibers, indicating co-trafficking to peripheral and central terminals. The immunolocalization evidence presented here provides valuable information on the physiological relevance of crosstalks between ionotropic and metabotropic ATP receptors in sensory pathways, yet much work is still needed to fully comprehend the role of nucleotide signaling in pain, especially in pathological conditions. Changes in P2Y2 receptor expression in response to inflammation have been documented (Malin et al., 2008), therefore it will be worth investigating the functional impact of P2Y2-P2X3 interactions on ATP signaling under chronic pain conditions.

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Authorship Contributions

Participated in research design: Mo, Séguéla

Conducted experiments: Mo, Peleshok

Performed data analysis: Mo, Peleshok, Cao, Ribeiro-da-Silva, Séguéla

Contributed to the writing of the manuscript: Mo, Peleshok, Cao, Séguéla

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Footnote

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Figure Legends

Figure 1. Application of UTP inhibits P2X3-mediated currents in DRG neurons.

(A) Sample traces of endogenous P2X3 receptor currents induced by 10 µM α,β-meATP in rat DRG neurons. Compared to control conditions (left), the current recorded after treatment with 100 µM UTP (middle) was significantly smaller in amplitude, and partially recovered back to control levels after a 3 min washout (right). (B) Normalized peak current amplitudes of responses under the different conditions show that treatment with UTP produced reversible inhibition of P2X3 response (N = 7 to 8, *** $p<0.001$).

Figure 2. P2X2/3 receptor-mediated currents are not sensitive to UTP treatment.

(A) Representative traces of 10 µM α,β-meATP-evoked currents in rat DRG neurons exhibiting significant P2X2/3 like currents. Currents were recorded in control condition (left), after 3 min treatment with 100 µM UTP that produced no inhibition of P2X2/3 mediated current (middle), and after washout with normal ECS (right). (B) Normalized sustained current amplitude of control response, after 3 min treatment with 100 µM UTP, and after washout confirms the absence of any significant effect of UTP on P2X2/3 receptor activity (N = 5, ns $p>0.05$).

Figure 3. UTP-activated P2Y receptor-mediated inhibition of P2X3 function involves phospholipase C.

(A) Sample traces of P2X3 currents from DRG neurons recorded with 10 µM of PLC inhibitor U73122 in the electrode solution (left). Application of intracellular U73122 was able to prevent the inhibitory effect of 100 µM UTP on P2X3 current (middle), and washout was able to further recover the P2X3 current from inhibition (right). (B) Pooled normalized P2X3 responses show

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that the inhibitory effect of UTP was significantly attenuated in presence of U73122. This was not the case for the inactive enantiomer U73343 (N = 7 to 8, ** $p<0.01$, ns $p>0.05$).

Figure 4. Exogenous PIP_2 reverses P2X3 inhibition mediated by UTP-activated P2Y receptors.

(A) Typical P2X3 current, induced by 10 µM α,β-meATP, recorded with 200 µM diC8-PIP_2 in the electrode solution (left). Addition of intracellular PIP_2 prevents UTP-induced inhibition of P2X3 activity (middle), no difference was observed after washout (right). (B) Pooled analysis of normalized current amplitudes of response demonstrates that addition of PIP_2 is able to fully reverse the UTP-induced inhibition of P2X3 activity back to control levels (N = 5 to 9, ** $p<0.01$).

Figure 5. P2Y1 receptors do not contribute to UTP-induced P2X3 inhibition.

(A) Pooled normalized P2X3 currents from DRG neurons treated with P2Y1-selective agonist MRS2365. After 3 min treatment with 5 nM MRS2365, P2X3 currents were not significantly different from currents recorded under control conditions, or after a 3 min washout (N = 3 to 6, ns $p>0.05$). (B) Pooled analysis of P2X3 currents recorded in presence of the P2Y1 antagonist MRS2279 at 500 nM. Following 3 min application of 100 µM UTP, the P2Y1 antagonist did not have any significant impact on the inhibition of P2X3 activity (N = 3 to 5, ns $p>0.05$).

Figure 6. P2X3 and P2Y2 receptors are co-expressed in rat DRG sensory neurons.

(A) DRG sections labeled for P2Y2 (green) and P2X3 (red) and merged (yellow). P2Y2 immunoreactivity is broadly distributed in small-diameter neuronal somata and co-localized with P2X3 (arrows). P2Y2 immunoreactivity can be detected in cells negative for P2X3 (arrow heads). (B) P2Y2 (green) and P2X3 (red) are also colocalized (yellow) in peripheral nerve fibers.

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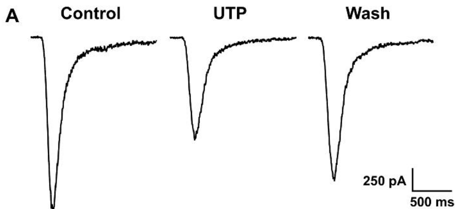
Figure 1



B

Normalized Peak Current (% of 1st Response)

0
20
40
60
80
100

***

Control
UTP
Wash

Figure 2

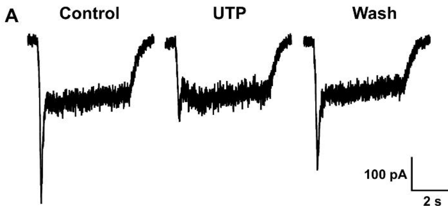
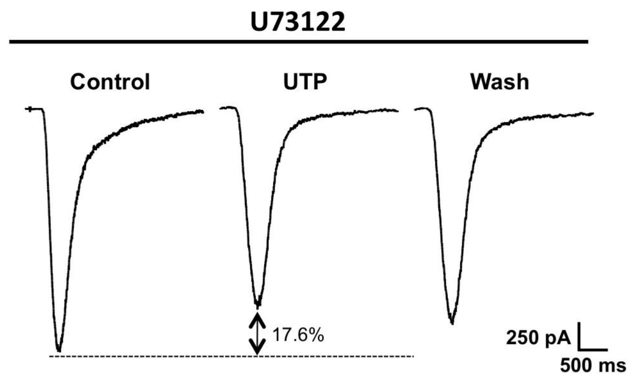


Figure 3



B

ns

**

Normalized Peak Current (% of 1st Response)

0
20
40
60
80
100

Control + U73122
UTP
UTP + U73122
UTP + U73343
Wash + U73122

Figure 4

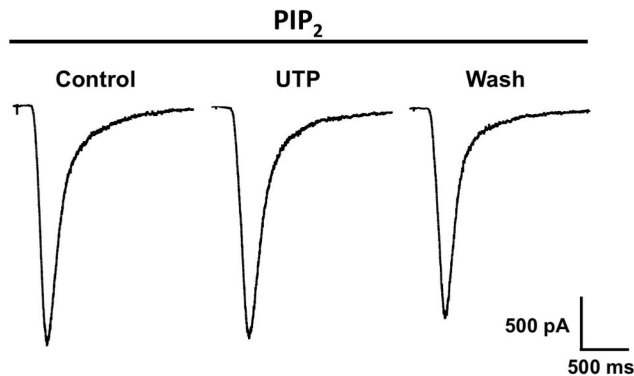
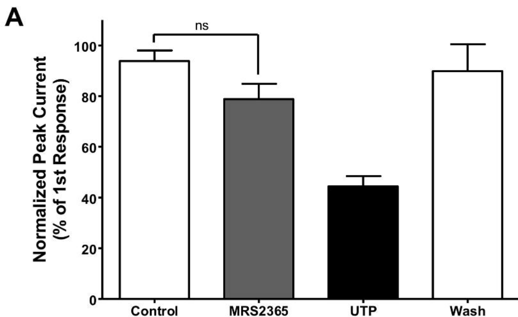


Figure 5



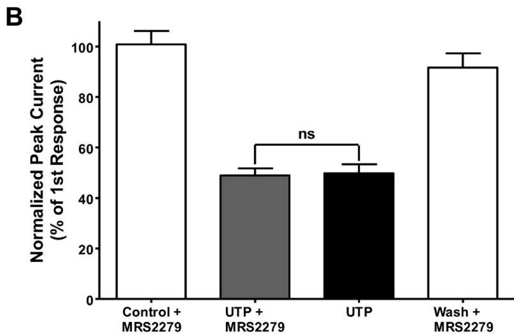


Figure 6