Brief Communication

Possible Involvement of P2Y$_2$ Metabotropic Receptors in ATP-Induced Transient Receptor Potential Vanilloid Receptor 1-Mediated Thermal Hypersensitivity

Tomoko Moriyama,[1] **Tohko Iida,**[1,2] **Kimiko Kobayashi,**[3] **Tomohiro Higashi,**[1] **Tetsuo Fukuoka,**[3] **Hideki Tsumura,**[4] **Catherine Leon,**[5] **Noboru Suzuki,**[4] **Kazuhide Inoue,**[6,7] **Christian Gachet,**[5] **Koichi Noguchi,**[3] **and Makoto Tominaga**[1]

[1]Department of Cellular and Molecular Physiology, Mie University School of Medicine, Tsu, Mie 514-8507, Japan, [2]Inoue Foundation for Science, Tokyo 150-0033, Japan, [3]Department of Anatomy and Neuroscience, Hyogo College of Medicine, Hyogo 663-8501, Japan, [4]Institute of Laboratory Animals, Mie University, Tsu, Mie 514-8507, [5]Institut National de la Santé et de la Recherche Médicale Unité 311, 67065 Strasbourg, France, [6]Division of Biosignaling, National Institute of Health Sciences, Tokyo 158-8501, and [7]Department of Molecular and System Pharmacology, Graduate School of Pharmaceutical Sciences, Kyushu University, Fukuoka 812-8582, Japan

The capsaicin receptor transient receptor potential V1 (TRPV1; also known as vanilloid receptor 1) is a sensory neuron-specific ion channel that serves as a polymodal detector of pain-producing chemical and physical stimuli. It has been reported that extracellular ATP potentiates the TRPV1 currents evoked by capsaicin or protons and reduces the temperature threshold for its activation through metabotropic P2Y receptors in a PKC-dependent pathway, suggesting that TRPV1 activation could trigger the sensation of pain at normal body temperature in the presence of ATP. Here, we show that ATP-induced thermal hyperalgesia was abolished in mice lacking TRPV1, suggesting the functional interaction between ATP and TRPV1 at a behavioral level. However, thermal hyperalgesia was preserved in P2Y$_1$ receptor-deficient mice. Patch-clamp analyses using mouse dorsal root ganglion neurons indicated the involvement of P2Y$_2$ rather than P2Y$_1$ receptors. Coexpression of TRPV1 mRNA with P2Y$_2$ mRNA, but not P2Y$_1$ mRNA, was determined in the rat lumbar DRG using *in situ* hybridization histochemistry. These data indicate the importance of metabotropic P2Y$_2$ receptors in nociception through TRPV1.

Key words: pain; thermal hyperalgesia; capsaicin receptor; P2Y receptor; ATP; UTP

Introduction

The sensation of pain allows us to recognize injury and triggers appropriate protective responses. A specific population of primary afferent neurons called nociceptors are known to be involved in the detection of noxious thermal, mechanical, or chemical stimuli and can be distinguished by their sensitivity to capsaicin, the pungent ingredient in hot chili peppers (Szallasi and Blumberg, 1999; Wood and Perl, 1999; Woolf and Salter, 2000). The capsaicin receptor transient receptor potential V1 (TRPV1; also known as vanilloid receptor 1), a member of the transient receptor potential ion channel superfamily (Minke and Cook, 2002), is a nonspecific cation channel with six transmembrane domains expressed predominantly in unmyelinated C fibers and activated not only by capsaicin but also by noxious heat (>43°C) or protons (acidification), both of which cause pain *in vivo* (Caterina et al., 1997; Tominaga et al., 1998; Caterina and Julius, 2001). Recently, TRPV1 has been reported to respond to

anandamide (Zygmunt et al., 1999), lipoxygenase products (Hwang et al., 2000), and *N*-arachidonoyl-dopamine (Huang et al., 2002). Furthermore, analyses of mice lacking TRPV1 have shown that TRPV1 is essential for selective modalities of pain sensation and for tissue injury-induced thermal hyperalgesia, further suggesting a critical role for TRPV1 in the detection or modulation of pain (Caterina et al., 2000; Davis et al., 2000).

Tissue damage associated with infection, inflammation, or ischemia produces an array of chemical mediators that activate or sensitize nociceptor terminals to elicit or exacerbate pain at the site of injury in addition to the release of the mediators from the nociceptor terminals themselves. One of the important components of this acute proalgesic response is ATP released from the cytosol of the damaged or ruptured cells and sensory neurons (Sawynok and Sweeney, 1989; Lazarowski et al., 2000; Dunn et al., 2001). Extracellular ATP excites nociceptor endings (Cook and McCleskey, 2002), evoking a sensation of pain (Sawynok and Sweeney, 1989; Dunn et al., 2001). In these neurons, the most widely studied targets of extracellular ATP have been ionotropic ATP (P2X) receptors (North and Barnard, 1997; Ralevic and Burnstock, 1998; Dunn et al., 2001). Indeed, several P2X receptor subtypes have been identified in sensory neurons, including one (P2X$_3$) whose expression is primarily confined to these cells (Chen et al., 1995; Lewis et al., 1995). In contrast, possible roles for metabotropic ATP (P2Y) receptors in nociceptive signalings

have received limited attention (von Kugelgen and Wetter, 2000). However, there is increasing evidence that P2Y receptors are involved in nociception (Lang et al., 2002; Molliver et al., 2002; Zimmermann et al., 2002). Furthermore, ATP has been found recently to potentiate the TRPV1 currents evoked by capsaicin or protons through metabotropic P2Y$_1$ receptor activation in a protein kinase C-dependent pathway (Tominaga et al., 2001). In the presence of extracellular ATP, the temperature threshold for TRPV1 activation was reduced from 42 to 35°C, such that normal body temperature is capable of activating TRPV1, which might cause pain sensation. Bradykinin has also been reported to reduce the temperature threshold for TRPV1 activation through a PKC-dependent pathway (Sugiura et al., 2002). These data suggest that phosphorylation of TRPV1 by PKC changes the agonist sensitivity of this ion channel. Indeed, direct phosphorylation of TRPV1 by PKC has been proven biochemically, and two serine residues as substrates for PKC-dependent phosphorylation have been identified (Numazaki et al., 2002).

Despite these findings, the involvement of metabotropic P2Y receptors in nociception has not yet been investigated in live animals. To address this question, we performed behavioral, electrophysiological, and histochemical analyses to explore the expression and function of P2Y subtypes in wild-type mice and mice lacking TRPV1. We observed a loss of ATP-induced thermal hyperalgesia in TRPV1 knock-out mice and found that P2Y$_2$ receptors, but not P2Y$_1$ receptors, could be the targets of extracellular ATP in TRPV1-mediated thermal hyperalgesia.

Materials and Methods

Primary cultures prepared from male adult C57BL/6 strain (wild-type) (20 gm body weight; Japan SLC, Shizuoka, Japan) or P2Y$_1$-deficient mice (obtained from Dr. Gachet, Institut National de la Santé et de la Recherche Médicale, Strasbourg, France) (Leon et al., 1999) dorsal root ganglion neurons were incubated in medium containing nerve growth factor (100 ng/ml). Whole-cell patch-clamp recordings were performed 1 d after preparation of DRG neurons as described previously (Caterina et al., 1997). Standard bath solution contained (in mM): 140 NaCl, 5 KCl, 2 MgCl$_2$, 5 EGTA, 10 HEPES, and 10 glucose, pH 7.4 (adjusted with NaOH). Pipette solution contained (in mM): 140 KCl, 5 EGTA, and 10 HEPES, pH 7.4 (adjusted with KOH). All patch-clamp experiments were performed at room temperature.

Male C57BL/6 strain mice, P2Y$_1$-deficient mice, or TRPV1-deficient mice (obtained from Dr. D. Julius, University of California, San Francisco, San Francisco, CA) were used for behavioral analyses. After 1 hr of adaptation, ATP (100 nmol), α,β-methylene ATP (αβmeATP) (20 nmol), or UTP (100 nmol) was injected intraplantarly into one hindpaw of each mouse, and response latencies to a radiant paw heating were measured as described previously (Caterina et al., 2000). In some experiments, mice were pretreated with 2′,3′-O-(2,4,6-trinitrophenyl) (TNP)–ATP (50 nmol) or saline (control) 10 min before αβmeATP or UTP injection. All procedures involving the care and use of mice and rats were performed in accordance with Mie University and Hyogo College of Medicine institutional guidelines and the National Institutes of Health *Guide for the Care and Use of Laboratory Animals.*

Total RNA was isolated from male adult C57BL/6 strain mice and reverse transcribed using Superscript II (Invitrogen, Gaithersburg, MD). The gene-specific primers (5′-GTTCAATTTGGCTCTGGCCG-3′ and 5′-CTGATAGGTGGCATAAACCC-3′ for P2Y$_1$; 5′-CTTCAACGAGGA-CTTCAAGTACGTGC-3′ and 5′-CATGTTGATGGCGTTGAGGGTG-TGG-3′ for P2Y$_2$) were designed from mouse P2Y$_1$ and P2Y$_2$ sequences. PCR was performed with 35 cycles of the following protocol: 94°C, 10 sec; 55°C (for P2Y$_1$) or 60°C (for P2Y$_2$), 30 sec; and 68°C, 45 sec. Precise amplification of the expected fragment was confirmed by sequencing.

For *in situ* hybridization, L5 DRGs of four male Sprague Dawley rats were quickly removed and frozen in powdered dry ice. Sections (5 μm thick) were cut with a cryostat, thaw-mounted onto Matsunami adhesive silane-coated slides (Matsunami, Osaka, Japan), fixed in phosphate-buffered 4% formaldehyde for 20 min, and treated with 5 μg/ml proteinase-K in 50 mM Tris-HCl and 5 mM EDTA for 3 min. The sections were acetylated with 0.25% acetic anhydride in 0.1 M triethanolamine, dehydrated in ascending ethanol series, and air dried. Two ^{35}S-labeled RNA probes were prepared by *in vitro* transcription of P2Y$_1$ cDNA (GenBank accession number U22830, nucleotides 653–980) fragment and P2Y$_2$ cDNA (GenBank accession number L46865, nucleotides 493–868) in linearized pGEM-T Easy vector (Promega, Madison, WI) by using T7 or SP6 RNA polymerase (Promega) and ^{35}S-UTP (PerkinElmer Life Sciences, Natick, MA), giving a specific activity of $1.0-1.5 \times 10^9$ cpm/μg. A digoxigenin (DIG)-labeled RNA antisense probe was prepared by *in vitro* transcription of TRPV1 cDNA (GenBank accession number AF029310, nucleotides 149–486) fragment in linearized pGEM-T Easy vector by using a DIG RNA labeling Kit SP6/T7 (Roche Diagnostics, Mannheim, Germany). The procedures for hybridization, washing, and visualization using DAB reaction and autoradiography have been described previously (Hashimoto et al., 2001). In brief, sections were hybridized overnight at 55°C in humidified boxes with each of ^{35}S-labeled RNA probes (3×10^6 cpm) and with a DIG-labeled probe (300 ng) in 300 μl of the hybridization buffer. All of these sections were rinsed in 5× SSC and 5 mM DTT for 30 min at 58°C and then washed in high-stringency buffer for 30 min at 58°C. Quantification of the labeled neurons was performed using randomly selected tissue profiles with bright-field illumination for DAB-stained neurons or dark-field illumination for silver grains over ^{35}S-labeled neurons (Fukuoka et al., 2001). At least 200 neurons from an L5 DRG section of each of four rats were measured.

All the chemicals were obtained from Sigma (St. Louis, MO).

Results

To confirm the interaction between ATP and TRPV1 in the context of ATP-induced hyperalgesia *in vivo*, we performed a behavioral analysis using wild-type mice and TRPV1-deficient mice. Nociceptive responses such as licking and biting lasted for ∼2 min after ATP injection in wild-type mice. After cessation of the responses induced by intraplantar ATP injection, mice were subjected to radiant paw heating. A significant reduction in paw withdrawal latency was observed for 5–30 min after ATP injection in wild-type mice. In contrast, TRPV1-deficient mice developed no such thermal hypersensitivity in response to ATP injection, suggesting a functional interaction between ATP and TRPV1 (Fig. 1*A*). Next, we injected αβmeATP to examine the possibility that P2X receptors are involved in ATP-induced thermal hyperalgesia (Fig. 1*B*). Interestingly, thermal hyperalgesia was also observed after injection of αβmeATP. However, the αβmeATP-induced thermal hyperalgesia appeared and disappeared more rapidly than ATP and was blocked by TNP–ATP, a blocker of P2X receptors (Tsuda et al., 1999). Furthermore, we examined the effect of αβmeATP in TRPV1-deficient mice. Small but significant thermal hypersensitivity was observed only 5 min after αβmeATP injection (Fig. 1*B*), suggesting that TRPV1 does not mediate primarily αβmeATP-induced thermal hyperalgesia. In addition, TNP–ATP failed to inhibit the ATP-induced thermal hyperalgesia (Fig. 1*C*). These results suggest that both P2X and P2Y receptors are involved in ATP-induced thermal hyperalgesia, although P2Y receptors seem to be involved more predominantly and contribute to the protracted component of the ATP-induced thermal hyperalgesia. A pharmacological analysis of ATP-induced potentiation of TRPV1 currents evoked by capsaicin in human embryonic kidney 293 (HEK293) cells expressing TRPV1 suggested the involvement of the P2Y$_1$ subtype of metabotropic ATP receptors (Tominaga et al., 2001). Therefore, we extended our behavioral analyses to P2Y$_1$-deficient mice, which were reported to show defective platelet aggregation and increased resistance to thrombosis (Leon et al., 1999). Surpris-

ingly, after ATP injection, mice lacking $P2Y_1$ exhibited a reduction in heat-evoked withdrawal latency similar to that observed in wild-type mice, indicating that $P2Y_1$ receptors are not involved in ATP-induced thermal hyperalgesia in mice (Fig. 1A).

To explore the identity of the P2Y subtypes responsible for ATP-induced thermal hyperalgesia in mice, we first examined the effects of ATP on the capsaicin (low-dose)-evoked response in isolated mouse DRG neurons. In DRG neurons of wild-type mice, extracellular ATP caused significant increases in low-dose capsaicin-evoked currents, as observed in rat DRG neurons (1.07-fold $\pm$ 0.26-fold increase for controls vs 4.01-fold $\pm$ 0.92-fold increase with ATP; $p < 0.05$) (Fig. 2A,B,E) (Tominaga et al., 2001). In some of the DRG neurons examined, rapidly desensitized inward currents at -60 mV were observed after ATP application, indicative of activation of P2X receptors (data not shown). DRG neurons without such ATP-evoked currents were used to examine the effects of ATP on capsaicin-evoked responses to simplify the pathway involved, although the ATP-induced potentiation of the capsaicin-evoked responses was also observed in the cells showing ATP-evoked inward currents (data not shown). Similar potentiation of capsaicin-evoked currents was observed in $P2Y_1$-deficient mice (4.37-fold $\pm$ 0.74-fold; $p < 0.01$ vs control) (Fig. 2C,E), suggesting lack of involvement of $P2Y_1$ receptors in mouse DRG neurons, consistent with our behavioral analyses (Fig. 1A). We subsequently examined the effect of another ATP-related molecule, UTP, because this molecule is thought to be a relatively selective agonist of $P2Y_2$ and $P2Y_4$ receptors (Ralevic and Burnstock, 1998). UTP potentiated the capsaicin-evoked current responses to an extent similar to that seen for ATP (6.24-fold $\pm$ 1.59-fold; $p < 0.05$ vs control) (Fig. 2D,E), suggesting the involvement of $P2Y_2$ or $P2Y_4$ subtypes. Finally, the fact that suramin, which blocks $P2Y_2$ but not $P2Y_4$, abolished the potentiation by UTP (0.95-fold $\pm$ 0.57-fold; $p < 0.05$ vs UTP in wild type) implicates $P2Y_2$ as the most likely P2Y subtype involved in the potentiation of capsaicin-evoked current responses in mouse DRG neurons.

To examine the functional importance of $P2Y_2$ receptors, we next investigated the expression of $P2Y_2$ mRNA. A reverse transcriptase-PCR study showed that $P2Y_2$ as well as $P2Y_1$ mRNA expression in mouse DRG neurons (Fig. 3A) was consistent with the pharmacological analyses described above. To further support the functional relationship of P2Y receptors with TRPV1 in the patch-clamp experiments, we examined the coexpression of $P2Y_1$ receptor mRNA and $P2Y_2$ receptor mRNA with TRPV1 mRNA in the rat lumbar DRG using *in situ* hybridization histochemistry. In this experiment, 23.5 $\pm$ 4.3, 22.5 $\pm$ 2.5, and 44.6 $\pm$ 5.2% of rat DRG neurons expressed $P2Y_1$, $P2Y_2$, and TRPV1 mRNAs, respectively. Coexpression of $P2Y_1$ mRNA and TRPV1 mRNA was quite rare (Fig. 3B). Thus, most of the ^{35}S-labeled $P2Y_1$ mRNA-expressing neurons were not stained by DAB (2.6 $\pm$ 2.0% of $P2Y_1$ mRNA-expressing cells were TRPV1 mRNA-positive) (Fig. 3B, a,b, arrowheads) and vice versa (1.6 $\pm$ 1.4% of TRPV1 mRNA-expressing cells were $P2Y_1$ mRNA-positive). However, a significant population of DRG neurons coexpressed
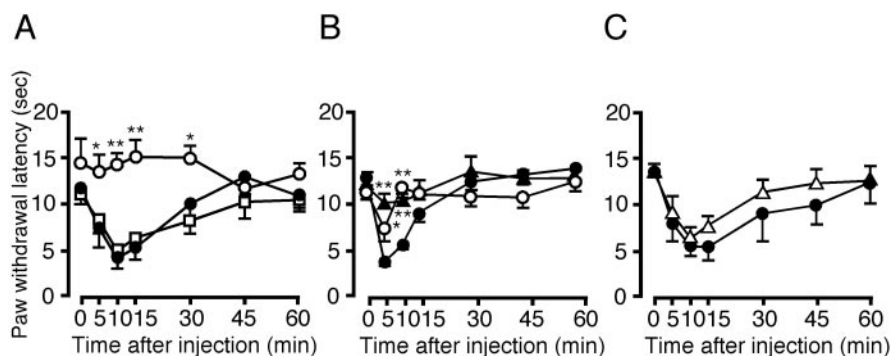
Figure 1. TRPV1 is essential for the development of ATP-induced thermal hypersensitivity *in vivo*, and P2Y receptors are predominantly involved. *A*, Wild-type (●), TRPV1 $^{-/-}$ (○), or $P2Y_1$ $^{-/-}$ mice (□) were injected intraplantarly with ATP (100 nmol), and the response latency to radiant heating of the hindpaw was measured at various time points after injection. Values are expressed as mean $\pm$ SE; $n = 6$ for each group.*$p < 0.05$ and **$p < 0.01$ versus wild-type and $P2Y_1$ $^{-/-}$ mice; two-tailed unpaired t test. *B*, Behavioral analyses in response to $\alpha\beta$meATP (20 nmol) injection similar to *A* in wild-type (●, ▲) or TRPV1 $^{-/-}$ mice (○). In some experiments, mice were pretreated with TNP–ATP (50 nmol) (▲). Values are expressed as mean $\pm$ SE; $n = 6$ for each group. *$p < 0.05$ and **$p < 0.01$ versus wild type without TNP–ATP pretreatment; two-tailed unpaired t test. *C*, Behavioral analyses in response to ATP (100 nmol) injection similar to *A* in wild-type mice pretreated with TNP–ATP (50 nmol) (△) or saline (●). Values are expressed as mean $\pm$ SE; $n = 6$ for each group.

$P2Y_2$ mRNA and TRPV1 mRNA (33.6 $\pm$ 10.7% of $P2Y_2$ mRNA-expressing cells were TRPV1 mRNA-positive and 15.9 $\pm$ 5.2% of TRPV1 mRNA-expressing cells were $P2Y_2$ mRNA-positive) (Fig. 3B, c,d, arrows). These data clearly suggest that $P2Y_2$ receptors, but not $P2Y_1$ receptors, can functionally interact with TRPV1 in DRG neurons.

To confirm this $P2Y_2$ receptor involvement, we examined the effect of UTP in mice (Fig. 4). UTP was found to cause thermal hyperalgesia with a time course similar to that observed in ATP injection. Furthermore, TNP–ATP could not prevent the UTP-induced thermal hyperalgesia, suggesting that $P2Y_2$ receptors are involved in ATP-induced thermal hyperalgesia in mice.

Discussion

In this study, we demonstrate the *in vivo* interaction of ATP with TRPV1. ATP could be involved in TRPV1-mediated thermal hyperalgesia through both P2X and P2Y receptors. However, P2Y receptors seem to be involved more predominantly, because the hyperalgesia was little affected by the P2X receptor inhibitor TNP–ATP. The existence of such a mechanism between P2Y receptors and TRPV1 is consistent with the observation that ATP-evoked nociceptive behavior in mice is only partially reduced by disruption of the $P2X_3$ gene (Cockayne et al., 2000; Souslova et al., 2000). It has been well documented that signaling by ATP through sensory neuron-specific $P2X_3$ and $P2X_{2/3}$ receptors mediates an acute nociceptive pathway contributing to the pain of tissue damage (North and Barnard, 1997; Ralevic and Burnstock, 1998; Tsuda et al., 2000; Dunn et al., 2001). ATP could act on both ionotropic and metabotropic receptors to initiate and maintain the nociceptive responses in concert.

In TRPV1-transfected HEK293 cells, pharmacological studies have concluded that $P2Y_1$ receptors are predominantly involved in ATP-induced potentiation or sensitization of TRPV1 (Tominaga et al., 2001). Our present results indicated that $P2Y_2$ rather than $P2Y_1$ receptors are likely to mediate the ATP-induced potentiation of capsaicin-evoked responses in mouse DRG neurons and in ATP-induced thermal hypersensitivity *in vivo*. Consistent with this hypothesis, $P2Y_2$ mRNA, but not $P2Y_1$ mRNA, is coexpressed with TRPV1 mRNA in rat DRG neurons (Fig. 3), and UTP injection causes thermal hyperalgesia in mice (Fig. 4). Together, these data suggest that $P2Y_2$ receptors mediate ATP-
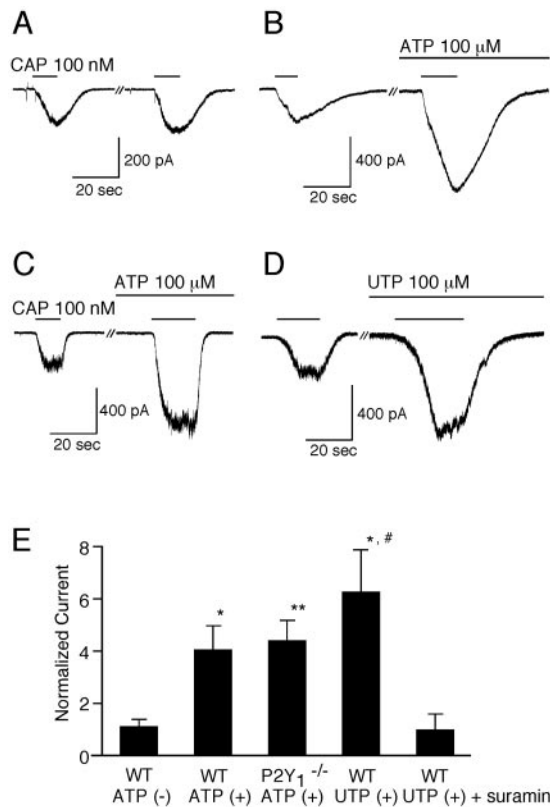
Figure 2. Extracellular ATP or UTP potentiates capsaicin-evoked currents in DRG neurons from wild-type or P2Y₁-deficient mice. *A–C*, Representative traces of increases in the capsaicin (CAP)-activated currents by extracellular ATP (100 μM) in DRG neurons from wild-type (WT) (*B*) or P2Y₁-deficient (*C*) mice. *A* shows a control trace without ATP pretreatment. Holding potential was −60 mV. *D*, A representative trace of increases in the capsaicin-activated currents by UTP (100 μM) in DRG neurons from wild-type mice. *E*, Effects of ATP, UTP, or UTP plus suramin (50 μM) on the capsaicin-activated currents. Currents were normalized to the currents initially evoked by capsaicin (100 nM) in the absence of the additives. Normalized currents in the absence of ATP in wild-type mice, in the presence of ATP in wild-type mice, in the presence of ATP in P2Y₁-deficient mice, in the presence of UTP in wild-type mice, or in the presence of UTP plus suramin in wild-type mice were 1.07 ± 0.26 ($n = 3$), 4.01 ± 0.92 ($n = 8$), 4.37 ± 0.74 ($n = 4$), 6.24 ± 1.58 ($n = 4$), or 0.95 ± 0.57 ($n = 3$), respectively. *$p < 0.05$ and **$p < 0.01$ versus absence of ATP in wild type; #$p < 0.05$ versus presence of UTP and suramin in wild type; two-tailed unpaired *t* test. WT, Wild type.

induced hypersensitivity in mammals. A behavioral analysis with P2Y₂-deficient mice would confirm our conclusion. P2Y₂ receptor activation leads to TRPV1 potentiation in a relatively short time course. In addition, ATP and UTP have been reported recently to cause cAMP response element-binding protein (CREB) phosphorylation, which is likely to activate gene expression through P2Y₂ receptor activation (Molliver et al., 2002). P2Y₂ receptor activation could increase intracellular Ca²⁺ levels, leading to various outcomes including CREB phosphorylation, activation of phospholipase A₂, and the consequent liberation of arachidonic acid, the rate-limiting step in prostaglandin formation (Molliver et al., 2002; Zimmermann et al., 2002). This Ca²⁺ mobilizing process could occur more slowly than the effects on TRPV1. Both fast and slow signals downstream of P2Y₂ receptor activation might exist in native cells and contribute coordinately to hyperalgesia.

P2Y₂ receptors confer responsiveness to UTP and ATP to a similar extent, suggesting a possible role for UTP as an important component of proalgesic response in the context of tissue injury. UTP has been reported to be released from ruptured cells
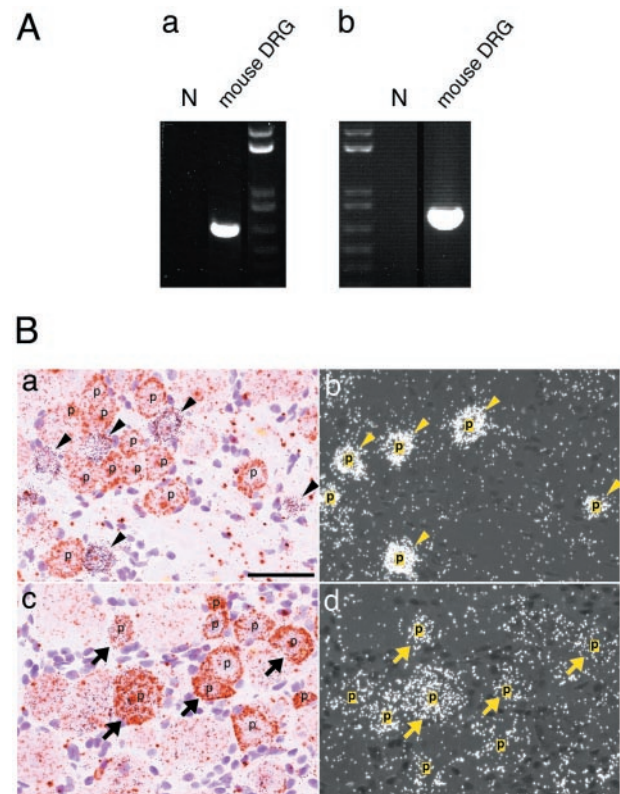
Figure 3. P2Y₂ mRNA, but not P2Y₁ mRNA, is coexpressed with TRPV1 mRNA in DRG. *A*, PCR amplification of P2Y₁ (*a*) and P2Y₂ (*b*) cDNA fragments from the RNAs of mouse DRG neurons. The expected sizes of the DNA fragments for mouse P2Y₁ and P2Y₂ are 651 and 781 bp, respectively. N, Negative control. *B*, Coexpression of TRPV1 mRNA with P2Y₁ and P2Y₂ mRNAs in rat DRG neurons. Double *in situ* hybridization histochemistry was performed on the sections. *b* and *d* are dark-field photomicrographs of *a* and *c*, respectively. The TRPV1 mRNA-expressing neurons were hybridized by a DIG-labeled antisense probe and visualized as DAB staining (brown cells in *a* and *c*). The P2Y₁ mRNA-expressing (*b*) and P2Y₂ mRNA-expressing (*d*) neurons were hybridized by the appropriate ³⁵S-labeled antisense probe and visualized as clusters of silver grains. p, Positively labeled neurons. Arrowheads indicate P2Y₁ mRNA-expressing neurons that do not express TRPV1 mRNA, whereas arrows indicate the neurons expressing both TRPV1 and P2Y₂ mRNAs.
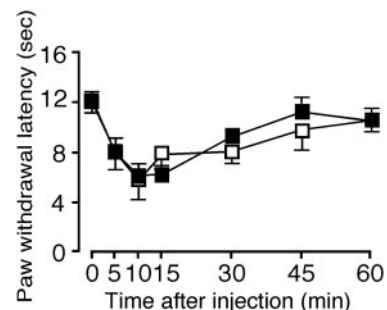
Figure 4. UTP induces thermal hyperalgesia in mice. Behavioral analyses in response to UTP (100 nmol) injection similar to Figure 1 in wild-type mice pretreated with TNP–ATP (50 nmol) (□) or saline (■). Values are expressed as mean ± SE; $n = 6$ for each group.

(Anderson and Parkinson, 1997; Lazarowski et al., 2000). In addition to non-neuronal cells, ATP can be released from a subset of small primary afferent nerves in response to capsaicin (Sawynok and Sweeney, 1989), suggesting possible autocrine and paracrine mechanisms for the exacerbation of pain. A similar context can be imagined for the action of UTP in tissue damage. Therefore, UTP as well as ATP should be taken into account when purinergic

contributions through P2Y receptors to pain sensation are examined.

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