Nucleotides control the excitability of sensory neurons via two P2Y receptors and a bifurcated signaling cascade

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Nucleotides contribute to the sensation of acute and chronic pain, but it remained enigmatic which G protein-coupled nucleotide (P2Y) receptors and associated signaling cascades are involved. To resolve this issue, nucleotides were applied to dorsal root ganglion neurons under current- and voltage-clamp. Adenosine triphosphate (ATP), adenosine diphosphate (ADP), and uridine triphosphate (UTP), but not uridine diphosphate (UDP), depolarized the neurons and enhanced action potential firing in response to current injections. The P2Y₂ receptor preferring agonist 2-thio-UTP was equipotent to UTP in eliciting these effects. The selective P2Y₁ receptor antagonist MRS2179 largely attenuated the excitatory effects of ADP, but left those of 2-thio-UTP unaltered. Thus, the excitatory effects of the nucleotides were mediated by 2 different P2Y receptors, P2Y₁ and P2Y₂. Activation of each of these 2 receptors by either ADP or 2-thio-UTP inhibited currents through KV7 channels, on one hand, and facilitated currents through TRPV₁ channels, on the other hand. Both effects were abolished by inhibitors of phospholipase C or Ca²⁺-ATPase and by chelation of intracellular Ca²⁺. The facilitation of TRPV₁, but not the inhibition KV7 channels, was prevented by a protein kinase C inhibitor. Simultaneous blockage of KV7 channels and of TRPV₁ channels prevented nucleotide-induced membrane depolarization and action potential firing. Thus, P2Y₁ and P2Y₂ receptors mediate an excitation of dorsal root ganglion neurons by nucleotides through the inhibition of KV7 channels and the facilitation of TRPV₁ channels via a common bifurcated signaling pathway relying on an increase in intracellular Ca²⁺ and an activation of protein kinase C, respectively.

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1. Introduction

Sensory dorsal root ganglion (DRG) neurons mediate the sensation of acute and chronic pain. Acute pain is elicited by thermal, mechanical, or chemical stimuli, whereas chronic pain results from the sensitization of sensory neurons most commonly due to inflammatory reactions. The sensitization is mediated by an "inflammatory soup" containing a plethora of signaling molecules, such as cytokines, neurotrophins, bradykinin, prostaglandins, and nucleotides. Interestingly, most analgesics target the synthesis, the release, or the receptors of these inflammatory mediators, as exemplified by inhibitors of cyclooxygenases, such as acetylsalicylic acid [4].

Adenosine triphosphate (ATP) and other nucleotides are released from all cells in response to a variety of stimuli and mediate their effects via a class of receptors termed P2 [25]: P2X receptors are ATP-gated cation channels composed of 3 of a repertoire of at least 7 different subunits (P2X1 to P2X7) that assemble in homomers or heteromers. P2Y receptors are G protein-coupled receptors, and 8 different subtypes have been identified (P2Y1,2,4,6,11,12,13,14) [1,13]. Sensory neurons express both P2X and P2Y receptors; in DRG neurons, the presence of all known P2X subunits but P2X7 has been reported, and the predominating receptors are P2X3 homomers and P2X2 homomers [13,47]. Furthermore, the expression of P2X3 is altered under conditions of chronic pain [41,48]. Therefore, P2X3 receptors are considered promising targets for novel analgesics [12,44].

For P2Y receptors, expression of P2Y1,2,4,6,11,12,13,14 has been reported in DRG neurons [24,28,35]. Activation of P2Y₁ receptors excites DRG neurons, but the underlying mechanisms remained obscure [30]. The respective agonists, ATP and uridine triphosphate (UTP), trigger increases in intracellular Ca²⁺ and the release of calcitonin gene-related peptide [36]. UTP has also been reported to activate cutaneous C-fibers [38]. Activation of P2Y₁ receptors, in contrast, was suggested to mediate analgesic effects through an inhibition of voltage-activated Ca²⁺ channels (VADCs), which results in a reduction of transmitter release onto dorsal horn neurons.

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[20]. However, more recent data suggest that inhibition of VACCs is rather mediated by P2Y12 instead of P2Y1 receptors [28]. Moreover, P2Y receptors were reported to mediate hyperalgesia [28], and this effect was proposed to be mediated by a sensitization of TRPV1 channels [40]. Later on, however, the P2Y receptor involved in the sensitization of TRPV1 channels by nucleotides was reported to be P2Y2 [27,31]. In addition, nucleotides were suggested to enhance nociception by modulating voltage-activated Na+ channels in DRG neurons [33]. Thus, it remained quite controversial which P2Y receptor subtypes control the functions of DRG neurons and whether the modulation of K+ channels might contribute to this effect, and what the underlying signaling mechanisms might be.

2. Materials and methods

2.1. Materials

All the nucleotides (adenosine diphosphate [ADP], ATP, uridine diphosphate [UDP], UTP), capsaicin, and 1,2-bis[2-aminophenoxy]ethane-N,N,N',N'-tetraacetic acid tetrakis acetylomethyl ester (BAPTA-AM), along with bulk chemicals, were obtained from Sigma–Aldrich (Vienna, Austria). Specific P2Y agonists and antagonists (2-thio-UTP and MRS 2179), iso-5-iodoresiniferatoxin, U73122, and tetrodotoxin (TTX) was obtained from Latoxan (Rosans, France); cangrelor was a generous gift of AstraZeneca R&D Charnwood (Loughborough, UK).

2.2. Primary cultures of DRG neurons

Primary cultures of dissociated DRG neurons from young rats were prepared as described previously [32] with minor modifications. Briefly, ganglia were dissected from 10- to 12-day-old Sprague–Dawley rats that had been killed by decapitation in full accordance with all rules of the Austrian animal protection (see http://www.ris.bka.gv.at/GeltendeFassung.wxe?Abfrage=Bundesnormen&Gesetzesnummer=20003541) and the Austrian animal experiment by-laws (see http://www.ris2.bka.gv.at/Dokumente/BgbIlPfd/2000_198_2/2000_198_2.pdf). Dissected ganglia were incubated in collagenase (1.5 mg/mL; Sigma) and dispase (3.0 mg/mL; Boehringer Mannheim, Vienna, Austria) for 30 minutes at 37°C. Then, they were incubated in trypsin (0.25% trypsin; Worthington, Lakewood, NJ) for 15 minutes at 37°C, dissociated by trituration, and resuspended in Dulbecco modified Eagle medium (Invitrogen, Lofer, Austria) containing 4.5 g/L glucose, 10 mg/L trituration, and resuspended in Dulbecco modified Eagle medium at 37°C. Then, they were incubated in trypsin (0.25% trypsin; Worthington, Lakewood, NJ) for 15 minutes at 37°C, dissociated by trituration, and resuspended in Dulbecco modified Eagle medium (Invitrogen, Lofer, Austria) containing 4.5 g/L glucose, 10 mg/L insulin, 25 000 IU/L penicillin, and 25 mg/L streptomycin (Invitrogen), 50 µg/L nerve growth factor (R&D Systems Inc., Minneapolis, MN), and 5% fetal calf serum (Invitrogen). Finally, dissociated cells were seeded onto 35-mm culture dishes coated with poly-D-lysine (Sigma). The cultures were kept in a humidified 5% CO2 atmosphere at 37°C. The medium was exchanged at least once per week.

2.3. Electrophysiology

All currents or potentials were recorded at room temperature (20°C to 24°C) from small-diameter DRG neurons after 1 to 2 days in culture using an Axopatch 200b amplifier and the pCLAMP 8.1 hard- and software (Molecular Devices, Sunnyvale, CA). All of the neurons investigated displayed capsaicin-induced currents (Icaps) and were thus assumed to provide nociceptive functions. In a few experiments, capsaicin-insensitive neurons were used for comparison. Membrane excitability and currents through K7 channels (Iks) or TRPV1 channels (Icaps) of DRG neurons were determined in the perforated patch clamp mode, voltage-activated Ca2+ currents in the whole-cell configuration. Signals were low-pass filtered at 5 kHz, digitized at 10 to 50 kHz, and stored on an IBM-compatible computer. Traces were analyzed off-line by the Clampfit 8.2 program (Molecular Devices). Patch electrodes were pulled (Flaming-Brown puller, Sutter Instruments, Novato, CA) from borosilicate glass capillaries (Science Products, Frankfurt/Main, Germany). For perforated patch recordings, the internal solution contained (mM) K2SO4 (75), KCl (55), MgCl2 (8), and HEPES (10), adjusted to pH 7.3 with KOH. The resulting electrode resistances were between 1.5 and 3.5 MΩ. The external (bathing) solution routinely contained (mM) NaCl (140), KCl (6.0), CaCl2 (2.0), MgCl2 (2.0), glucose (20), HEPES (10), adjusted to pH 7.4 with NaOH. For the recordings of Iks and Icaps, TTX (0.5 µM) was included to suppress voltage-activated Na+ currents. For Ca2+ current recordings, the external solution contained (mM) tetraethylammonium chloride (155), MgCl2 (0.8), CaCl2 (2.5), glucose (11), HEPES (10), and TTX (0.0005), adjusted to pH 7.3 with KOH. Tip resistances were between 1.5 and 3.5 MΩ. The resulting liquid junction potentials were below ±10 mV and were not taken into further consideration during experimentation or data evaluation. Drugs were applied via a DAD-12 drug application device (Adams & List, Westbury, NY), which permits a complete exchange of solutions surrounding the cells being investigated within less than 100 ms [5].

To test for the effects of different nucleotides on membrane excitability, DRG neurons were challenged by 5 increasing current injections (0.1 to 0.5 nA), each lasting for 2 seconds. Changes in excitability were evaluated by counting the number of action potentials fired during these 5 current injections in the absence and presence of nucleotides, respectively.

To quantify currents through K7 channels (Iks), nonactivating outward currents were activated by depolarizing the membrane to −30 mV. Once every 10 seconds, cells were hyperpolarized to −55 mV for 1-second periods to deactivate K7 channels; the differences between current amplitudes 20 ms after the onset of hyperpolarisations and 20 ms before re-depolarization were taken as a measure for Iks [6]. Currents through TRPV1 channels were evoked by 15-second applications of capsaicin (Icaps) to cells held at a potential of −70 mV once every 2 minutes, and the peak amplitudes of these currents were quantified. Ca2+ currents were elicited by 30-ms depolarizations from a holding potential of −80 to −10 mV at a frequency of 4 per minute. Leakage currents were corrected for by an online leak subtraction protocol that applies 4 hyperpolarizing pulses before the depolarization to −10 mV; currents were quantified by measuring peak current amplitudes.

Recordings obtained in the presence of nucleotides (b) were compared with those measured before (a) and after (c) the presence of the nucleotides; changes in current amplitudes were then calculated as percent of control = 200 ⋅ b/(a + c) or as percent inhibition = 100 − [200 ⋅ b/(a + c)]. For statistical comparison, such sequences were recorded, but nucleotide solutions were replaced by solvent.

2.4. Statistics

All data are given as arithmetic means ± SEM; n values give the numbers of single cells. Differences between 2 data points were evaluated by the nonparametric Mann–Whitney U test or the
Wilcoxon signed rank test. Comparisons between multiple data points were evaluated by the nonparametric Kruskal–Wallis analysis of variance followed by a Dunn post-hoc test.

3. Results

3.1. Nucleotides increase the excitability of DRG neurons

The excitability of DRG neurons is well known to be enhanced in the presence of a variety of inflammatory mediators, such as bradykinin [26] and prostaglandins [18], which results in increased action potential firing frequencies. Moreover, changes in membrane excitability of DRG neurons correlate well with changes in nociceptive behavior [26]. Therefore, DRG neurons were investigated in perforated patch recordings (to maintain the integrity of cytosolic signaling components) in current clamp mode. To test for the excitability of the neurons, 2-second current injections were applied in increments of 100 pA as described, for instance, in investigations on the effects of prostaglandins [18].

A total of 64 cells had an average membrane potential of $-65.1 \pm 0.8$ mV. In response to the injection of 100 pA for 2 seconds, none of the neurons fired any action potential; with increasing current injections, however, the number of elicited action potentials increased; with 500 pA injections, for instance, the neurons fired 1.4 $\pm 0.1$ action potentials per 2 seconds ($n = 64$; Fig. 1A). Subsequently, the total number of action potentials fired in response to all 5 current injections will be evaluated. In the presence of 10 $\mu$M ATP, the neurons were depolarized by about 10 mV, and the number of action potentials elicited by the 5 consecutive periods of current injection increased to $>40$. After removal of ATP, the membrane potential and the number of elicited action potentials returned to the levels observed prior to the application of the nucleotide (Fig. 1A). Thus, ATP significantly and reversibly enhanced the excitability of DRG neurons.

For comparison, 10 $\mu$M of ADP, UDP, UTP, or thio-UTP were applied to DRG neurons instead of ATP; ADP, UTP, and 2-thio-UTP caused effects equivalent to those observed for ATP, whereas UDP hardly caused any change (Fig. 1B). The effects of the nucleotides on both the membrane potential and the number of elicited action potentials were concentration dependent, with ATP, UTP, and thio-UTP being more potent than ADP (Fig. 1C and D).

3.2. The effects of nucleotides are mediated by 2 separate receptors

None of the cloned P2Y receptors accept ADP and UTP as agonists acting at a similar range of concentrations [43]. Moreover, 2-thio-UTP is a P2Y$_2$-preferring agonist [17], and ADP does not activate P2Y$_3$ [43]. Therefore, we assumed that the actions of the 4 nucleotides, as described above, were mediated by at least 2 separate P2Y receptors. Because ADP is an agonist at P2Y$_1$ receptors, and these receptors are expressed in a large number of different neurons [21], ADP was applied together with 30 $\mu$M of the specific P2Y$_1$ antagonist MRS 2179 [43]. In fact, the number of action potentials triggered by current injections in the presence of 10 $\mu$M ADP was significantly reduced by MRS 2179 (Fig. 1E), whereas this antagonist had no effect on the number of action potentials triggered in the presence of 10 $\mu$M 2-thio-UTP (Fig. 1F). Thus, ADP and 2-thio-UTP acted via different receptors. Nevertheless, the excitatory effects mediated by these 2 receptors were not additive, as the combination of ADP and 2-thio-UTP raised the number of action potential to a similar extent as the separated application of these nucleotides (Fig. 1G).

![Image](https://example.com/image1.png)

**Fig. 1.** Increase in membrane excitability of rat sensory neurons caused by different nucleotides. Membrane potential was measured in perforated patch current clamp mode, and five 2-second current injections of increasing amplitudes (as shown in A) were applied. Nucleotides were present for 2 minutes before determining their effects. (A) Original traces obtained before (ctrl), during (ATP), and after (wash) the application of 10 $\mu$M ATP. (B) Total number of action potentials fired in response to the 5 current injections in the presence of ADP, ATP, UDP, UTP, and thio-UTP (all at 10 $\mu$M; $n = 5$ to 7). **Significant difference vs the effects of all other nucleotides at $P < .01$ (Kruskal–Wallis test). (C) Concentration response curves for the number of action potentials fired in the presence of nucleotides ($n = 5$ to 8). Calculated EC$_{50}$ values were: 0.45 $\mu$M (ATP), 7.5 $\mu$M (ADP), 0.3 $\mu$M (UTP), and 0.63 $\mu$M (thio-UTP). (D) Concentration response curves for the membrane depolarization in the presence of nucleotides ($n = 5$ to 8). Calculated EC$_{50}$ values were: 0.8 $\mu$M (ATP), 2.2 $\mu$M (ADP), 0.5 $\mu$M (UTP), 1.2 $\mu$M (thio-UTP). (E) Total number of action potentials fired in response to current injections in the presence of ADP (10 $\mu$M) either alone or together with MRS2179 (30 $\mu$M; $n = 5$). **Significant difference at $P < .01$ (Mann–Whitney $U$ test). (F) Total number of action potentials fired in response to current injections in the presence of UTP (10 $\mu$M) either applied alone or together with MRS2179 (30 $\mu$M; $n = 5$). (G) Total number of action potentials fired in response to current injections in the presence of ADP (10 $\mu$M), thio-UTP (10 $\mu$M), or ADP co-applied with thio-UTP (both at 10 $\mu$M; $n = 5$). ADP = adenosine diphosphate; ATP = adenosine triphosphate; tUTP = 2-thio-UTP; UDP = uridine diphosphate; UTP = uridine triphosphate.
3.3. Two separate receptors mediate an inhibition of K<sub>V7</sub> channels by nucleotides

Knowing that ADP and 2-thio-UTP elicited excitatory effects via 2 separate P2Y receptors, ADP was used first to further investigate the underlying mechanisms. In current clamp experiments, ADP (10 μM) slowly reduced nondesensitizing outward currents at holding potentials more positive than −60 mV (not shown), as previously observed in sympathetic neurons [6]: this is indicative of an effect on K<sub>V7</sub> channels. Therefore, the gating of these channels was assessed by applying hyperpolarizing voltage steps from a holding potential of −30 mV where K<sub>V7</sub> channels are open to −55 mV in order to close the channels (Fig. 2A). ADP (10 μM) slowly reduced the holding currents at −30 mV as well as the slow deactivation of the current through K<sub>V7</sub> channels (M current; I<sub>M</sub>) as observed at −55 mV (Fig. 2A). The reduction of outward currents at −30 mV by ADP reached a maximum after 30 seconds, and these currents returned to control values within 1 minute after removal of the nucleotide (Fig. 2B). This effect was also seen with 10 μM ATP and UTP, as well as with 10 μM of the selective P2Y<sub>2</sub> agonist 2-thio-UTP (Fig. 2C). In concentration-response curves for the inhibition of I<sub>M</sub>, ATP turned out to be more potent than ADP, UTP, and 2-thio-UTP (Fig. 2E).

At least ATP might also induce inward currents in DRG neurons through an activation of P2X receptors. To reveal whether P2X might be involved in the slow induction of apparent inward currents at −30 mV (Fig. 2B), the nucleotides were also applied to neurons clamped at −70 mV where K<sub>V7</sub> channels are entirely inactivated [10]. Under these conditions, ATP, but not ADP, UTP, nor 2-thio-UTP (each at a concentration of 10 μM), caused rapidly activating inward currents that reached maximal amplitudes within <100 ms and largely desensitized within the next 2 seconds (Supplementary Fig. 1A and B). Thus, the slow effects on K<sub>V7</sub> channels described above can be easily discerned from actions mediated by P2X receptors. Moreover, ATP did reduce I<sub>M</sub> at concentrations (such as 0.1 μM; Fig. 2E) that were too low to activate P2X receptors (not shown).

To learn whether the inhibition of K<sub>V7</sub> channels by nucleotides is specific for nociceptive neurons, ADP and 2-thio-UTP were also applied to capsaicin-insensitive neurons. There, the reduction of I<sub>M</sub> deactivation amplitudes was as pronounced as in capsaicin-sensitive neurons (compare Fig. 2C and D).

The inhibition of I<sub>M</sub> by ADP (Fig. 2F), but not that by 2-thio-UTP (Fig. 2G), was largely reduced by the P2Y<sub>2</sub> antagonist MRS 2179. Thus, 2 separate P2Y receptors mediate an inhibition of K<sub>V7</sub> channels in sensory neurons. Nevertheless, the effects of ADP and 2-thio-UTP on I<sub>M</sub> were not additive (Fig. 2H).

3.4. Phospholipase C and increase in intracellular Ca<sup>2+</sup> mediate the inhibition of K<sub>V7</sub> channels by nucleotides

To investigate the underlying signaling mechanisms, ADP and 2-thio-UTP were used as agonists for the 2 different receptors. All P2Y receptors with the exception of P2Y<sub>12,13,14</sub> couple to G<sub>q</sub> proteins and thereby to phospholipase C (PLC) [43]. When DRG neurons were treated with 1 μM of the PLC inhibitor U73122 [23] for 15 minutes, the inhibition by either ADP or 2-thio-UTP was significantly smaller than prior to this treatment. Furthermore, treatment of the neurons with 1 μM of the Ca<sup>2+</sup>-<wbr/>ATPase inhibitor thapsigargin [39] for 15 minutes or with 10 μM of the cell

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**Fig. 2.** Inhibition of currents through K<sub>V7</sub> channels by different nucleotides. Currents were determined in perforated patch voltage clamp mode; neurons were clamped to −30 mV and hyperpolarized to −55 mV for 1-second periods. (A) Original traces obtained before (control), during (ADP), and after (wash) the presence of 10 μM ADP. The dotted line indicates the zero current level. (B) Time course of the inhibition of the current measured at −30 mV by 10 μM ADP; current amplitudes were normalized to the first amplitude determined in the same neuron (n = 5). (C) Inhibition of currents through K<sub>V7</sub> channels (I<sub>M</sub>) determined by the deactivation of currents at −55 mV in the presence of ATP, ADP, UTP, and tUTP (all at 10 μM; n = 9). “Significant difference vs the effects of all other nucleotides at P < .01 (Kruskal–Wallis test).” (D) Inhibition of currents through K<sub>V7</sub> channels (I<sub>M</sub>) in capsaicin-insensitive neurons in the presence of ADP and tUTP (both at 10 μM; n = 5). (E) Concentration response curves for the inhibition of currents through K<sub>V7</sub> channels by different nucleotides (n = 5 to 9); All values of inhibition were normalized to that evoked by 10 μM ADP in the very same cell. Calculated EC<sub>50</sub> values were: 1.6 μM (ADP), 0.2 μM (ATP), 2.3 μM (UTP), and 1.1 μM (tUTP). (F) Inhibition of currents through K<sub>V7</sub> channels (I<sub>M</sub>) by ADP (10 μM) either applied alone or together with MRS2179 (30 μM; n = 8). “Significant difference at P < .01 (Mann–Whitney U test).” (G) Inhibition of currents through K<sub>V7</sub> channels (I<sub>M</sub>) by ATP (10 μM) either applied alone or together with MRS2179 (30 μM; n = 5). (H) Inhibition of currents through K<sub>V7</sub> channels (I<sub>M</sub>) by ADP (10 μM), tUTP (10 μM), or ADP co-applied with tUTP (both at 10 μM; n = 5). ADP = adenosine diphosphate; ATP = adenosine triphosphate; tUTP = 2-thio-UTP; UDP = uridine diphosphate; UTP = uridine triphosphate.
permeant Ca$^{2+}$-chelator BAPTA-AM also reduced the inhibition of $I_M$ by the 2 nucleotides when compared with the pretreatment situation (Fig. 3B and C). Thus, inhibition of PLC, depletion of intracellular Ca$^{2+}$ stores, and chelation of intracellular Ca$^{2+}$ prevented the inhibition of $K_v7$ channels by nucleotides.

The PLC-mediated synthesis of inositol trisphosphate together with increases in intracellular Ca$^{2+}$ can lead to the activation of Ca$^{2+}$-dependent protein kinase C (PKC) isozymes that might be involved in the inhibition of $K_v7$ channels. However, the specific PKC inhibitor GF 109203X [29] at a concentration of 1 μM did not alter the inhibition of $K_v7$ channels by either ADP or 2-thio-UTP (Fig. 3B and C).

3.5. Two separate receptors mediate a sensitization of TRPV$_1$ channels by nucleotides

The sensitization of TRPV$_1$ channels by inflammatory mediators is believed to contribute to the development of chronic pain [22]; nucleotides have been reported to cause sensitization of TRPV$_1$ channels. Indeed, currents evoked by 0.3 μM capsaicin were markedly enhanced in the presence of 10 μM ADP (Fig. 4A). This effect of ADP developed slowly and reached its maximum after 94.7 ± 19.5 seconds (n = 4). In concentration-response curves for capsaicin-evoked currents, this enhancement was revealed to be due to a leftward shift in the presence of the nucleotide; 2-thio-UTP caused a leftward shift similar to that observed for ADP (Fig. 4A). The depletion of intracellular Ca$^{2+}$ stores by thapsigargin and the chelation of intracellular Ca$^{2+}$ by BAPTA-AM also abolished the sensitization of TRPV$_1$ channels by the nucleotides. Likewise, when PKC was inhibited by GF 109203X, the facilitation of capsaicin-evoked currents by either ADP or 2-thio-UTP was largely reduced (Fig. 5A–C).

3.6. Phospholipase C, increases in intracellular Ca$^{2+}$, and protein kinase C mediate the sensitization of TRPV$_1$ channels by nucleotides

TRPV$_1$ channels are believed to be tonically inhibited by phosphatidylinositol 4,5-bisphosphate (PIP$_2$), and a depletion of PIP$_2$ in the membrane thus leads to sensitization of the receptors. Alternatively, the sensitization can occur via an activation of PKC [34]. To analyze the signaling cascades that link the 2 P2Y receptors to TRPV$_1$ channels, the manipulations used to investigate the inhibition of $K_v7$ channels were used again. As observed in those experiments, inhibition of PLC by 1 μM U73122 prevented the facilitation of capsaicin-evoked currents by either ADP or 2-thio-UTP (Fig. 5A–C).

The depletion of intracellular Ca$^{2+}$ stores by thapsigargin and the chelation of intracellular Ca$^{2+}$ by BAPTA-AM also abolished the sensitization of TRPV$_1$ channels by the nucleotides. Likewise, when PKC was inhibited by GF 109203X, the facilitation of capsaicin-evoked currents by either ADP or 2-thio-UTP was largely reduced (Fig. 5B and C).

3.7. The increase in the excitability of DRG neurons involves $K_v7$ and TRPV$_1$ channels

To test whether the increase in the excitability of DRG neurons observed in the presence of nucleotides is brought about by the inhibition of $K_v7$ channels and/or by the sensitization of TRPV$_1$ channels, the nucleotides were applied together with either flupirine, an activator of $K_v7$ channels [10], XE991, an inhibitor of $K_v7$ channels [10], and/or iodoresiniferatoxin, an antagonist at TRPV$_1$ channels [2]. Flupirine 100 μM hyperpolarized DRG neurons by about 5 mV (Fig. 6C), but failed to significantly reduce the small number of action potentials fired in response to the injection of depolarizing currents with increasing amplitudes (0.1 to 0.5 mA) in the absence of nucleotides (Fig. 6A and B). However, flupirine entirely prevented action potential firing in the presence of either 100 μM ADP (Fig. 6A and B) or 10 μM 2-thio-UTP (Fig. 6B). In addition,
flupirtine abolished the depolarizations caused by either of these 2 nucleotides (Fig. 6C).

XE991 at 10 μM, a concentration that fully blocks currents through KV7 channels in DRG neurons [26], enhanced the action potential firing and caused depolarization of DRG neurons to about the same extent as ADP or 2-thio-UTP (Fig. 6D and E). Nevertheless, in the presence of 10 μM XE991, the 2 nucleotides were still able to further depolarize the neurons and to facilitate action potential firing (Fig. 6D and E).

Iodoresiniferatoxin (0.3 μM), in contrast, did not alter action potential firing in the absence of nucleotides, but significantly reduced the number of action potentials fired in response to current

Fig. 4. Potentiation of currents through TRPV1 channels by different nucleotides. Currents were determined in perforated patch voltage clamp mode; neurons were clamped to -70 mV, and capsaicin was applied for 15-second periods in the absence or presence of the indicated concentrations of nucleotides. (A) Original traces of currents evoked by 0.3 μM capsaicin (caps) before (ctrl), during (ADP), and after (wash) the presence of 10 μM ADP; capsaicin was applied as indicated by the bars. (B) Concentration response curves for the peak amplitudes of inward currents induced by the indicated concentrations of capsaicin in the absence (ctrl) or presence of 10 μM of ADP or tUTP (n = 5 to 6). All values were normalized to the current evoked by 1 μM capsaicin in the absence of nucleotides in the very same cell [Icaps normalized]. (C) Concentration response curves for the potentiation of currents induced by capsaicin (0.3 μM) in the presence of the indicated concentrations of nucleotides (n = 4 to 6). Amplitudes in the presence of nucleotides were expressed as percentage of the amplitudes recorded in their absence (% Icaps). (D) Potentiation of capsaicin-induced currents (Icaps) by ADP (10 μM) either applied alone or together with MRS 2179 (30 μM; n = 6). **Significant difference at P < .01 (Mann–Whitney U test). (E) Potentiation of capsaicin-induced currents (Icaps) by tUTP (10 μM) either applied alone or together with MRS 2179 (30 μM; n = 5). (F) Potentiation of capsaicin-induced currents (Icaps) by ADP (10 μM), tUTP (10 μM), or ADP co-applied with tUTP (n = 5). ADP = adenosine diphosphate; tUTP = 2-thio-UTP.

Fig. 5. The potentiation of currents through TRPV1 channels by nucleotides involves phospholipase C, increases in intracellular Ca2+, and protein kinase C. Currents were determined in perforated patch voltage clamp mode; neurons were clamped to -70 mV. (A) Original traces obtained with 0.3 μM capsaicin (caps) in the absence (ctrl) or presence of tUTP; these recordings were performed before and at the end of a 15-minute application of U73122 (1 μM). (B and C) Effects of U73122 (1 μM), thapsigargin (1 μM), BAPTA-AM (10 μM), and GF109203X (1 μM) on the potentiation of currents through TRPV1 channels (Icaps) by either ADP (10 μM in B) or tUTP (10 μM in C); currents were determined before (pre) and at the end (post) of 15-minute applications of U73122, thapsigargin (Thaps), or GF109203X (GFX) or of 30-minute applications of BAPTA-AM (n = 4 to 7); **Significant differences between the results obtained before and after the application of U73122, thapsigargin, BAPTA-AM, or GF 109203X at P < .05 (Mann–Whitney U test). ADP = adenosine diphosphate; tUTP = 2-thio-UTP.
injections in the presence of either 100 μM ADP (Fig. 7A and B) or 10 μM 2-thio-UTP (Fig. 7B). Moreover, iodoresiniferatoxin per se did not alter the membrane potential, but significantly reduced the depolarizing effect of either ADP or 2-thio-UTP (Fig. 7C). Finally, when applied in the continuing presence of XE991 (10 μM), iodoresiniferatoxin prevented any depolarization and any enhancement of action potential firing by either ADP or 2-thio-UTP (Fig. 7D and E). Thus, the blockade of Kv7 channels and of TRPV1 channels is sufficient to abolish the excitation of DRG neurons by nucleotides.

3.8. P2Y12 receptors mediate an inhibition of voltage-gated Ca2+ channels in DRG neurons

Nucleotides have been suggested to reduce neurotransmission from DRG neurons onto dorsal horn neurons by an inhibition of voltage-activated Ca2+ channels, but it remained controversial whether this effect was mediated by P2Y1 [20] or P2Y12 [28] receptors. To clarify this issue, voltage-activated Ca2+ currents were recorded in the absence and presence of ADP. The nucleotide prevented any depolarization and any enhancement of action potential firing by either ADP or 2-thio-UTP applied either alone or in combination. (Significant difference vs the effect of ADP or tUTP applied alone at P < .05 (Mann–Whitney U test; n = 6 to 8). ADP = adenosine diphosphate; tUTP = 2-thio-UTP.

4. Discussion

Although it is firmly established that ionotropic ATP receptors containing P2X2 subunits do contribute to the well-known algetic effects of this nucleotide, the role of metabotropic P2Y receptors has remained at least controversial, if not unclear [44]. Here, we demonstrate that activation of both P2Y1 and P2Y2 receptors leads to a marked increase in the excitability of DRG neurons; such an
Inhibition of voltage-activated Ca\(^{2+}\) currents in sensory neurons by ADP. Currents were determined in whole-cell voltage clamp mode; neurons were clamped to \(-80\) mV and depolarized to \(-10\) mV for 30 ms at a frequency of 4 min\(^{-1}\). (A) Original traces obtained before, during (ADP), and after the application of 10 \(\mu\)M ADP in either the absence (control) or presence of MRS2179 (30 \(\mu\)M) or cangrelor (1 \(\mu\)M). (B) Effects of ADP on peak amplitudes of Ca\(^{2+}\) currents in either the absence (no ligand) or presence of MRS2179 (30 \(\mu\)M) or cangrelor (1 \(\mu\)M; \(n = 8\)). **, ***: Significant difference vs the currents determined in the absence of ADP at \(P < .05\) and \(P < .01\), respectively (Wilcoxon signed rank test). ADP = adenosine diphosphate; tUTP = 2-thio-UTP.

These results reveal that both an inhibition of Kv7 channels and a potentiation of TRPV1 channels are involved in the excitation of DRG neurons via these 2 nucleotide receptors. The same signaling mechanisms are involved in the excitatory actions of bradykinin in DRG neurons [11,26,46]. ADP, ATP, UTP, and 2-thio-UTP enhanced action potential firing in response to current injections with the following order of agonist potency: ATP = UTP = 2-thio-UTP > ADP. ADP is an agonist at P2Y\(_1,12,13\) receptors [43], and the effects of ADP were largely attenuated by the selective P2Y\(_1\) receptor antagonist MRS 2179 [14], which demonstrates the participation of this latter receptor. The other nucleotides used are either poor or not at all agonists at P2Y\(_1\) receptors [43]. Hence, another nucleotide receptor must have contributed, and this was P2Y\(_2\) for the following reasons: (1) UDP did not mimic the effects of UTP; thus, a role of P2Y\(_6\) can be excluded [43]. (2) ATP and UTP were about equipotent as observed for rat P2Y\(_2\) and P2Y\(_4\) receptors [43]. (3) UTP and 2-thio-UTP were equipotent; this only holds true for P2Y\(_2\), but not for P2Y\(_4\) receptors [17]. Thus, the nucleotides enhanced the excitability of DRG neurons via both P2Y\(_1\) and P2Y\(_2\) receptors. Recombinant P2Y\(_{1,2,4,6}\) receptors mediate an inhibition of Kv7 channels [7], and DRG neurons express Kv7.2, Kv7.3, and Kv7.5 [10]. Most recently, acute nociception induced by bradykinin was shown to involve inhibition of Kv7 channels and activation of a Ca\(^{2+}\)-dependent chloride current [26]. Here, all the nucleotides mentioned above reduced currents through Kv7 channels, and the action of ADP, but not that of 2-thio-UTP, was attenuated by the P2Y\(_1\) antagonist MRS 2179. Thus, the activation of both P2Y\(_1\) and P2Y\(_2\) receptors contributed to the closure of Kv7 channels. However, we found no evidence for an induction of chloride currents by any of the nucleotides tested (not shown). The inhibition of Kv7 channels by both P2Y\(_1\) and P2Y\(_2\) has also been observed with receptors heterologously expressed in sympathetic neurons [9].

TRPV1 channels mediate acute and chronic pain [22], and ADP as well as 2-thio-UTP enhanced currents through these receptors; this effect of ADP, but not that of 2-thio-UTP, was again antagonized by MRS 2179. Hence, in contrast to what has been indicated by previous experiments, it is not P2Y\(_1\) [40] or P2Y\(_2\) [31,27], but both receptors that contribute to the sensitization of TRPV1 channels by nucleotides. The most likely reason for the conflicting interpretations of previous results is the fact that a full range of selective agonists and antagonists for these 2 receptors as employed here had not been used. Alternatively, the reason might be species differences between rats and mice.

Both P2Y\(_1\) and P2Y\(_2\) receptors are linked to Gq proteins and thereby to PLC [43]. In accordance with this, the PLC inhibitor U73122 [23] prevented the effects of both ADP and 2-thio-UTP, on Kv7 channels as well as on TRPV1 channels. The actions of these nucleotides on Kv7 and TRPV1 channels were also abolished when Ca\(^{2+}\)-ATPases had been inhibited by thapsigargin [39] or when intracellular Ca\(^{2+}\) had been chelated by BAPTA-AM; thus, increases in intracellular Ca\(^{2+}\) were involved in both of these effects. Downstream of Ca\(^{2+}\), however, the signaling cascades linking the 2 P2Y receptors to either Kv7 channels or TRPV1 channels differed from each other: the sensitization of TRPV1 channels was mediated by an activation of protein kinase C, as indicated by the inhibitory effect of GF 109203X, whereas the inhibition of Kv7 channels was not altered by this PKC inhibitor. This bifurcated signaling pathway was shared by both receptors, P2Y\(_1\) and P2Y\(_2\). As a consequence, the simultaneous activation of both receptors by ADP and 2-thio-UTP did not achieve larger effects than the activation of only one of these receptors. These results lead to the question of how P2Y receptor activation could modulate Kv7 channels, on one hand, and TRPV1 channels, on the other hand. The inhibition of neuronal Kv7 channels by G-protein coupled receptors involves either depletion of membranePIP2, phosphorylation via PKC, or binding of intracellular Ca\(^{2+}\) to channel-bound calmodulin. In sympathetic neurons, the first 2 mechanisms were shown to mediate Kv7 channel inhibition via M\(_1\) muscarinic receptors [16]; the latter mechanism has been found to mediate the inhibition via bradykinin and P2Y receptors [8,49]. The present results support this latter idea, as manipulations that prevented increases in intracellular Ca\(^{2+}\) abolished the effects of nucleotides on Kv7 channels, but inhibition of PKC failed to alter the effect of P2Y receptor activation.

Obviously, the facilitation of currents through TRPV1 channels via P2Y receptors cannot involve Ca\(^{2+}\)/calmodulin, as this signaling entity, if anything, mediates the desensitization of TRPV1, but not a sensitization [34]. However, PLC-mediated depletion of membrane PIP2 [15] as well as activation of Ca\(^{2+}\)-dependent PKC [42] are both known to potentiate currents evoked by low, but not those evoked by high, capsaicin concentrations. In accordance with both mechanisms, the nucleotides caused a leftward shift in the concentration-response curves of capsaicin-evoked currents. However, the fact that prevention of increases in intracellular Ca\(^{2+}\) as well as inhibition of PKC abolished the sensitization of TRPV1 channels by ADP or 2-thio-UTP demonstrates a role of Ca\(^{2+}\)-dependent PKC.

Because this latter result proves that activation of either P2Y\(_1\) or P2Y\(_2\) leads to the activation of Ca\(^{2+}\)-dependent PKC, which then phosphorylates TRPV1, one has to ask why activated PKC did not contribute to the inhibition of Kv7 channels as previously reported for the signaling cascade linking M\(_1\) muscarinic receptors to this ion channel family [16]. The most likely explanation is offered by the finding that Ca\(^{2+}\)/calmodulin disrupts the association of Kv7 channels and AKAP79/150, which is a prerequisite for the phosphorylation of Kv7 channels by PKC [3].

The increase in action potential firing in the presence of ADP or 2-thio-UTP was completely abolished in the presence of the nonopioid analgesic flupirtine, which is known to activate Kv7 channels [45]. This appears to indicate that the entire increase in excitability was mediated by the inhibition of the Kv7 channels. However, the hyperpolarization of the neurons caused by flupirtine...
might also counteract the excitatory effects of the nucleotides independently of the interaction at Kv7 channels. Hence, this set of data does not unequivocally demonstrate that Kv7 channels were involved in the effects of the nucleotides on excitability and membrane potential. However, blockage of Kv7 channels by XE991 depolarized DRG neurons and enhanced their excitability to a similar extent as the activation of the 2 nucleotide receptors; this verifies that the closure of Kv7 channels is sufficient to depolarize DRG neurons and to enhance their excitability.

Nevertheless, the excitatory actions of ADP or 2-thio-UTP were additive to that of XE991, which hints to a role of an additional mechanism. In line with this conclusion, the TRPV1, antagonist iodoresiniferatoxin also attenuated the nucleotide-induced increase in membrane excitability. Moreover, the effects of the nucleotides on action potential firing and membrane potential in the presence of XE991 were entirely abolished by iodoresiniferatoxin. Hence, the inhibition of Kv7 channels as well as the sensitization of TRPV1 channels both contributed to the increase in excitability triggered by the activation of either P2Y1 or P2Y2 receptors.

In contrast to the excitatory effects shown above, ADP has been reported to elicit analgesic rather than proalgesic effects, and an inhibition of transmembrane Ca2+ entry in DRG neurons was reported to elicit analgesic rather than proalgesic effects, and an inhibition of PVACCs was first suggested to be mediated by P2Y1, whereas a later report demonstrated a role of P2Y12 and/or P2Y13 receptors [28]. In accordance with that latter result, we found that the inhibition of PVACCs by ADP was attenuated by the P2Y12 antagonist cangrelor, but not by MRS2179. This confirms that P2Y1 contributes to the sensation of pain in concert with P2Y2, whereas analgesic effects are mediated by P2Y2 receptors coupled to inhibitory G proteins [28].

Taken together, our results reveal that ATP may cause pain not only by the activation of ionotropic P2X receptors, but also via P2Y receptors. In addition, the ATP degradation product ADP also contributes to the sensation of pain as it activates P2Y1 receptors. These 2 metabotropic nucleotide receptors share a bifurcated signaling cascade to enhance the excitability of DRG neurons through a twofold mechanism: an inhibition of Kv7 channels through Ca2+/calmodulin and a sensitization of TRPV1 channels via Ca2+-dependent PKC. Because this bifurcated mechanism is also associated with bradykinin B2 receptors, it may well be a general signal cascade employed by painful mediators [11].

Conflict of interest statement
The authors declare no conflict of interest.

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Appendix A. Supplementary data
Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.pain.2011.04.016.

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