A1 Adenosine Receptor Activation Inhibits P2X3 Receptor–Mediated ATP Currents in Rat Dorsal Root Ganglion Neurons

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Abstract

Purinergic signaling is involved in multiple pain processes. P2X3 receptor is a key target in pain therapeutics, while A1 adenosine receptor signaling plays a role in analgesia. However, it remains unclear whether there is a link between them in pain. The present results showed that the A1 adenosine receptor agonist N6-cyclopentyladenosine (CPA) concentration dependently suppressed P2X3 receptor–mediated and α,β-methylene-ATP (α,β-meATP)–evoked inward currents in rat dorsal root ganglion (DRG) neurons. CPA significantly decreased the maximal current response to α,β-meATP, as shown a downward shift of the concentration–response curve for α,β-meATP. CPA suppressed ATP currents in a voltage-independent manner. Inhibition of ATP currents by CPA was completely prevented by the A1 adenosine receptor antagonist KW-3902, and disappeared after the intracellular dialysis of either the Gi/o protein inhibitor pertussis toxin, the adenylate cyclase activator forskolin, or the cAMP analog 8-Br-cAMP. Moreover, CPA suppressed the membrane potential depolarization and action potential bursts, which were induced by α,β-meATP in DRG neurons. Finally, CPA suppressed the membrane potential depolarization and action potential bursts induced by α,β-meATP in DRG neurons. These results indicated that CPA inhibited the activity of P2X3 receptors in rat primary sensory neurons by activating A1 adenosine receptors and its downstream cAMP signaling pathway, revealing a novel peripheral mechanism underlying its analgesic effect.

Keywords A1 adenosine receptor · P2X3 receptor · Electrophysiology · Dorsal root ganglion neuron · Nociceptive behavior

Introduction

Purines, including adenosine, ATP and ADP, are involved in multiple pain processes through their cognate receptors [1]. Among them, adenosine/A1 adenosine receptor signaling has been shown to have analgesic effects [2, 3]. Adenosine can alleviate pain behavior in a variety of pain models by activating A1 adenosine receptors [3, 4]. A1 adenosine receptors are expressed in the afferent pathway of pain, including primary sensory neurons and spinal cord dorsal horns [3, 5]. Studies have shown that A1 adenosine receptor activation significantly reduces postoperative, visceral, neurological, and inflammatory pain [6–10]. Acupuncture and various natural compounds produce analgesia by increasing local adenosine and/or activating A1 adenosine receptors [11–16]. Positive allosteric modulators of A1 adenosine receptors have also efficacy in pain relief [17, 18]. On the contrary, down-regulation of adenosine/A1 adenosine receptor signaling contributes to neuropathic pain [19]. A1 adenosine receptor-deficient mice display greater nociceptive responses [20, 21]. Peripheral A1 adenosine receptors are involved in the analgesic effects of adenosine signaling. For example, the A1 adenosine receptor agonists including adenosine can reduce nociceptive responses when they are injected into the hind paws of animals [22, 23]. It has recently been reported that activation of peripheral adenosine A1 receptors significantly relieves glutamate-induced nociceptive behaviors [24]. The findings that analgesic
effects of acetaminophen and tramadol are reversed by locally administration of selective A1 adenosine receptor antagonists indicate the contribution of peripheral A1 adenosine receptors to analgesia [25, 26]. Although previous studies have shown the role of peripheral A1 adenosine receptors in analgesia, the exact mechanisms underlying analgesia remain to be determined.

Apart from A1 adenosine receptor, other purinergic receptors, such as P2X3 receptor, are also expressed in nociceptive afferent pathway, including in terminals of nociceptive fibers [27, 28]. P2X3 receptor has been shown to participate in multiple pain processes [29, 30]. Antagonists, antisense oligonucleotide, and siRNA of P2X3 receptors are effective in pain relief [31–34]. Mice lacking P2X3 receptors display attenuated spontaneous nociceptive behaviors to ATP administration or in formalin test [35, 36]. Since a variety of purinergic signals have been shown to play roles in pain, is there a functional interaction between them in pain process? Previous studies have shown that activation of metabotropic P2Y receptors can inhibit ionotropic P2X3 receptors [37–39]. However, it is still unclear whether P2X3 receptors can be modulated by the activation of A1 adenosine receptors. The present study reported that the A1 adenosine receptor agonist N6-cyclopentyladenosine (CPA) did not only inhibit the electrophysiological activity of P2X3 receptors in rat dorsal root ganglion (DRG) neurons via an intracellular cAMP pathway, but also relieved P2X3 receptor–mediated nociceptive behaviors in rats by activating peripheral A1 adenosine receptors.

Materials and Methods

Preparation of DRG Neurons

All experimental protocols were approved by the Animal Research Ethics Committee of Hubei University of Science and Technology (2016–03-005). Sprague–Dawley rats (5–6 weeks old) were anesthetized and sacrificed. The DRGs from the rats were removed and chopped. The minced ganglia were transferred to a test tube containing Dulbecco’s modified Eagle’s medium (DMEM) and incubated with shaking for 25–30 min at 35 °C. The incubation solution contained 1.0 mg/ml of collagenase, 0.5 mg/ml of trypsin, and 0.1 mg/ml of IV DNase. Trypsin digestion was terminated by adding 1.25 mg/ml of soybean trypsin inhibitor. The cells were cultured for 12–24 h at 37 °C in DMEM containing nerve growth factor (100 ng/ml) and fetal bovine serum (10%).

Electrophysiological Recordings

Electrophysiological experiments were performed as described previously [40]. An EPC-10 patch clamp amplifier (HEKA Electronic, Lambrecht, Germany) was used for the whole-cell patch clamp recordings. The isolated DRG neurons were transferred to a 35-mm culture dish and kept in a normal external solution for at least 60 min before electrophysiological recordings. The external solution contained the following (in mM): 150 NaCl, 5 KCl, 2 MgCl2, 2.5 CaCl2, 10 HEPES, and 10 D-glucose. The pH and osmolarity were adjusted to 7.4 with NaOH and 330 mOsm/l with sucrose, respectively. The recording pipettes were pulled using a Sutter P-97 puller (Sutter Instruments, CA, USA), whose resistance was in the range of 3–6MΩ. The micro-pipette solution contained the following (in mM): 140 KCl, 2 MgCl2, 11 EGTA, 10 HEPES, 4 ATP, and 0.3Na2GTP. The pH and osmolarity were adjusted to 7.2 with KOH and 310 mOsm/l with sucrose, respectively. After whole-cell configuration was established, 70–80% series resistance and membrane capacitance current were compensated. The recorded currents were sampled at 10 kHz and filtered at 2 kHz. Only small- and medium-sized nociceptive DRG neurons (15–40 μm in diameter) were used for the electrophysiological recordings. The membrane potential of the neurons was clamped at −60 mV. Only DRG neurons with a resting membrane potential less than −50 mV were used for current-clamp recordings.

Drug Application

All drugs were obtained from Sigma-Aldrich (St. Louis, MO, USA). The working concentration of drugs was freshly prepared in normal external solution. Each working drug was stored in a series of independent reservoirs and subjected to gravity. The distance between the drug exit and the recorded neurons was approximately 30 μm. To block G-protein and intracellular signaling, some antagonists or blockers were dissolved in the internal solution and applied for intracellular dialysis through recording patch pipettes as described previously [40]. To ensure that dialysis drugs are infused into the cell interior, current recordings were performed at least 30 min after cell membrane rupture.

Nociceptive Behavior Induced by α,β-meATP in Rats

Rats were first habituated for 30 min in a Plexiglas chamber during the nociceptive behavioral experiment. The rats in eight different groups received intraplantar injections of 50 μl vehicle, and different doses (0.1, 1, and 10 ng) of CPA, 30 ng KW-3902 +10 ng CPA, 30 ng
KW-3902, 20 ng SQ22536, and 20 ng H-89 respectively. After 10 min, another experimenter injected α,β-methylene-ATP (α,β-meATP, 50 μg in 50 μl) into the ipsilateral hind paw and tested the nociceptive behavior. The assessor of the behavioral measures was blinded to the prior treatment conditions. Nociceptive behaviors (that is, number of flinches) were monitored over a 10-min period starting after the injection of α,β-meATP. The rats in three different groups received intraplantar injections of 50 μl vehicle, 10 ng CPA, and 30 ng KW-3902 + 10 ng CPA. After 10 min, another experimenter injected α,β-methylene-ATP (α,β-meATP, 50 μg in 50 μl) into the ipsilateral hind paw. The rats in the control group received two intraplantar injections of 50 μl vehicle. After another 10 min, thermal hyperalgesia was assessed by measuring paw withdrawal latency to heat stimulation. The heat source was focused on a portion of the hind paw. The stimulus shut off when the hind paw moved (or after 30 s to prevent tissue damage). In group vehicle, 10 ng CPA, 30 ng KW-3902 + 10 ng CPA, mechanical allodynia was measured by paw withdrawal threshold (PWT). PWT of the ipsilateral hind plantar was detected by using a series of von Frey filaments (Stoelting, Wood Dale, IL) at 0.5, 2.5, and 24h after α,β-meATP injection.

Data Analysis

All data were expressed as mean ± S.E.M, and statistically compared using Student’s t-test or analysis of variance (ANOVA), followed by Bonferroni’s post hoc test. The non-linear curve-fitting program ALLFIT was used for statistical analysis of the concentration–response data.

Results

CPA Concentration-Dependently Inhibits P2X3 Receptor–Mediated ATP Currents in Rat DRG Neurons

In the majority of tested DRG cells (76.5%, 13/17), an application of 100 μM α,β-methylene-ATP (α,β-meATP) or 100 μM ATP for 5 s evoked rapid inward currents (I\text{ATP}) under holding potentials of −60 mV conditions (Fig. 1A).

![Fig. 1](image_url) Inhibition P2X3 receptor–mediated ATP currents by CPA in DRG neurons. A In a representative DRG neuron, application of either α,β-meATP (100 μM) or ATP (100 μM) produced an inward current, which was completely blocked by the P2X3 and P2X2/3 receptor antagonist A-317491 (100 μM), but not by the P2X4 receptor antagonist PSB-12062 (10 μM) and the P2X7 receptor antagonist A438079 (1 μM). Membrane potentials were clamped at −60 mV. B The 100 μM α,β-meATP- and ATP-induced currents were similarly inhibited by pre-application of 100 nM CPA, a selective A1 adenosine receptor agonist, for 5 min to a recorded DRG cell. C The sequential current traces illustrate that the amplitude of 100 μM α,β-meATP-activated currents progressively decreased as the concentration of CPA increased from 3 to 300 nM in a representative DRG cell. D The graph shows the concentration-effect curve of CPA on 100-μM α,β-meATP-induced currents (I\text{ATP}) with an IC\text{50} value of 39.40 ± 3.18 nM. Each point represents the mean ± S.E.M. of 7–9 cells.
The $I_{\text{ATP}}$ was completely blocked by the specific P2X3 or P2X2/3 receptor antagonist A-317491 (100 μM), but not by the P2X4 receptor antagonist PSB-12062 (10 μM) and the potent P2X7 receptor antagonist A438079 (1 μM), indicating that the $I_{\text{ATP}}$ was mediated by P2X3 or P2X2/3 receptors.

In some DRG neurons (69.2%, 9/13), we observed that the peak amplitude of 100 μM α,β-meATP- and ATP-activated currents decreased when the A1 adenosine receptor agonist CPA (100 nM) was pre-treated to DRG cells for 5 min prior to the next recording (Fig. 1B). The decrease of the $I_{\text{ATP}}$ amplitude depended on the concentration of CPA. The sequential current traces in Fig. 1C illustrate that a gradual decrease in the amplitude of $I_{\text{ATP}}$ was evoked by 100 μM α,β-meATP with an increase in CPA concentration from 3 to 300 nM in a representative DRG cell. Figure 1D shows the concentration-effect curve of CPA on $I_{\text{ATP}}$ with an IC$_{50}$ (half-maximal effective concentration) value of 39.40 ± 3.18 nM. These results suggest that CPA inhibited P2X3 receptor–mediated ATP currents in a concentration-dependent manner.

**Concentration–Response and Current–Voltage Relationships for α,β-meATP in the Absence and Presence of CPA**

We studied whether the suppression of CPA depends on the concentration of α,β-meATP. Figure 2A shows that pre-application of CPA (100 nM for 5 min) decreased the amplitudes of ATP currents evoked by 3, 30, and 300 μM α,β-meATP, respectively. The concentration–response curves in Fig. 2B were plotted using a series of concentration of α,β-meATP in the absence and presence of 100 nM CPA, which were fit with the Hill equation. The concentration–response curve for α,β-meATP in the presence of 100 nM CPA was downwardly shifted with a decrease of 38.44 ± 5.87% in maximal current response, which was evoked by 300 μM α,β-meATP ($P < 0.01$, Bonferroni’s post

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hoc test). However, the Hill coefficients or the slopes were not significantly different between the two curves, which were $1.36 \pm 0.31$ and $1.28 \pm 0.26$ in the absence and presence of CPA, respectively ($P > 0.1$, Bonferroni’s post hoc test). In addition, CPA pre-treatment did also not change the EC$_{50}$ of α,β-meATP for P2X3 receptors, which were $29.23 \pm 3.06$ μM and $27.42 \pm 2.94$ μM in the absence and presence of CPA, respectively ($P > 0.1$, Bonferroni’s post hoc test). These results suggest that CPA inhibited the maximum response to α,β-meATP, but not shifted the sensitivity of P2X3 receptors to α,β-meATP.

We then investigated whether the suppression of ATP currents by CPA depends on the clamping potentials of recorded DRG cells. Figure 2C shows that pre-application of CPA (100 nM for 5 min) decreased the amplitudes of ATP currents evoked by 30 μM α,β-meATP when the membrane potential was clamped at $-80$ mV, $-40$ mV, and $+20$ mV, respectively. Figure 2D shows the current–voltage (I–V) curves for α,β-meATP in the absence and presence of 100 nM CPA, which were plotted under a series of clamping potentials conditions. CPA pre-treatment decreased the slope of the I–V curve with no significant difference in the CPA-induced suppression on ATP currents at different clamping potentials from $-80$ to $20$ mV ($P > 0.1$, Bonferroni’s post hoc test). CPA did not change the reversal potential (near 0 mV). The results indicate that CPA suppressed P2X3 receptor–mediated ATP currents in a voltage-independent manner.

**Participation of A1 Adenosine Receptors, G$i/o$ Proteins and cAMP Signaling in the CPA-Induced Inhibition of ATP Currents**

To determine whether A1 adenosine receptor is involved in the CPA-induced inhibition of α,β-meATP-evoked currents, the A1 adenosine receptor antagonist KW-3902 was co-treated with CPA to tested DRG cells. Figure 3A and B show that CPA failed to suppress $I_{ATP}$ when KW-3902 was co-applied with it. The amplitude of $I_{ATP}$ decreased from $1.36 \pm 0.13$ nA to $0.79 \pm 0.09$ nA in 8 DRG cells pre-treated with 100 nM CPA alone ($P < 0.01$, one-way ANOVA followed by post hoc Bonferroni’s test). In contrast, the amplitude of $I_{ATP}$ was $1.22 \pm 0.11$ nA in 8 DRG neurons co-treated with 300 nM KW-3902 and 100 nM CPA, which was significantly different from the $I_{ATP}$ amplitude after CPA pre-treatment alone ($P < 0.01$, one-way ANOVA followed by post hoc Bonferroni’s test, $n = 8$). In addition, application of 300 nM KW-3902 alone had no effect on the amplitude

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of $I_{\text{ATP}}$ (data not shown). These results indicate that CPA inhibited ATP currents in DRG neurons through A1 adenosine receptors.

A1 adenosine receptor is coupled to $G_{i/o}$ proteins, which, once activated, can trigger intracellular signaling events. We then identified which intracellular signaling of A1 adenosine receptor activation contributed to the suppression of ATP current by CPA. Firstly, pertussis toxin (PTX, 1 μg/ml), a $G_{i/o}$ protein inhibitor, was delivered intracellularly into tested DRG cells through the recording micropipettes before CPA treatment, resulting in a significant decrease in CPA-induced suppression of $I_{\text{ATP}}$ amplitude (Fig. 3C and D).

Secondly, to further explore intracellular signaling involving in the CPA-induced suppression, the adenylate cyclase activator forskolin and the cAMP analog 8-Br-cAMP were contained in the internal solution. Unlike under the normal internal solution conditions, CPA (100 nM for 5 min) caused a decrease of 41.78 ± 2.76% in $I_{\text{ATP}}$ amplitude. Intracellular dialysis of forskolin (0.1 μM) or 8-Br-cAMP (1 mM) prevented the CPA-induced suppression of $I_{\text{ATP}}$, the amplitude of $I_{\text{ATP}}$ only decreased 3.04 ± 2.92% and 9.59 ± 1.79%, respectively, after CPA treatment ($P < 0.01$, compared with normal internal solution, one-way ANOVA followed by post hoc Bonferroni’s test, n = 8; Fig. 3C and D). These data collectively indicate that CPA suppressed ATP currents via a $G_{i/o}$ proteins and downstream cAMP signaling pathway.

**CPA-Induced Suppression of α,β-meATP-Evoked Membrane Excitability of Rat DRG Neurons**

We further investigated the effect of CPA on the membrane excitability triggered by α,β-meATP. In the same DRG cell, 10 μM α,β-meATP did not induce a large inward current, but also triggered bursts of action potentials (APs) under current-clamp conditions (Fig. 4A and B). Consistent with the results under voltage-clamp conditions, pre-application of CPA (100 nM for 5 min) significantly decreased the number of α,β-meATP-evoked APs from 4.83 ± 0.60 of control condition to 2.17 ± 0.48 of CPA pre-treatment in 6 recorded DRG neurons ($P < 0.01$, paired t-test, n = 6; Fig. 4B). In contrast, the number of α,β-meATP-evoked APs was 4.50 ± 0.62 after co-application of 100 nM CPA and 300 nM KW-3902 to the DRG cells for 5 min, suggesting the effect of CPA on APs was reversed by KW-3902.

We then observed the effect of CPA on the membrane potential of DRG neurons in the presence of TTX (1 μM) to block Na+ channel-mediated APs. As shown in Fig. 4C, pre-application of CPA (100 nM for 5 min) also decreased the

**Fig. 4 CPA-induced suppression of α,β-meATP-evoked membrane excitability of rat DRG neurons.** Original traces in A show that application of 100 μM α,β-meATP to the same DRG cell caused an inward current and bursts of action potentials (APs) under voltage-clamp and current-clamp conditions, respectively. Original traces in B show that application of 100 μM α,β-meATP to the same DRG cell caused an inward current and depolarization of membrane potentials in the presence of TTX (1 μM) to block Na+ channel-mediated APs under voltage-clamp and current-clamp conditions, respectively. Data in C and D show that application of CPA (100 nM for 5 min) significantly decreased the number of α,β-meATP-induced APs and the magnitude of membrane depolarization ($\Delta V_m$) in six DRG neurons. The suppressing effects of CPA on α,β-meATP-evoked membrane excitability were reversed by the co-application of the 300 nM KW-3902. * * $P < 0.01$, paired t-test, n = 6 cells.
membrane depolarization evoked by 100 μM α,β-meATP. In 6 tested neurons, the magnitude of membrane depolarization (ΔVm) significantly decreased from 24.04 ± 1.86 mV to 17.64 ± 1.63 mV after 100 nM CPA pre-treatment for 5 min (paired t-test, *P < 0.01, *n = 7; Fig. 4D). In contrast, ΔVm was 23.56 ± 1.72 mV after co-application of 100 nM CPA and 300 nM KW-3902 for 5 min. These results indicate that CPA suppressed α,β-meATP-induced membrane excitability of rat DRG neurons through A1 adenosine receptors.

**Relief of α,β-meATP-Evoked Nociceptive Behaviors by CPA in Rats**

Finally, we investigated whether the suppression of P2X3 receptors by CPA in vitro plays a role in α,β-meATP-induced nociceptive behaviors in vivo. Figure 5A shows intraplantar injection of α,β-meATP (50 μg in 50 μl) caused an intense spontaneous flinching/shaking response in rats, which was relieved by intraplantar pre-treatment of CPA. Quantitative analysis showed that CPA dose-dependently (0.1, 1 and 10 ng) decreased the number of flinches induced by α,β-meATP (*P < 0.05 and 0.01, one-way ANOVA followed by post hoc Bonferroni’s test, *n = 8). Figure 5A shows that the relief of α,β-meATP-induced nociceptive behaviors by 10 ng CPA was blocked by co-treated the A1 adenosine receptor antagonist KW-3902 (30 ng; *P < 0.01, one-way ANOVA followed by post hoc Bonferroni’s test, *n = 8). In contrast, intraplantar administration of 30 ng KW-3902 alone had no effects on α,β-meATP-induced flinching behaviors (Fig. 5B). In addition, α,β-meATP-induced nociceptive behaviors were

![Fig. 5](image-url)

**Fig. 5** Relief of α,β-meATP-evoked nociceptive behaviors by CPA in rats. A Intraplantar injection of α,β-meATP (50 μg in 50 μl) caused spontaneous flinching behaviors in rats. Intraplantar pre-treatment with CPA (0.1, 1, and 10 ng in 50 μl) dose-dependently decreased the number of α,β-meATP-induced flinching behaviors. The analgesic effect of CPA (10 ng) was completely prevented by co-treatment with the A1 adenosine receptor antagonist KW-3902 (30 ng). B Intraplantar administration of 30 ng KW-3902 alone had no effects on α,β-meATP-induced flinching behaviors. C Intraplantar injection of α,β-meATP (50 μg in 50 μl) caused thermal hyperalgesia. The decreased paw withdrawal latency was prolonged by intraplantar pre-treatment with CPA (10 ng in 50 μl) in rats treated with α,β-meATP. The analgesic effect of CPA was blocked by co-treatment with KW-3902 (30 ng in 50 μl). D Intraplantar injection of α,β-meATP (50 μg in 50 μl) also caused a remarkable decrease in paw withdrawal thresholds (PWT, in g) at 0.5 and 2.5 h after injection, which was recovered at 24 h. The α,β-meATP-induced mechanical allodynia was significantly relieved by intraplantar pretreatment with CPA (10 ng in 50 μl), but not co-treatment with CPA (10 ng in 50 μl) and KW-3902 (30 ng in 50 μl). Bonferroni’s post hoc test, n.s., no significant; *P < 0.05, **P < 0.01. Each column represents the mean ± S.E.M. of 8 rats. # P < 0.05. ## P < 0.01, compared with CPA + α,β-meATP group; 8 rats/each group in D.
not affected if 10 ng CPA was injected into the contralateral paws (data not shown). Figure 5B shows the number of α,β-meATP-induced flinching behaviors was significantly decreased by intraplantar pre-treatment of the adenylyl cyclase inhibitor SQ22536 (20 ng in 50 μl) or PKA inhibitor H-89 (20 ng in 50 μl), suggesting they could inhibit the anti-nociceptive effects of CPA. Intraplantar injection of α,β-meATP (50 μg in 50 μl) caused thermal hyperalgesia, as shown a decreased paw withdrawal latency in rats treated with α,β-meATP. Figure 5C shows intraplantar pretreatment of CPA (10 ng in 50 μl) significantly prolonged the decreased paw withdrawal latency in rats treated with α,β-meATP (P < 0.01, Bonferroni’s post hoc test, n = 8 rats). The effect of CPA was blocked by co-treatment of KW-3902 (30 ng in 50 μl). We finally observed the effects of CPA on α,β-meATP-induced mechanical allodynia in rats. Figure 5D shows intraplantar injection of α,β-meATP (50 μg in 50 μl) resulted in a significant decrease in the paw withdrawal threshold (PWT) within 0.5 and 2.5 h after injection, which was recovered at 24 h. Intraplantar pretreatment with CPA had also an analgesic effect on the mechanical allodynia. The α,β-meATP-induced mechanical allodynia significantly decreased within 0.5 and 2.5 h after 10 ng CPA pretreatment (P < 0.05 and 0.01, Bonferroni’s post hoc test, compared with vehicle + α,β-meATP group, n = 8 rats; Fig. 5D). The analgesic effect of CPA was completely blocked by co-treated 30 ng KW-3902 (P < 0.05 and 0.01, Bonferroni’s post hoc test, compared with CPA + α,β-meATP group, n = 8 rats; Fig. 5D). Together, these results suggest that CPA relieved the α,β-meATP-induced nociceptive behaviors in vivo through activating peripheral A1 adenosine receptors.

Discussion

The present data demonstrated that the A1 adenosine receptor antagonist CPA suppressed the functional activity of peripheral P2X3 receptors. CPA decreased the amplitude of ATP currents and α,β-meATP-induced membrane excitability in rat DRG neurons, which involved A1 adenosine receptors, PTX-sensitive Gi/o proteins, and cAMP signaling cascades. Behaviorally, CPA also relieved α,β-meATP-induced nociceptive behaviors in rats through peripheral A1 adenosine receptors.

In the present experiments, α,β-meATP-induced ATP currents were recorded in small- and medium-sized DRG nociceptive neurons, where P2X3 homomeric and P2X2/3 heteromeric receptors are abundantly expressed [27, 28]. These recorded ATP currents were blocked by specific antagonist of P2X3 and P2X2/3 receptors, but not by antagonists of P2X4 receptors and P2X7 receptors, indicating they were P2X3 or P2X3-containing receptor-mediated currents. Moreover, α,β-meATP can only activate P2X3 and P2X1 receptors [41]. The present study showed that CPA concentration-dependently suppressed the ATP currents through A1 adenosine receptors, indicating P2X3 receptor was a downstream regulatory target of CPA/A1 adenosine receptor signaling. Consistent with current results, other ion channels are also regulated by activation of A1 adenosine receptors, which may be a potential mechanism underlying its antinociceptive effect. For example, the A1 adenosine receptor agonists modulate the function of GABA_A and glycine receptors in rat primary sensory and spinal cord neurons [42, 43]. Voltage-dependent Ca^{2+} channels and inward rectifier K^{+} channels are also inhibited by activation of A1 adenosine receptors [44, 45]. We observed that the CPA-induced inhibition did not alter the sensitivity of P2X3 receptor to α,β-meATP, but significantly decreased the maximum response to α,β-meATP. Activation of A1 adenosine receptors has been shown to reduce AMPA receptor surface expression through internalization [46]. It remains to be further studied whether CPA inhibited P2X3 receptors through a similar mechanism.

The CPA-induced suppression of ATP currents was completely blocked by the A1 adenosine receptor antagonist KW-3902, indicating the effect of CPA was dependent upon the activation of A1 adenosine receptors. Both A1 adenosine receptors and P2X3 receptors have been shown to be present in DRG neurons [3, 5, 19, 27, 28]. In addition, only some, but not all, ATP currents were inhibited by CPA, which may be related to the degree of the co-expression of A1 adenosine receptors and P2X3 receptors in rat DRG neurons. On the contrary, CPA failed to change ATP currents in some DRG neurons, and one possible explanation was that these DRG cells only express P2X3 receptors, but not A1 adenosine receptors. Therefore, we believed that CPA indirectly acted on P2X3 receptors, which only occurred in these DRG neurons co-expressing A1 adenosine receptors and P2X3 receptors, although the evidence of morphological co-existence remains to be elucidated.

The A1 adenosine receptor is coupled to G_{i/o} member of the G protein family, through which it can inhibit adenylyl cyclase activity and decrease intracellular cAMP levels [47–49]. The present data showed that CPA-induced inhibition of ATP currents was lacking after the DRG neurons were intracellularly diazied with the G_{i/o} protein inhibitor PTX, the adenylyl cyclase activator forskolin, or the cAMP analog 8Br-cAMP, indicating involvement of G_{i/o} proteins and intracellular cAMP signaling. Our recent studies have shown that CPA suppresses acid-sensing ion channels via A1 adenosine receptors and intracellular G_{i/o} proteins and cAMP signaling cascades in rat DRG neurons [50]. Consistent with current results, previous studies have shown that ATP-induced currents are modulated by intracellular cAMP-PKA signaling [51, 52]. Cannabinoids inhibit ATP-activated currents in rat trigeminal ganglionic neurons by activating
CB1 receptors and inhibiting the adenylate cyclase-cAMP-PKA signaling pathway [53]. Recent studies have reported that blockade of HCN channels inhibits the function of P2X2 and P2X3 receptors in rat DRG neurons via a cAMP-PKA signaling pathway [54]. Leu-enkephalin inhibits P2X3 currents in DRG neurons through G_{i/o} proteins, while it increases P2X3 currents via a PLC signaling pathway after pre-treatment of the neurons with a G_{i/o} protein inhibitor PTX [55]. The present and previous studies suggested P2X3 receptor was a downstream target of PTX-sensitive G_{i/o} proteins and intracellular cAMP signaling cascades.

P2X3 receptor is a cation-permeable channel. Once activated, it evokes an inward current, which is sufficient to result in membrane potential depolarization and even bursts of APs [56]. Under the current-clamp conditions, CPA also suppressed α,β-meATP-induced membrane excitability of rat DRG neurons, including APs and membrane potential depolarization. These two results corroborated each other in the current-clamp and voltage-clamp experiments. P2X3 receptors are expressed in peripheral nociceptive sensory nerve endings, along with the soma of DRG neurons [27, 28]. ATP causes pain by activating P2X3 receptors when injected into the skin [35]. Injection of α,β-meATP into a hind paw also evokes spontaneous nociceptive behaviors in rats, such as licking, biting, and lifting of the injected paw, which was significantly blocked by the antagonists, antisenses, and gene deletion of P2X3 receptors [31, 33, 35]. These data indicate that P2X3 receptors play an important role in the generation of pain at peripheral nerve endings. Within the periphery, A1 adenosine receptors are also localized on sensory nerve endings [3, 5]. The present results showed that peripheral pre-treatment of the CPA relieved the α,β-meATP-triggered nociceptive behaviors. The effects of CPA occurred locally by directly activating peripheral A1 adenosine receptors, since the anti-nociceptive effect of CPA was completely blocked by intraplantar administration of the A1 adenosine receptor antagonist KW-3902. These behavioral findings apparently confirmed the aforementioned electrophysiological results that CPA suppressed α,β-meATP-evoked ATP currents, membrane potential depolarization, and bursts of APs in DRG neurons through A1 adenosine receptors.

Purinergic signaling plays a well-established role in the processing of nociceptive information in different pain models. The same purine molecule can regulate pain through different purinoceptors, also through the interaction between purinoceptors. For example, ATP can activate ionotropic P2X3 and metabotropic P2Y receptors. Activation of P2Y receptors has been shown to reversibly inhibit inward currents mediated by P2X3 receptors in rat DRG neurons [37–39]. The present results that inhibition of P2X3 receptors by A1 adenosine receptor activation indicated that different purine molecules, such as adenosine and ATP, regulate pain not only through cognate A1 adenosine receptors and P2X3 receptors, respectively, but also through the interaction between the two different purinoceptors. Adenosine is mainly metabolized from ATP, and also is a precursor for ATP synthesis in vivo. Under inflammatory conditions, adenosine levels can reach up to 100 µM [48, 57]. A functional crosstalk between A1 adenosine receptors and P2X3 receptors would provide a homeostatic mechanism to prevent excessive ATP signaling through P2X3 receptors. Clinically, adenosine and its receptors represent a target for pharmacological treatment of pain. But A1 adenosine receptor is widely expressed in the central nervous system, the heart, and adipose tissue; its agonists may elicit dose-limiting side effects such as bradycardia [58]. The present results that suppression of P2X3 receptors by CPA in primary sensory neurons provided a novel peripheral mechanism for analgesics targeting peripheral A1 adenosine receptors. CPA can exert its analgesic effects by inhibiting periphery P2X3 receptors, indicating P2X3 receptor may be a therapeutic target for peripheral A1 adenosine receptor analgesia.

**Conclusions**

In summary, our findings indicated a functional link between two purine signaling, the A1 adenosine receptor and the P2X3 receptor. Activation of A1 adenosine receptors suppressed the electrophysiological activity of P2X3 receptors and also relieved α,β-meATP-induced nociceptive behaviors.

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**Author Contribution** WPH designed this research. JWH, WLQ, QL, SW, XML, TTL, and CYQ performed the experiments. JWH, WLQ, and QL participated in data analysis. JWH and WPH wrote the paper.

**Data Availability** All data generated during this study are included in this article or are available on reasonable request from the corresponding authors.

**Declarations**

**Ethics Approval** The animal study was reviewed and approved by the Animal Research Ethics Committee of Hubei University of Science and Technology (2016–03-005).

**Consent to Participate** Not applicable.

**Consent for Publication** All authors consent to publication of this manuscript.

**Competing Interests** The authors declare no competing interests.
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