A1 adenosine receptor activation inhibits P2X3 receptor-mediated ATP currents in rat dorsal root ganglion neurons

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Research Article

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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 N^6-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 of α,β-meATP, as shown a downward shift of its concentration-response curve. The CPA-induced suppression was independent on the clamping-voltageof the membrane. 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 $G_{i/o}$-protein inhibitor pertussis toxin, the adenylate cyclase activator forskolin, or the cAMP analog 8-Br-cAMP. Morover, CPA suppressed the membrane potential depolarization and action potential burst induced by α,β-meATP in DRG neurons. Finally, CPA relieved α,β-meATP-induced nociceptive behaviors in rats by activating peripheral A1 adenosine receptors in dose-dependent manner. These results indicated that CPA inhibited P2X3 receptor activity in rat primary sensory neurons by activating A1 adenosine receptors, $G_{i/o}$-proteins and intracellular cAMP signaling, revealing a novel peripheral mechanism underlying its analgesic effect.

1 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 an analgesic effect [2, 3]. Adenosine activates A1 adenosine receptors to alleviate pain behavior in a variety of pain models [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 pruduce analgesia via a local increase in adenosine and/or activation of 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 has been reported to contribute to neuropathic pain [19]. A1 adenosine receptor-deficient mice display a greater nociceptive response [20, 21]. Peripheral A1 adenosine receptors are involved in the analgesic effect of adenosine signaling. For example, A1 adenosine receptor agonists including adenosine can reduce nociceptive responses when they are injected into the hindpaws of animals [22, 23]. It has recently been reported that activation of peripheral adenosine A1 receptors significantly relieves glutamate-induced nociceptive behavior [24]. The contribution of peripheral A1 adenosine receptors to analgesia is further supported by the results the analgesic effects of acetaminophen and tramadol are blocked by local administration of selective A1 adenosine receptor antagonists [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 receptors, other purinergic receptors such as P2X3 receptors 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 pain behaviors to administration of ATP 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 receptor can be modulated by the activation of A1 adenosine receptors. The present study reports that the A1 adenosine receptor agonist N^6-cyclopentyladenosine (CPA) did not only inhibit the electrophysiological activity mediated by 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.

2 Materials And Methods

2.1 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 adding1.25 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%).

2.2 Electrophysiological recordings

Electrophysiological experiments were performed as described previously [40]. An EPC-10 patch clamp amplifier (HEKA Electronic, Lambrecht, Germany) were used for the whole-cell patch clamp recordings. The isolated DRG neurons weretransferred to a 35- mm culture dish and kept in normal external solution for at least 60 min before electrophysiological recordings. The external solution contained the following (in mM): 150 NaCl, 5 KCl, 2 $MgCl_2$, 2.5 $CaCl_2$, 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 micropipette solution contained the following (in mM): 140 KCl, 2 $MgCl_2$, 11 EGTA, 10 HEPES, 4 ATP, and 0.3Na_2GTP. 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 (15–40μm 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.

2.3 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 recording was performed at least 30 min after cell membrane rupture.

2.4 Nociceptive behavior induced by α,β-meATP in rats

Rats were first habituated for 30 minutes in a Plexiglas chamber during the nociceptive behavioral experiment. The rats in six different groups received intraplantar injections of 50μl vehicle, different doses (0.1, 1 and 10ng) of CPA, 30ng KW-3902 + 10ng CPA, respectively. After 10 min, another experimenter injected α,β-methylene-ATP (α,β-meATP, 50μg in 50μl) 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.

2.5 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 nonlinear curve-fitting program ALLFIT was used for statistical analysis of the concentration–response data.

3 Results

3.1 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 sec evoked a rapid inward currents (I_{ATP}) under holding potentials of − 60 mV conditions (Fig. 1A). The I_{ATP} was completely blocked by the specifical P2X3 or P2X2/3 receptor antagonist A-317491(100 μM), but not by the P2X4 receptor antagonist PSB-12062 (10μM) and the potent P2X7 receptor antagonist A438079 (1μM), indicating that the I_{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 5min prior to the next recording (Fig. 1B). The decrease of the I_{ATP} amplitude depended on the concentration of pre-treated CPA. The sequential current traces in Fig. 1C illustrate that a gradual decrease in the amplitude of I_{ATP} evoked by 100 μM α,β-meATP with an increase in CPA concentration from 3 nM to 300 nM in a representative DRG cell. Figure 1D shows the concentration-effect curve of CPA on 100 μM α,β-meATP evoked I_{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.

3.2 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 shifted downwards 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 hoc test). However, the Hill coefficients or the slopes were not significantly different between the two curves, which were 1.36 ± 0.31 and 1.28 ± 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 ± 3.06 μM and 27.42 ± 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 clamping potentials. 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 that 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 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.

3.3 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 receptors are 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 applied with it. The amplitude of I_{ATP} decreased from 1.36 ± 0.13 nA to 0.79 ± 0.09 nA in 8 DRG cells pre-treated with 100nM CPA alone for 5 min ($P < 0.01$, one-way ANOVA followed by post hoc Bonferroni's test). In contrast, the amplitude of I_{ATP} was 1.22 ± 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). 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_{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_{ATP} amplitude. Intracellular dialysis of forskolin (0.1 μM) or 8-Br-cAMP (1 mM) prevented the CPA-induced suppressio of I_{ATP}, the amplitude of I_{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 through a $G_{i/o}$ proteins and downstream cAMP signaling pathway.

3.4 CPA-induced suppression of α,β-meATP-evoked membrane excitability of rat DRG neurons

We further investigated the effect of CPA on membrane excitability triggered by α,β-meATP. In the same DRG cell, 10 μM α,β-meATP induced a large inward current, and burst of action potentials (APs) under voltage-clamp and current-clamp conditions, respectively (Fig. 4A and B). Consistent with the results under voltage-clamp conditions, pre-application of CPA (100 nM for 5 min) 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).

We then observed the effect of CPA on 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 membrane depolarization evoked by 100μM α,β-meATP. In 6 tested neurons, the magnitude of membrane depolarization (ΔVm) 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). These results indicate that CPA suppressed α,β-meATP-induced membrane excitability of rat DRG neurons.

3.5 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 the nociceptive behaviors induced by α,β-meATP *in vivo*. Figure 5A shows intraplantar injection of α,β-meATP (50μg in 50μl) 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 10ng) 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 5 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 addition, α,β-meATP-induced nociceptive behaviors were not affected if 10 ng CPA was injected into the contralateral paws (data not shown). The results suggest that CPA relieved the α,β-meATP-induced nociceptive behaviors *in vivo* through activating peripheral A1 adenosine receptors.

4 Discussion

The present data demonstrated that the A1 adenosine receptor agonist CPA suppressed the functional activity of peripheral P2X3 receptors. CPA decreased the amplitude of ATP currents and the membrane excitability induced by α,β-meATP 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 [28, 27]. 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 is only an activator of P2X3 and P2X1 receptors [41]. The present study showed that CPA concentration-dependently suppressed the recorded 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 regulated by activation of A1 adenosine receptors, which may be a potential mechanism underlying its anti-nociceptive 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 [19, 3, 5, 28, 27]. Although the evidence of morphological co-existence remains to be elucidated, it was possible that CPA decreased ATP currents in some DRG neurons, where A1 adenosine receptors and P2X3 receptors are co-locate in the same cells. On the contrary, CPA failed to change ATP currents in some DRG cells, one possible explanation was that the DRG neurons only express P2X3 receptors, but not A1 adenosine receptors.

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 lack after the DRG neurons were intracellularly dialyzed with the $G_{i/o}$ protein inhibitor PTX, the adenylate cyclase activator forskolin, or the cAMP analog 8Br-cAMP, indicating involvement of $G_{i/o}$-proteins and intracellular cAMP signaling. Our recent study has 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 blockage 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 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 burst 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 activates P2X3 receptors and causes pain 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 significantly blocked by the P2X3 receptor antagonists, P2X3 receptor antisenses, and P2X3 gene deletion [33, 35, 31]. These data indicate that P2X3 receptor plays an important role in generating 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 dose-dependently 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 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 ATP-evoked currents, membrane potential depolarization and bursts of APs in DRG neurons through A1 adenosine receptors.

Purinergic signaling play a well-established role in the processing of nociceptive sensory signals in different pain models. The same purine molecule can regulate pain by activating different purinoceptors, also through the interaction between activated purinoceptors. For example, ATP can activate ionotropic P2X3 and metabotropic P2Y2 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 study showed that different purine molecules, such as adenosine and ATP, regulate pain not only by activating cognate A1 adenosine receptors and P2X3 receptors, respectively, but also through the interaction between the two different purinoceptors. Adenosine is mainly metabolized from ATP, also is a precursor for ATP synthesis *in vivo*. Under inflammatory conditions, adenosine levels can reach up to 100 μM [57, 48]. 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 not only in the central nervous system but in 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 therapeutic target for peripheral A1 adenosine receptor analgesia.

5 Conclusions

In summary, our findings indicated a functional link between two purine signaling, A1 adenosine receptor and P2X3 receptor. Activation of A1 adenosine receptors suppressed P2X3-mediated the electrophysiological activity and pain. Adenosine/A1 adenosine receptor signaling exerts its peripheral analgesic effects by targeting P2X3 receptors.

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.

Availability of data and materials

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

Competing interests

All authors declare no conflicts of interest.

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Authors' contributions

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. All authors contributed substantially to this research and reviewed this manuscript.

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Figures

Figure 1

Inhibition P2X3 receptor-mediated ATP currents by CPA in DRG neurons

(A) In a representative DRG neuron, α,β-meATP (100 μM) or ATP (100 μM) can produce an inward current, which was completely blocked by the P2X3 and P2X2/3 receptor antagonist A-317491 (100μM), but not by the P2X4 receptor antagonist PSB-12062 (10μM) and the P2X7 receptor antagonist A438079 (1μM). 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 CPA, a selective A1 adenosine receptor agonist, (100 nM 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 CPA concentration increased from 3 nM to 300 nM in a representative DRG cell. (D) The graph shows that the concentration-effect curve of CPA on 100 μM α,β-meATP-induced currents (I_{ATP}) with an IC_{50} value of 39.40 ± 3.18 nM. Each point represents the mean ± S.E.M. of 7-9 cells.

Figure 2

Concentration–response and current–voltage (I–V) relationships for α,β-meATP in the absence and presence of CPA

(A) The sequential current traces illustrate pre-application of CPA (100 nM for 5 min) has inhibitory effects on three representative ATP currents evoked by 3, 30, and 300 μM α,β-meATP, respectively. (B) Concentration-response curves for α,β-meATP in the absence (●) and presence (●) of CPA. 100 nM CPA pre-treatment decreased the maximum response to α,β-meATP, as shown a downward shift of the concentration-response curve. Each point represents the mean ± S.E.M. of 7-9 cells. All current values from the same cell were normalized to the current response, which was induced by 300 μM α,β-meATP applied alone in the absence of CPA (marked with asterisk). (C) The sequential current traces illustrate that pre-application of CPA (100 nM for 5 min) decreased the peak amplitudes of 100 μM α,β-meATP-induced currents at three different clamped potentials (-80, -40 and +20 mV). (D) The I–V curves for 100 μM α,β-meATP-induced currents (I_{ATP}) in the absence (●) and presence (●) of CPA (100nM). All current values from the same cell were normalized to the current response induced by 100 μM α,β-meATP applied alone at the holding potential of −60 mV (marked with asterisk). Each point represents the mean ± S.E.M. of 7-9 cells. The experiment was carried out using recording pipettes filled with CsCl containing internal solution.

Figure 3

Participation of A1 adenosine receptors, $G_{i/o}$ proteins and cAMP signaling in the CPA-induced inhibition of ATP currents

Representative current traces in (A) and the bar graph in (B) show that 100 μM α,β-meATP-induced currents were inhibited by 100 nM CPA pre-applied alone for 5 min in DRG cells, and the CPA-induced inhibition was blocked by the co-application of the 300 nM KW-3902, an A1 adenosine receptor antagonist. *P < 0.05, **P < 0.01; Bonferroni's post hoc test, n = 8 in each column. Unlike a significant inhibition under normal internal solution conditions, the current traces in (C) and the bar graph in (D) show that CPA (100 nM) had little effect on I_{ATP} in recording pipettes filled with internal solution containing PTX (1 μg/ml), forskolin (0.1 μM) or 8-Br-cAMP (1 mM). **P < 0.01, Bonferroni's post hoc test, compared with normal column. n = 8 in each column.

Figure 4

CPA-induced suppression of α,β-meATP-evoked membrane excitability of rat DRG neurons

Original traces in (A) show that application of 100μM α,β-meATP to the same DRG cell caused an inward current and 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 (100nM for 5 min) significantly decreased the number of α,β-meATP-induced APs and membrane depolarization (ΔVm) in six DRG neurons.**P <0.01, paired *t*-test, n = 6 cells.

Figure 5

Relief of α,β-meATP-evoked nociceptive behaviorsby CPA in rats

Intraplantar injection of α,β-meATP (50μg in 50μl) caused spontaneous flinching behaviors in rats. Intraplantar pre-treatment of CPA (0.1, 1 and 10 ng in 50μl) dose-dependently decreased the number of α,β-meATP-induced flinching behaviors. The anti-nociceptive effect of CPA (10 ng) on the flinching behaviors was completely prevented by co-treatment of the A1 adenosine receptor antagonist KW-3902 (30 ng). Bonferroni's post hoc test, *P < 0.05, **P < 0.01. Each column represents the mean ± S.E.M. of 8 rats.

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