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ORIGINAL PAPER

CB_1 Receptors Mediated Inhibition of ATP-Induced $[Ca^{2+}]i$ Increase in Cultured Rat Spinal Dorsal Horn Neurons

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Abstract Spinal cannabinoid receptor 1 (CB_1R) and purinergic P2X receptors (P2XR) play a critical role in the process of pathological pain. Both CB_1R and P2XR are expressed in spinal dorsal horn (DH) neurons. It is not clear whether CB_1 receptor activation modulates the function of P2X receptor channels within dorsal horn. For this reason, we observed the effect of CP55940 (cannabinoid receptor agonist) on ATP-induced Ca^{2+} mobilization in cultured rat DH neurons. The changes of intracellular calcium concentration ($[Ca^{2+}]i$) were detected with confocal laser scanning microscopy using fluo-4/AM as a calcium fluorescent indicator. 100 μM ATP caused $[Ca^{2+}]i$ increase in cultured DH neurons. ATP-evoked $[Ca^{2+}]i$ increase in DH neurons was blocked by chelating extracellular Ca^{2+} and P2 purinoceptor antagonist PPADS. At the same time, ATP-γ-S (a non-hydrolyzable ATP analogue) mimicked the ATP action, while P2Y receptor agonist ADP failed to evoke $[Ca^{2+}]i$ increase in cultured DH neurons. These data suggest that ATP-induced $[Ca^{2+}]i$ elevation in cultured DH neurons is mediated by P2X receptor. Subsequently, we noticed that, in cultured rat DH neurons, ATP-induced Ca^{2+} mobilization was inhibited after pretreated with CP55940 with a concentration-dependent manner, which implies that the opening of P2X receptor channels are down-regulated by activation of cannabinoid receptor. The inhibitory effect of CP55940 on ATP-induced Ca^{2+} response was mimicked by ACEA (CB_1R agonist), but was not influenced by AM1241 (CB_2R agonist). Moreover, the inhibitory effect of CP55940 on ATP-induced Ca^{2+} mobilization was blocked by AM251 (CB_1 receptor antagonist), but was not influenced by AM630 (CB_2 receptor antagonist). In addition, we also observed that forskolin (an activator of adenylate cyclase) and 8-Br-cAMP (a cell-permeable cAMP analog) reversed the inhibitory effect of CP55940, respectively. In a summary, our observations raise a possibility that CB_1R rather than CB_2R can downregulate the opening of P2X receptor channels in DH neurons. The reduction of cAMP/PKA signaling is a key element in the inhibitory effect of CB_1R on P2X-channel-induced Ca^{2+} mobilization.

Keywords CB_1 receptors · ATP · P2X receptor · Spinal dorsal horn neurons

Introduction

The endocannabinoid system is composed of two main cannabinoid receptors (CB_1 and CB_2) and two main classes of endogenous ligands or endocannabinoids. It is reported that CB_1 and CB_2 receptor are abundantly expressed in the central nervous system, particularly in the cortex, basal ganglia, hippocampus, cerebellum and spinal cord [1, 2]. More and more experimental evidence show that endogenous cannabinoid mechanisms play an important regulatory role in the nociceptive information processing in various areas of the nervous system including the spinal dorsal horn. Farquhar-Smith et al. [3] found that CB_1R was strongly expressed in the superficial spinal dorsal horn, including primary afferents and spinal neurons. Immunocytochemical experiments show that more than 30% glutamatergic and approximately

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20% GABAergic spinal interneurons is endowed with CB_1R, respectively [4]. Intrathecal pretreatment with AM251 (CB_1 receptor antagonist) blocked Aβ-fiber stimulation induced-inhibition of the evoked excitatory postsynaptic currents in both excitatory and inhibitory substantia gelatinosa neurons in spinal nerve ligation rats, which implies that activation of CB_1R contributes to suppression of spinal nociceptive transmission [5].

On the other hand, more and more evidence suggest that activation of spinal P2X receptors (P2XR) is involved in the induction of nociceptive responses, mechanical hyperalgesia, and the excitation of sensory spinal neurons [6]. It is well known that the fast ionotropic effects are exerted through a family of P2X ATP-gated channels expressed in spinal cord. Presynaptic P2X receptors have been proposed to play a role in modulating glutamate release from the first sensory synapse in the spinal cord [7, 8]. Several ATP receptor subtypes including $P2X_{2,5,6}$ are expressed in spinal dorsal horn neurons [9, 10]. Jo et al. [11] also reported that about 50–60% of dorsal spinal cord neurons possess functional P2X receptors. Increasing evidence suggests that the activation of postsynaptic P2X receptors mediates excitatory transmission within the spinal cord. Patch clamp recording revealed that, in transverse neonatal rat spinal cord slices, ATP-activated excitatory postsynaptic currents are mediated by P2X receptors in a subpopulation of spinal cord lamina II neurons [6]. A further study found that neurons in the superficial layer revealed long-lasting enhancement of depolarization by ATP through P2X receptors during the slow repolarization phase at a single neuron level [10].

It is well known that some different kinds of protein kinases play an important role in the regulation of P2X receptor function. The ATP-induced $P2X_1$ and $P2X_3$ currents are potentiated by an activator of protein kinase C (PKC) [12–14]. Protein kinase A (PKA) has been suggested to play a role in the prostaglandin E2-induced potentiation of $P2X_3R$ currents in DRG neurons [15]. Our previous studies indicate that corticosterone rapidly inhibits P2X receptors-induced Ca^{2+} elevation and inward currents through activating PKA in DRG neurons [16, 17]. Han et al. [18] also reported that corticosterone modulated P2X receptor-medicated Ca^{2+} influx through a membrane-initiated, non-genomic and PKA-dependent pathway in HT4 cells. On the other hand, activation of the Gi/Go-protein-coupled CB_1 receptor can reduce PKA activity through reducing the activity of adenylate cyclase [19]. Based on these reports, we hypothesized that CB_1R activation could negatively regulate cAMP/PKA signaling and downregulate the opening of the P2X receptor channel in dorsal horn neurons. Therefore, in the present study, we examined whether P2X receptor-induced Ca^{2+} influx in cultured rat spinal dorsal horn neurons could be influenced after CB_1 receptor activation by using confocal laser scanning microscopy.

Materials and Method

Purification and Culture of Dorsal Spinal Cord Neurons

The technique for preparing primary cultures of spinal dorsal horn neurons has been described in detail elsewhere [20]. Briefly, the dorsal one-third of the spinal cord was cut into pieces and incubated in 0.125% trypsin (Sigma) for 25 min at 37 °C in a humidified 5% CO_2 −95% air atmosphere. The enzymatic digestion was stopped by adding 3 ml DMEM/F12 containing bovine serum albumin (1 mg/ml; Sigma) and DNase (0.01%; Sigma). The mechanical dissociation was performed by passing through a fire-polished glass pipette. After centrifugation (600 g min for 10 min), the supernatant was removed and replaced with culture medium containing Neurobasal medium (without phenol red and glutamate-free), glutamine (0.5 mM) and B27 (Life Technologies Inc., Gaithersburg, MD). The viability of the dissociated cells was determined by trypan blue exclusion (> 95%). The cells were plated onto sterile glass coverslips pre-coated with 10 μg/ml poly-d-lysine (Sigma) followed by 10 μg/ml Laminin-I (Sigma) and maintained in a 95% air and 5% CO_2 humidified incubator at 37 °C for 2–3 days. One day after the cells were seeded, cytosine arabinoside (5 μM) was added to the culture medium for 24 h to reduce glial proliferation. All experiments were performed on neurons after 2–3 days in vitro.

$[Ca^{2+}]i$ Measurement

$[Ca^{2+}]i$ measurement was carried out according to methods previously described [20, 21]. Changes of $[Ca^{2+}]i$ in neurons were detected with confocal laser scanning microscopy using fluo-4/AM (Dojindo, Kumamoto, Japan) as a calcium fluorescent indicator that could monitor real-time alterations of $[Ca^{2+}]i$. All fluorescence measurements were made from subconfluent areas of the dishes so that individual neurons could be readily identified. Prior to recording, neurons were loaded with the Ca^{2+} -sensitive fluorescent dye fluo-4/AM (2 μM) for 30 min in a 95% air and 5% CO_2 humidified incubator at 37 °C. Subsequently, the coverslips were thoroughly rinsed with D-Hanks medium lacking fluo-4/AM to remove extracellular traces of the dye and to complete de-esterification. Finally, the coverslips were mounted cell-side-up in the free bottom of the chamber and placed on the stage of the confocal microscope. The dye in the selected cytoplasmic part of cells was excited at wavelength 494 nm and fluorescence images were captured at 516 nm at 2 s intervals with an intensified charge-coupled device camera controlled by a computer. All fluorescence measurements were made at room temperature (20–22 °C). Image data were analyzed off-line. The change in $[Ca^{2+}]i$ was represented by relative

fluorescence intensity [F1/F0, %] (F0, control; F1, administration of drugs).

Drug Application

ATP, adenosine 5′-*O*-(3-thiotriphosphate) (ATP-γ-S), pyridoxal phosphate-6-azophenyl-2′,4′ -disulfonic acid (PPADS) and 8-Br-cAMP were made with 0.01 M PBS. CP55940, ACEA, AM1241, AM251, AM630, forskolin and Fluo-4/AM was prepared in 100% DMSO and then diluted to various concentrations with 0.01M PBS. Ca^{2+} -free solution was made up of 0.2 mM calcium with the addition of 1 mM EGTA (NP-ethyle-neglycoltetraacetic acid). All drugs were added directly to bath solutions. The presence of dimethyl sulfoxide (<0.1%) alone did not affect the Ca^{2+} fluorescence intensity. All chemicals except Fluo-4/AM were purchased from Sigma. Fluo-4/AM was purchased from Dojindo, Kumamoto, Japan.

Statistical Analysis

All data were presented as mean ± SD. Statistical analysis was performed with one-way analysis of variance (ANOVA) followed by LSD post hoc test when multiple comparisons were made (SPSS13.0, USA). IC50 was obtained by using GraphPad Prism 4 Software (USA). Differences at the $P < 0.05$ level were considered statistically significant.

Results

Involvement of P2X Receptor in ATP-Induced $[Ca^{2+}]$i Increase in Cultured Rat DH Neurons

We observed the effect of 100 μM ATP on $[Ca^{2+}]$i in cultured DH neurons by using confocal laser scanning microscopy. ATP at 100 μM caused $[Ca^{2+}]$i increase in approximately 50% of tested neurons. However, ATP can be rapidly degraded into ADP, AMP and adenosine by ectonucleotidases in the extracellular space [22]. It is not clear whether P2X or P2Y receptors are involved in Ca^{2+} mobilization induced by ATP. Then, ATP-γ-S (a non-hydrolyzable ATP analogue) and ADP (P2Y receptor agonist) were used [23]. We found that ATP-induced $[Ca^{2+}]$i increase was mimicked by exogenous ATP-γ-S. In a given neuron, the ATP and ATP-γ-S increased Ca^{2+} fluorescence intensity had similar traces (Fig. 1c, d). On the other hand, no detectable $[Ca^{2+}]$i increase was observed in neurons stimulated with ADP (Fig. 1c, d). Zeng et al. also reported that ADPbetaS (non-hydrolysable ADP analogue) had no effect on cultured DH neuronal Ca^{2+} fluorescence intensity [24]. In addition, pretreated with PPADS (the selective antagonist for P2X1, 2, 3, 5, 7 receptor) or chelating extracellular Ca^{2+} with EGTA eliminated ATP-induced $[Ca^{2+}]$i responses (Fig. 1a, b, d). It seems that P2X receptor activation is required for ATP-induced Ca^{2+} response.

The Effect of CP55940 on ATP-Induced $[Ca^{2+}]$i Increase in Cultured Rat DH Neurons

In order to identify whether the synthesized CB receptor agonist CP55940 regulates ATP-evoked Ca^{2+} response, cultured DH neurons were pretreated with CP55940 for 10 min prior to ATP application. CP55940 at 1 μM had no effect on basal Ca^{2+} fluorescence intensity in neurons at quiescence state. However, as shown in Fig. 2a, b, CP55940 at 1 μM notably decreased ATP-induced $[Ca^{2+}]$i increase in these neurons. We also noticed that the inhibitory action of CP55940 was concentration-dependent. CP55940 inhibited ATP-induced $[Ca^{2+}]$i increase to 98.20 ± 9.85, 90.17 ± 8.06, 63.37 ± 3.45, 53.40 ± 2.37% at 1, 10, 100 nM and 1 μM, respectively (Fig. 2c). The IC50 of the CP55940 effect was 35.7 nM.

CP55940 Modulates ATP-Induced $[Ca^{2+}]$i Increase via CB1 Receptor in Cultured DH Neurons

To find out which type of cannabinoid receptor is responsible for the effect of CP55940 on ATP-induced $[Ca^{2+}]$i increase, AM251 (selective CB_1R antagonist) and AM630 (selective CB_2R antagonist) was applied, respectively. According to previous studies, some other off-target effects may prevail if the concentration of CB receptor agonist is greater than 1 μM [25]. We noticed that AM251 and AM630 at 1–10 μM are frequently used in order to establish the involvement of cannabinoid receptor signalling in cellular responses [26, 27]. For this reason, in our experiments, cells were exposed to CP55940 (1 μM) with or without AM251 (10 μM) and AM630 (10 μM) pretreatment, respectively. In the presence of AM251 (10 μM for 10 min), the inhibitory effect of CP55940 on ATP-induced $[Ca^{2+}]$i elevation was abolished (Figs. 3a, 4). In contrast, the inhibition of CP55940 was not affected by pretreatment with AM630 (10 μM for 10 min) (Figs. 3b, 4). Moreover, the inhibitory action of CP55940 on ATP-induced Ca^{2+} response was mimicked by selective CB_1R agonist ACEA (3 μM for 10 min) (Figs. 3c, 4). Pretreatment with selective CB_2R agonist AM1241 (1 μM for 10 min) failed to inhibit ATP-induced Ca^{2+} response (Figs. 3d, 4). These observations suggest that the effect of CP55940 on ATP-induced Ca^{2+} response in cultured DH neurons is mediated by CB_1R rather than by CB_2R.

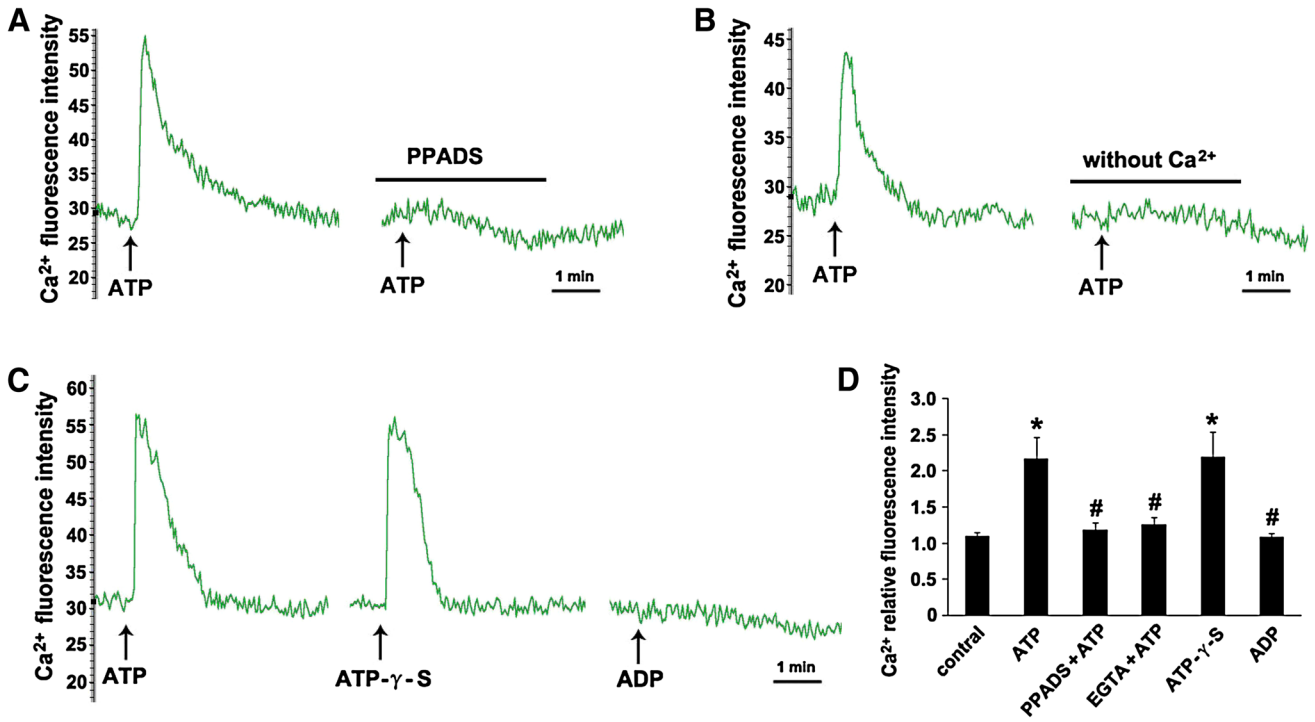

Fig. 1 The influence of Ca^{2+}-free solution and PPADS on ATP-induced Ca^{2+} response in cultured DH neurons. **a** The trace is from a single neuron and shows that 100 μM ATP increased $[Ca^{2+}]i$ in DH neurons, with features of a rapid rise after ATP application (arrow) followed by a slower recovery. Pretreatment with PPADS (50 μM for 10 min) significantly blocked the ATP-induced Ca^{2+} response. **b** Ca^{2+}-free solution eliminated $[Ca^{2+}]i$ increase response to ATP. **c** ATP-γ-S (100 μM), a non-hydrolyzable analog of ATP, mimicked the effect of ATP in DH neurons. In contrast, ADP (100 μM) stimulation failed to induce $[Ca^{2+}]i$ elevation in DH neurons. **d** A statistical analysis of the influence of Ca^{2+}-free solution and PPADS on ATP -induced $[Ca^{2+}]i$ increase. Data represent means obtained from 3 independent experiments, each including 20–30 cells. $^{*}P<0.01$, compared with control; $^{\#}P<0.01$ compared with ATP

Involvement of cAMP/PKA in the Inhibitory Effect of CP55940 on ATP-Activated $[Ca^{2+}]i$ Elevation

It is reported that binding of cannabinoids to the CB_1R suppressed the activity of adenylate cyclase through the intermediacy of the inhibitory Gi/o protein [19]. Subsequently, the fall of intracellular concentration of cAMP may then contribute to decreased PKA protein phosphorylation in many different types of cells [28–30]. To investigate a possible role of cAMP/PKA pathway in the effect of CP55940 on the ATP-activated $[Ca^{2+}]i$ increase, forskolin (an activator of adenylate cyclase, 10 μM) and 8-Br-cAMP (cell-permeable cAMP analog, 100 μM) were used. 10 μM forskolin completely eliminated the inhibitory effect of CP55940 on the ATP-evoked Ca^{2+} responses (Figs. 3e, 4). At the same time, 8-Br-cAMP also reversed the inhibitory effect of CP55940 (Figs. 3f, 4). These results largely confirm that the down-regulation of cAMP/PKA signaling may be involved in the inhibitory effect of CP55940 on ATP-evoked Ca^{2+} responses in cultured DH neurons.

Discussion

The extracellular mediator ATP, by activating ionotropic P2X and metabotropic P2Y receptors, participates in the generation and modulation of various forms of pain [31–33]. It has been postulated that ATP is released from the sensory neurons themselves or from damaged cells and activates various subtypes of P2X and P2Y receptors expressed along the nociceptive pathways [10, 34]. Extracellular ATP increases $[Ca^{2+}]i$ in many different types of cells through binding to purinergic P2X receptor ion channel or G protein-coupled P2Y receptor. P2X activation by ATP always results in rapid influx of extracellular Ca^{2+} [34]. P2Y receptor-induced $[Ca^{2+}]i$ increase is not dependent upon extracellular Ca^{2+} and may result from Ca^{2+} release from an internal store [35]. Agreement with these suggestions, we also found that ATP caused $[Ca^{2+}]i$ increase in approximately 50% of cultured rat DH neurons. Non-hydrolyzable ATP analogue ATP-γ-S mimicked the ATP action, while P2Y receptor agonist ADP had no effect on cultured DH neuronal $[Ca^{2+}]i$. ATP-evoked

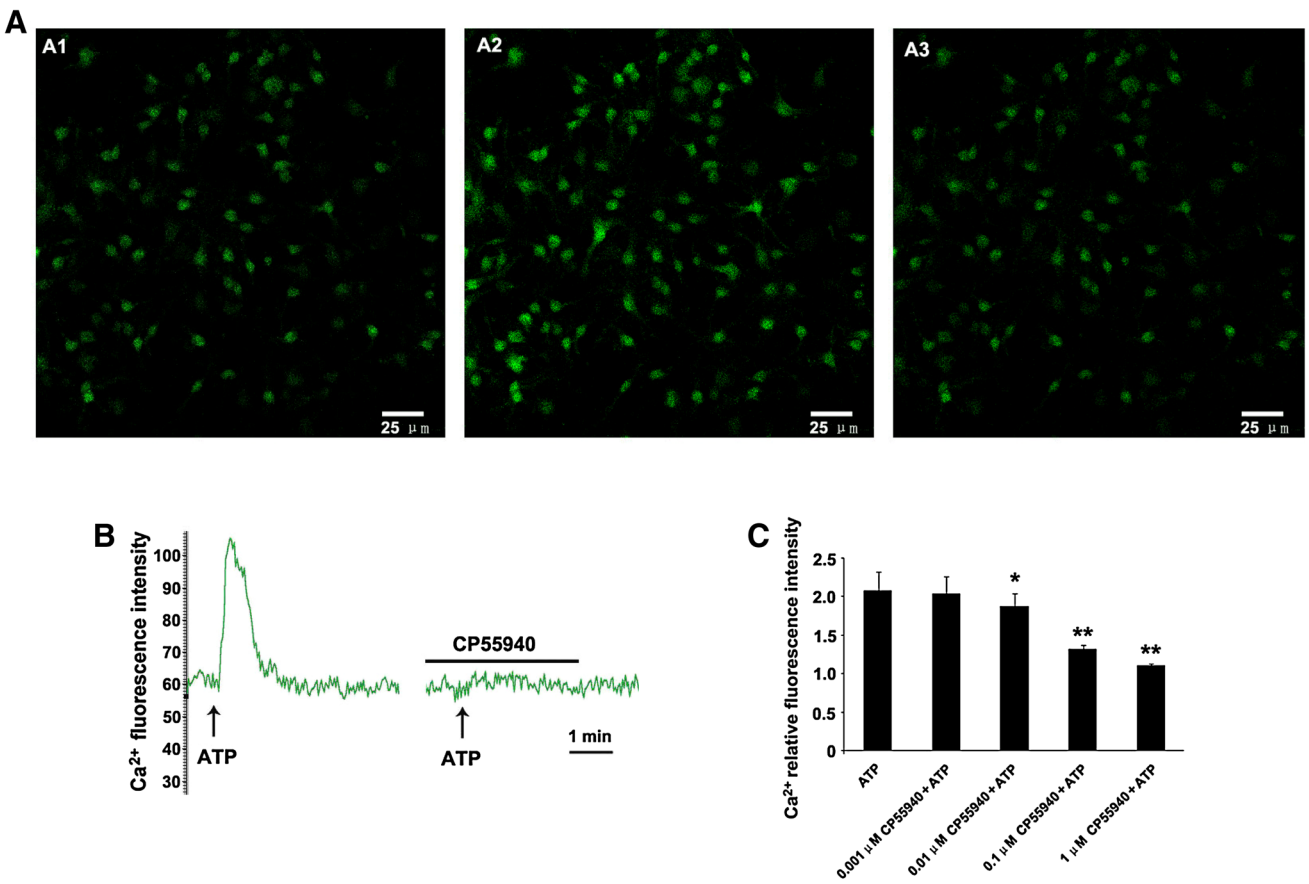

Fig. 2 Concentration-dependent inhibitory effect of CP55940 on ATP-induced Ca^{2+} response in cultured DH neurons. a Fluorescence images of Ca^{2+} response from cultured DH neurons. (*A1*) Control: basal Ca^{2+} fluorescence intensity in neurons; (*A2*) The effect of 100 μM ATP on neurons; (*A3*) The effect of 1 μM CP55940 on ATP-induced $[Ca^{2+}]$i increase in neurons. **b** The trace is from a single DH neuron and shows that CP55940 pretreatment (1 μM for 10 min) inhibited ATP-induced $[Ca^{2+}]$i increase. **c** CP55940 (0.001–1 μM) inhibited 100 μM ATP-stimulatory action on $[Ca^{2+}]$i in cultured DH neurons with a dose-dependent manner. $^{*}P<0.05$, $^{**}P<0.01$, compared with ATP

Ca^{2+} response in these neurons was also blocked by chelating extracellular Ca^{2+}. It appears that ATP-induced $[Ca^{2+}]$i elevation in cultured DH neurons is mediated by P2X receptor. In addition, previous studies suggest that P2Y receptor activation can evoke $[Ca^{2+}]$i increase in dorsal spinal cord astrocytes and microglia cells in vitro [36, 37]. We also noticed that nearly all cells exhibited Ca^{2+} mobilization was not stimulated by ADP, which also implies that dorsal spinal cord neurons can be isolated and cultured with high purity in the experiment. It was reported that $P2X_2$, $P2X_5$ and $P2X_6$ subunits are expressed in superficial dorsal spinal cord neurons. Among them, $P2X_2$ subunit is the most abundant in the dorsal horn neurons [9, 10]. Bardoni's study also shows that the P2X receptors on dorsal horn neurons are nondesensitizing, insensitive to α,β-methylene ATP and sensitivity to the antagonists suramin and PPADS (the selective antagonist for $P2X_{1,2,3,5,7}$), which also means that the composition of P2X receptors [6]. In our experiments, PPADS largely blocked the ATP-evoked Ca^{2+} responses, which presumes that ATP-induced $[Ca^{2+}]$i elevation in cultured DH neurons is possibly mediated by P2X receptors. Agreement with our suggestion, some previous studies also reported that ATP excites a subset of DH neurons in culture [6, 38] and in spinal cord slices [6, 10, 39] through activation P2X receptors.

Recently, increasing evidence suggests that the dorsal spinal cord is an important site contributing to CB_1 and CB_2 receptor-mediated analgesia [5, 40, 41, 43]. We noticed that ATP-induced Ca^{2+} mobilization in cultured DH neurons was inhibited after pretreated with CP55940 with a concentration-dependent manner, which implies that the opening of P2X-channels is down-regulated by the activation of cannabinoid receptors. It is clear that CB_1 receptors are expressed in spinal dorsal horn neurons [3–5]. In addition, Burston et al. found that the CB_2 receptor expression was increased in spinal dorsal horn neurons and microglia cells in a rat osteoarthritis model [40]. However, Romero-Sandoval et al. reported that The CB_2R was localized to microglia and perivascular cells at the rat spinal dorsal horn

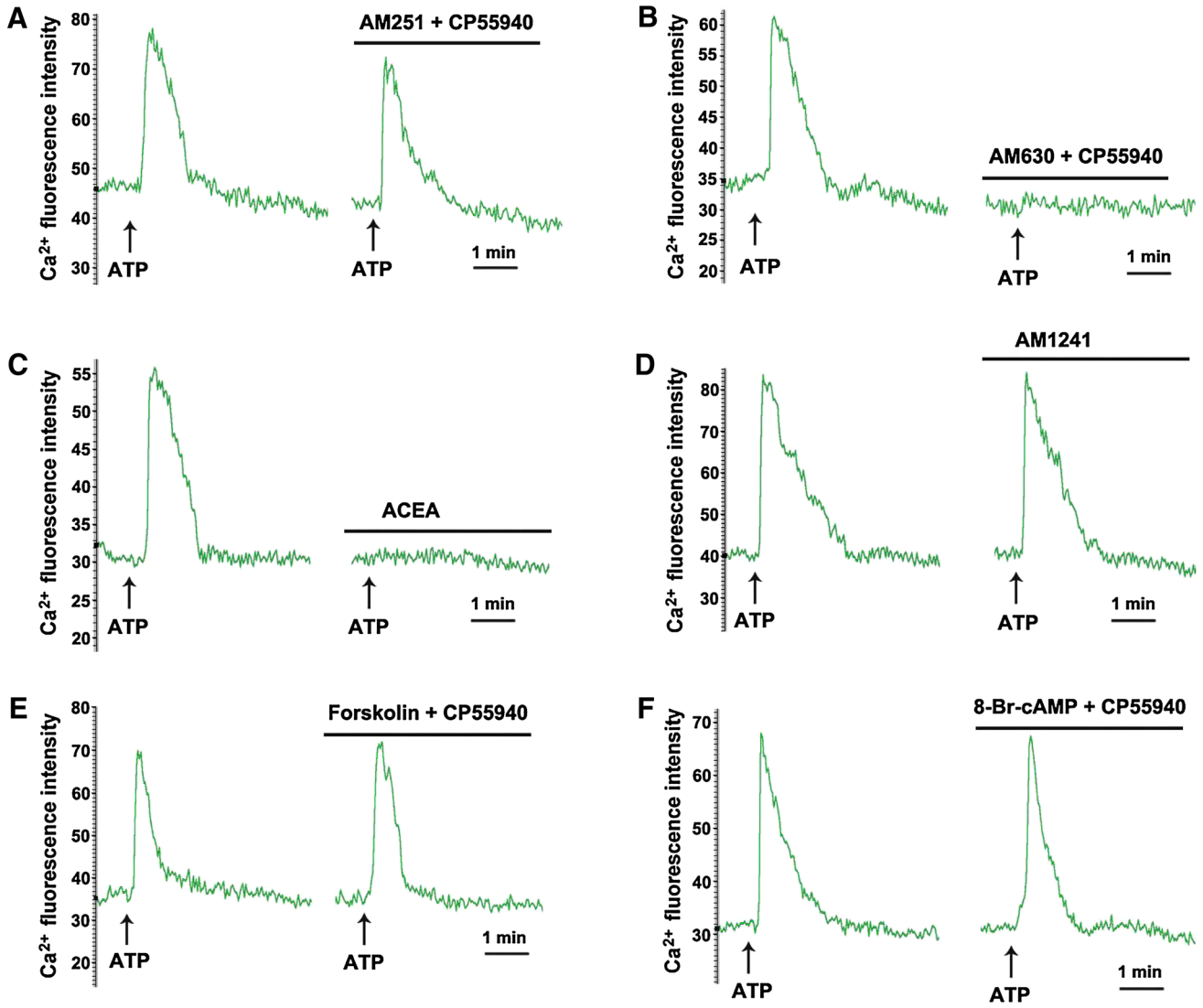

Fig. 3 CP55940 inhibited ATP-induced $[Ca^{2+}]i$ increase via CB_1 receptor in cultured DH neurons. **a** The trace is from a single DH neuron and shows that CB_1R antagonist AM251 (10 μM for 10 min) treatment blocked the inhibitory effect of CP55940. **b** The trace from another DH neuron and shows that CB_2R antagonist AM630 (10 μM for 10 min) treatment fails to block the inhibitory effect of CP55940. **c** The trace shows that CB_1R agonist ACEA pretreatment (3 μM for 10 min) inhibited ATP-induced $[Ca^{2+}]i$ increase. **d** The trace shows that CB_2R agonist AM1241 pretreatment (1 μM for 10 min) failed to inhibit ATP-induced $[Ca^{2+}]i$ increase. **e** The trace shows that forskolin (10 μM, co-incubated with CP55940) completely eliminated the inhibitory effect of CP55940. **f** The trace shows that 8-Br-cAMP (100 μM, co-incubated with CP55940) markedly suppressed the inhibitory effect of CP55940

[41]. In this experiment, the inhibitory action of CP55940 was mediated by CB_1 receptors since pretreatment with the CB_1 receptor antagonist AM251 blocked this effect, while the CB_2 receptor antagonist AM630 was ineffective. Earlier data indicate that activation of the CB_1 receptors inhibit the slow response to ATP mediated by $P2X_2$ and $P2X_{2/3}$ receptors in primary sensory neurons [42]. To the further study, CB_2R in rat spinal dorsal horn have inhibitory effects in neuropathic, but not sham-operated rats [43]. Moreover, in cultured spinal neurons, CB_2 receptor is not involved in AEA (endocannabinoid anandamide)-induced potentiation of Gly receptor-mediated responses [44]. WIN55212-2 suppressed heat-evoked activity and decreased the receptive field areas of isolated rat dorsal horn neurons. Pretreatment with the cannabinoid CB_1 receptor antagonists SR141716A or AM251, but not the CB_2 antagonist SR144528, blocked the effects [45]. These observations support the notion that, at the spinal cord level, CB_2 receptor does not participate in regulation of neuronal function under physiological conditions. In agreement with these suggestions, we also found that, in cultured rat DH neurons, CP55940's inhibition on ATP-induced Ca^{2+} response was mimicked by CB_1R agonist

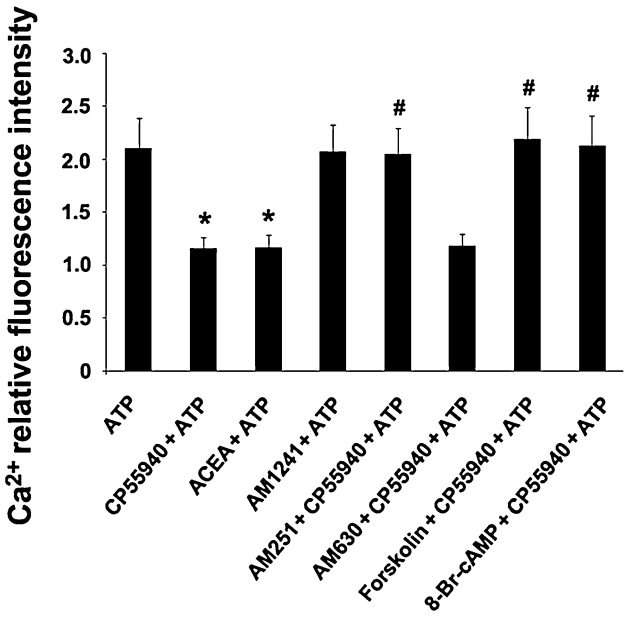

Fig. 4 The pharmacological profile of the effect of CP55940 on ATP-induced Ca^{2+} response in cultured DH neurons. CP55940 (1 μM) inhibited ATP-induced Ca^{2+} elevation in cultured DH neurons. The effect of CP55940 on ATP-induced Ca^{2+} mobilization was mimicked by CB_1R agonist ACEA, but not by CB_2R agonist AM1241. In addition, the inhibitory effect of CP55940 on ATP-induced Ca^{2+} mobilization was blocked by AM251 (CB_1 receptor antagonist), but was not influenced by AM630 (CB_2 receptor antagonist). Forskolin (an activator of adenylate cyclase) and 8-Br-cAMP (a cell-permeable cAMP analog) reversed the inhibitory effect of CP55940, respectively. Data represent means obtained from 3 independent experiments, each including 20–30 cells. $^{*}P < 0.01$, compare with ATP; $^{\#}P < 0.01$, compare with CP55940 +ATP

ACEA, but not by CB_2R agonist AM1241. It appears that the inhibitory effect mainly mediated through CB_1R rather than CB_2R.

As a member of the seven transmembrane domain G-protein-coupled receptors family, CB_1R can mediate its effects via activation or inhibition of adenylate cyclase [19, 46]. Inhibition of adenylate cyclase plays an important role in several aspects of cannabinoid function. For example, activation of CB_1R can reduce the activity of transient receptor potential vanilloid type 1 in rat cultured primary sensory neurons through inhibiting cAMP/PKA activity [47]. Cannabinoid receptor agonist WIN55212-2, which binds to the CB_1R, inhibits ATP-activated currents in rat trigeminal ganglionic neurons via inhibition of the AC-cAMP-PKA signaling pathway [48]. In the present study, the addition of adenylate cyclase activator and cAMP analog reversed the inhibition of CP55940 on ATP-induced Ca^{2+} mobilization in cultured DH neurons. It is reasonable to deduce that the corresponding intracellular signal transduction pathway is as follows: CP55940, after binding to the CB_1 receptors, activates Gi which inhibits the activity of AC and results in the reduction of cAMP levels and PKA activity. The inhibition of CP55940 on ATP-induced Ca^{2+} mobilization may result from decreased phosphorylation of P2X receptor by CB_1R-induced reduction of PKA activity.

P2X receptors have been shown to be regulated by both PKA and PKC activation, which suggests that they might be substrates for phosphorylation [49]. Chow et al. report that the intracellular carboxyl terminus of $P2X_2$ receptor contains several consensus phosphorylation sites for cAMP-dependent PKA, suggesting that the function of the $P2X_2$ purinoceptor could be regulated by the protein phosphorylation [50]. It was reported that CB_1R activation inhibited forskolin-stimulated cAMP accumulation in spinal locomotor networks and hippocampal slices [51, 52]. In the present study, in cultured dorsal spinal cord neurons, CB_1R activation significantly suppressed P2X receptor-evoked Ca^{2+} mobilization through down-regulating cAMP/PKA signaling pathway, which also strongly supports the notion that cAMP/PKA is an important downstream targets for the CB_1 receptor signaling. In addition, we suspect that those neurotransmitter receptors contain several consensus phosphorylation sites for cAMP-dependent PKA can be modulated by CB_1 receptor through the tuning down of PKA activity. For example, Roscioni et al. found that the PKA activator 6-Bnz-cAMP and the Epac activator 8-pCPT-2′-O-Me-cAMP significantly increased bradykinin-induced IL-8 release, which means that bradykinin receptor may have phosphorylation sites for cAMP-dependent PKA [53]. Moreover, ACEA (selective CB1 receptor agonist) reduced the mechanical hyperalgesia induced by bradykinin [54]. In the present study, CB_1 receptor activation can effectively suppress ATP-induced $[Ca^{2+}]i$ increase mediated by P2X receptor in cultured dorsal spinal cord neurons. The inhibition of CP55940 on ATP-induced Ca^{2+} mobilization may result from decreased phosphorylation of P2X receptor by CB_1R-induced reduction of PKA activity. In additon, Walter et al. report that ATP-induced 2-Arachidonoylglycerol (2-AG, an endogenous agonist of the cannabinoid receptor) production is mainly regulated by $P2X_7$ receptor in cultured mouse astrocytes [55]. Witting et al. suggest that ionotropic, and not metabotropic, purinergic receptors control 2-AG production in cultured microglial cells [56]. In the present study, activation of CB_1 receptor can effectively suppress ionotropic P2X receptor-induced $[Ca^{2+}]i$ increase in cultured dorsal spinal cord neurons. Furthermore, in the primary afferent neurons, the analgesic effect of CB_1 receptor activation is mediated by a negative modulation of the $P2X_3$ receptor [54]. Based on these reports, we hypothesized that whether CB_1R-mediated reduction in P2XR function is a feed-back mechanism to down-regulate 2-AG release in the CNS. It looks likely that P2XR signaling may lead to 2-AG production, which in turn may decrease the neuronal excitability and synaptic strength under pathological

circumstances. However, the next important question, whether glial-derived 2-AG decreases neuronal excitability and synaptic strength under pathological circumstances in situ, awaits resolution.

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