

The agonists for nociceptors are ubiquitous, but the modulators are specific: P2X receptors in the sensory neurons are modulated by cannabinoids

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Received: 23 March 2006 / Accepted: 18 April 2006 / Published online: 2 June 2006
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Abstract P2X2 and P2X3 receptors expressed in mammalian sensory neurons participate in nociception. Cannabinoid receptors modulate nociceptive processing in various models of pain. They are also expressed in nociceptive sensory neurons. We have examined the effect of cannabinoids on the slow P2X2 and P2X2/3 receptors in the cells isolated from nodosal and dorsal root ganglia of rat. The study was carried out by means of the whole-cell patch clamp and rapid superfusion methods. We have found that both endogenous and synthetic cannabinoids (anandamide, WIN55,212-2, and (*R*)-(+)-methanandamide) inhibit the slow response to ATP mediated by P2X2 and P2X2/3 receptors in a majority of tested neurons. This inhibition was significant but only partial: anandamide (0.5–1 μ M) inhibited the response to 51 \pm 21% of control. In the remaining minority of tested neurons, the response was transiently facilitated. The effect of cannabinoids appears to be mediated via cannabinoid CB₁ receptors: it was reversibly inhibited by selective CB₁ antagonist, SR141716A (10 μ M). Introduction of cyclic AMP (0.5 mM) into the cell potentially facilitated the inhibitory effect of cannabinoids: the ATP-activated current was inhibited to 13 \pm 10% of control. These data indicate that cannabinoids may inhibit nociceptive responses produced by P2X receptors.

Keywords Cannabinoids · P2X receptors · ATP · Pain

Introduction

There is one more anniversary “hidden” in the shadow of patch-clamp glory: 25 years have passed since the discovery of the receptor for protons in mammalian sensory neurons [22]. The early hypothesis that these receptors may participate in nociception [23] has been confirmed in numerous publications and now seems to be settled in the paradigm. First cloning of these receptors [45] has resulted in identification of a family of acid-sensing ionic channels (ASICs) with some of them specific for mammalian sensory system [25]. Initially, it was hard to imagine that the ligand as ubiquitous and simple as proton could be specific for gating decently selective Na⁺ channels. However, the search for some other, possibly more specific, ligands for these channels (in the course of which the extracts of different tissues were applied to the sensory neurons) resulted in a discovery of one more mechanism employing almost as ubiquitous agonist, ATP. ATP-activated conductance was found in sensory neurons [24]. While purinergic mechanisms were already known in smooth muscles [1], this was the first indication on their existence in the nervous tissue. The cloning of ATP-gated ionic channels [42] has resulted in the identification of a family of P2X receptors/channels. Two of them, P2X2 and P2X3, extensively expressed in sensory neurons, are also important players in the processes of nociception [2].

It seems understandable that natural selection has picked up ubiquitous ligands to sense pain. The nature of metabolism is such that its disruptions lead to the tissue acidification in the overwhelming majority of cases (ischemia, inflammation, etc.), while the interstitial level of ATP is subjected to great variations both in normal function (like muscle work) and in numerous pathological states. Together with vanilloid receptors gated by the factor

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“most ubiquitous of all”, temperature, ASICs, and P2X receptors comprise a triad of major nociceptors.

It seemed natural to search whether some of the receptors involved are subjected to specific modulation by “global” antinociceptive systems. Recently, we have found that P2X receptors in sensory neurons are subjected to opioid control [7]. In this study, we report that cannabinoid receptors also modulate P2X receptors in the sensory neurons.

Out of the seven P2X receptor/channel subunits identified up to date, P2X2 and P2X3 are expressed in nociceptive sensory neurons and form functional ionic channels in their membrane [4, 26]. Mounting evidence indicates that these ATP-activated conductance mechanisms are involved in primary nociception. ATP released as a result of tissue injury, visceral distension, or sympathetic activation [3, 6] excites nociceptive primary sensory afferents by activating homomeric and heteromeric combinations of P2X3 and P2X2 receptors [14, 19]. Double knockout of these subunits leads to a total disappearance of ATP-activated conductance in the sensory neurons [9].

Experiments with KO animals provide the evidence for a direct physiological role of homomeric and heteromeric P2X receptors expressed in nociceptive neurons. P2X3 receptor null-mutant mice respond normally to acute noxious, thermal, and mechanical stimuli but display attenuated responses to nonnoxious “warming” stimuli and show reduced formalin-induced nocifensive behaviors [8, 40]. Furthermore, in the models of neuropathic and inflammatory pain, inhibition of development of mechanical hyperalgesia, as well as significant reversal of established hyperalgesia, was observed after inhibition of P2X3 receptors with antisense oligonucleotides [8]. Both P2X2^{-/-} and P2X2/P2X3(Dbl^{-/-}) mice had reduced pain-related behaviors in response to intraplantar injection of formalin. P2X3^{-/-}, P2X2^{-/-}, and P2X2/P2X3(Dbl^{-/-}) mice had reduced urinary bladder reflexes and decreased pelvic afferent nerve activity in response to bladder distension [9].

Cannabinoid receptors are present in many sensory neurons [36]. Their activation inhibits pain responses. Site-specific administration of agonists and antagonists suggests that both CB₁ and CB₂ cannabinoid receptors inhibit pain responses by acting at peripheral sites [27]. Cannabinoid agonists attenuate depolarization-dependent calcium influx in primary afferent neurons of adult rat [21]. However, the molecular targets responsible for the antinociceptive effects of peripherally applied cannabinoids are still unclear. In this study, we show that cannabinoids inhibit the activity of slow ATP-activated current in the sensory neurons isolated from nodosal and dorsal root ganglia (DRG) of rat. This effect is mediated by the activity of CB₁ cannabinoid receptors.

Materials and methods

The neurons were isolated from nodosal and DRG of 5–10 days old rats. Isolation procedure is described elsewhere [34]. The experiments were performed on the cells after 24–48 h in a primary culture, after they were attached to the surface. The majority of data have been obtained in the experiments on nodose neurons due to much more frequent occurrence in these neurons of slow ATP-induced current (mediated by P2X2/3 and P2X2 receptors).

Desensitization properties of P2X receptors imply the necessity of rapid removal of agonist after the onset of response. For this purpose, we used rapid “square-pulse” application techniques combining two separate U-tubes [24] targeted at the surface-attached cell. The rate of solution change was 40–60 ms. Rapidly terminating agonist applications (100–200 ms long) ensured sufficiently long recording of the ATP-elicited responses without their significant run-down.

Electrophysiological experiments were conducted at room temperature (20–22°C) using the whole-cell patch-clamp technique. The patch pipettes with resistance of 3–9 MΩ were made from Harvard borosilicate glass tubing (1.4/1.6 mm outer diameter, 0.8/1.0 mm boron with 0.15 mm fiber attached to the inside wall). The Axopatch 200A amplifier (Axon Instruments) was used.

The patch pipettes were filled with the solution containing (in millimolars) 120 KCl, 10 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), 10 ethylene glycol bis(2-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid, 0.3 GTP, and 5 MgATP, pH 7.2. The extracellular solution contained (in millimolars) 120 NaCl, 2 CaCl₂, 5 KCl, 10 glucose, and 10 HEPES, pH 7.4. After entering the whole-cell recording configuration, the neurons were allowed to equilibrate for 2–3 min before measurements were started. In the experiments with intracellular administration of cyclic AMP (cAMP), the measurements of ATP-activated current were started in less than 1 min after breaking into the cell. Stock solution of anandamide (10 mM) was prepared in 100% DMSO. Solutions of WIN 55,212-2 and SR141716A were prepared daily from a 10-mM stock solution in ethanol diluted with extracellular bathing medium. All chemicals except SR141716A was purchased from Tocris; SR141716A was purchased from SRI International. Data were captured and stored on a computer hard drive using DigiData 1200 interface. The data analysis was performed off-line using the Origin software (version 6.0). The data were presented as the mean±SD; statistical significance was determined using a paired or independent Student's *t* test.

Results

Effect of cannabinoids on the ATP-activated current

Nodose neurons express robust slowly desensitizing component of ATP-activated current mediated by P2X2 and P2X2/3 receptors [15, 34]. This evidence has been recently decisively confirmed in the experiments with double KO animals [9]. In this study, we have concentrated on the effects of cannabinoids on these receptors: the major part of evidence has been collected from the experiments with nodose neurons.

The use of square-pulse application technique (the agonist is removed in 1 s after its application) allows avoiding desensitization of slow ATP-activated current. In these conditions, the

response to ATP can be repeatedly evoked (typically every 30 s) for up to 1 h without essential run-down of its amplitude. This procedure ensures total lack of fast P2X3 receptor-mediated component (especially prominent in DRG neurons) because it needs more than 15 min to be restored after initial desensitization [35].

We have found that extracellular application of cannabinoid agonists (2-AG, WIN 55,212-2, or anandamide) to a nodose neuron leads to the inhibition (Fig. 1a) or transient potentiation (Fig. 1b) of ATP-activated currents.

About 15% out of 54 tested nodose neurons responded to the cannabinoid agonists with transient potentiation, while in the great majority of cases we observed the inhibitory effect.

The time course of inhibitory action of anandamide is demonstrated in Fig. 1a. The current reached a new steady-

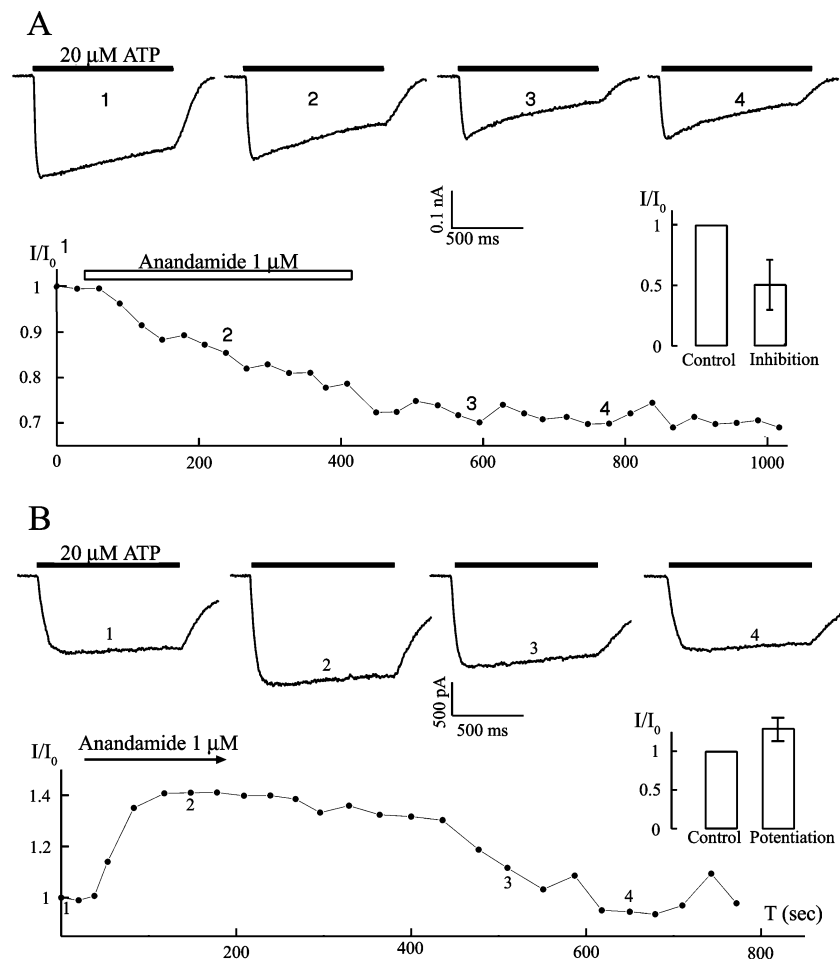


Fig. 1 Endogenous cannabinoid agonist, anandamide (AEA), modulates the activity of P2X receptors. **a** Application of anandamide (1 μ M) inhibits the response of nodosal neuron to ATP. The effect developed within 5 min, the current was inhibited by 50%. The inhibition is almost irreversible. The numbers below current traces (upper graph) correspond to the timing indicated in the low graph.

This type of response to cannabinoid is typical: it has been recorded in 36 out of 50 tested neurons. Holding voltage, -100 mV. **b** Facilitation for 30% has been found in nine neurons. This effect was transient: anandamide was present in the superfusion saline during all the duration of the measurements, while the current declined to a control level after a phase of facilitation. Holding voltage, -100 mV

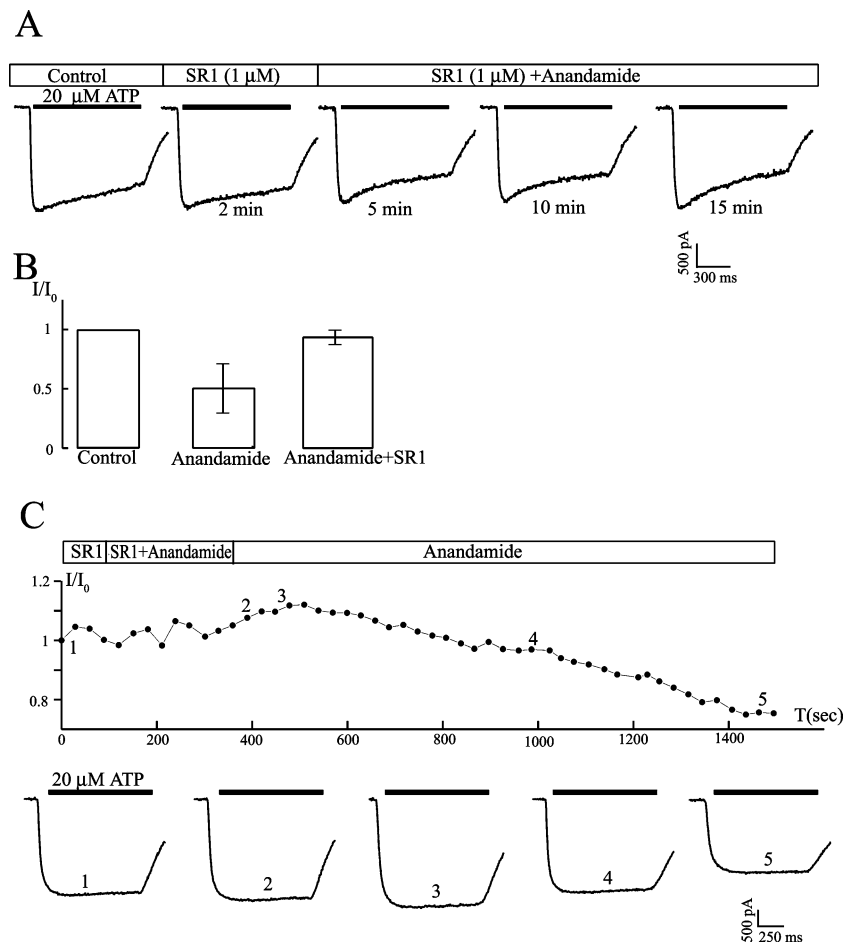


Fig. 2 Effects of anandamide are antagonized by CB₁ cannabinoid receptor blocker. **a** Anandamide fails to produce any effect when applied in the presence of CB₁ receptor blocker, SR141716A (later referred as SR1), 1 μM. The *numbers* below current traces correspond to the timing of application of SR1 and SR1 + anandamide. Holding voltage, -90 mV. **b** Mean data: The maximum current amplitude under anandamide + SR1 is 0.87 ($n=4$, $p>0.2$) of the control value, as

compared to a twofold inhibition by anandamide. **c** Anandamide exerts its inhibitory action after SR1 is removed. Slow time course of this inhibition may be attributed to the slow washout of hydrophobic substance (SR1) from the membrane. The *numbers* below current traces (*low graph*) correspond to the timing indicated in the *upper graph*. Holding voltage, -90 mV

state level within 4–6 min and did not show any further change for the next 5–20 min. The mean inhibition of the peak current by 0.5–1 μM of anandamide was 0.5 ± 0.21 ($p < 0.001$, $n=12$) (see the inset in Fig. 1a). In five tested DRG neurons demonstrating slow response to ATP, anandamide (1 μM) inhibited this current for 0.46 ± 0.06 ($p < 0.001$, $n=3$).

Other cannabinoid receptor agonists (2-AG and WIN 55,212-2) demonstrated similar inhibitory effect (0.46 ± 0.14 , $p < 0.001$, $n=7$ for 1 μM of 2-AG and 0.54 ± 0.25 , $p < 0.001$, $n=6$ for 1 μM of WIN 55,212-2). Washout of cannabinoids demonstrated only a slight tendency for a recovery of ATP-activated current (for about 5% within 5–10 min after removal of the drugs). It should be noted that the probability of run-down in the course of these experiments was absolutely minimized by the control experiments in which

the current remained stable (within 10–15%) for 15–30 min of repetitive activation of P2X receptors (see also Fig. 4).

Figure 1b demonstrates a rare example of transient potentiation of ATP-activated currents by anandamide. The amplitude of current was increased within 2–3 min, remained stable for 3–4 min, and slowly returned to initial level within further 7–10 min (Fig. 1b, low panel). The mean facilitation of the peak current was 1.3 ± 0.15 ($p < 0.01$, $n=4$) for 1 μM of anandamide (see the inset in Fig. 1b). Other cannabinoid receptor agonists (2-AG and WIN 55,212-2) caused similar facilitation of the ATP-activated currents (1.32 ± 0.17 , $p < 0.01$, $n=3$ for 2-AG and 1.28 ± 0.25 , $p < 0.01$, $n=2$ for WIN 55,212-2). Both effects of cannabinoids were not accompanied by any noticeable changes in the kinetics of desensitization or activation of ATP-activated currents.

Cannabinoids modulate ATP-induced current via CB₁ receptor

Two cannabinoid receptors have been characterized in mammalian tissues: CB₁ [28] and CB₂ [31]. CB₁ receptor is distributed mainly in the central nervous system and is also present in the peripheral nerve terminals, while CB₂ receptor occurs in certain nonneuronal tissues, particularly in immune cells. We have used selective CB₁ cannabinoid receptor antagonist SR141716A [38] to find out which type of cannabinoid receptor is responsible for the effect of anandamide on the ATP-induced current. Figure 2a (upper panel) demonstrates that the amplitude of ATP-activated current is not affected by anandamide when the latter is coapplied with SR141716A. The peak inward current measured on the background of anandamide and SR141716A was $0.87 \pm 0.11\%$ ($p < 0.014$, $n = 4$) of the control value, as compared with a twofold inhibition by the sole anandamide (Fig. 2b). The control experiment presented in Fig. 2c demonstrates that anandamide reveals its inhibitory action after the washout of SR141716A, though the time course of this inhibition is significantly slower (Fig. 2c). The latter observation can be attributed to the slow washout of strongly hydrophobic SR141716A from the membrane.

Similar effect of the antagonist was observed with other cannabinoid receptor agonists (WIN 55,212-2, $n = 2$ and 2AG, $n = 2$). Thus, SR141716A prevents the inhibition of ATP-induced currents by anandamide.

(*R*)-(+)-methanandamide modulates ATP-activated currents in nodose and DRG neurons

Endogenous cannabinoid agonist anandamide is transported into the cells via a specific mechanism [32]. Once within the cell, anandamide is hydrolyzed to arachidonic acid and ethanolamine by the microsomal enzyme, fatty acid amide hydrolase (FAAH) [13, 41]. The question arises whether the observed effects are due solely to the interaction with CB₁ receptor or comprise also the effects of intracellular metabolites [11].

(*R*)-(+)-methanandamide is a metabolically stable anandamide analogue which is not processed by FAAH, and thus does not decay to arachidonic acid and ethanolamine [33].

We have found that (*R*)-(+)-methanandamide inhibits ATP-induced currents in nodose (Fig. 3a) and DRG neurons. As compared to anandamide, its effect was stronger in nodose neurons: inhibition of the peak inward current after application of (*R*)-(+)-methanandamide (1 μ M)

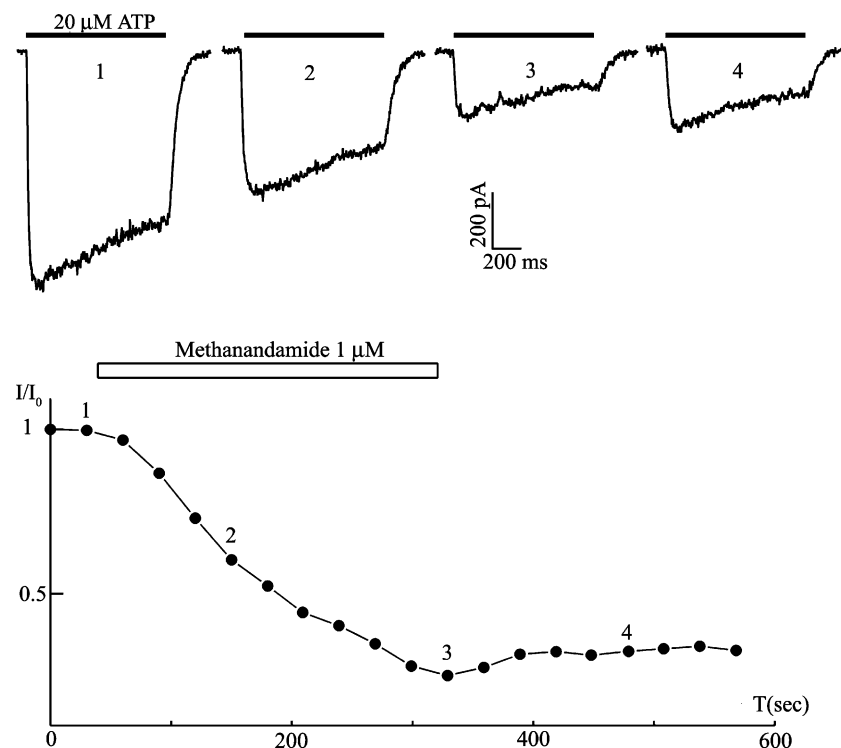


Fig. 3 Nonhydrolyzable cannabinoid agonist, (*R*)-(+)-methanandamide, modulates the activity of P2X receptors. Application of (*R*)-(+)-methanandamide (1 μ M) inhibits the response of nodosal neuron to ATP. The effect developed within several minutes, the current was

inhibited by 75%. The inhibition is almost irreversible. The numbers below current traces (*upper graph*) correspond to the timing indicated in the *low graph*. Holding voltage, -100 mV

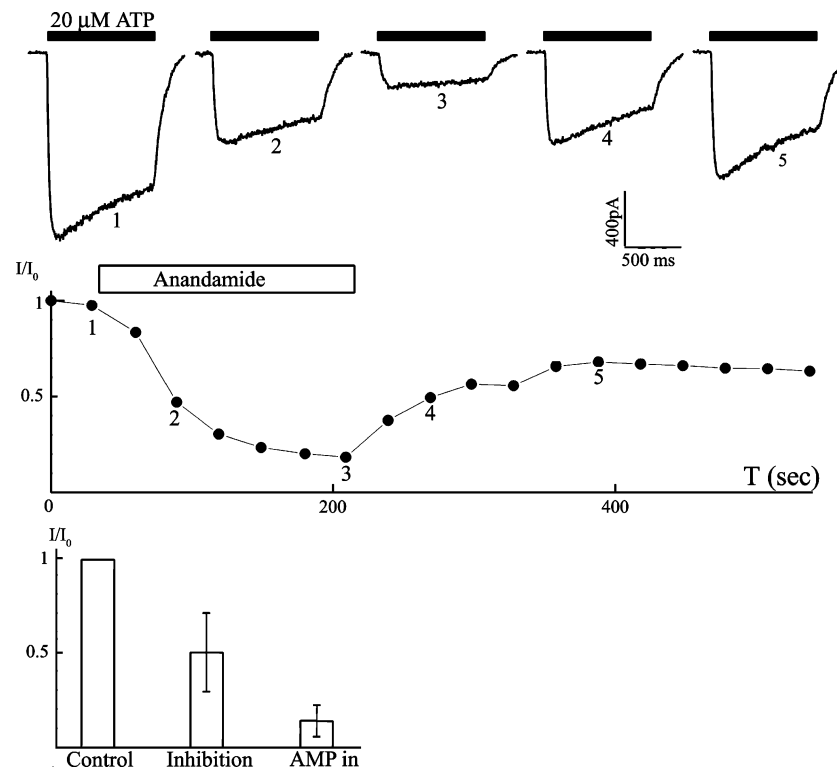


Fig. 4 Intracellular cAMP facilitates the effect of extracellular cannabinoid. Anandamide has been applied to the cell loaded with cAMP (0.5 mM). The current demonstrates rapid decline for almost 80% and also rapid (though partial) recovery. The numbers below

current traces (*upper graph*) correspond to the timing indicated in the *low graph*. Holding voltage, -90 mV. Cumulative data are presented at the *lower graph*

was 0.19 ± 0.17 ($p < 0.01$, $n = 3$). In DRG neurons, (*R*)-(+)-methanandamide inhibited ATP-induced current with approximately the same efficiency as anandamide (0.4 ± 0.1 , $p < 0.01$, $n = 3$).

Intracellular cAMP facilitates the inhibitory effect of cannabinoids on the ATP-activated currents

CB₁ is a G-protein-coupled receptor. Its activity includes ligand-induced decrease in the cAMP production inside the cells due to the inhibition of adenylyl cyclase [20]. To investigate a possible role of cAMP in the effect of cannabinoids on the ATP-activated current, this substance was added to the intracellular solution in supposedly saturating concentration of 0.5 mM. In 60% of the tested cells ($n = 11$), cAMP dramatically facilitated the effect of cannabinoids on the ATP-activated current. Figure 4 demonstrates that application of anandamide to the cAMP-loaded cell leads to much stronger inhibition of ATP-activated current (down to 0.13 ± 0.1 , $p < 0.001$, $n = 7$). This effect was more readily reversible: the washout of anandamide from extracellular solution led to a rapid (within 2–3 min) and significant (for about 50%) recovery of ATP-activated current.

SR141716A was equally effective in the cAMP-loaded cells (data not shown).

Discussion

In our recent study on rat sensory neurons, it was found that the agonists of μ -opioid receptors affect P2X receptors via G-protein-dependent mechanisms [7]. In this study, we demonstrate that the ATP-activated current mediated by P2X2 and P2X2/3 receptors is also modulated by both endogenous (anandamide and 2-AG) and synthetic (WIN 55,212-2) cannabinoids. These data are in concert with the mounting evidence indicating that opioid and cannabinoid receptors share a multiplicity of effects at biochemical and behavioral levels [12, 44].

In the case of peripheral P2X receptors, the effect of opioids is biphasic in the majority of neurons with a phase of transient potentiation followed by the irreversible inhibition [7]. However, when G_i/G_o proteins were inactivated by pertussis-like toxin [17], the current was only potentiated like it was found in a minority of the cells in the presence of cannabinoids. It should be noted that potentiation of current by cannabinoids cannot be due to the

increase in the affinity of P2X receptors to ATP because this effect was observed at the saturating concentration of this agonist.

The experiments with cannabinoid receptor antagonist SR141716A allow to suggest that cannabinoids affect slow responses to ATP via CB₁ receptors (Fig. 2a,b). In principle, these data cannot exclude a possibility that the products of anandamide catabolism (arachidonic acid and ethanolamine) directly affect P2X receptors because SR141716A also reduces FAAH-independent intracellular uptake of anandamide [32]. However, because nonhydrolyzable analogue of anandamide, (*R*)-(+)-methanandamide, inhibited ATP-activated currents more effectively than anandamide (Fig. 3), we may conclude that the observed inhibition is mediated by CB₁ receptor.

CB₁ receptor, a member of the seven transmembrane domain G-protein-coupled receptors family, can mediate its effects via activation or inhibition of adenylyl cyclase depending on adenylyl cyclase isoform type [16, 18, 29]. Inhibition of adenylyl cyclase plays an important role in several aspects of cannabinoid function including modulating conductances of voltage-dependent K⁺ and Ca⁺⁺ channels [5]. We have found that CB₁ receptors are especially effective in inhibiting P2X receptors activity when the changes in adenylyl cyclase activity are buffered by a high level of intracellular cAMP. This suggests that cAMP promotes operational link between CB₁ and P2X receptors, which is mediated by some still unspecified mechanism [20]. Intracellular carboxyl terminus of P2X receptor contains several consensus phosphorylation sites for cAMP-dependent protein kinase and protein kinase C, suggesting that the function of the P2X purinoceptor could be regulated by the protein phosphorylation [43]. Neuroprotective effect of cannabinoids can be mediated by cAMP-independent phosphatidylinositol 3-kinase (PI3-K) signaling pathway [30]. Thus, cannabinoid agonists may trigger a series of both cAMP-dependent and cAMP-independent reactions within the cells, including activation of PKA and PI3-K. The cAMP dependence of inhibitory action of anandamide on ATP-activated currents (Fig. 4) can be due to the phosphorylation of P2X2 and P2X2/3 receptors by PKA (or some other cAMP-dependent process), while the inhibitory action itself is mediated by other kinase(s). Anyway, the mechanisms underlying the interaction(s) between CB₁ and P2X receptors still have to be elucidated.

Our data indicate that endogenous cannabinoids play an important role in primary nociception processes. They are in concert with earlier data indicating that CB₁ antagonist SR141716A completely blocks all of the pain-relieving effects of THC and related cannabinoids in various animal models of pain [10, 39]. Furthermore, animals receiving an intrathecal injection of SR141716A demonstrated significant thermal hyperalgesia [37].

The existence of cannabinoid modulatory system affecting peripheral nociceptors adds a new opportunity for the development of blood–brain impermeable cannabinoid agonists.

Acknowledgements This work has been supported by CRDF and HHMI.

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