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Short communication

Metabotropic $P2Y_1$ receptors inhibit $P2X_3$ receptor-channels in rat dorsal root ganglion neurons

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Abstract

Whole-cell patch-clamp recordings from cultured rat dorsal root ganglion neurons demonstrated that the $P2Y_1$ receptor agonists adenosine 5′-O-2-thiodiphosphate (ADP-β-S) and 2-methylthio adenosine 5′-diphosphate (2-MeSADP) inhibit the α,β-methylene adenosine 5′-triphosphate (α,β-meATP)-induced $P2X_3$ receptor-currents. This effect could be antagonized by the wide-spectrum G protein blocker GDP-β-S and the $P2Y_1$ receptor antagonist MRS 2179. The $P2Y_{12,13}$ receptor antagonist AR-C6993MX and pertussis toxin, a blocker of $G\alpha_{i/o}$, did not interact with the effect of ADP-β-S. Hence, the results indicate that ADP-sensitive $P2Y_1$ receptors of rat dorsal root ganglion neurons inhibit ionotropic $P2X_3$ receptors via G protein-activation.

Keywords: Pain; Sensory neuron; Electrophysiology

1. Introduction

Extracellular adenosine 5′-triphosphate (ATP) is co-released with classic neurotransmitters and functions as a neurotransmitter through the activation of ionotropic P2X and metabotropic P2Y receptors (Illes and Ribeiro, 2004). Currently, seven ionotropic P2X ($P2X_{1-7}$) and eight G protein-coupled P2Y receptors ($P2Y_{1,2,4,6,11,12,13,14}$) have been characterized in humans (Burnstock, 2004). The $P2X_3$ receptors are selectively expressed at high levels in small-diameter nociceptive neurons of the dorsal root ganglion (Chen et al., 1995) and activation of these receptors mediates the noxious effects of ATP (Chizh and Illes, 2001; Kennedy et al., 2003). However, several types of metabotropic P2Y receptors have also been found in primary sensory neurons and recent data indicated the additional involvement of P2Y receptors in pain transmission (Gerevich and Illes, 2004). The adenosine 5′-diphosphate (ADP) analog ADP-β-S was reported to cause analgesia in the tail-flick test when applied intrathecally to rats (Gerevich et al., 2004). The possible mechanism of action of ADP-β-S is the inhibition of N-type voltage-sensitive calcium channels in dorsal root ganglion cells through $P2Y_1$ receptors, an effect which may depress the release of glutamate from the central terminals of these neurons and subsequently inhibit pain transmission from the primary sensory neuron to the spinal cord cells (Borvendeg et al., 2003; Gerevich et al., 2004).

Here, we present an additional explanation for the analgesic effect of ADP. We found that activation of the ADP-sensitive $P2Y_1$ receptors, G protein-dependently, inhibits the ionotropic $P2X_3$ receptor-mediated currents in rat dorsal root ganglion neurons. Hence, ADP, produced by enzymatic degradation of extracellular ATP and acting at G protein-coupled $P2Y_1$ receptors is able to inhibit the noxious effects of ATP mediated by $P2X_3$ receptors.

2. Materials and methods

2.1. Preparation of dorsal root ganglion neuronal cultures

One-day-old Wistar rats were killed by CO_2 and decapitated to obtain cell cultures of dorsal root ganglion

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neurons. All of the animal use procedures were approved by the Committee of Animal Care and Use of the relevant local governmental body in accordance with the law of experimental animal protection. The isolation and culturing procedures of thoracic and lumbar dorsal root ganglion cells have been described in detail previously (Himmel et al., 2002).

2.2. Whole-cell patch-clamp recordings in dorsal root ganglion neurons

Membrane currents were recorded at a holding potential of −70 mV using the whole-cell configuration of the patch-clamp technique by means of an Axopatch 200B amplifier (Axon Instruments, Foster City, CA, U.S.A.). Patch pipettes (3–5 MΩ) were filled with intracellular solution of the following composition (mM): 135 CsCl, 2 $MgCl_2$, 20 HEPES, 11 EGTA, 1 $CaCl_2$, 1.5 Mg-ATP, and 0.3 Li-GTP, pH 7.3 adjusted with CsOH. When indicated, GTP was substituted with guanosine 5′-O-(2-thiodiphosphate) (GDP-β-S) (0.3 mM). In some experiments, activated pertussis toxin (1 μg/ml) was applied intracellularly via the patch pipette. We have shown recently that this procedure functionally uncouples $P2Y_{13}$ receptors from transfected rabbit N-type Ca^{2+} channels in HEK293 cells (Wirkner et al., 2004).

The external recording solution consisted of (in mM) 140 NaCl, 5 KCl, 2 $MgCl_2$, 2 $CaCl_2$, 10 HEPES, and 11 glucose, pH 7.4 adjusted with NaOH. To block the firing of cells during agonist application, tetrodotoxin (0.5 μM) or lidocaine *N*-ethyl bromide (2 mM) was given to the extracellular and intracellular solutions, respectively. A pressure-operated, computer-controlled, rapid drug application device (DAD-12; Adams and List, Westbury, NY, U.S.A.) was used for drug administration. Concentration-response curves of α,β-meATP (0.3-100 μM) and adenosine 5′-O-2-thiodiphosphate (ADP-β-S; 3-300 μM) to induce inward currents were constructed by applying increasing concentrations of the respective agonists for 1 s every 5 min onto single dorsal root ganglion cells. In subsequent experiments, α,β-meATP at 10 μM was applied 6-times again for 1 s and with 5-min intervals. After the recording of two α,β-meATP-induced inward currents, ADP-β-S or 2-methylthio adenosine 5′-diphosphate (2-MeSADP) were applied to selectively stimulate the P2Y receptors for 7.5 min, starting 2.5 min after the second α,β-meATP administration. Two α,β-meATP currents were recorded during and two further ones after the application of the P2Y receptor agonists. Data are presented as percentage±S.E.M. of the amplitude of the second α,β-meATP current immediately before starting the application of P2Y receptor agonists (reference value). To estimate the effects of ADP-β-S and 2-MeSADP on the $P2X_3$ receptor currents, the forth α,β-meATP current was compared to the reference value and expressed as a percentage value. Concentration-response curves were fitted to Hill's three-parameter sigmoidal equation and IC_{50} values were determined by non-linear regression analysis using the Sigma Plot 7.0 software (SPSS, Erkrath, Germany). The parameters obtained were I_{max}, the maximal current at infinite agonist concentrations; n, the Hill coefficient; and EC_{50}, the concentration of agonist producing 50% of I_{max}.

2.3. Drugs

The drugs used were: N^6-(2-methylthioethyl)-(3,3,3-trifluoropropylthio)-β,γ-dichloromethylene-ATP (AR-C6993MX; cangrelor) (Jayne Prats, The Medicines Company, Waltham, MA, U.S.A.); α,β-methylene adenosine 5′-triphosphate (α,β-meATP), 2-methylthio adenosine 5′-diphosphate (2-MeSADP), adenosine 5′-O-2-thiodiphosphate (ADP-β-S), pertussis toxin, GDP-β-S, dithiothreitol, β-nicotinamide adenine dinucleotide (NAD) (Sigma, Deisenhofen, Germany); 2′deoxy-N^6-methyladenosine-3′,5′-diphosphate (MRS 2179) (Tocris, Bristol, UK).

2.4. Statistics

Multiple comparisons with the control value were performed by one-way analysis of variance (ANOVA) followed by the parametric Bonferroni's *t*-test. A probability level of 0.05 or less was considered to be statistically significant.

3. Results

The $P2X_{1,3}$ receptor agonist α,β-meATP evoked rapidly desensitizing currents in cultured small-diameter (20–35 μm) dorsal root ganglion neurons (Fig. 1A). Then, concentration-response curves were constructed for inward currents caused by α,β-meATP (0.3–100 μM) and ADP-β-S (3–300 μM). The relevant parameters calculated were for α,β-meATP (I_{max}=727.3±41.6 pA; Hill coefficient=2.0±3.9; EC_{50}=4.5±6.0 μM; DF=5), and for ADP-β-S (I_{max}=178.4±0.8 pA; Hill coefficient=4.2±0.5; EC_{50}=33.1±0.4 μM; DF=5). Hence, ADP-β-S induced current responses at higher concentrations and with a lower potency than α,β-meATP. It is noteworthy that the threshold concentration of ADP-β-S was at 30 μM.

In all subsequent experiments we applied every 5 min a constant, submaximal concentration of α,β-meATP (10 μM); the peak amplitude measured was 472.2±34.6 pA (n=102). The responses to α,β-meATP were reproducible, indicating that complete recovery from receptor desensitization occurred during an interval of 5 min. To determine whether ADP-sensitive, G protein-coupled P2Y receptors can modulate the α,β-meATP currents, we superfused the $P2Y_{1,12,13}$ receptor agonists ADP-β-S (0.03–10 μM) and 2-MeSADP (0.01–3 μM) (Fig. 1A). These concentrations of the P2Y receptor agonists did not evoke any inward currents. Both agonists significantly and concentration-dependently

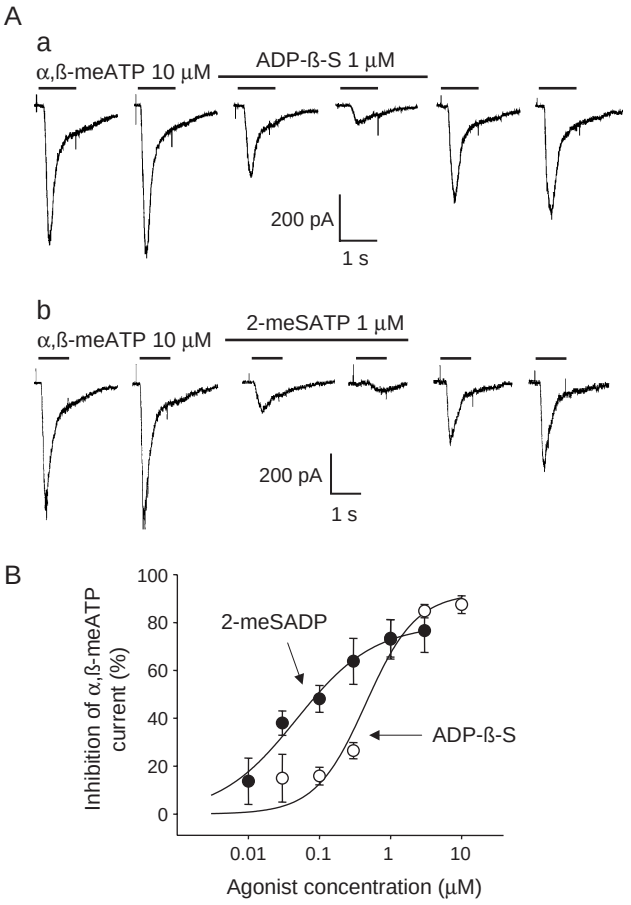

Fig. 1. Inhibition of α,β-meATP-evoked currents by ADP-β-S and 2-MeSADP in dorsal root ganglion neurons. (A) Representative recordings from two different dorsal root ganglion neurons. Drug applications are shown by horizontal bars. The $P2X_3$ receptor agonist α,β-meATP (10 μM) was applied for 1 s at 5 min intervals. The $P2Y_1$ agonists ADP-β-S (Aa) and 2-MeSADP (Ab) were applied for 7.5 min, starting 2.5 min after the second α,β-meATP current. (B) Concentration-response relationships for the inhibition of the α,β-meATP-evoked current by 2-MeSADP and ADP-β-S; the fourth response to α,β-meATP in the presence of the P2Y receptor agonists was expressed as a percentage of the second response to α,β-meATP. Each point represents the mean±S.E.M. of 4–7 experiments.

inhibited the α,β-meATP currents, although 2-MeSADP was approximately 10-times more potent than ADP-β-S (Fig 1B). The IC_{50} values for ADP-β-S and 2-MeSADP were 450.3±166.5 and 47.7±13.6 nM ($DF=5$), respectively. The inhibitory effects of both agonists developed slowly, with an apparent maximum 7.5 min after the beginning of their application (Figs. 1A, B and 2A).

In order to clarify the mechanism of the P2Y/P2X receptor interaction, ADP-β-S was applied at a submaximal inhibitory concentration of 1 μM. When GDP-β-S (300 μM) known to block all G protein-mediated reactions replaced the normal GTP in the pipette solution (Sternweis and Pang, 1990), ADP-β-S no longer depressed the α,β-meATP response (Fig. 2). Then, we tried to find out which G protein α-subunit is involved, and therefore applied activated pertussis toxin (1 μg/ml), a blocker of $G\alpha_{i/o}$ into the intracellular solution. Since pertussis toxin did not interfere with the ADP-β-S effect, the participation of $G_{i/o}$ is unlikely (Fig. 2).

To define the P2Y receptor subtype activated by ADP-β-S, various P2Y receptor antagonists were applied 5 min before the first α,β-meATP-induced current and were maintained until the end of the experiment. The $P2Y_1$ receptor selective antagonist MRS 2179 (10 μM; von Kügelgen and Wetter, 2000) prevented the effect of ADP-β-S (Fig. 2), suggesting that the inhibition was mediated by the $P2Y_1$ receptor subtype. In contrast, AR-C69931 MX (1 μM), a selective antagonist at $P2Y_{12,13}$ receptors did not interfere with the effect of ADP-β-S. Hence, it can be concluded that of the ADP-sensitive P2Y receptors ($P2Y_{1,12,13}$) only the $P2Y_1$ subtype mediates the inhibition of $P2X_3$ receptors.

4. Discussion

The present experiments demonstrate that agonists at ADP-sensitive P2Y receptor-subtypes ($P2Y_{1,12,13}$) inhibit

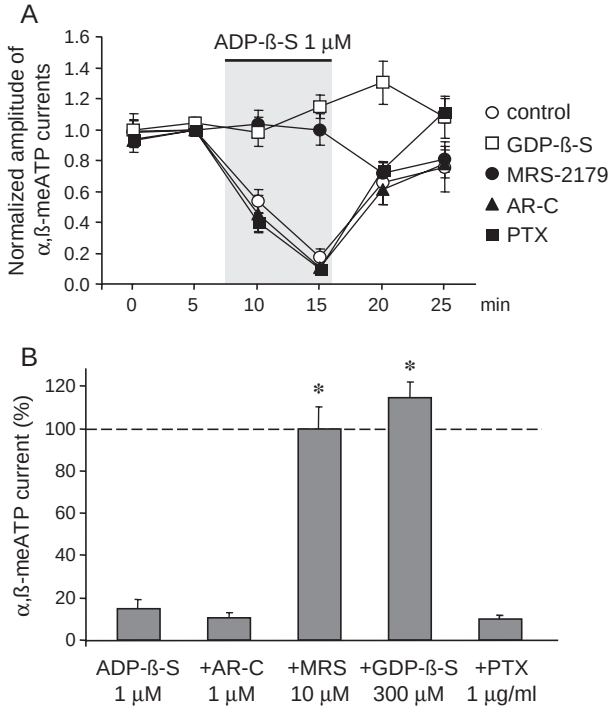

Fig. 2. Effects of P2Y receptor antagonists and G protein blockers on the ADP-β-S-induced inhibition of α,β-meATP currents in dorsal root ganglion neurons. (A) Peak α,β-meATP current amplitudes normalized with respect to the second response of each individual experiment. Data are mean±S.E.M. of 4–9 experiments. ADP-β-S was applied for the period indicated by the horizontal bar and the grey panel. GDP-β-S and pertussis toxin (PTX) were included into the patch pipette. MRS 2179 and AR-C6993MX were applied extracellularly, 5 min before the first α,β-meATP current and were maintained until the end of the experiment. (B) Summary data. Means±S.E.M. of the forth α,β-meATP currents in a series, expressed as a percentage of the second responses. *$P<0.05$; statistically significant difference from single ADP-β-S treatment.

$P2X_3$ receptor-mediated currents of dorsal root ganglion neurons. We used α,β-meATP to stimulate homomeric $P2X_3$ receptors of rat dorsal root ganglion cells; this agonist also activates $P2X_1$ receptors, which have, however, no functional significance in these neurons (Chizh and Illes, 2001). A direct blockade or a desensitization of $P2X_3$ receptors by ADP-β-S or 2-MeSADP as only reasons of the depression of α,β-meATP currents (Pratt et al., 2005) could be excluded by two pieces of evidence. First, the ADP-β-S-induced inhibition was counteracted by the highly selective $P2Y_1$ receptor antagonist MRS 2179 (but not the $P2Y_{12,13}$ receptor-selective antagonist AR-C69931 MX; Barnard and Simon, 2001); and second, GDP-β-S, a blocker of G protein-mediated reactions also abolished the inhibitory effect of ADP-β-S.

Hence, an interaction between the $P2Y_1$ and $P2X_3$ receptors of dorsal root ganglion neurons appear to be the most likely explanation for the depression of the α,β-meATP current by ADP-β-S (and 2-MeSADP). Ligand-gated cationic ($P2X_2$/nicotinic acetylcholine; Searl et al., 1998; Khakh et al., 2000; $P2X_2$/serotonin (5-hydroxytryptamine; $5\text{-}HT_3$) Barajas-Lopez et al., 2002) and anionic channels ($P2X_3$/γ-amino butyric acid ($GABA_A$) Sokolova et al., 2001) were repeatedly shown to negatively interact with each other. In addition, P2X receptors were also shown to cross-talk with G protein-coupled metabotropic receptors ($P2X_3$/tachykinin NK_1, Paukert et al., 2001; $P2X_2$/$GABA_C$, Boue-Grabot et al., 2004a). The most frequent mechanism of such interactions is via diverse protein kinases which are activated by one of the receptors and then, phosphorylate and thereby alter the conductance of the other receptor. More recently a direct protein–protein cross-talk has also been demonstrated, which can lead to a decreased affinity towards agonists (Puntambekar et al., 2004), a decrease of ionic conductance (Boue-Grabot et al., 2004a), or an altered expression of receptors in the plasma membrane due to changes in receptor trafficking (Boue-Grabot et al., 2004b).

With respect to the present $P2Y_1$/$P2X_3$ receptor interaction, the second-messenger mechanisms coupled to the $P2Y_1$ receptor appear to be initiated by $G\alpha_q$, since pertussis toxin which selectively ADP-ribosylates $G\alpha_{i/o}$ (Zamponi and Snutch, 1998), was ineffective. In view of the fact that intracellular GDP-β-S, which was described to block all types of G protein-mediated reactions, abolished the depression of α,β-meATP-induced currents by ADP-β-S, $G\alpha_q$ rather than $G_{i/o}$ seems to be involved. This assumption accords with the finding that the transduction mechanism of $P2Y_1$ receptors is the G_q/phospholipase C/proteinkinase C or G_q/phospholipase C/inositol 1,4,5-trisphosphate/Ca^{2+} pathway (Ralevic and Burnstock, 1998). Although we did not search for the second-messenger mechanism of $P2Y_1$ receptors to interact with $P2X_3$ receptors, phosphorylation by protein kinase C or calmodulin kinase II is a definite possibility.

P2Y receptor agonists cause analgesia on intrathecal application (Gerevich et al., 2004). This effect may be due on the one hand to the blockade of N-type voltage-sensitive Ca^{2+} channels (Borvendeg et al., 2003; Gerevich et al., 2004) and on the other hand to the blockade of $P2X_3$ receptor-channels (present study), both situated at the central terminals of primary afferent fibers in the spinal cord dorsal horn. Inhibition of $I_{Ca(N)}$ and depression of $P2X_3$ receptor-currents may unavoidably lead to a decrease of glutamate release and in consequence an interruption of pain transmission to higher order centers in the brain. Hence, the sequence of events may be the following: First, ATP may cause pain by directly stimulating $P2X_3$ receptors (Chizh and Illes, 2001) or indirectly stimulate TRPV1 receptors via $P2Y_2$ receptor-activation (Moriyama et al., 2003); second, the enzymatic degradation of ATP generates ADP which may cause analgesia by interrupting pain transmission.

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