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Pharmacological and electrophysiological characterization of rat P2X currents

Hai Ying Li, Seog Bae Oh*, and Joong Soo Kim*

Department of Physiology and Program in Molecular and Cellular Neuroscience, School of Dentistry and Dental Research Institute, Seoul National University, Seoul 110-749 Korea.

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Adenosine 5'-triphosphate (ATP) is an important extracellular signaling molecule which is involved in a variety of physiological responses in many different tissues and cell types, by acting at P2 receptors, either ionotropic (P2X) or G protein-coupled metabotropic receptors (P2Y). P2X receptors have seven isoforms designated as $P2X_1$-$P2X_7$. In this study, we investigated the electrophysiological and pharmacological properties of rat $P2X_1$-$P2X_4$ currents by using whole-cell patch clamp technique in a heterologous expression system. When ATP-induced currents were analyzed in human embryonic kidney (HEK293) cells following transient transfection of rat $P2X_1$-$P2X_4$, the currents showed different pharmacological and electrophysiological properties. ATP evoked inward currents with fast activation and fast desensitization in $P2X_1$- or $P2X_3$-expressing HEK293 cells, but in $P2X_2$- or $P2X_4$-expressing HEK293 cells, ATP evoked inward currents with slow activation and slow desensitization. While PPADS and suramin inhibited $P2X_2$ or $P2X_3$ receptor-mediated currents, they had little effects on $P2X_4$ receptor-mediated currents. Ivermectin potentiated and prolonged $P2X_4$ receptor-mediated currents, but did not affect $P2X_2$ or $P2X_3$ receptor-mediated currents. We suggest that distinct pharmacological and electrophysiological properties among P2X receptor subtypes would be a useful tool to determine expression patterns of P2X receptors in the nervous system including trigeminal sensory neurons and microglia.

Key words: ATP, ivermectin, P2X receptors, PPADS, surarmin.

*Corresponding author: Seog Bae Oh, D.D.S & Ph.D and Joong Soo Kim, D.D.S & Ph.D. Department of Physiology, School of Dentistry, Seoul National University 28 Yeongun-dong, Jongno-gu, Seoul, 110-749, Republic of Korea. Tel.: +82-2-740-8656 and 82-740-8657; Fax.: +82-2-762-5107; E-mail: odolbae@snu.ac.kr and joongkim@snu.ac.kr

Introduction

Adenosine 5'-triphosphate (ATP) is an important extracellular signalling molecule and its release evoke a variety of physiological responses in many different tissues and cell types (Khakh & North, 2006; Ralevic *et al.,* 1998). Many of the biological effects of extracellular ATP are mediated by P2 receptors, either the nucleotide-gated ion-channel (P2X) or G protein-coupled (P2Y) receptors (Boeynaems *et al.,* 2005; Khakh & North, 2006; Ralevic *et al.,* 1998). Seven mammalian P2X receptor subunits ($P2X_1$–$P2X_7$) have been identified and the mRNA for P2X receptors is expressed in a wide range of tissues and cells, and the properties of P2X receptor subtypes have been studied in a number of neurons, including sensory, sympathetic, parasympathetic, myenteric and central neurons (Burnstock, 2007b). Besides, with P2X receptors cloned, the properties of P2X receptors have been widely investigated. Homomeric $P2X_1$, $P2X_2$, $P2X_3$, $P2X_4$, $P2X_5$, and $P2X_7$ channels and some heteromeric P2X channels such as $P2X_{2/3}$ and $P2X_{1/5}$ have been characterized in a hete- rologous expression system (Bianchi *et al.,* 1999; Burnstock, 2006, 2007a & 2007b; Evans *et al.,* 1995; North, 2002).

However, lack of selective agonists and antagonists of P2X receptors still limits the thorough characterization of P2X receptors. In addition, there are several controversial results from different groups (North, 2002). For example, the mouse $P2X_4$ was found to be blocked by PPADS (Jones *et al.,* 2000), whereas the currents evoked by ATP at the mouse $P2X_4$ receptor were actually increased by PPADS (Townsend-Nicholson *et al.,* 1999). It has also been demonstrated that suramin has different effects on P2X currents in different species (Ikeda, 2007; Sim *et al.,* 2007). To date, these discrepancies remain to be resolved.

Thus, in the present study, we set out to characterize rat P2X receptor currents in terms of their electrophysiological and pharmacological properties. Following transient transfection

of rat homomeric $P2X_1$-$P2X_4$ receptors in HEK293 cells, ATP-induced currents were analyzed by using whole-cell patch clamp technique, respectively. It was also thought that electrophysiological and pharmacological characterization of rat P2X receptor subtypes would be helpful to identify expression patterns of P2X receptors in the nervous system including trigeminal sensory neurons and microglia.

Materials & methods

Transient transfection of $P2X_1$-$P2X_4$ in HEK293 cells

cDNAs encoding the $P2X_1$-$P2X_4$ subunits were expressed individually in HEK 293 cells. HEK293 cells were cultured in Dulbecco's modified Eagle's medium and 10% heat-inactivated fetal bovine serum containing penicillin and streptomycin. Cells reaching 60-80% confluency were used for transient cDNAs of $rP2X_1$-$P2X_4$ transfections with the cationic liposome method with 1 μg of supercoiled plasmid cDNA per 1×10^5 cells mixed with 2.5 μl enhancer and 3 μl WelFect-EX^{TM} in 0.5 ml of serum-free medium. Green fluorescence protein (GFP) was co-transfected with P2X receptors as an expression marker. After 5 h at 37°C, the medium was replaced with normal HEK293 media, and cells were incubated for another 28~36 h before electrophysiological experiments.

Electrophysiological recordings

Whole-cell voltage-clamp recordings were performed for the measurement of ATP-evoked currents with an Axopatch-1C amplifier (Axon Instruments, Union City, CA, USA). The patch electrodes and puffer pipettes were pulled from borosilicate capillaries (Chase Scientific Glass, Inc., Rockwood, TN, USA). When the pipettes were filled with the solution, their resistance was 2~4 MΩ. The extracellular solution contained (mM): NaCl 147, KCl 3, $MgCl_2$ 1, $CaCl_2$ 2, Glucose 13, HEPES 10, and pH adjusted to 7.4 with NaOH. The pipette solution contained (mM): NaCl 147, HEPES 10, and EGTA 10, pH adjusted to 7.3 with NaOH. The resting potential was held at −60 mV.

Drugs

Adenosine-5'-triphosphate (ATP), suramin, pyridoxal-5-phosphate-6-azophenyl-2',4'-disulfonic acid (PPADS), and ivermectin were purchased from Sigma. ATP were applied via a glass 'puffer' pipette (1 μm tip diameter, 5 psi) using a Picopump (Medical Systems Corporation, PLI-100, Greenvale, NY, USA). The tip of the puffer pipette was positioned downstream from the cell with respect to the direction of flow of the superfusing solution, and temporarily repositioned to a point about 15 μm from the cell only for the period of application. After a single application of ATP to any cell, a subsequent application was performed. In ivermectin experiments, many times of applications of ATP were performed. We normalized the second current to the first one to analyze the effects of P2X receptor antagonists, suramin and PPADS, on ATP-evoked currents. Antagonists were applied by superfusing for 170s before the second application of ATP, and the ATP-evoked currents were compared with those observed in other cells without antagonist pretreatment. The perfusion rate of bath solution was continuous (4 ml/min) during the experiment.

Statistical analyses

Data are expressed as mean± SEM. ANOVA and Student's t test was used to determine the differences using the software Origin 6.0 (Northampton, MA, USA). Differences were considered to be significant when p was less than 0.05.

Results

Electrophysiological properties of ATP-induced currents in $P2X_1$-$P2X_4$ expressing-HEK293 cells

10 μM ATP was used to induce inward currents at the holding potential of −60 mV in $P2X_1$-$P2X_4$ expressing HEK293 cells (Fig. 1). $P2X_1$ and $P2X_3$ receptor-mediated currents underwent fast desensitization. Recovery from desensitization is extremely slow, and the second application of ATP did not elicit as large currents as the first application (Fig. 1B). As to $P2X_1$, almost no currents were recorded by the second application of ATP. In contrast, $P2X_2$ and $P2X_4$ receptor-

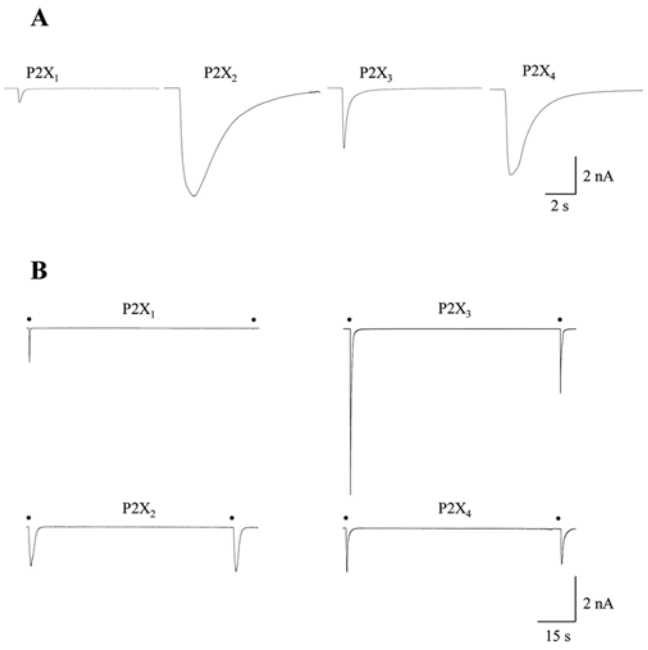

Fig. 1. Electrophysiological recordings of $P2X_1$-$P2X_4$ receptors-mediated currents activated by 10 μM ATP at the holding potential of −60 mV in HEK293 cells. A, Average current traces activated by 10 μM ATP in $P2X_1$-$P2X_4$–expressing HEK293 cells; n = 9 ($P2X_1$), n = 45 ($P2X_2$), n = 60 ($P2X_3$), and n = 35 ($P2X_4$). B, Records of $P2X_1$-$P2X_4$ receptors-mediated currents activated by twice applications of 10 μM ATP with the interval of 90s in HEK293 cells. Black points indicate the time point of ATP application.

mediated currents underwent slow desensitization, the second application of ATP-induced currents were almost unchanged compared to the first ATP-induced currents (Fig. 1B).

Pharmacological property of $P2X_2$ receptor-mediated currents in HEK293 cells

In order to perform pharmacological characterization, we used two non-selective P2X receptor antagonists, suramin and PPADS (North & Surprenant, 2000). Both suramin and PPADS inhibited 3 μM ATP-induced currents in $P2X_2$-expressing HEK293 cells (Fig. 2A). The inhibitory effect of PPADS (79± 6%, n = 6) on ATP-induced currents was bigger than suramin (33± 5%, n = 8) ($p<0.05$) (Fig. 2B), indicating that $P2X_2$ was more sensitive to PPADS than suramin. Ivermectin did not affect ATP-induced currents (n = 5, data not shown).

Phamacological property of $P2X_3$ receptor-mediated currents in HEK293 cells

Both suramin and PPADS inhibited 3 μM ATP-induced currents in $P2X_3$-expressing HEK293 cells (Fig. 3A). Unlike in $P2X_2$ receptor, the inhibitory effects of PPADS (91± 9%, n = 7) and suramin (79± 9%, n = 8) on $P2X_3$ receptor-mediated currents were not significantly different ($p>0.05$) (Fig. 3B), indicating that the sensitivity of $P2X_3$ to PPADS was similar to suramin. Ivermectin was also not found to affect the $P2X_3$ receptor-mediated currents (n = 5, data not shown).

Phamacological property of $P2X_4$ receptor-mediated currents in HEK293 cells

Differently from $P2X_2$ and $P2X_3$ receptor, neither suramin (n = 7, data not shown) nor PPADS (Fig. 4A, n = 5) inhibited $P2X_4$ receptor-mediated currents in $P2X_4$-expressing HEK293 cells. In addition, ivermectin potentiated and prolonged 3 μM ATP-induced currents in $P2X_4$-expressing HEK293 cells (Fig. 4B, n = 6).

Discussion

We tested the electrophysiological and pharmacological properties of $P2X_1$-$P2X_4$ receptors-mediated currents in HEK293 cells, which is currently one of the most common heterologous expression systems for studying ligand-gated ion channels. ATP-gated channels, which are not endogenously expressed in HEK293 cells, can be expressed well in HEK293 cells after transfection with the P2X subunit cDNA (Dutton *et al.,* 2000; Woodward *et al.,* 2004). When we tested the effects of ATP (3 μM, 10 μM, 30 μM and 100 μM) in naive HEK293 cells, no currents were observed (data not shown). However, 3 μM, 10 μM, 30 μM and 100 μM ATP evoked inward currents in all $P2X_1$, $P2X_2$, $P2X_3$, and $P2X_4$-expressing HEK293 cells, respectively.

In all $P2X_1$-$P2X_4$-expressing HEK293 cells, 10 μM ATP

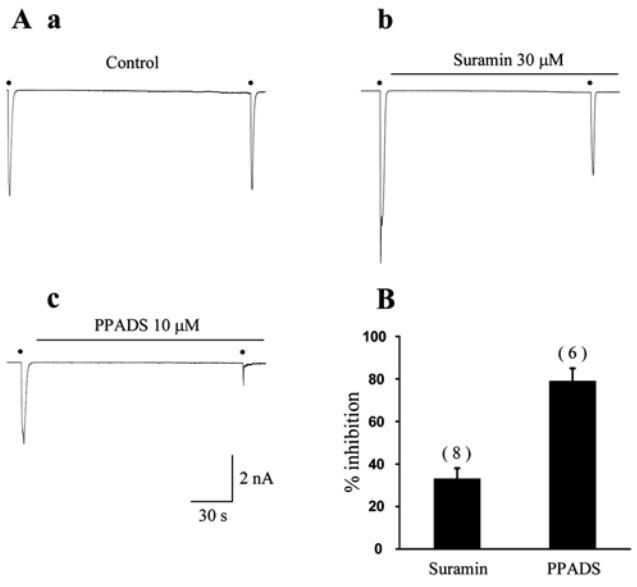

Fig. 2. Pharmacology of $P2X_2$ receptor-mediated currents in HEK293 cells. Records of currents activated by twice applications of 3 μM ATP with the interval of 3 min under control (Aa), 30 μM suramin (Ab), and 10 μM PPADS (Ac). Suramin and PPADS were pretreated for 170s before the second ATP application. Black points indicate the time point of ATP application. B, Summary of the inhibitory effects of 30 μM suramin and 10 μM PPADS on $P2X_2$ receptor-mediated currents in HEK293 cells. The effect of suramin is significantly smaller than PPADS (mean ± SEM, $p< 0.05$). The number in parentheses represents the number of cells studied.

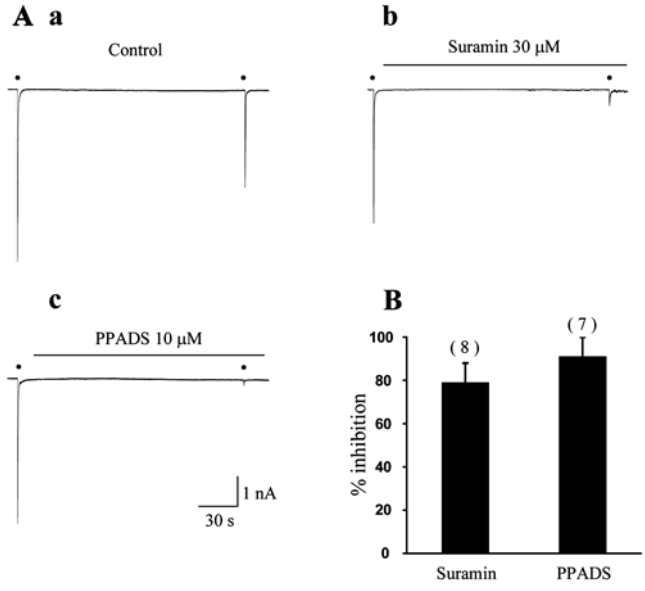

Fig. 3. Pharmacology of $P2X_3$ receptor-mediated currents in HEK293 cells. Records of currents activated by twice applications of 3 μM ATP with the interval of 3 min under control (Aa), 30 μM suramin (Ab), and 10 μM PPADS (Ac). Suramin and PPADS were pretreated for 170s before the second ATP application. Black points indicate the time point of ATP application. B, Summary of the inhibitory effects of 30 μM suramin and 10 μM PPADS on $P2X_3$-receptor-mediated currents in HEK293 cells. The effects of suramin and PPADS are not significantly different (mean ± SEM, $p>0.05$). The number in parentheses represents the number of cells studied.

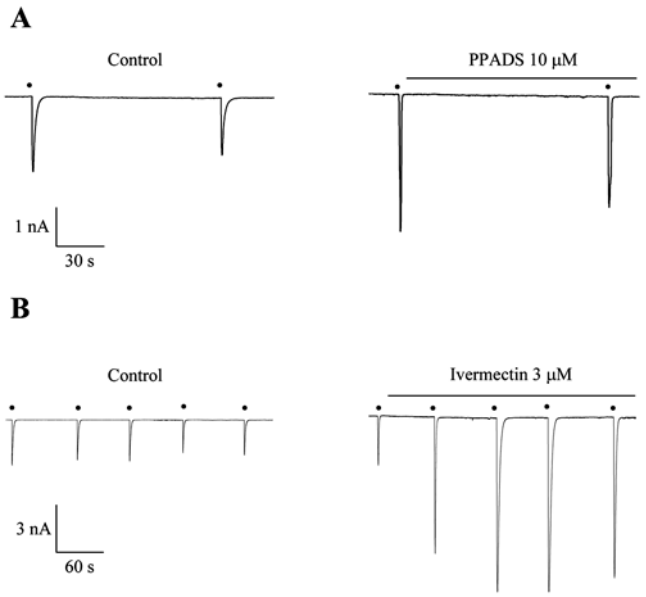

Fig. 4. Pharmacology of $P2X_4$ receptor-mediated currents in HEK293 cells. A, records of currents activated by twice applications of 3 μM ATP with the interval of 3 min under control (left) and 10 μM PPADS (right), B, ATP-induced currents by 5 times applications of ATP during control (left) and 3 μM ivermectin (right). Black points indicate the time point of ATP application.

evoked inward currents at a holding potential of −60 mV. $P2X_1$ and $P2X_3$-mediated currents showed fast activation and fast desensitization, while $P2X_2$ and $P2X_4$-mediated currents showed slow activation and slow desensitization. Recovery from desensitization was extremely slow in $P2X_1$ and $P2X_3$ so that the second application of ATP induced smaller currents than the first one. Especially in $P2X_1$, almost no currents were recorded by the second application of ATP. In $P2X_2$ and $P2X_4$, slow desensitization was observed compared to $P2X_1$ and $P2X_3$ receptors-mediated currents. These data were in good agreement with previous studies (North, 2002).

Both suramin and PPADS are non-selective antagonists of P2X receptors (North & Surprenant, 2000). In $P2X_2$-$P2X_4$-expressing HEK293 cells, we tested the effects of suramin and PPADS on 3 μM ATP-induced currents based on previous studies on antagonists of P2X receptors (Hamilton, 2002). $P2X_1$ receptor was reported to be inhibited by suramin and PPADS (Evans *et al.,* 1995). However, because of the fast desensitization of $P2X_1$ receptor-mediated current, we did not test the pharmacological properties of $P2X_1$ receptor. Previous studies on the effects of suramin and PPADS had controversial results in different or even in the same species (Bo *et al.,* 1995; Jones *et al.,* 2000; North, 2002; Townsend-Nicholson *et al.,* 1999). For example, the mouse $P2X_4$ was found to be blocked by PPADS (Jones *et al.,* 2000), while in another group, the currents evoked by ATP at the mouse $P2X_4$ receptor were actually found to be increased by PPADS (Townsend-Nicholson *et al.,* 1999). In addition, in $P2X_4$-expressing *Xenopus* oocytes, it was found that both antagonists enhanced ATP-induced currents (Bo *et al.,* 1995). It has also been demonstrated that suramin has different potency to block $P2X_4$ receptors in different species such as rat, mouse, and human (North, 2002). $P2X_4$ receptors are relatively unaffected by suramin in rat, while blocked in human and increased in mouse. In addition, it was found that suramin did not inhibit the ATP-induced currents in mouse macrophages and mouse megakaryocytes (Ikeda, 2007; Sim *et al.,* 2007). In the present study, both suramin and PPADS inhibited ATP-induced currents in $P2X_2$ and $P2X_3$ expressing HEK293 cells, and $P2X_3$ was more sensitive to suramin and PPADS than $P2X_2$. In addition, the inhibitory effect of PPADS on $P2X_3$ receptor-mediated currents was bigger than suramin, indicating that $P2X_3$ was more sensitive to PPADS than suramin, while the inhibitory effects of both antagonists on $P2X_2$ receptor-mediated currents were not significantly different. The effects of suramin and PPADS on $P2X_2$ and $P2X_3$ are almost consistent with the previous studies (North, 2002). Different from $P2X_2$ and $P2X_3$, neither suramin nor PPADS affected ATP-induced currents in $P2X_4$ expressing HEK293 cells, which is consistent with the previous study on the effect of suramin on rat $P2X_4$ (North, 2002), but different from the studies on the effect of PPADS on mouse and *Xenopus* oocytes (Jones *et al.,* 2000; Townsend-Nicholson *et al.,* 1999; Bo *et al.,* 1995).

The effect of ivermectin, which is an allosteric modulator of several receptor channels including mammalian $P2X_4$ (Toulmé *et al.,* 2006), was also tested. Ivermectin produced profound potentiation and prolongation of the $P2X_4$-mediated currents in HEK293 cells, but had no effects on $P2X_2$ and $P2X_3$-mediated currents. These data are consistent with previous studies (Khakh & North, 2006; Priel & Silberberg, 2004).

Our results demonstrate that the electrophysiological properties of $P2X_1$-$P2X_4$ are consistent with previous studies, while pharmacological properties are clearly distinctive among different P2X receptor subtypes. The development of new selective agonists and antagonists of P2X receptors will further help the thorough characterization of P2X receptors. It is thought that distinctive electrophysiological and pharmacological properties of rat P2X receptor subtypes would provide an insight into the identification of the expression patterns of P2X receptors in the nervous system including trigeminal sensory neurons and microglia (Burgard *et al.,* 1999; Sim *et al.,* 2007), which are the crucial cellular components in pain information processing under normal and pathological pain conditions (Burnstock, 2007a).

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