P2X7 on Mast Cells Participates in Peripheral Pain and Serves as A Potential Target for Salicylic Acid and Aspirin Analgesia

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Research

Keywords: ATP, mast cell, P2X7, salicylic acid and aspirin, analgesia

Posted Date: May 20th, 2020

DOI: https://doi.org/10.21203/rs.3.rs-28934/v1

Abstract

Background Pain is an unpleasant sensory and emotional experience associated with actual or potential tissue damage. ATP is an important molecule closely related to many important physiological and pathologic functions. ATP-gated cation channel P2X receptors are widely distributed in various tissues of body, including nervous system and immune system. As an important member of P2X receptors, P2X7 is not only involved in pain, epilepsy, Parkinson's diseases, but also in the formation of blood, respiratory and digestive diseases. In this study, we investigated the role of P2X receptors in mast cells for peripheral pain and the analgesic mechanism of salicylic acid and aspirin.

Methods P2X receptors were examined and identified in mouse peritoneal mast cells by RT-PCR, intracellular calcium measurement and electrophysiology. The inflammatory mediators released from the activated mast cell were examined by real-time PCR and ELISA. Paw swelling, mechanical stimulation threshold and histopathological changes were tested to evaluate the peripheral pain in mice.

Results The results showed that P2X1, P2X4, P2X7 receptors were expressed in mouse peritoneal mast cells. Mast cell was activated in a concentration-dependent manner by extracellular ATP, and the activation could be blocked by specific ion channel antagonists. In addition, high concentrations of ATP also induced mast cells to release inflammatory mediators such as histamine, IL-1β and CCL3 through P2X7 receptor. Furthermore, peripheral pain of the extracellular high concentration ATP to induce could be alleviated by P2X7 blockers or mast cell defects. We also found that salicylic acid and its derivation aspirin could inhibit high concentration ATP-induced inward current, release of inflammatory factors in mast cells, as well as the peripheral pain caused by high concentration ATP.

Conclusions Together with these, we concluded that extracellular ATP with high concentration could not only activate neurons directly, but also activate P2X7 receptor on mast cells, and induce peripheral pain via neuro-immune crosstalk. Additionally, salicylic and aspirin could inhibit the activity of P2X7, therefore, P2X7 receptor may be one of the potential targets for salicylic acid and aspirin analgesia.

Background

Extracellular ATP is a "signal of danger" or damage-associated molecular pattern mediated by P2 purinergic receptors. P2 receptors have two types, i.e. P2X (P2 × 1–7) and P2Y receptors [1]. P2X receptors carry out many important functions in the central and peripheral nervous system, such as rapid synaptic transmission, neurotransmitter release and the generation of pain signals [2]. Compelling evidences have shown that some P2X receptors such as P2 × 2, P2 × 3, P2 × 4 and P2 × 7 are involved in the pathogenesis of central pain [3]. P2 × 7 purinoceptor, as a ligand-gated cation channel, exists in neurons and glial cells of central and peripheral nervous, but is mainly expressed in cells of immune origin [4]. The absence of P2 × 7 receptor would completely eliminate the inflammatory and neuropathic hypersensitivity to both mechanical and temperature stimulation in mice [5, 6]. P2 × 7 receptor of microglia also plays an important role in chronic neuropathy and inflammatory pain by releasing IL-1β [7].

Besides IL-1β, cytokines as IL-6, CCL2, TNFα and CCL3 could also be mediated by P2 × 7 receptor in neutrophil and monocyte [8–12]. However, the expression of P2 × 7 in mast cells and how it participates in the pain process are still unclear.

As a part of innate and adaptive immune systems, mast cells defend against invading foreign organisms by instantly releasing chemical mediators or attracting other cellular entities. In addition, mast cells are in close proximity to afferents innervating the periphery, visceral organs, meninges and establishing dynamic interactions with pain-activating nociceptors [13]. Performed and newly synthesized mediators released from the activated mast cells, including histamine, protease, lipid mediators, various inflammatory factors and chemokines, which could contribute to pain via the nervous system or other immune cells [14]. Therefore mast cells play important roles in the pathological process of pain [15]. The classic pathway of mast cell activation is mediated by IgE receptor (FcεRI). Mast cells are also activated by a wide variety of triggers, such as substance P, lipid mediators, interleukins and microbial products [16]. Extracellular ATP and the related nucleotides could stimulate mast cells and induce different inward currents through P2X receptors [17]. Although activation of P2 × 4 receptor could augment the degranulation mediated by the FcεRI in mouse bone marrow-derived mast cells, only P2 × 7 receptor contributes to degranulation in human mast cell line LAD2 [18, 19]. In particular, the mechanism of pain caused by the release of inflammatory factors by activating P2 × 7 receptor on mast cells is rarely studied. The relationship between P2 × 7 receptors expressed in mast cells and peripheral pain is also unclear.

Chronic pain is a global problem affecting more than two-thirds of the population. So far, there are many difficulties in the treatment of chronic pain patients, and there is no effective drug for some patients with inflammatory and neuropathic pain. Salicylate has been used for pain management as early as 4,000 years ago. Aspirin, derived from salicylic acid, is a peripheral analgesic drug inhibiting cyclooxygenase-1 (COX-1) in clinical treatment. The analgesic effect of salicylic acid and its derivatives is apparently, however, the target is not very clear. Previous studies indicated that salicylates might be associated with mast cells. Kim HM et al. reported that salicylic acid could significantly inhibit histamine release from rat peritoneal mast cells activated by compound 48/80 or anti-DNP IgE [20]. The relationship between aspirin and mast cells remains unclear. There are some evidences to show that the anaphylaxis triggered by aspirin is due to eosinophils and mast cells [21].Yet, the direct relationship between aspirin and mast cells is still controversial. High concentrations of aspirin such as 10 mM can activate mast cells directly [22]. However, the effect of normal concentrations of aspirin on mast cells remains unknown.

Based on the analgesic effect of salicylic acid (or aspirin) and the distribution of P2 × 7, we suggested a hypothesis that P2 × 7 receptor may be an important candidate target for salicylic acid and aspirin. Therefore, in this study, we evaluated the effects of several P2X receptor subtypes in mast cells, especially the P2 × 7 receptor. Meanwhile, we also studied the participation of a mast cells and P2 × 7 receptor for the occurrence of peripheral pain. Final, we investigated whether the analgesia effects of salicylic acid and aspirin are related to P2 × 7 receptors in mast cells.

Methods

Animals

All procedures were performed under protocols approved by the Animal Care and Use Committee of Nanjing University of Chinese Medicine (Approval no. A190401). Adult male mice used were 22–26 g males in a C57BL/6 background (Qinglongshan, China). Animals were housed at constant humidity (40–60%) and temperature (22 ± 2℃) on a 12 h light/dark cycle and allowed free access to food and water. C-kit mutant genetically mast cell-deficient Kit (W-sh) "sash" mice were donated by Johns Hopkins.

Chemicals

ATP (Sigma-Aldrich, St. Louis, MO, United States), PPADS (10 μM, Tocris Bioscience, Missouri, USA, a non-selective P2 purinergic receptor antagonist), NF449 (1 μM, Cayman, Ann Arbor, Michigan, USA, P2 × 1 receptor antagonist), 5-BDBD (1 μM, Sigma-Aldrich, St. Louis, MO, United States, P2 × 4 receptor antagonist), AZ10606120 (1 μM, Tocris Bioscience, Missouri, USA, P2 × 7 receptor antagonist), recombinant mouse SCF protein (10 ng/mL, R&D Systems, Minneapolis, MN, USA), Penicillin and streptomycin (100 μg/mL; Gibco, Waltham, MA, USA), fibronectin (30 μg/mL; Sigma-Aldrich, St. Louis, MO, United States), Fluo 4-AM (Solarbio, Beijing, China, calcium indicator), Histamine ELISA Kit (Yifeixue, Nanjing, China), Trizol (Vazyme Biotech, Nanjing, China), HiScript II Q RT SuperMix for qPCR (Vazyme Biotech, Nanjing, China), Taq MasterMix (Vazyme Biotech, Nanjing, China), AceQ qPCR SYBR Green Master Mix (Vazyme Biotech, Nanjing, China), salicylic acid (300 μM, Yuanye Biotech, China), aspirin (1 Mm, Tocris Bioscience, Missouri, USA).

P815 cell culture and mouse peritoneal mast cell purification

Mouse Mastocytoma Cells (P815) was cultured in 1640 complete medium (90% 1640 medium, 10% fetal bovine serum, 100 μg/ml penicillin and 100 μg/ml streptomycin). Cells were maintained at 37 °C in a humidified atmosphere of 5% CO2 incubator.

Mouse peritoneal mast cells were established from C57BL/6 mice as Xinzhong Dong described [23]. Briefly, the mouse peritoneal cells were collected with mast cell dissociation media MCDM (HBSS with 10 mM HEPES and 3% fetal bovine serum, pH 7.2), and then centrifuged at 200 g for 5 min. The pellet was resuspended and layered over 70% percoll suspension, and then centrifuged at 500 g for 20 min. Pipetted off supernatant carefully and the mast cells were washed with fresh MCDM. Mast cells were resuspended in DMEM containing 10% fetal bovine serum and 10 ng/mL recombinant mouse stem cell factor.

P2X purinoceptors RT-PCR screen

Trizol method was employed to isolate total RNA from mouse peritoneal cells. 10–500 ng RNA was used for RT reactions by using HiScript II Q RT SuperMix for qPCR Kit according to the manufacturer's

instructions, and then PCR screen explored by Taq MasterMix. 10 μL of the PCR reaction product was used for agarose gel electrophoresis and stained with Gold View and then observed by ChemiDoc MP (Bio-rad, California, USA). All of the PCR primers were synthesized by Genescipt Biotechnology (Nanjing, China). The primer sequence and product size are shown in Table 1.

Table 1
The sequence of primers

Gene	Primer sequence (5′-3′)	Product size
P2 × 1	Forward: GCCCAAGGTATTCGCACAGG	496 bp
	Reverse: GACGACGGTTTGTCCCATTCT	
P2 × 2	Forward: ACCTGCCATTTAGATGACGACTG	241 bp
	Reverse: TGTTGCCCTTGGAGAACTTGA	
P2 × 3	Forward: GCTTCGGACGCTATGCCAACA	490 bp
	Reverse: AAATCCTGCCCAGCAAACTTAA	
P2 × 4	Forward: GTGCTCGGGTCCTTCCTGTTC	154 bp
	Reverse: CCGTTTCCTGGTAGCCCTTTT	
P2 × 5	Forward: TGTAGCGGGACACGGACTGA	209 bp
	Reverse: TTTCTAGCACATTGGCTTTGGA	
P2 × 6	Forward: GGTACAACTTCAGGACAGCCAATC	207 bp
	Reverse: CATACAGTAGCAGCAGGTCACAGAG	
P2 × 7	Forward: AACATCTTGCCAACTATGAACGG	132 bp
	Reverse: TCCTCCCTGAACTGCCACCT	

Intracellular calcium measurement

The mouse peritoneal mast cells were isolated and plated on the glass cover slips, which coated by 30 μg/mL fibronectin for two hours of incubation at 37 °C, 5% CO_2. After that, mast cells were incubated by 1μL/mL Fluo-4 calcium ion indicator along with 0.02% Pluronic F-127 for 30 minutes at room temperature. Then cells immediately washed for 3 times by calcium imaging buffer (125 mM NaCl, 3 mM KCl, 2.5 mM $CaCl_2$, 0.6 mM $MgCl_2$, 20 mM glucose, 10 mM HEPES, 20 mM sucrose, 1.2 mM $NaHCO_3$, pH 7.4). Finally, cells were used for imaging within two hours.

Electrophysiology

The whole cell recording of the patch-clamp technique was used. Patch pipettes typically had a resistance of 6–8 MΩ. Osmolality was adjusted to 300–310 mOsM (adjusted by sucrose as necessary). The standard pipette solution contained 135 mM CsCl, 8 mM NaCl, 10 mM EGTA, 3.6 mM $CaCl_2$, 10 mM HEPES, PH 7.3 (adjusted by CsOH). The standard external solution contained 147 mM NaCl, 2 mM KCl, 2 mM $CaCl_2$, 1 mM $MgCl_2$.6H2O, 10 mM HEPES, 16 mM glucose, pH 7.3 (adjusted by NaOH). In addition, low divalent external solution contained 147 mM NaCl, 10 mM HEPES, 13 mM glucose, 0.2 mM $CaCl_2$, 2 mM KCl, pH 7.3 (adjusted by NaOH).

All the experiments were performed at room temperature. Whole-cell currents were record by using Multiclamp 700 B and Digidata 1440 A (Molecular Devices, Inc., San Jose, USA), capacitance transients and series resistance were minimized by using the capacitance neutralization circuits on the amplifier. Experiments were performed with a perfusion system, and drugs were directly added to the recording chamber with a pipette. The cells were usually evoked by holding the membrane potential, and applied voltage commands to a range of potentials with 10 mV steps from − 130 mV to + 130 mV in 100 ms. In addition, currents were evoked by ramping the membrane potential from − 90 mV to + 100 mV in 300 ms. The currents were digitized (sampled at a frequency of 10 kHz and filtered at 0.1 kHz for analysis), stored and subsequently analyzed by using Clampex 10.3 (Molecular Devices, Inc., San Jose, USA).

Quantitative Real-time PCR

Total RNA from Mouse Mastocytoma Cells (about 10^5-10^6 cells) stimulated by different concentration of ATP for 4 hours, tissues (10–50 mg) isolated from paw treated by 100 mM ATP or saline were prepared by using Trizol reagent. cDNA was generated by HiScript II Q RT SuperMix for qPCR Kit according to the manufacturer's instructions. Real-time qPCR was performed using AceQ qPCR SYBR Green Master Mix and GAPDH were used as internal controls. The primer sequences are shown in Table 2.

Table 2
The sequence of primers

Gene	Primer sequence (5′-3′)
IL-6	Forward: GTTGCCTTCTTGGGACTGAT
	Reverse: CTGGCTTTGTCTTTCTTGTTAT
IL-1β	Forward: AAATCTCGCAGCAGCACATC
	Reverse: AGCAGGTTATCATCATCATCCC
CCL2	Forward: GGCCTGCTGTTCACAGTTGC
	Reverse: CAGAAGTGCTTGAGGTGGTTG
CCL3	Forward: GGCCTGCTGTTCACAGTTGC
	Reverse: CAGGCATTCAGTTCCAGGTCAG
GAPDH	Forward: GCACAGTCAAGGCCGAGAAT
	Reverse: GCCTTCTCCATGGTGGTGAA

Histamine ELISA

Histamine ELISA prepared according to the manufacturer's protocol. Briefly, mouse peritoneal mast cells were stimulated with different concentration of ATP (1 μM, 100 μM, 1 mM and 5 mM), and the supernatants were harvested at time point of 0.5 h and stored at 80℃ until used for ELISA.

Behavioral assays

As references, the Von Frey behavioral assays were performed in a blinded manner. In briefly, different groups of mice were put in a transparent plastic box, which was placed on a metal mesh for about 30 min. Then the value of threshold was measured with a time interval of 1 h, 3 h, 5 h after 100 mM ATP treatment. Each mouse was tested by more than 10 times at a specific force manually. At last, we determined the threshold by the lowest force, which elicited responses with more than 50% of the time.

Histological

The paw skin was isolated and washed with PBS, then fixed with 4% paraformaldehyde for 24 h and 30% sucrose for 48 h. The tissue was embedded in OCT and sliced to a thickness of 10 microns, followed by hematoxylin–eosin (HE) staining and toluidine blue staining. Images of each section were obtained by using the Nikon ECLIPSE 80i microscope (Nikon, Tokyo, Japan) with a magnification of 200.

Quantification and statistical analysis

The data were analyzed by GraphPad 6.0 and presented as mean ± SEM, $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, $^{\#}p < 0.05$, $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$ was considered statistically significant. Statistical analysis of the results was performed by two-tailed, unpaired Student's T-test or ANOVA analysis.

Results

P2X receptors expression and ATP-induced calcium response in mouse peritoneal mast cells

To identify P2X receptors on mast cell, we explored the P2X expression in mouse peritoneal mast cells by RT-PCR screen using specific primers. As shown in Fig. 1A, we found that mouse peritoneal mast cells expressed several different ionotropic P2X receptors including P2 × 1, P2 × 3, P2 × 4 and P2 × 7.

P2X receptor is a non-selective cation channel, which has permeability to Na^+, K^+ and Ca^{2+}, but the most obvious permeability to Ca^{2+}. Hence, we examined whether purinergic receptors could induce calcium influx in mouse peritoneal mast cells. The experimental results indicated that there were transient increases of intracellular calcium in mast cells treated by different concentrations of ATP from 0.01 to 5000 μM (Fig. 1B(a)). As shown in Fig. 1B(c-d), the ratios of fluorescence intensity were varied with different ATP concentrations, and the EC50 was about 6.5 μM. In addition, there was some difference in the reaction durations. For instance, high concentration of ATP (1 mM and 5 mM) could lead to longer lasting changes in intracellular calcium than the low concentration of ATP (1 μM) as Fig. 1B (b) showed. Therefore, we concluded that different concentrations of ATP could activate mast cells through different P2X receptors.

Next, we further confirmed the type of P2X receptors involved in this calcium influx by using special P2X channel antagonists. As shown in Fig. 1D-F, extracellular ATP could lead to Ca^{2+} response with a concentration-dependent manner. The increasing calcium influx could be partially blocked by a non-selective P2 purinergic receptor antagonist PPADS (10 μM, pre-incubation for 5 minutes). In addition, calcium influx caused by 1 μM ATP was inhibited by P2 × 1 receptor antagonist NF449 (1 μM, pre-incubation for 5 minutes). And the transient increase of the intracellular calcium concentration induced by 100 μM ATP was blocked by 5-BDBD (1 μM, pre-incubation for 5 minutes), which is a specific P2 × 4 receptor antagonist. Furthermore, the specific P2 × 7 receptor antagonist AZ10606120 (1 μM, pre-incubation for 5 minutes) had the ability of reducing the calcium influx caused by high concentration ATP such as 1 mM and 5 mM. These results indicated that P2 × 1, P2 × 4 and P2 × 7 might contribute to the activation of mast cells.

Electrophysiological characteristics of mouse peritoneal mast cells induced by extracellular ATP

According to previously published literature, human mast cells are sensitive to ATP in a concentration-dependent manner [17]. However, the relationship between inward currents and different concentrations of ATP in mouse peritoneal mast cells has not been reported so far. Therefore we explored the relationship with reference to Wareham's reports. Our experimental results showed that about 85% of mast cells were sensitive to 1 μM ATP (Fig. 2A, n = 17) and the mean amplitude of inward current was

73.9 ± 18.1 pA (Fig. 2E). We also found that the current could also be induced by 100 μM ATP (Fig. 2B, n = 16). The amplitude of the inward current induced by 100 μM ATP was 154.0 ± 40.7 pA, which was not significantly different from 1 μM ATP (Fig. 2E). However, the current duration evoked by 100 μM ATP was shorter (0.88 ± 0.14 s, n = 16) than that of 1 μM ATP (2.67 ± 0.56 s, n = 17) (Fig. 2F). When we increased the concentration of extracellular ATP to a high level, we found that both 1 mM and 5 mM ATP had the ability to repeatedly induce the inward currents (Fig. 2C, n = 9; Fig. 2D, n = 16). Although the current amplitude (Fig. 2E) as well as the duration (Fig. 2F) were different for 1 mM ATP and 5 mM ATP, the inward currents had some similar characteristics such as “run-up” tendency (Fig. 2G, F). The current growth rate of second ATP application had no difference as Fig. 2I shown (current growth rate of 1 mM ATP and 5 mM ATP were 1.2 ± 0.19, 1.36 ± 0.38, respectively). The growth rate is defined as the difference between the magnitude of the second current minus the magnitude of the first current divided by the magnitude of the first current. In addition, the inward current evoked by 1 mM ATP was voltage-dependent and could be washed out by normal extracellular buffer (Fig. 2J-L). The activate curves and inactivate curves induced by 5 mM ATP were shown in Fig. 2M. According these curves, the conductance curve was calculated as shown in Fig. 2N, which indicated that the current induced by 5 mM ATP had characteristics with faster activation and slower inactivation.

Inward currents induced by extracellular ATP with different concentrations could be blocked by P2 × 1, P2 × 4 and P2 × 7 antagonist

Electrophysiological results showed that there were obvious changes for currents corresponding to ATP with different concentrations. Therefore, we concluded that some types of the P2X receptor subtypes probably contributed to the mast cells activation progress referred as Wareham described [17]. In Wareham's research, P2 × 1,P2 × 4 and P2 × 7 receptors in LAD2 were activated by 1 μM ATP, 100 μM ATP and high concentrations of ATP (1 mM, 5 mM), respectively. In order to further explore which types of the P2X receptor are involved in the formation of inward currents, we utilized several functional blockers. The data indicated showed that 1 μM ATP hardly induced inward current when 10 μM PPADS (a non-selective P2 antagonist) (Fig. 3A, H, n = 13) or 1 μM NF449 (the blocker of P2 × 1 receptor) (Fig. 3A, H, n = 9) was applied. In addition, the current induced by 100 μM ATP was blocked by 1 μM NF449 (Figs. 3B, 3I, n = 15) and 1 μM PPADS (Fig. 3B, I, n = 13, $p = 0.0009$). However, neither the activation of current nor the tendency of “run-up” was affected by NF449 (data not shown). The current caused by 1 mM ATP declined by using 10 μM PPADS (Fig. 3C, J) or 1 μM AZ 10606120 (the blocker of P2 × 7 receptor) (Fig. 3D, K), and the blocking effects could be washed. Moreover, the voltage-dependent currents from − 130 mV to + 130 mV could also be blocked by AZ 10606120 as Fig. S1(C) shown. Furthermore, the current induced by 5 mM ATP could be inhibited by AZ 10606120 (Fig. 3E, L). At the same time, the conductance induced by 5 mM ATP was also affected by AZ 10606120 (Fig. S1D, n = 4). It should be noted that P2 × 7 receptor was sensitive to divalent cation as North described [24]. Consequently, our results indicated that the inward current induced by 5 mM ATP in the external solution of the low divalent cation was greater than that in

normal external solution (Figs. 3F, G, M). This conclusion further confirmed the existence of P2 × 7 channel in mouse peritoneal mast cells. The above results were in line with the calcium imaging results. Results indicated that P2 × 1, P2 × 4 and P2 × 7 in mouse peritoneal mast cells are involved in the progress activation induced by extracellular ATP.

Activation of P2 × 7 receptor on mouse-derived mast cell could lead to degranulation and release cytokines

Degranulation is one of important indicators of mast cell activation. Consistent with the research by Wareham et al in human mast cell line LAD2 [18], our results indicated that there is no detectable histamine release at lower concentrations of ATP such as 1 μM ATP and 100 μM ATP. However, histamine release was significantly increased at higher concentrations of ATP. Besides histamine, mast cells are effective producers of inflammatory cytokines in response to various stimuli. According to some previous literatures, cytokines secretion especially IL-1β induced by the extracellular ATP has been widely studied in different immune cells [8–12]. However, there are few studies on the release of cytokines from mast cells induced by extracellular ATP. In order to examine the potential mediator release in mouse-derived mast cells, we detected a serious of mediators such as IL-6, IL-1β, CCL2 and CCL3 by using quantitative real-time PCR at 4 h after ATP treatment. Since a large number of cells are required in this experiment and the primary cells derived from mice are too few. Hence, we turn to study the mouse mastocytoma cells P815, which also expressed P2 × 1, P2 × 4 and P2 × 7 receptors (data not shown). Data in Fig. 4B-E demonstrated that the release of inflammatory mediators was related to the concentration as well. Furthermore, ATP with high concentrations could induce the release of several inflammatory cytokines, such as IL-1β and CCL3. There was no significant cytokine release at low concentrations. AZ10606120, a specific P2 × 7 receptor antagonist (5 μM, pre-incubation for 5 minutes), almost completely inhibited the release of IL-1β and CCL3 induced by 1 mM ATP (Fig. 4F). Therefore, we concluded that the P2 × 7 receptor in mast cells could mediate mast cell degranulation accompanied the release of histamine. At the same time, the P2 × 7 receptor promoted de novo synthesis of inflammatory factors such as IL-1β and CCL3.

High concentration of ATP could induce peripheral pain in mice by activating P2 × 7 channel on mast cells.

Our experimental results indicated that P2 × 7 receptor had the functions of activating mast cells and releasing mediators, which might contribute to pain via neuro-immune interactions. Therefore, we assumed that mast cells and P2 × 7 receptor promoted the peripheral pain induced by high concentration ATP. In order to prove this hypothesis, we utilized the mast cell-deficient Kit (W-sh) Sash mutant mice and P2 × 7 receptor antagonist. Firstly, results showed that high concentration of ATP (100 mM) did induce paw swelling, pain behavior, inflammatory cells infiltration and mast cells degranulation at the same time (Fig. 5A,B). As our expected, Sash mutant mice could alleviate ATP-induced pain including paw swelling,

mechanical withdrawal threshold and the infiltration of inflammatory cells (Fig. 5A-D). To further explore the mechanism, we also tested the mediators related with mast cells for both of the two genotype mice. The RT-PCR data showed that the mRNA expression levels of IL-6 (8.13 ± 2.32, * $p < 0.05$, C57/BL-saline vs C57/BL-ATP), IL-1β (13.65 ± 2.39, * $p < 0.01$, C57/BL-saline vs C57/BL-ATP), CCL2 (6.81 ± 1.19, * $p < 0.01$, C57/BL-saline vs C57/BL-ATP) and CCL3 (9.17 ± 1.06, * $p < 0.01$, C57/BL-saline vs C57/BL-ATP) were significantly upregulated for the C57/BL mice. However, only IL-6 (7.11 ± 2.02, * $p < 0.05$, Sash-saline vs Sash-ATP) and CCL3 (5.15 ± 0.96, * $p < 0.05$, Sash-saline vs Sash-ATP) increased for the Sash mice. In comparison with the results for the C57/BL mice, the degree of upregulation of IL-1β (2.49 ± 0.49, $^{\#\#}$ $p < 0.05$, Sash-ATP vs C57/BL-ATP), CCL2 (2.74 ± 0.85, $^{\#}$ $p < 0.05$, Sash-ATP vs C57/BL-ATP) and CCL3 (5.15 ± 0.96, $^{\#}$ $p < 0.05$, Sash-ATP vs C57/BL-ATP) for the Sash mice was reduced (Fig. 5E). At the same time, we have also studied the function of P2 × 7 receptor in high concentration ATP-induced peripheral pain. Our experimental results showed that AZ10606120 (5 mg/kg, pre-administration 1 h) could significantly reduce the pain behavior and paw thickness at 4 h after ATP treatment.

P2 × 7 on mast cells is a potential target for salicylic acid and aspirin analgesia

Salicylic acid and its derivative aspirin are commonly used peripheral analgesic drugs. Although the analgesic effect is obvious, yet the target has not been clear. We found that salicylic acid and aspirin have the function of partially inhibiting the inward current generated by the P2 × 7 channel as Fig. 6A shown. Compared with the current amplitude induced by first ATP application, 300 μM salicylic acid (266.3 ± 31 pA vs 214 ± 27.96 pA, n = 21), 500 μM salicylic acid (321.4 ± 30.47 pA vs 248.4 ± 42.38 pA, n = 14), 1 mM salicylic acid (256.4 ± 18.57 pA vs 164.4 ± 22.88 pA, n = 16, * $p < 0.05$), 500 μM aspirin (229.4 ± 27.19 pA vs 220 ± 28.18 pA, n = 8) or 1 mM aspirin (323.7 ± 49.82 pA vs 287 ± 41.85 pA, n = 20) could slightly inhibit the current induced amplitude by 5 mM ATP (Fig. 6B-D, Fig. 6F-H). It should be noted that the current induced by 5 mM ATP had a “run-up” tendency as shown in Fig. 2H. This phenomenon indicated that the current growth rate should be studied. Our experimental data showed that the current growth rate of second ATP application was significantly inhibited by salicylic acid and aspirin. The growth rate of control was 0.8894 ± 0.281, the growth rate of 300 μM, 500 μM and 1 mM salicylic acid were − 0.1141 ± 0.1, -0.2197 ± 0.1138 and − 0.3218 ± 0.0948, respectively. The growth rate of 300 μM, 500 μM and 1 mM salicylic acid were 0.1627 ± 0.0917, 0.015 ± 0.1358 and − 0.023 ± 0.01, respectively (Fig. 6E). Furthermore, intercellular Ca^{2+} concentration assay results also showed that 300 μM salicylic acid and 1 mM aspirin could inhibit 5 mM ATP-induced calcium influx (Fig. 6F), which was consistent with the electrophysiological results. At the same time, we also explored the effects of drugs on the release of inflammatory mediators from mast cells. As expected, salicylic acid and aspirin blocked the up-regulation of IL-1β and CCL3 mediated by P2 × 7 receptor, especially IL-1β (Fig. 6G). Results from vonfrey as shown in Fig. 6H indicated that salicylic acid (50 mg/kg) and aspirin (50 mg/kg) could also alleviate peripheral pain induced by high concentration of ATP. These experimental results suggested that P2 × 7 on mast cells might be a potential target for salicylic acid and aspirin analgesia.

Discussion

It's well-known that P2X receptors such as P2 × 3, P2 × 4 and P2 × 7 in the nervous system were involved in the pathogenesis of neuropathic pain [3, 25]. Recently, accumulating evidence indicated that P2X receptors also exist in immune cells such as mast cells, macrophages, neutrophil and so on [9, 10, 17]. The P2R most involved in inflammation and immunity is the P2 × 7 receptor. P2 × 7 is widely expressed by different immune cells including monocytes, macrophages, neutrophils, lymphocytes, and mast cells. In neuroinflammatory and neurodegenerative diseases, P2 × 7 upregulation and function appears to contribute to disease progression [26]. P2 × 7R mediates NLRP3 inflammasome activation, cytokine and chemokine release, T lymphocyte survival and differentiation, transcription factor activation, and cell death [27]. The P2 × 7R promotes release of pro-inflammatory factors, such as IL-1β, IL-6, CCL2, TNFα and CCL3 [8–12]. Hence, P2 × 7R is an appealing target for anti-inflammatory therapy. However, the relationships between P2 × 7 receptor expressed in mast cells and the pathogenesis of pain are still not very clear. Our study aimed to explore the effects of extracellular ATP on the mouse-derived mast cells. At the same time, the functions of mast cells and P2 × 7 receptor in ATP-mediated peripheral pain also were explored.

Firstly, we found that several functional P2X receptors including ionotropic P2 × 1, P2 × 4 and P2 × 7 existed in mouse peritoneal mast cells. The expression profile was similar to that previously observed in mouse bone marrow mast cells, LAD2 and human lung mast cells. Furthermore, we have also explored the response of calcium ions to the extracellular ATP with different concentration. The results demonstrated that ATP could also activate mouse peritoneal mast cells in a concentration-dependent manner. The blocker results indicated that P2 × 1, P2 × 4 and P2 × 7 were involved in the calcium influx caused by 1 μM ATP, 100 μM ATP and high concentration ATP respectively. Interestingly, inhibitors of P2X receptor only partially inhibited the increase of concentration of the intracellular calcium. It indicated that this process also be associated with other receptors such as P2Y. For example, previous literatures have shown that besides P2 × 4 receptors, P2Y2 receptors could be stimulated by ATP with a concentration of 100 μM [19]. In addition, we also found P2 × 3 and P2 × 6 existed in mouse peritoneal mast cells. The function of P2 × 3 and P2 × 6 in mast cells is not clear, we will continue to study it in the future.

Just as other cell models, extracellular ATP provoked distinct inward currents in mouse peritoneal mast cells as well. P2 × 1 receptor was characterized by fast activation and desensitization [24]. Our results demonstrated the current induced by 1 μM ATP was closely resemble for P2 × 1-like current, which could be eliminated by non-selective P2 antagonists PPADS as well as P2 × 1-selective antagonist NF449 (Fig. 2A). In addition, the current evoked by 100 μM ATP could be blocked by PPADS and 5-BDBD (Fig. 2B), indicating that P2 × 4 receptor was involved. However, unlike LAD2 and HLMC [17], the P2 × 1-like and P2 × 4-like current in mouse peritoneal mast cells did not exhibit 'run-down' phenomenon upon repeated application. The reason for this may be due to the more rapid receptor internalization because of the heterogeneous of mast cells [28]. Our results showed that both 1 mM ATP and 5 mM ATP could induced P2 × 7-like currents with 'run-up' characteristic, which is in line with the conclusion reported by other literatures [17, 24]. Although there were some differences in the inward currents caused by 1 mM

ATP and 5 mM ATP, AZ10606120 could block both of two currents. Moreover, current induced by 1 mM ATP could not be affected by NF449 and 5-BDBD, hence, we concluded that P2 × 7 receptor was involved in the activation by 1 mM ATP and 5 mM ATP.

Compelling of researches pointed out several substances including histamine, tryptase, cytokines and chemokines released from mast cells could contribute to pain either directly or indirectly [15]. We also explored the mediators released from mast cells induced by different concentrations of ATP. The data showed that the higher concentration of ATP could induce histamine release, which was consistent with previous report [18, 19]. In addition to degranulation, extracellular ATP also promoted inflammatory mediators expression and secretion via trigging intracellular signaling cascades such as MAPKs. For instance, IL-1β, IL-6, CCL2, TNFα and CCL3 could be mediated by ATP in microglial cells, neutrophil and monocyte [8–12]. Our study demonstrated that high concentration ATP could up-regulate IL-1β and CCL3, which were almost completely inhibited by AZ10606120. These results indicated that P2 × 7 receptor mediated mouse-derived mast cells degranulation and release of inflammatory mediators.

Neuron-immune crosstalk plays important roles in many inflammatory diseases, we suggested that not only neurons directly but also mast cells indirectly participated in peripheral pain as demonstrated in Fig. 7. Histamine released from mast cells plays a critical role in neurogenic inflammation and pain transmission via specific receptors in a bidirectional manner [15, 29]. What's more, increased levels of IL-1β and IL-6 were found in inflammation-associated pain such as migraine, complex regional pain syndrome and fibromyalgia [30]. IL-1β had been studied clearly in the context of neuropathic pain. IL-1β can modulate neuronal activity directly, in addition, IL-1β can also mediate pain sensitization via IL-1 receptor. Blocking spinal IL-1 signaling could alleviate neuropathic pain as expect [31, 32]. Furthermore, the roles of inflammatory chemokines could regulate synaptic transmission, especially CCL2/CCR2 and CCL3/CCR1 signaling [33, 34]. The behavioral results further confirmed the important role of mast cells in high concentration ATP-mediated peripheral pain. Of note, compared to C57/BL mice, the percentage of withdrawal in Sash is higher, which might result from the aberrant immune cells because of c-Kit mutation [35]. Moreover, in our study, P2 × 7 receptor was confirmed to be involved in high concentration ATP-induced peripheral pain. Interestingly, peripheral analgesic drugs salicylate acid and aspirin could inhibit the calcium influx, inward current, release of inflammatory factors and peripheral pain induced by high concentration ATP.

Conclusion

In summary, we found that mouse-derived mast cells could be activated by extracellular ATP via P2 × 1, P2 × 4 and P2 × 7 receptors. Furthermore, mast cells and P2 × 7 receptor on mast cells played important roles in peripheral pain caused by high concentration ATP. In addition, we also found that peripheral P2 × 7 receptor may be a potential target for analgesic drugs salicylate acid and aspirin.

Abbreviations

ATP: Adenosine triphosphate

SCF: Stem cell growth factor

IL-6: Interleukin 6

IL-1β: Interleukin 1β

SA: Salicylic acid

ASA: Acetylsalicylic acid

Declarations

Ethics approval and consent to participate

All procedures were performed under protocols approved by the Animal Care and Use Committee of Nanjing University of Chinese Medicine (Approval no. A190401). This article does not contain any studies with human participants performed by any of the authors.

Consent for publication

Not applicable.

Availability of data and materials

The datasets used and analyzed during the current study are available from the corresponding author on reasonable request.

Competing interests

The authors declare no conflicts of interest.

Funding

This work was supported by the Project of National Natural Science Foundation of China (No. 31771163).

Author contributions

Zongxiang Tang designed and wrote the paper, Yucui Jiang, Fan Ye and Ying Du performed experiments, analyzed the data, and wrote part of the paper. All authors read and approved the final manuscript.

Acknowledgments

The authors thank Prof. Dong in Johns Hopkins for kindly providing the Mast cell-deficient Kit (W-sh) "Sash" mutant mice.

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Figures

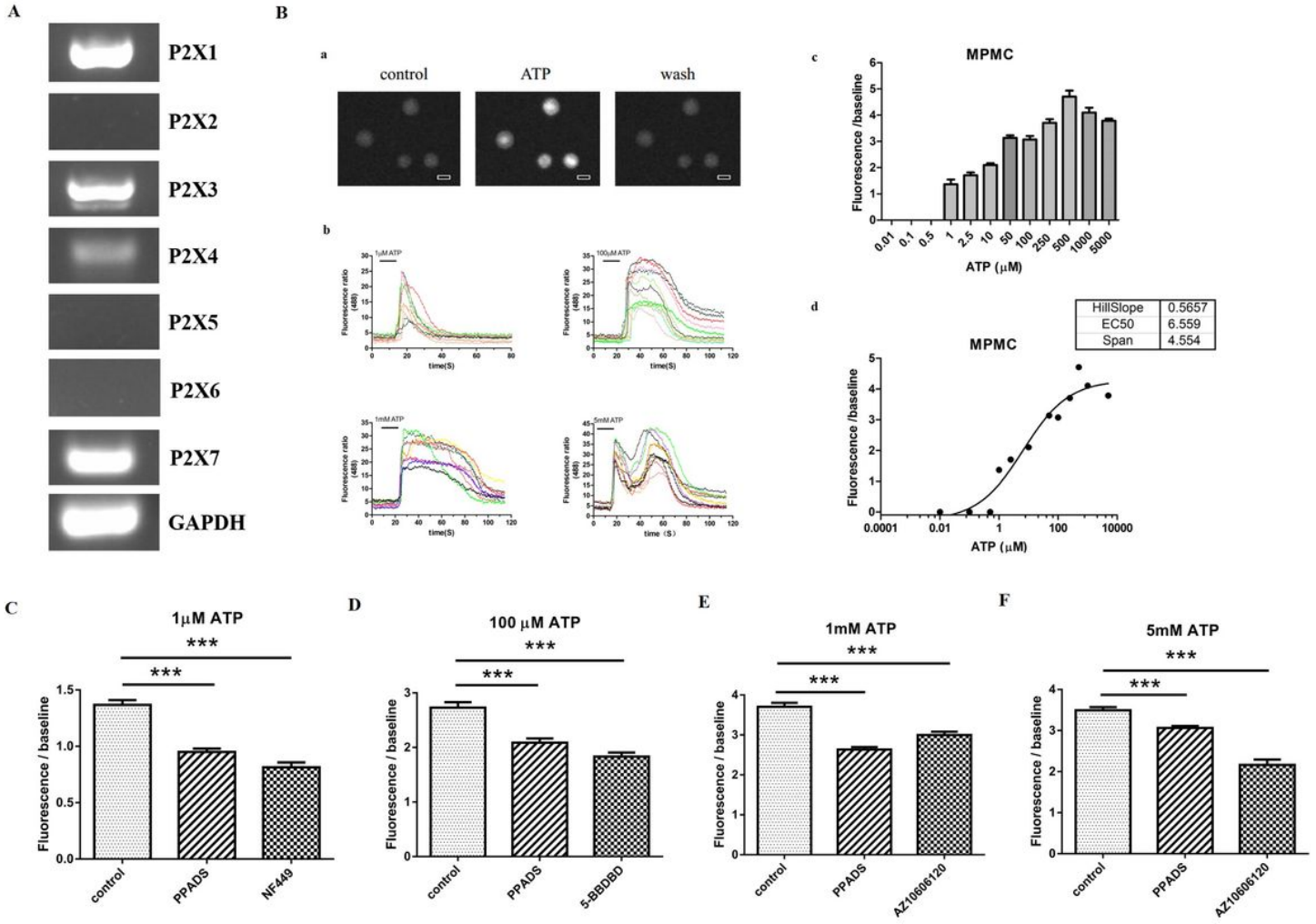

Figure 1

P2X receptors expression and ATP-induced calcium response in mouse peritoneal mast cells. (A) Expression of P2X1, P2X4 and P2X7 receptors in mouse peritoneal mast cells. (B) Calcium influx mediated by extracellular ATP. (a) Representative heat map images showing the changes of calcium ions induced by application of ATP. Scale bar is 10 μm. (b) Calcium imaging traces with different concentrations of ATP. (c-d) The quantification of fluorescence intensity, EC50 was about 6.5 μM. (C) The calcium influx induced by 1 μM ATP was reduced by PPADS or NF449 (*** $p < 0.001$, control vs PPADS; *** $p < 0.001$, control vs NF449). (D) The calcium influx induced by 100 μM ATP was blocked by PPADS or 5-BDBD (*** $p < 0.001$, control vs PPADS; *** $p < 0.001$, control vs 5-BDBD). (E) The calcium influx induced by 1 mM ATP was reduced by PPADS or AZ10606120 (*** $p < 0.001$, control vs PPADS; *** $p < 0.001$, control vs AZ10606120). (F) The calcium influx induced by 1 mM ATP was blocked by PPADS or AZ10606120 (*** $p < 0.001$, control vs PPADS; *** $p < 0.001$, control vs AZ10606120).

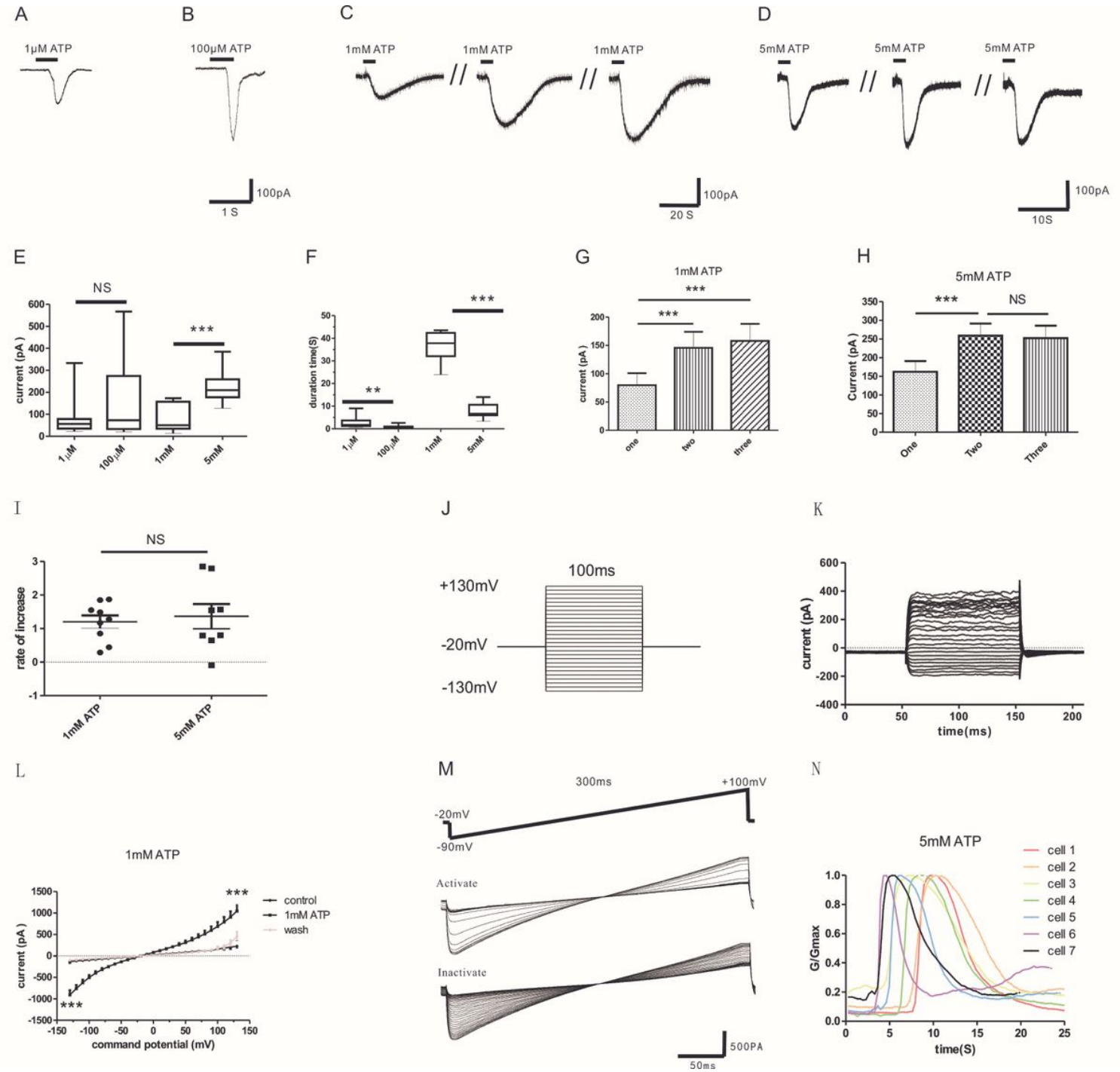

Figure 2

The inward currents evoked by ATP in mouse peritoneal mast cells recorded by the whole cell patch clamp. (A-D) Different types of currents induced by different concentrations of ATP (1 μM, 100 μM, 1mM and 5mM ATP respectively). (E) The amplitude of inward currents. There is no obvious difference in current amplitude for 1 μM and 100 μM ATP ($p=0.076$, 1 μM ATP vs 100 μM ATP, n=17 or 16 respectively), but the current amplitude were different between 1mM ATP and 5 mM ATP (*** $p < 0.001$, 1mM ATP vs 5 mM ATP, n=9 or 16 respectively). (F) The time duration of inward currents. There is an obvious difference in time duration for 1 μM and 100 μM ATP (** $p < 0.01$, 1 μM ATP vs 100 μM ATP, n=17 or 16 respectively), the current amplitude were also different between 1mM ATP and 5 mM ATP (*** $p < 0.001$,

1mM ATP vs 5 mM ATP, n=9 or 16 respectively). (G, H) The “run-up” tendency for 1 mM ATP and 5 mM ATP. (I) There was no significant difference in the second current growth rate with 1 mM ATP and 5 mM ATP respectively (p= , 1mM ATP vs 5 mM ATP, n=9 or 16 respectively). (J-L) The relationship between the voltage and the current induced by 1mM ATP. (M) The activate curves and inactivate curves induced by 5 mM ATP. (N) The conductance curve of 5 mM ATP. * p < 0.05, ** p < 0.01, *** p < 0.001.

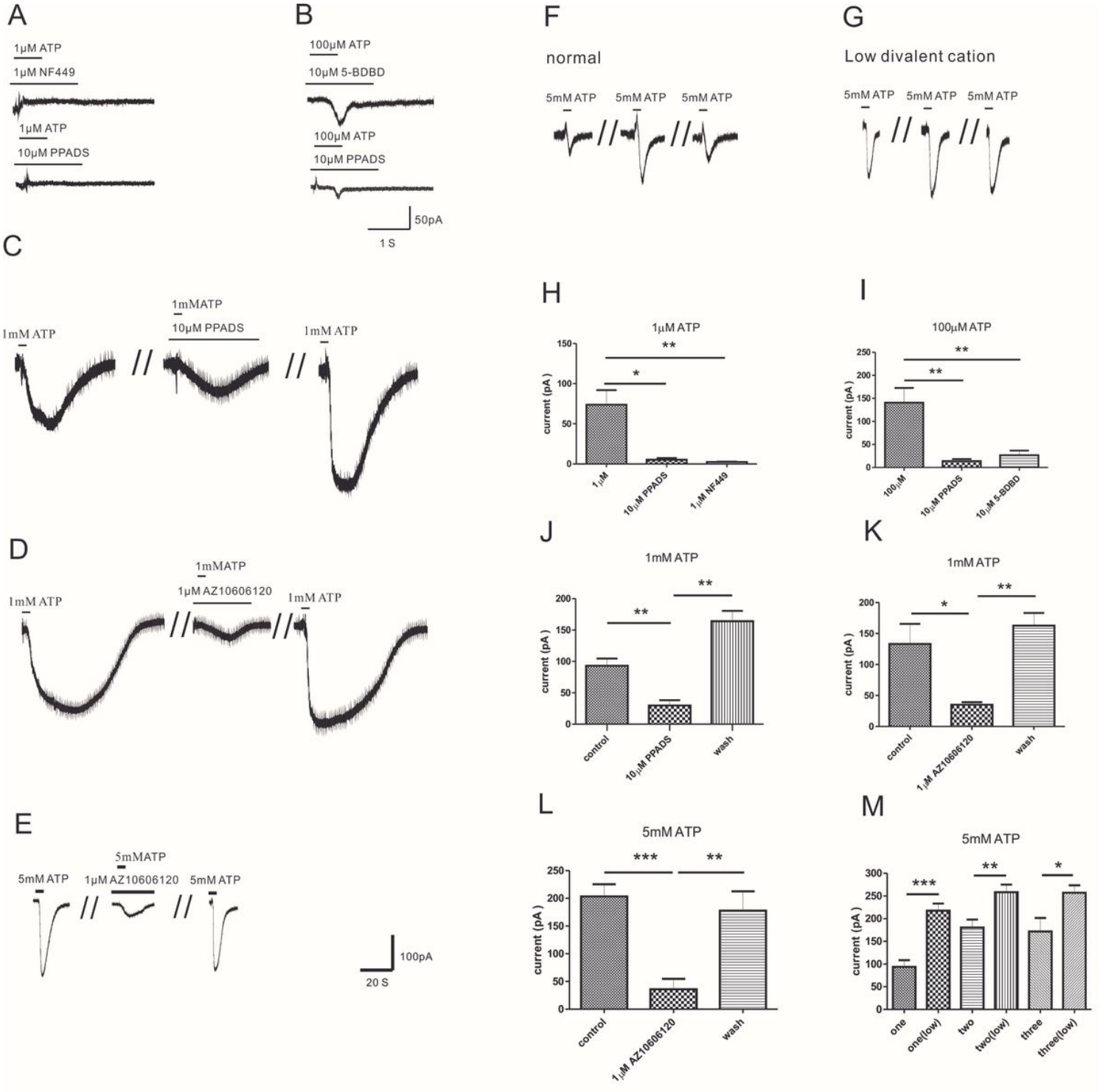

Figure 3

The effects of different blockers on the currents activated by extracellular ATP. (A, H) Cell with 10 μM PPADS and 1 μM NF449 almost impossible to induce current by 1 μM ATP treatment (* $p < 0.05$, 1 μM ATP vs 10 μM PPADS, n=13) (** $p < 0.01$, 1 μM ATP vs 1 μM NF449, n=9). (B, I) The current induced by 100 μM ATP could be blocked by 10 μM PPADS and 1 μM 5-BDBD (** $p < 0.01$, 100 μM ATP vs 10 μM PPADS, n=15) (*** $p < 0.001$, 100 μM ATP vs 1 μM 5-BDBD, n=13). (C, D, J, K) The current induced by 1mM ATP could be blocked by 10 μM PPADS or 1 μM AZ10606120 (** $p < 0.01$, 1mM ATP vs 10 μM PPADS, n=4) (* $p < 0.05$, 1mM ATP vs 1 μM AZ10606120, n=5). (E, L) 1 μM AZ10606120 could inhibit the current evoked by 5 mM ATP (*** $p < 0.001$, 5mM ATP vs 1 μM AZ10606120, n=6). (F, G, M) The current amplitude induced by 5mM ATP in the external solution of low divalent cation was greater than that in the normal external solution. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

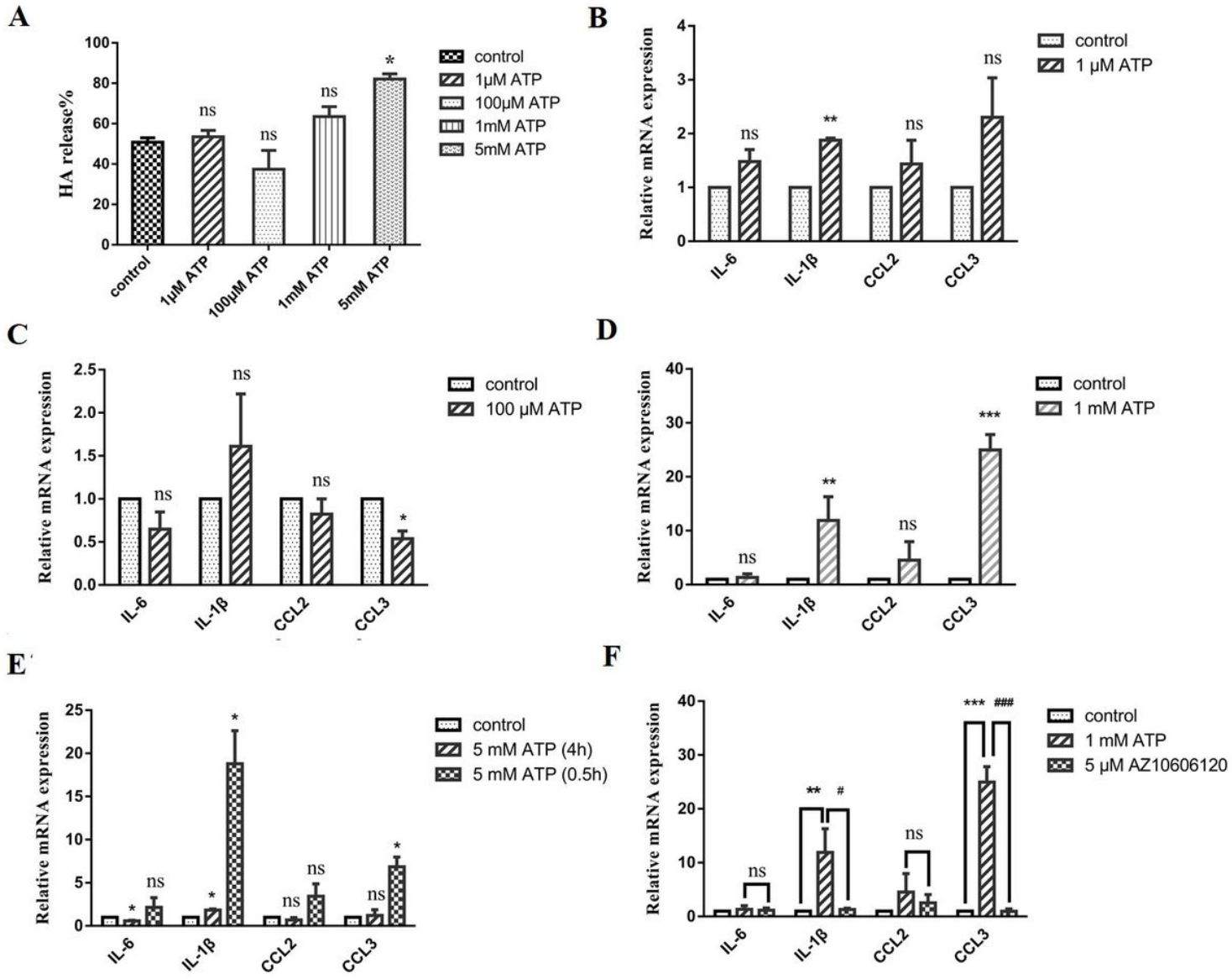

Figure 4

The release of several mediators induced by different concentration of extracellular ATP. (A) Histamine release significantly increased by adopting high concentrations of ATP (* $p < 0.05$, control vs 5 mM ATP).

(B-E) The relative mRNA expression levels of IL-1β, IL-6, CCL2 and CCL3 were regulated by different concentrations of ATP. After 4 hours of ATP treatment, the mRNA expression levels were not or slightly up-regulated induced by 1 μM ATP and 100 μM ATP (C), the mRNA expression levels of IL-1β and CCL3 were up-regulated significantly induced by 1 mM ATP (D) 5 mM ATP (E). (F) The up-regulation of IL-1β and CCL3 caused by 1 mM ATP was blocked by specific P2X7 receptor antagonist AZ10606120. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$; # $p < 0.05$, ## $p < 0.01$, ###$p < 0.001$.

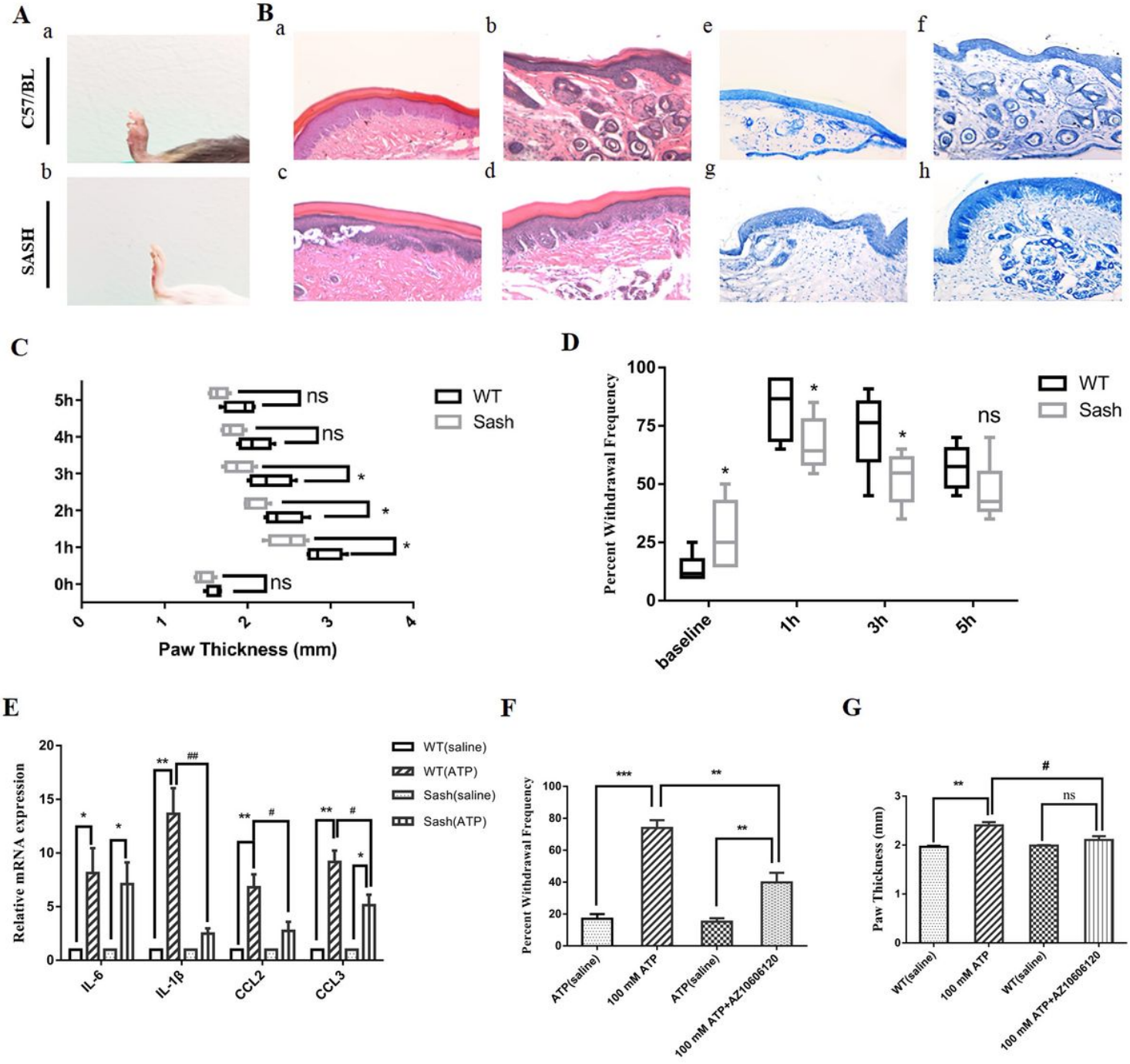

Figure 5

Mast cell deficient and AZ10606120 alleviated high concentration ATP-induced pain. (A-F) Compared with C57/BL mice, sash mice alleviated the paw swelling (A), inflammatory cells infiltration (B) (a-b, HE

staining of saline and ATP groups in C57/BL mice; c-d, HE staining of saline and ATP groups in Sash mice; e-f, toluidine blue staining of saline and ATP groups in C57/BL mice; g-h, toluidine blue staining of saline and ATP groups in Sash mice, 200X), paw thickness (C) (* $p < 0.05$, WT group vs Sash group, n=8 and 4 respectively), mechanical withdrawal threshold (D) (* $p < 0.05$, WT group vs Sash group, n=8 and 4 respectively), the mRNA expression upregulation of IL-1β, CCL2 and CCL3 (E) (n=4). (F-G) AZ10606120 significantly relieved pain behavior (G) and paw swelling (H) (n=6). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$; # $p < 0.05$, ## $p < 0.01$, ###$p < 0.001$.

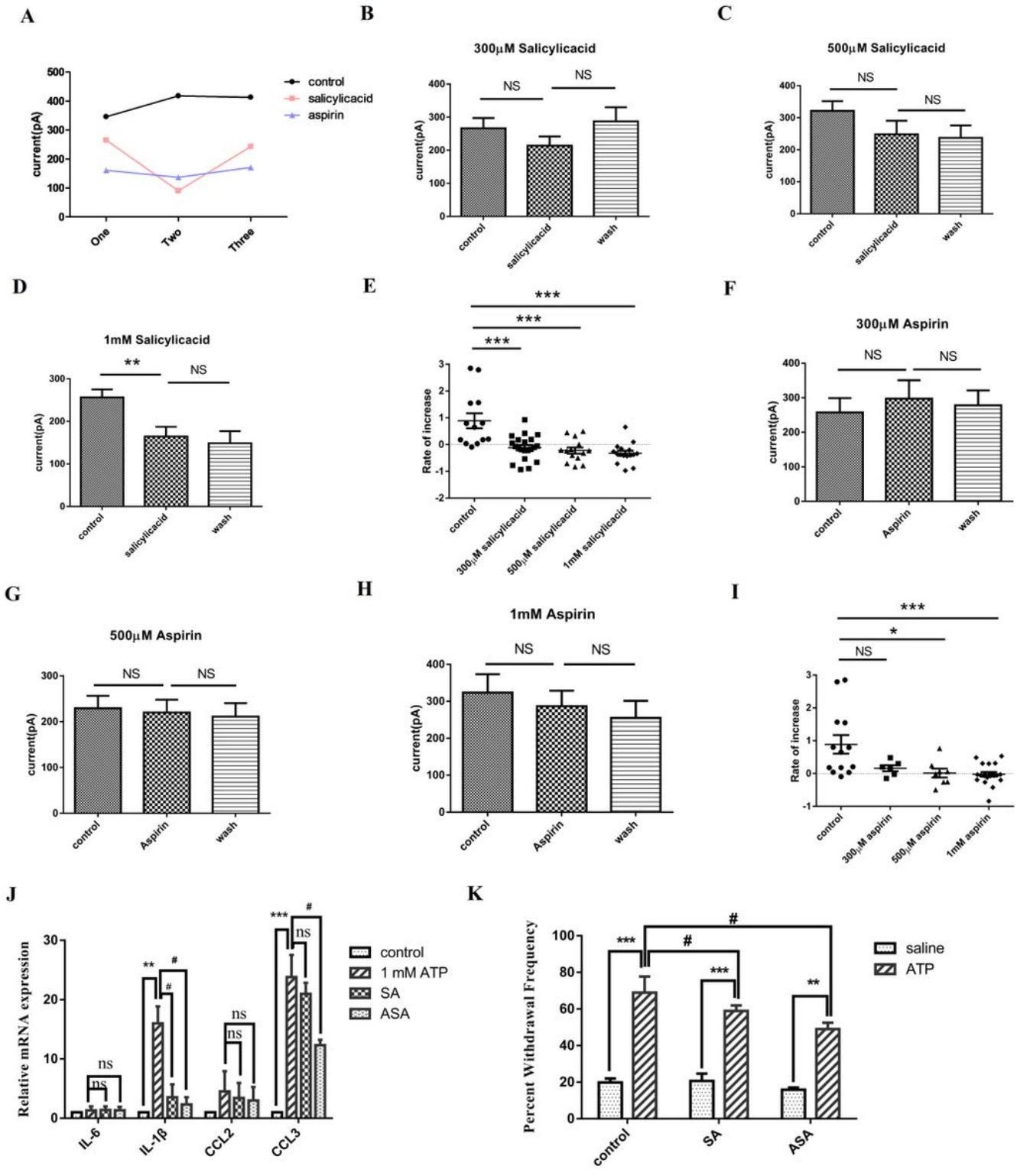

Figure 6

The effects of salicylic acid and its derivative aspirin on P2X7 receptor on mast cells. (A-E) Salicylic acid and aspirin inhibited the current induced by 5 mM ATP (A). 300 μM salicylic acid (B), 500 μM salicylic acid (C) and 1 mM aspirin (D) slightly inhibited the current induced by second ATP application compared with that induced by the first ATP application. The current growth rate of the second ATP application was significantly inhibited by different concentrations of salicylic acid and aspirin (E) (*** $p < 0.001$, control vs). (F) 5mM ATP-induced calcium influx was inhibited by 300 μM salicylic acid and 1 mM aspirin. (G) Salicylic acid and aspirin blocked the up-regulation of cytokines mediated by P2X7 receptor. (H) Salicylic acid and aspirin alleviate peripheral pain induced by ATP with high concentration. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$; # $p < 0.05$, ## $p < 0.01$, ###$p < 0.001$.

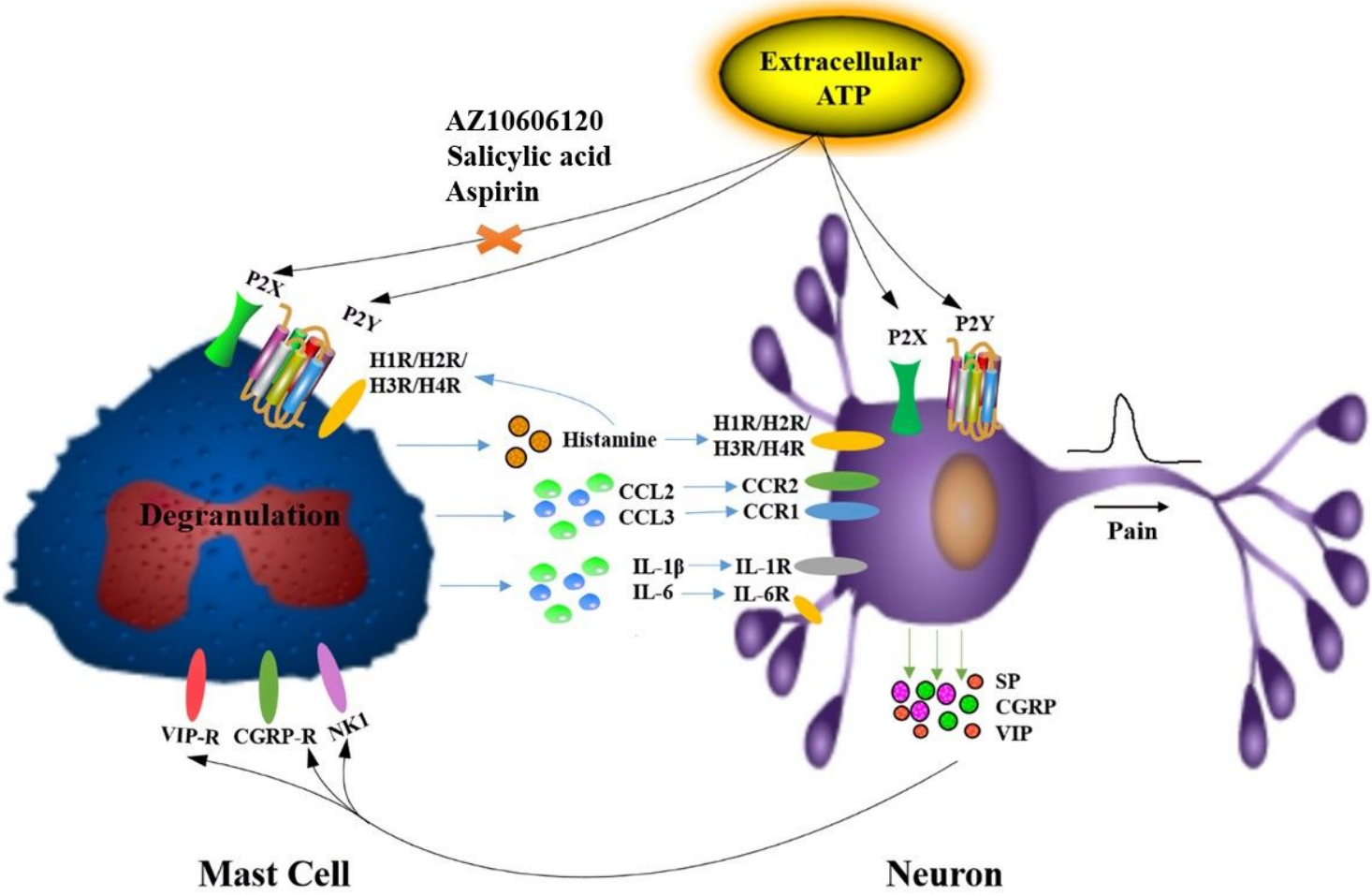

Figure 7

The mechanism of pain induced by extracellular ATP via neuron-immune crosstalk.