P2X7 on mast cells participates in peripheral pain and serves as a potential target for salicylic acid and aspirin analgesia

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Abstract

Background Extracellular ATP signaling through excitatory and calcium-permeable P2X receptor channels is considered as a critical player in pain generation and maintenance. P2X7 has attracted much attention over the past decade because of its prominent role in driving inflammatory processes. The role of P2X7 in mast cells in peripheral pain remains unclear.

Methods P2X expression in mouse peritoneal mast cells was detected by RT-PCR. The subtypes of P2X receptors in mouse peritoneal mast cells were determined with a series of blockers by using calcium imaging and electrophysiology. The regulation of inflammatory factors mediated by different P2X subtypes were detected by ELISA and real-time PCR. The role of mast cells and P2X7 receptor in peripheral pain was explored by behavioral assays, pathological analysis and real-time PCR. Several anti-inflammatory small molecules were screened based on P2X7 in mast cells by using calcium imaging, electrophysiology and molecular docking.

Results We found that ATP was significantly increased in inflammatory pain. Mouse peritoneal mast cells expressed P2X1, P2X3, P2X4 and P2X7 and could be activated by different concentrations of extracellular ATP, which could be blocked by specific ion channel antagonists. In particular, high concentration of ATP could induce mast cells to release inflammatory mediators such as histamine, IL-1 β and CCL3 through P2X7 receptor. Furthermore, peripheral pain induced by high concentration of ATP could be alleviated by P2X7 blockers or mast cell defects. Interestingly, salicylic acid and aspirin could attenuate the inward current, the release of inflammatory factors and peripheral pain induced by ATP with high concentration. Furthermore, salicylic acid and aspirin also inhibited the inward current evoked by P2X7 agonist BZATP. Molecular docking results showed that salicylic acid and aspirin had affinity to the cytoplasmic GDP-binding region of P2X7.

Conclusions We concluded that P2X7 on mast cells involved in peripheral pain. Salicylic acid and aspirin could inhibit the activity of P2X7 via interacting with the GDP binding region. P2X7 receptor was a potential target for salicylic acid and aspirin analgesia.

Background

Extracellular ATP is a “signal of danger” mediated by P2 purinergic receptors. P2 receptors have two types, P2X (P2X1-7) and P2Y receptors [1]. P2X receptors carry out many important functions in the central and peripheral nervous system [2]. Compelling evidences have shown that P2X3, P2X4 and P2X7 receptors are involved in the pathogenesis of chronic pain [3]. P2X7 exists in neurons and glial cells of nervous, but is mainly expressed in cells of immune origin [4]. The absence of P2X7 receptor can completely eliminate the inflammatory and neuropathic hypersensitivity to both mechanical and temperature stimulation [5, 6]. P2X7 receptor of microglia also plays an important role in chronic neuropathy and inflammatory pain by releasing IL-1 β [7]. Besides IL-1 β , cytokines such as IL-6, CCL2,

TNF α and CCL3 are also mediated by P2X7 receptor in neutrophil and monocyte [8–12]. Hence, P2X7 receptor represents a promising target for pain intervention.

Mast cells are in close proximity to afferents and establishing dynamic interactions with pain-activating nociceptors [13]. Performed and newly synthesized mediators released from mast cells 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 (Fc ϵ RI). Mast cells are also activated by a wide variety of triggers, such as substance P, lipid mediators and interleukins [16]. Extracellular ATP also can stimulate mast cells and induce different inward currents through P2X receptors [17]. Although activation of P2X4 can augment the degranulation mediated by the Fc ϵ RI in mouse bone marrow-derived mast cells, only P2X7 contributes to degranulation in human mast cell line LAD2 [18, 19]. As yet, the relationship between P2X7 receptor on mast cell and pain remains unclear.

Chronic pain is a global problem affecting more than two-thirds of the population. Extensive research has been conducted to find appropriate methods of relieving pain and improving the quality of life. Salicylic acid, an extract of willow bark, has been used for pain management as early as 4,000 years ago. Aspirin, derived from salicylic acid, is a common peripheral analgesic drug via inhibiting cyclooxygenase-1 (COX-1). The analgesic effect of salicylates is apparently, however, the targets are still not entirely clear. Previous literatures indicate that salicylates are associated with mast cells. Salicylic acid can 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 controversial. There are some evidences show that the anaphylaxis triggered by aspirin is due to eosinophils and mast cells [21]. High concentrations of aspirin such as 10 mM can activate mast cells directly [22], but the effect of normal concentrations of aspirin on mast cells remains unclear.

In this study, we evaluated the roles of several P2X receptor subtypes on mast cells, especially the P2X7 receptor. In addition, we also explored the relationship between salicylates and P2X7 channel.

Materials And Methods

Ethics statement

All procedures were performed under protocols approved by the Animal Care and Use Committee of Nanjing University of Chinese Medicine.

This article does not contain any studies with human participants performed by any of the authors.

Animals

Adult male mice used were 22–28 g males in a C57BL/6 background (Qinglongshan, China). Animals were housed at constant humidity (40–60%) and temperature ($22 \pm 2^\circ\text{C}$) 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 and MrgprB2Cre^{Td/tomato} mice were donated by Johns Hopkins.

Chemicals

ATP (Sigma-Aldrich, St. Louis, MO, United States), PPADS (20 μ M, Abcam, USA, a non-selective P2 purinergic receptor antagonist), NF449 (1 μ M, Cayman, USA, P2X1 receptor antagonist), AF-353 (0.1 μ M, donated by China Pharmaceutical University, P2X3 receptor antagonist), 5-BDBD (1 μ M, Sigma-Aldrich, United States, P2X4 receptor antagonist), AZ10606120 (1 μ M, Tocris Bioscience, USA, P2X7 receptor antagonist), BzATP (30 μ M, Alomone Labs, Israel, P2X7 receptor agonist), recombinant mouse SCF protein (10 ng/mL, R&D Systems, USA), penicillin and streptomycin (100 μ g/mL; Gibco, USA), fibronectin (30 μ g/mL; Sigma-Aldrich, United States), Fluo 4-AM (Solarbio, China, calcium indicator), Histamine ELISA Kit (Yifeixue, China), Trizol (Vazyme Biotech, China), HiScript II Q RT SuperMix for qPCR (Vazyme Biotech, China), Taq MasterMix (Vazyme Biotech, China), AceQ qPCR SYBR Green Master Mix (Vazyme Biotech, China), ATP Content Assay Kit (Yifeixue, China), salicylic acid (Yuanye Biotech, China), aspirin (Yuanye Biotech, China).

ATP measurement

ATP was measured through an ATP Content Assay Kit (Yifeixue, China) following manufacturer's instructions. The paw tissue (about 10 mg) homogenate was centrifuged at 8000g for 10 min at 4°C, and the supernatant was collected. Then treated with chloroform, and the supernatant was collected for ATP content detection.

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 μ g/ml penicillin and 100 μ g/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 Dong's Lab 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. As supplementary Fig.S1 shown, the extracted mouse peritoneal mast cell was identified by toluidine blue staining, and the purity was about 90%.

P2X purinoceptors RT-PCR screen

Trizol method was used to isolate total RNA from mouse peritoneal mast cells or the mouse mastocytoma cells. 10–500 ng RNA was used for reverse transcription reaction by using HiScript II Q RT SuperMix for qPCR Kit according to the manufacturer's instructions. Polymerase chain reaction conditions were as follows: 95°C for 5 min, 40 cycles of 15 s at 95°C, 30 s at 60°C, and 1 min at 72°C, and 10 min at 72°C. 10 μ L 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 Genescript 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
P2X1	Forward: GCCCAAGGTATTCGCACAGG	496 bp
	Reverse: GACGACGGTTTGTCCCATTCT	
P2X2	Forward: ACCTGCCATTTAGATGACGACTG	241 bp
	Reverse: TGTTGCCCTTGGAGAACTTGA	
P2X3	Forward: GCTTCGGACGCTATGCCAACA	490 bp
	Reverse: AAATCCTGCCCAGCAAACCTTAA	
P2X4	Forward: GTGCTCGGGTCCTTCCTGTTC	154 bp
	Reverse: CCGTTTCCTGGTAGCCCTTTT	
P2X5	Forward: TGTAGCGGGACACGGACTGA	209 bp
	Reverse: TTTCTAGCACATTGGCTTTGGA	
P2X6	Forward: GGTACAACCTTCAGGACAGCCAATC	207 bp
	Reverse: CATACAGTAGCAGCAGGTCACAGAG	
P2X7	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. After two hours of incubation, mast cells were incubated by 1 µL/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 CaCl₂, 0.6 mM MgCl₂, 20 mM glucose, 10 mM HEPES, 20 mM sucrose, 1.2 mM NaHCO₃, pH 7.4). Finally, cells were imaged at 488-nm excitation to detect intracellular free calcium within two hours. Each experiment was done at least three times, and at least 200 cells were analyzed each time.

Whole-cell current-clamp recordings of mouse peritoneal mast cells

The patch pipettes typically had a resistance of 4–6 MΩ, and the 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, 2 mM Mg-ATP (added when detecting P2X7 channel), PH 7.3 (adjusted by CsOH). The standard external solution contained 147 mM NaCl, 2 mM KCl, 2 mM CaCl_2 , 1 mM $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$, 10 mM HEPES, 16 mM glucose, pH 7.3 (adjusted by NaOH). 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 or 0.5 hours (5 mM ATP), tissues (10–50 mg) isolated from paw treated with 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-1 β	Forward: AAATCTCGCAGCAGCACATC
	Reverse: AGCAGGTTATCATCATCATCCC
CCL2	Forward: GGCCTGCTGTTACAGTTGC
	Reverse: CAGAAGTGCTTGAGGTGGTTG
CCL3	Forward: GCTCCCAGCCAGGTGTCATT
	Reverse: CAGGCATTCAAGTCCAGGTCAG
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 μ M, 100 μ M, 1 mM and 5mM), and the supernatants were harvested at time point of 0.5 h and stored at 80°C until used for ELISA.

Behavioral assays

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 30min. 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 at least 10 times at a specific force (0.16g) manually.

Histological

The paw skin was isolated and washed, and then fixed with 4% paraformaldehyde for 24 h, and then treated with 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.

Molecular docking

The discovery Studio 2016 4.0 software was used to verify the molecular docking. We downloaded the three-dimensional structure of salicylic acid (PubChem CID: 338) and aspirin (PubChem CID: 2244) from

the NCBI PubChem Compound database (<http://www.ncbi.nlm.nih.gov/pccompound>) and the monomeric crystal structure of P2X7 (PDB ID: 6U9V) from the Research Collaboratory for Structure Bioinformatics (RCSB) Protein Data Bank (<http://www.rcsb.org/pdb>).

Quantification and statistical analysis

The data were analyzed by GraphPad 6.0 and presented as mean $\pm$ 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 or paired Student's T-test or ANOVA analysis.

Results

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

Upon for instance tissue stress, hypoxia or inflammation, ATP is released from cells into the extracellular environment leading to the increase in extracellular ATP. Our results also showed that ATP was significantly increased in inflammatory pain such as CFA ($0.4913 \pm 0.04 \mu\text{mol/g}$ vs $1.116 \pm 0.09 \mu\text{mol/g}$, *** $p < 0.001$, Supplementary Fig. S2). Because mast cells play important roles in the pathological process of pain, we speculate that P2X receptors on mast cells involved in this function.

First, to identify P2X receptors on mast cell, we explored the P2X expression in mouse peritoneal mast cells by RT-PCR screen. As shown in Fig. 1A, we found that mouse peritoneal mast cells expressed several different ionotropic P2X receptors including P2X1, P2X3, P2X4 and P2X7. P2X receptor is a non-selective cation channel, the most obvious permeability to Ca^{2+} . Hence, we examined the calcium influx in mouse peritoneal mast cells induced by ATP. The results showed that there were transient increases of intracellular calcium in mast cells treated by different concentrations of ATP (Fig. 1B, C). The ratios of fluorescence intensity were varied with different ATP concentrations, and EC₅₀ was about $6.5 \mu\text{M}$ (Fig. 1C). In addition to fluorescence intensity, the reaction durations also existed differences (Fig. 1B), which indicated that different concentrations of ATP could activate mast cells through different P2X ionotropic receptors.

To confirm that, we used special P2X channel antagonists. As shown in Fig. 1D-H, the calcium influx caused by different concentrations of ATP could be partially blocked by a non-selective P2 purinergic receptor antagonist PPADS ($20 \mu\text{M}$, pre-incubation for 5 minutes). In addition, calcium influx caused by $1 \mu\text{M}$ ATP was inhibited by P2X1 receptor antagonist NF449 ($1 \mu\text{M}$, pre-incubation for 5 minutes) (Fig. 1D). AF-353 (P2X3 receptor antagonist, $0.1 \mu\text{M}$, pre-incubation for 5 minutes) could reduce the calcium influx caused by $10 \mu\text{M}$ ATP (Fig. 1E). And the transient increase of the intracellular calcium induced by $100 \mu\text{M}$ ATP was blocked by 5-BDBD ($1 \mu\text{M}$, pre-incubation for 5 minutes) (Fig. 1F), which is a specific P2X4 receptor antagonist. Furthermore, the specific P2X7 receptor antagonist AZ10606120 ($1 \mu\text{M}$, 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 (Fig. 1G, H). These results indicated that P2X1, P2X3, P2X4 and P2X7 might contribute to the activation of mouse peritoneal 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]. Our experimental results proved that mouse peritoneal mast cells had the same characteristics. About 85% of mast cells were sensitive to 1 μ M ATP (Fig. 2A, n = 17). The inward current could also be induced by 100 μ M ATP (Fig. 2B, n = 21). When we increased the concentration of extracellular ATP to a high level, we found that both 1mM and 5 mM ATP had the ability to induce the inward currents repeatedly (Fig. 2C, n = 9; Fig. 2D, n = 8). As Fig. 2E, F shown, the current characteristics evoked by various concentrations of extracellular ATP were different from each other, including the amplitude of the inward current and the current durations. Although the current amplitude as well as the duration were different between 1mM ATP and 5 mM ATP, the inward currents had some similar characteristics such as “run-up” tendency (Fig. 2G, H). The current growth rate of second ATP application had no difference as Fig. 2I shown. The current growth rate is defined as. Furthermore, the inward current evoked by 1mM ATP was voltage-dependent (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 P2X1, P2X3, P2X4 and P2X7 antagonist

Electrophysiological results showed that there were different currents corresponding to ATP with different concentrations. Therefore, we speculated that several P2X receptor subtypes contributed to the activation progress. As Wareham described, P2X1, P2X4 and P2X7 receptors in LAD2 were activated by 1 μ M ATP, 100 μ M ATP and high concentrations of ATP, respectively [17]. In our study, we also found that 1 μ M ATP hardly induced inward current when 20 μ M PPADS (a non-selective P2 antagonist) (Fig. 3A, G, n = 9) or 1 μ M NF449 (the blocker of P2X1 receptor) (Fig. 3A, G, n = 13) was applied, which indicated that P2X1 was activated by 1 μ M ATP. As Fig. 3B, H shown, 20 μ M PPADS (n = 9) or 0.1 μ M AF-353 (the blocker of P2X3 receptor, n = 10) could reduce the current evoked by 10 μ M ATP, which indicated that P2X3 was activated by 10 μ M ATP. In addition, P2X4 was involved in the current induced by 100 μ M ATP, which was blocked by 1 μ M 5-BDBD (Fig. 3C, I, n = 10) or 20 μ M PPADS (Fig. 3C, I, n = 13). The current caused by 1mM ATP were inhibited by using 1 μ M AZ 10606120 (the blocker of P2X7 receptor) (Fig. 3D, J, n = 5). The current induced by 5 mM ATP could also be inhibited by AZ 10606120 (Fig. 3E, K, n = 6). These results demonstrated that P2X7 receptor was activated by high concentrations of ATP such as 1 mM and 5 mM. Interestingly, we found that 20 μ M PPADS could partially inhibit the current induced by 1 mM ATP (Supplementary Fig. S3A, S3C), but had no effect on the current evoked by 5 mM ATP (Supplementary Fig. S3B, S3D), which indicated that PPADS might have limited inhibitory effect on P2X7. Furthermore, P2X7 receptor was sensitive to divalent cation as North described [24]. Consequently, our results illustrated that the inward current induced by 5mM ATP in the low divalent cation was greater than that in normal external solution (Fig. 3F, L), which confirmed that the existence of P2X7 in mouse peritoneal

mast cells. Taken together, results confirmed that P2X1, P2X3, P2X4 and P2X7 expressed in mouse peritoneal mast cells and involved in the progress activation induced by extracellular ATP.

Activation of P2X7 receptor on mouse-derived mast cell could lead to degranulation and de novo synthesis of 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 μ M ATP and 100 μ M ATP. However, histamine released from mouse peritoneal mast cells was significantly increased at higher concentrations of ATP (Fig. 4A). Besides histamine, mast cells are effective producers of inflammatory cytokines in response to various stimuli. According to previous literatures, cytokines secretion especially IL-1 β induced by the extracellular ATP has been widely studied in different immune cells [8–12]. However, the release of cytokines from mast cells induced by extracellular ATP remains unclear. To examine the potential mediator release in mast cells, we detected a series of mediators such as IL-6, IL-1 β , CCL2 and CCL3. Due to the mouse peritoneal mast cells are too few to detect, we turned to mouse mastocytoma cells P815, which also expressed P2X1, P2X3, P2X4 and P2X7 receptors (Supplementary Fig. S4). Data in Fig. 4B-H demonstrated that the regulation of inflammatory mediators was related to the concentration as well. There was no significant change or up-regulated slightly in the expression of cytokines when treated with low concentration of ATP (Fig. 4B-D). ATP with high concentrations could significantly up-regulate the expression of a variety of inflammatory cytokines, such as IL-1 β and CCL3 (Fig. 4E, G). In addition, we found that cytokines were regulated slightly after treatment with 5mM ATP for 4 hours, but IL-1 β and CCL3 were significantly up-regulated after treatment for 0.5 hours, which might be related with the negative feedback caused by high ATP concentration (Fig. 4F, G). AZ10606120, a specific P2X7 receptor antagonist (5 μ M, pre-incubation for 5 minutes), almost completely inhibited the upregulation of IL-1 β and CCL3 induced by 1mM ATP (Fig. 4H). Therefore, we concluded that P2X7 receptor on mast cells could mediate mast cell degranulation and de novo synthesis of inflammatory factors such as IL-1 β and CCL3.

High concentration of ATP could induce peripheral pain in mice by activating P2X7 channel on mast cells

Our experimental results indicated that P2X7 receptor on mast cell had the function of releasing inflammatory mediators, which might contribute to pain via neuro-immune interactions. We assumed that mast cells and P2X7 receptor promoted the peripheral pain induced by high concentration of ATP. In order to prove this hypothesis, we utilized the mast cell-deficient Kit (W-sh) Sash mutant mice and P2X7 receptor antagonist. Firstly, results showed that high concentration of ATP (100 mM, 20 μ L, iH) did induce paw swelling (Fig. 5A, C), inflammatory cells infiltration (Fig. 5B), mast cells degranulation (Fig. 5D) and mechanical hyperalgesia (Fig. 5E). As our expected, mast cell-deficient mice could alleviate ATP-induced pain including paw swelling, mechanical withdrawal threshold and the infiltration of inflammatory cells (Fig. 5A-E). To further explore the mechanism, we also tested the inflammatory mediators. The RT-PCR data showed that the mRNA expression levels of IL-6, IL-1 β , CCL2 and CCL3 were significantly

upregulated for the C57/BL mice. However, only IL-6 and CCL3 were slightly upregulated for the Sash mice. In comparison with the C57/BL mice, the degree of upregulation of IL-1 β , CCL2 and CCL3 for the Sash mice was reduced (Fig. 5F). At the same time, we also studied the function of P2X7 receptor in high concentration of ATP-induced peripheral pain. Our experimental results showed that AZ10606120 (specific P2X7 receptor antagonist, 2 mg/kg, ip, pre-administration 1h) could significantly reduce the pain behavior and paw thickness at 4h after ATP treatment (Fig. 5G, H).

P2X7 on mast cells is a potential target for salicylic acid and aspirin analgesia

P2X7 is an appealing target for anti-inflammatory therapy, so we used P2X7 as an analgesic target to screen several anti-inflammatory substances monomers. We found that Matrine, Higenamine, Dictamnine, Prim-O-glucosylcimifugin, Liquiritin, Menthol, Ferulic Acid, 3-Hydroxy-4-methoxycinnamic acid, Isoginkgetin, Vanillic acid, Luteolin, Isoliquiritigenin or Aloeemodin had no inhibitory effect on the current evoked by high concentration of ATP (Supplementary Table 1). Interestingly, salicylic acid and aspirin could inhibit the inward current generated by high concentration of ATP as Fig. 6A shown. Compared with the current amplitude induced by first ATP application, 300 μ M, 500 μ M or 1 mM salicylic acid could slightly inhibit the current amplitude induced by second ATP application (Fig. 6B-D, n = 21, n = 14 and n = 16 respectively). It is worth noting that the current induced by 5 mM ATP had a “run-up” tendency, which indicated that the current growth rate should be studied. Data showed that the current growth rate of second ATP application was significantly inhibited by salicylic acid as Fig. 6E shown. 500 μ M or 1 mM aspirin could also inhibit the current amplitude induced by 5mM ATP (Fig. 6G-H, n = 6, n = 8 and n = 20 respectively). The current growth rate was significantly inhibited by 500 μ M or 1 mM aspirin (Fig. 6I). The intercellular Ca²⁺ concentration assay results also showed that 300 μ M salicylic acid or 1 mM aspirin could also inhibit 5mM ATP-induced calcium influx (Supplementary Fig. S5). At the same time, we also explored the effects of drugs on inflammatory mediators. As our expected, 300 μ M salicylic acid or 1 mM aspirin could attenuate the up-regulation of IL-1 β and CCL3 mediated by high concentration of ATP, especially IL-1 β (Fig. 6J). Results from behavioral test indicated that salicylic acid (50 mg/kg, ig) or aspirin (50 mg/kg, ig) could also alleviate the peripheral pain induced by high concentration of ATP (Fig. 6K). To further clarify the relationship between salicylates and P2X7 receptor, we used the P2X7 receptor agonist BzATP. Results showed that 300 μ M salicylic acid (n = 18) or 300 μ M aspirin (n = 7) significantly inhibited the current growth rate (Fig. 6L) and the calcium influx (Supplementary Fig. S6) evoked by BzATP. These experimental results suggested that P2X7 on mast cells might be a potential target for salicylic acid and aspirin analgesia.

GDP binding region is the critical for the combination of salicylic acid and aspirin with P2X7

Our experimental results have shown that the analgesic effect of salicylic acid and aspirin may be achieved by inhibiting P2X7 channel. Next, we want to know how salicylic acid and aspirin work in combination with P2X7. To uncover this mystery, molecular docking was used to analyze the interaction between salicylic acid or aspirin and P2X7 by Discovery Studio software. As shown in Fig. 7, salicylic acid (Fig. 7G-L) and its derivative aspirin (Fig. 7A-F) had affinity to the GDP-binding region of P2X7, including A: GDP 703, B: GDP703 and C: GDP704 ligands. Among these, aspirin has the highest affinity with A: GDP

703 ligand as Fig. 7A, B shown. The -COOH of aspirin formed two Electrostatic-bonds with Arg546 (R546) and Arg578 (R578) of A: GDP 703 ligand, and van der Waals-bonds with Ser589 (S589). Consistent with the electrophysiological and the intercellular Ca^{2+} concentration assay results, we considered that salicylic acid and aspirin could inhibit P2X7 activation by directly binding the receptor.

Discussion

It is well-known that P2X3 and P2X4 in the nervous system were involved in the pathogenesis of neuropathic pain [25]. A more recent player in chronic pain is the P2X7 receptor in immune cells. The high threshold activation gives P2X7 a role in damage-sensing, only triggering downstream effects when ATP concentration is pathologically elevated [26]. In neuroinflammatory and neurodegenerative diseases, P2X7 upregulation and function appears to contribute to disease progression [26]. P2X7 receptor mediates NLRP3 inflammasome activation, cytokine and chemokine release, T lymphocyte survival and differentiation, transcription factor activation, and cell death [27]. P2X7 promotes release of pro-inflammatory factors, such as IL-1 β , IL-6, CCL2, TNF α and CCL3 [8–12]. Hence, P2X7 is an appealing target for anti-inflammatory therapy. However, the relationship between P2X7 on mast cells and the pathogenesis of pain is still not illustrated. Our study aimed to explore the roles of P2X receptor subtypes on mast cells in inflammatory pain, especially the P2X7 receptor. At the same time, we also used P2X7 as an analgesic target to screen several anti-inflammatory small molecule compounds.

Firstly, we found that several functional P2X receptors including ionotropic P2X1, P2X3, P2X4 and P2X7 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 which express P2X1, P2X4 and P2X7. Furthermore, calcium image results demonstrated that ATP could also activate mouse peritoneal mast cells in a concentration-dependent manner. The blocker results indicated that P2X1, P2X3, P2X4 and P2X7 were involved in the calcium influx caused by 1 μM ATP, 10 μM ATP, 100 μM ATP and high concentration ATP (1 mM and 5 mM) respectively. Notably, specific P2X inhibitors only partially inhibited the increase of concentration of the intracellular calcium. It indicated that the process also be associated with other receptors. For example, previous literatures have shown that besides P2X4, P2Y2 could be stimulated by ATP with a concentration of 100 μM [19].

Just as other cell models, extracellular ATP provoked distinct inward currents in mouse peritoneal mast cells as well. P2X1 receptor was characterized by fast activation and desensitization [24]. Our results demonstrated the current induced by 1 μM ATP was closely resemble for P2X1-like current, which could be eliminated by non-selective P2 antagonists PPADS as well as P2X1-selective antagonist NF449. PPADS or P2X3-selective antagonist AF-353 could block the inward current induced by 10 μM ATP, which suggested that P2X3 participated in this activation process. In addition, the current evoked by 100 μM ATP could be blocked by PPADS or 5-BDBD, indicating that P2X4 receptor was involved. Unlike LAD2 and HLMC [17], the P2X1-like and P2X4-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 heterogeneous of mast cells [28]. Our results showed that both 1mM ATP and 5 mM ATP could induce P2X7-like currents with

'run-up' characteristic, which is in line with the conclusion reported by other literatures [17, 24]. Although the current evoked by 1mM ATP and 5 mM ATP existed some differences, AZ10606120 could block both of them. Hence, we confirmed that P2X7 was involved in the activation caused 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 ATP. The data showed that ATP with high concentration could induce histamine release, which was consistent with previous report [15, 19]. In addition to degranulation, high concentration of ATP also promoted the upregulation and secretion of inflammatory mediators. Our study showed that ATP with high concentration could significantly up-regulate IL-1 β and CCL3, which could be blocked by AZ10606120. These results indicated that P2X7 receptor was involved in mast cells degranulation and release of inflammatory mediators. Neuron-immune crosstalk plays important roles in many inflammatory diseases, we suggested P2X7 on mast cells could also induce peripheral pain indirectly as demonstrated in Fig. 8. Histamine plays a critical role in neurogenic inflammation and pain transmission via specific receptors in a bidirectional manner [15, 29]. IL-1 β can modulate neuronal activity directly, in addition, IL-1 β also mediate pain sensitization via IL-1 receptor. Blocking spinal IL-1 signaling could alleviate neuropathic pain [30, 31]. Furthermore, the roles of inflammatory chemokines could regulate synaptic transmission, especially CCL2/CCR2 and CCL3/CCR1 signaling [32, 33]. Consistent with the hypothesis, our results also demonstrated mast cell and P2X7 participated in the peripheral pain.

Chronic pain is a serious global health issue and a huge clinical challenge without available effective treatment. The commonly used pain-relieving agents are often associated with harmful side effects such as addiction. Therefore, the discovery of novel therapeutic alternatives with superior effectiveness and minimal adverse effects would be beneficial. Hence, we screened several anti-inflammatory molecule compounds based on P2X7 receptor. Interestingly, we found that salicylic acid and aspirin could inhibit the inward current and calcium influx induced by high concentration ATP or P2X7 agonist BZATP. Salicylic acid and aspirin could also attenuate the release of inflammatory factors and peripheral pain induced by high concentration ATP. Molecular docking results showed that salicylic acid and aspirin had affinity to the cytoplasmic GDP-binding region of P2X7. Therefore, the analgesic mechanism salicylic acid and aspirin may be the inhibition of P2X7 activity by binding to GDP-binding region. needs further exploration. The binding patterns and the binding sites of salicylic acid and aspirin to the P2X7 receptor still need to be further verified.

Conclusion

In summary, we found that mouse-derived mast cells could be activated by extracellular ATP via P2X1, P2X3, P2X4 and P2X7 receptors. Mast cells and P2X7 receptor on mast cells played important roles in peripheral pain via inflammatory mediators. In addition, we also found that P2X7 receptor may be a potential target for the analgesic drugs salicylate acid and aspirin.

Abbreviations

ATP: Adenosine-triphosphate

IL-1 β : Interleukin-1 β

IL-6: Interleukin-6

CCL2: C-C motif chemokine 2

CCL3: C-C motif chemokine 3

BZATP: 3'-O-(4-Benzoylbenzoyl)ATP;3'-O-(4-Benzoylbenzoyl)-ATP

GDP: Guanosine diphosphate)

MrgprB2: MAS-related GPR member B2

PPADS: 4-[[4-Formyl-5-hydroxy-6-methyl-3-[(phosphonooxy)methyl]-2-pyridinyl]azo]-1,3-benzenedisulfonic acid tetrasodium salt

NF449: 4,4',4'',4'''-[Carbonylbis(imino-5,1,3-benzenetriyl-bis(carbonylimino))]tetrakis-1,3-benzenedisulfonic acid

AF-353: 5-(5-iodo-2-isopropyl-4-methoxyphenoxy)pyrimidine-2,4-diamine

5-BDBD: 5-(3-Bromophenyl)-1,3-dihydro-2H-benzofuro[3,2-e]-1,4-diazepin-2-one

AZ10606120: 2-(1-adamantyl)-N-[2-[2-(2-hydroxyethylamino)ethylamino]quinolin-5-yl]acetamide

SCF: Stem cell growth factors

SA: Salcylic acid

ASA: Acetylsalicylic acid

Declarations

Consent for publication

Not applicable.

Availability of data and materials

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

Competing interests

The authors declare that they have no competing interests.

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Authors' 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.

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Table S1

Table S1

The effects of anti-inflammatory drugs on the current growth rate evoked by 5 mM ATP.

Drug Names	Drug concentrations	P value	N	Inhibitory
Matrine	100 µmol/L	0.188	3	No
Higenamine	50 µmol/L	0.3584	4	No
Dictamnine	10 µmol/L	0.7841	3	No
Prim-O-glucosylcimifugin	10 µmol/L	0.9592	3	No
Liquiritin	60 µmol/L	0.3069	3	No
Menthol	500 µmol/L	0.5496	3	No
Ferulic Acid	100 µmol/L	0.8702	3	No
3-Hydroxy-4-methoxycinnamic acid	100 µmol/L	0.6239	3	No
Isoginkgetin	20 µmol/L	0.101	4	No
Vanillic acid	2 µmol/L	0.1504	5	No
Luteolin	3 µmol/L	0.155	4	No
Isoliquiritigenin	30 µmol/L	0.6593	4	No
Aloeemodin	6 µmol/L	0.1903	6	No

Figures

Figure 1

P2X receptors expression and ATP-induced calcium response in mouse peritoneal mast cells. (A) P2X1, P2X3, P2X4 and P2X7 expressed in mouse peritoneal mast cells. (B) Calcium influx mediated by different concentrations of extracellular ATP. (C) Fluorescence intensity induced by different concentrations of ATP from 0.01 to 5000 μM is different, and the EC₅₀ was about 6.5 μM . (D) The calcium influx induced by 1 μM ATP was inhibited by PPADS or NF449 (***p* < 0.001, control vs PPADS or NF449). (E) The calcium influx induced by 10 μM ATP was blocked by PPADS or AF-353 (***p* < 0.001, control vs PPADS or AF-353). (F) The calcium influx induced by 100 μM ATP was blocked by PPADS or 5-BDBD (***p* < 0.001, control vs PPADS or 5-BDBD). (G) The calcium influx induced by 1 mM ATP was blocked by PPADS or AZ10606120 (***p* < 0.001, control vs PPADS or AZ10606120). (H) The calcium influx induced by 5 mM ATP was blocked by PPADS or AZ10606120 (***p* < 0.001, control vs PPADS or AZ10606120).

Figure 2

The inward currents evoked by ATP in mouse peritoneal mast cells. (A-D) Different types of currents induced by different concentrations of ATP (1 μ M, 10 μ M, 100 μ M, 1 mM and 5 mM ATP respectively). (E) The amplitude of inward currents evoked by various concentrations of extracellular ATP were different from each other (** p < 0.01, *** p < 0.001). (F) The time durations of inward currents evoked by various concentrations of extracellular ATP were different from each other (*** p < 0.001). (G, H) The “run-up” tendency for 1 mM and 5 mM ATP induced. (I) There was no significant difference in the current growth

rate between 1 mM ATP with 5 mM ATP induced. (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 induced. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

Figure 3

The effects of specific blockers on the currents activated by extracellular ATP. (A) Cell with 20 μM PPADS or 1 μM NF449 hardly evoke current by 1 μM ATP treatment. (B) Current evoked by 10 μM ATP could be blocked by 20 μM PPADS or 0.1 μM AF-353. (C) The current induced by 100 μM ATP could be blocked by

20 μ M PPADS or 1 μ M 5-BDBD. (D) The current induced by 1mM ATP could be blocked by 1 μ M AZ10606120. (E) 1 μ M AZ10606120 could inhibit the current evoked by 5 mM ATP. (F) 5mM ATP could evoke greater inward current in the low divalent cation external solution. (G) The current amplitude induced by 1 μ M ATP could be inhibited by PPADS or NF449 (* $p < 0.05$, control vs 20 μ M PPADS, $n=9$, ** $p < 0.01$, control vs 1 μ M NF449, $n=13$). (H) The current amplitude evoked by 10 μ M ATP was blocked by PPADS or AF-353 (** $p < 0.001$, control vs 20 μ M PPADS, $n=9$, * $p < 0.05$, control vs 0.1 μ M AF-353, $n=10$). (I) PPADS or 5-BDBD could inhibit the current amplitude evoked by 100 μ M ATP (** $p < 0.01$, control vs 20 μ M PPADS, $n=13$, *** $p < 0.001$, control vs 1 μ M 5-BDBD, $n=10$). (J) The current amplitude evoked by 1mM ATP was blocked by AZ10606120 (* $p < 0.05$, control vs 1 μ M AZ10606120, $n=5$). (K) The current amplitude evoked by 5mM ATP could also be blocked by AZ10606120 (** $p < 0.001$, control vs 1 μ M AZ10606120, $n=6$). (L) The current amplitude induced by 5mM ATP in the low divalent cation external solution ($n=8$) was greater than that in the normal external solution ($n=13$).

Figure 4

Mediators induced by different concentrations of extracellular ATP. (A) Histamine release significantly increased by adopting high concentrations of ATP (* $p < 0.05$, control vs 5 mM ATP). (B-H) The relative mRNA expression levels of IL-1 β , IL-6, CCL2 and CCL3 were regulated by different concentrations of ATP. After 4 hours of ATP treatment, there was no significant change in the expression of cytokines (B). IL-1 β was up-regulated slightly induced by 10 μ M ATP or 100 μ M ATP (C, D) (* $p < 0.05$, control vs 10 μ M ATP

or 100 μ M ATP). IL-1 β and CCL3 were up-regulated significantly induced by 1 mM ATP (E) (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, control vs 1 mM ATP). Cytokines were slightly up-regulated induced by 5 mM ATP for 4 hours (F) (* $p < 0.05$, control vs 5 mM ATP), however, IL-1 β and CCL3 were significantly up-regulated induced by 5 mM ATP for 0.5 hours (G) (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, control vs 5 mM ATP). (H) The up-regulation of IL-1 β and CCL3 caused by 1 mM ATP was blocked by specific P2X7 receptor antagonist AZ10606120. (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, control vs 1 mM ATP; # $p < 0.05$, ## $p < 0.01$, ### $p < 0.001$, 1 mM ATP vs 5 μ M AZ10606120).

Figure 5

Mast cell deficient and AZ10606120 alleviated high concentration ATP-induced pain. (A-C) Compared with C57/BL mice, sash mice alleviated the paw swelling (A), paw thickness (C) (* $p < 0.05$, WT group vs Sash group, $n=8$ and 4 respectively) and the 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, 200X). (D) High concentration of ATP induced mast cells degranulation (MrgprB2CreTd/tomato mice, tdTomato fluorescent protein was integrated into the MrgprB2 promoter, red represented mast cell, and the small red particles around mast cells represent degranulation). (E) Sash mice alleviated the mechanical withdrawal threshold induced by high concentration of ATP (* $p < 0.05$, WT group vs Sash group, $n=8$ and 4 respectively). (F) Mast cell deficient attenuated the up-regulation of several inflammatory factors including IL-6, IL-1 β , CCL2 and CCL3 (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, saline group vs ATP group, # $p < 0.05$, ## $p < 0.01$, ### $p < 0.001$, WT (ATP) group vs Sash (ATP) group). (G, H) AZ10606120 significantly relieved the paw swelling (G) and the mechanical withdrawal threshold (H) ($n=6$). (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, saline group vs ATP group or ATP+AZ10606120 group, # $p < 0.05$, ## $p < 0.01$, ### $p < 0.001$, ATP group vs ATP+AZ10606120 group).

Figure 6

The effects of salicylic acid and aspirin on P2X7 receptor on mast cells. (A) Schematic diagram of the effect of salicylic acid and aspirin on inward current. (B-D) Salicylic acid inhibited the current induced by 5 mM ATP. 300 μ M (B), 500 μ M (C) or 1 mM salicylic acid (D) inhibited the current induced by second ATP application compared with that of first ATP application. (E) The current growth rate was significantly inhibited by different concentrations of salicylic acid. (F-H) Aspirin inhibited the current induced by 5 mM

ATP. 300 μ M (F), 500 μ M (G) or 1 mM aspirin (H) inhibited the current induced by second ATP application. (I) The current growth rate was significantly inhibited by different concentrations of aspirin. (J) 300 μ M Salicylic acid or 1 mM aspirin blocked the up-regulation of cytokines mediated by P2X7 receptor. (K) Salicylic acid and aspirin alleviate peripheral pain induced by ATP with high concentration. (L) The current growth rate evoked by BzATP was significantly inhibited by 300 μ M salicylic acid or 300 μ M aspirin. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$; # $p < 0.05$, ## $p < 0.01$, ### $p < 0.001$.

Figure 7

Molecular docking of compounds with P2X7 receptor. (A, B) Docking process of aspirin with the A: GDP703 ligand of P2X7 receptor; (C, D) Docking process of aspirin with the B: GDP703 ligand of P2X7 receptor; (E, F) Docking process of aspirin with the C: GDP704 ligand of P2X7 receptor; (G, H) Docking process of salicylic acid with the A: GDP703 ligand of P2X7 receptor; (I, J) Docking process of salicylic acid with the B: GDP703 ligand of P2X7 receptor; (K, L) Docking process of salicylic acid with the C: GDP704 ligand of P2X7 receptor.

Figure 8

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

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