



Immunopharmacology and inflammation

P2X7 receptor antagonist activity of the anti-allergic agent oxatomide

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ABSTRACT

Activation of the P2X7 receptor by extracellular ATP is associated with various immune responses including allergic inflammation. Anti-allergic agents, such as H₁-antihistamines, are known to inhibit the effects of different chemical mediators such as acetylcholine and platelet-activating factor. Therefore, we hypothesized that some anti-allergic agents might affect P2X7 receptor function. Using N18TG2 and J774 cells, which express functional P2X7 receptors, the effects of several anti-allergic agents on P2X7 receptor function were investigated by monitoring the ATP-induced increase in intracellular Ca²⁺ concentrations ([Ca²⁺]_i). Among the various agents tested, oxatomide significantly inhibited P2X7 receptor-mediated [Ca²⁺]_i elevation in a concentration-dependent manner without affecting the P2Y₂ receptor-mediated response in both N18TG2 and J774 cells. Consistently, oxatomide inhibited P2X7 receptor-mediated membrane current and downstream responses such as mitogen-activated protein kinase activation, inflammation-related gene induction, and cell death. In addition, oxatomide inhibited P2X7 receptor-mediated degranulation in mouse bone marrow-derived mast cells. Whole cell patch clamp analyses in HEK293 cells expressing human, mouse, and rat P2X7 receptors revealed that the inhibitory effect of oxatomide on ATP-induced current was most prominent for the human P2X7 receptor and almost non-existent for the rat P2X7 receptor. The potent inhibitory effects of oxatomide on human P2X7 receptor-mediated function were confirmed in RPMI8226 human B cell-like myeloma cells, which endogenously express the P2X7 receptor. Our results demonstrated that the antihistamine oxatomide also acts as a P2X7 receptor antagonist. Future studies should thus evaluate whether P2X7 receptor antagonism contributes to the anti-allergic effects of oxatomide.

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1. Introduction

Extracellular ATP acts as important intercellular signalling molecule by activating membrane surface P2 purinoceptors. The P2 purinoceptors are divided into two families: the ionotropic P2X receptors and the G protein-coupled P2Y receptors. To date, seven P2X receptor subtypes (P2X1–7) and eight P2Y receptor subtypes (P2Y₁, 2, 4, 6, 11–14) have been identified and functionally characterized (Burnstock, 2006; Khakh et al., 2001). The P2X7 receptor has several unique characteristics among the various P2 receptor subtypes (Surprenant et al., 1996). For example, its activation requires high concentrations of ATP (> 500 μM). In addition, sustained activation of P2X7 receptors results in the formation of a cytolytic pore that allows the passage of molecules up to 900 Da. P2X7 receptors are highly expressed in immune cells such as lymphocytes, monocytes/macrophages, mast cells, and dendritic cells (Jacob et al., 2013), and have been found to be up-regulated in

response to pro-inflammatory stimuli (Humphreys and Dubyak, 1996, 1998). Therefore, P2X7 receptor signalling is implicated in inflammatory responses. Indeed, activation of P2X7 receptors induces rapid efflux of potassium ions from the cell (Taylor et al., 2008), which in turn leads to oligomerization of the inflammasome, activation of caspase-1, cleavage of the IL-1β precursor, and release of active IL-1β via secretory lysosomes (Piccini et al., 2008; Idzko et al., 2014; Jacob et al., 2013; Latz et al., 2013). Accordingly, blockade of P2X7 receptor activation with pharmacological antagonists or genetic deletion of the receptor gene has been shown to ameliorate the severity of inflammatory diseases in models of arthritis (Lopez-Castejon et al., 2010), neuropathic pain (Sorge et al., 2012), colitis (Kurashima et al., 2012), allergic airway inflammation (Müller et al., 2011), and allergic dermatitis (Weber et al., 2010). Therefore, there has been a great deal of research concerning the use of the P2X7 receptor as a therapeutic target for inflammatory diseases. Although several P2X7 receptor antagonists have been developed and evaluated as therapeutic agents in clinical trials (Keystone et al., 2012; Stock et al., 2012), none have so far made it to the clinic.

Recent studies have shown that P2X7 receptor activation plays an important role in allergic inflammation (Kurashima et al., 2012;

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Manthei et al., 2012). It is well known that many anti-allergic agents, including H₁-antihistamines and chemical mediator secretion inhibitors, possess multi-target activity. For example, first-generation H₁-antihistamines have considerable potency as muscarinic cholinergic antagonists. The newer, second-generation H₁-antihistamines have been shown to inhibit chemical mediator release from mast cells (Okayama et al., 1994) and have exhibited antagonist behaviour against platelet-activating factor (PAF) and leukotrienes (Archer et al., 1985). However, there is no information in the literature concerning the effect of H₁-antihistamines on P2X7 receptor signalling, except for the fact that clemastine potentiates the human P2X7 receptor by an allosteric mechanism (Nörenberg et al., 2011). We hypothesized that P2X7 receptor antagonism may contribute to the anti-allergic effect of certain H₁-antihistamines. In this study, we demonstrated that oxatomide significantly inhibits P2X7 receptor function.

2. Materials and methods

2.1. Materials

2',3'-O-(4-benzoyl)benzoyl-ATP (BzATP), fura-2-acetoxymethyl ester (fura-2 AM), fluo-4-acetoxymethyl ester (fluo-4AM), G418, 2,4-dinitrophenyl-human serum albumin (DNP-HSA), anti-DNP IgE (clone SPE-7), and *p*-nitrophenyl-*N*-acetyl-β-D-glucosaminide, uridine 5'-triphosphate (UTP) were purchased from Sigma-Aldrich Japan (Tokyo, Japan). *N*-[2-[(2-hydroxyethyl)amino]ethyl]amino]-5-quinoliny]-2-tricyclo [3.3.1.1^{3,7}]dec-1-yl acetamide dihydrochloride (AZ10606120) were purchased from Tocris (Bristol, UK). ATP, azelastine, cetirizine, chlorpheniramine, diphenhydramine, clemastine, cromogricate, fexofenadine, ketotifen, tranilast, oxatomide, histamine, and charbachol were purchased from Wako (Osaka, Japan). SYBR Premix EX Taq, random hexamer, and RNase inhibitor were purchased from Takara Bio (Ohtsu, Japan). Moloney murine leukaemia virus reverse transcriptase (M-MLV RT) and RNase-free DNase were purchased from Invitrogen Japan (Tokyo, Japan). Anti-extracellular signal-regulated kinase (ERK), anti-phospho ERK, anti-p38 mitogen-activated protein kinase (MAPK), and anti-phospho p38 MAPK were purchased from Cell Signalling Technology (Danvers, MA, USA). Fluorescein isothiocyanate (FITC)-conjugated anti-CD23 (clone M-L233) was purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Allophycocyanin-conjugated rat anti-mouse CD117 (c-Kit; clone 2B8) was purchased from BD Pharmingen (Tokyo, Japan). Phycoerythrin-conjugated mouse anti-mouse FcεR1α (clone MAR-1) was purchased from eBioscience (San Diego, CA, USA). Recombinant murine interleukin (IL)-3 and recombinant murine stem cell factor were purchased from Peprotech (London, UK). FuGENE and CytoTox 96 non-radioactive cytotoxicity assay kit were purchased from Promega (Tokyo, Japan). All other chemicals used were of reagent-grade or the highest quality available.

2.2. Cell culture

J774 and RAW264.7 murine macrophage cells and RPMI8226 human B cell-like myeloma cells were cultured in RPMI1640 medium containing 10% foetal bovine serum, 100 units/ml penicillin, and 100 µg/ml streptomycin. Mouse neuroblastoma N18TG2, human embryonic kidney (HEK) 293 cells, and human astrocytoma 1321N1 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% foetal bovine serum, 100 units/ml penicillin, and 100 µg/ml streptomycin. Rat P2X7 receptor-expressing 1321N1 cells were maintained in DMEM growth medium containing 0.7 mg/ml G418. Cultures of mouse bone marrow-derived mast cells (mBMMC) were established using bone marrow

from 6- to 12-week-old C57BL/6 mice as previously described (Ohmori et al., 2012). Briefly, bone marrow cells were cultured with complete RPMI1640 medium containing 10 ng/ml murine IL-3. After 2 weeks, cells were cultured with 10 ng/ml murine stem cell factor for 4–6 weeks. After this treatment, almost all (>95%) cells displayed a mast cell phenotype, as assessed by the expression of c-Kit and FcεR1 using a FACSCanto II flow cytometer (BD Biosciences, Tokyo, Japan).

2.3. Flow cytometry

Bone marrow cells were stained with allophycocyanin-conjugated rat anti-mouse CD117/c-Kit antibody and phycoerythrin-conjugated mouse anti-mouse FcεR1α antibody. Dead cells were stained using propidium iodide. Flow cytometric analysis was performed using a FACSCanto II flow cytometer.

2.4. Intercellular Ca²⁺ concentration ([Ca²⁺]_i) assay

Cells were collected and washed twice with Krebs-Ringer-HEPES (KRH) buffer consisting of 130 mM NaCl, 4.7 mM KCl, 4.0 mM NaHCO₃, 1.2 mM KH₂PO₄, 1.2 mM MgSO₄, 1.8 mM CaCl₂, 11.5 mM glucose, and 10 mM HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; pH 7.4) containing 0.1% bovine serum albumin (BSA). Cells were treated with 1 µM fura-2 AM or fluo-4 AM at 37 °C for 20 min. The treated cells were washed twice with KRH-BSA buffer and adjusted to 1–2 × 10⁵ cells/ml. The change in the fluorescence of fura-2 AM or fluo-4 AM was measured as previously described (Ito and Matsuoka, 2008).

2.5. Western blot

ATP-induced activation of ERK 1/2 and p-38 MAPK was evaluated by measuring the phosphorylation of the kinase proteins by western blot. N18TG2 cells were cultured in 12-well plates and washed with KRH buffer. The reaction was performed in KRH buffer and terminated by adding 0.1 ml of sample buffer (75 mM Tris-HCl pH 6.8, 2% SDS, 10% glycerol, 3% 2-mercaptoethanol, 0.003% bromophenol blue). The lysate was separated by 10% sodium dodecylsulphate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to Immobilon-P polyvinylidene fluoride (PVDF) membranes. Western blotting was performed as described previously (Tang et al., 2008). The antibodies were used at the following dilutions: phospho-ERK1/2 (1:2000), ERK1/2 (1:2000), phospho-p38 MAPK (1:2000), p38MAPK (1:2000), and horseradish peroxidase (HRP)-linked anti-rabbit IgG (1:5000).

2.6. Real-time quantitative polymerase chain reaction (qPCR)

Total RNA was isolated using the acid guanidinium thiocyanate-phenol-chloroform extraction method (Chomczynski and Sacchi, 1987). First-strand cDNA was synthesized using M-MLV RT with a 6-mer random primer. qPCR was performed using a SYBR green kit as previously described (Ito and Matsuoka, 2008).

2.7. Lactate dehydrogenase (LDH) assay

N18TG2 cells were cultured in 48-well plates under various conditions, and supernatants were collected. LDH activity in the supernatant was measured using a CytoTox 96 non-radioactive cytotoxicity assay kit following the manufacturer's instructions, and results were expressed as a percentage of the total amount of LDH.

2.8. Measurement of macrophage inflammatory peptide-2 (MIP-2)

J774 cells were seeded in a 48-well plate and grown to confluence. The cells were washed twice and incubated with serum-free RPMI 1640 medium at 37 °C for 3 h in the presence or absence of various agents. After incubation, each culture medium was collected and centrifuged at $300 \times g$ for 5 min at 4 °C to remove the cells. The amount of MIP-2 in the supernatant was assayed using an ELISA kit, according to the manufacturer's instructions (R&D Systems, Minneapolis, MN, USA).

2.9. mBMMC degranulation

Degranulation was evaluated by measuring β -hexosaminidase release. mBMBCs were sensitized with 250 ng/ml anti-DNP IgE (Sigma Aldrich; clone: SPE-7) overnight in RPMI1640 growth medium. Cells were washed twice and suspended in KRH-BSA buffer. Cells were then stimulated using various conditions for 5 min at 37 °C. Reactions were terminated by placing on ice and centrifuging at $1000 \times g$ for 5 min. Supernatants were collected and the cell pellets were then lysed in 1% Triton X-100. Supernatant and cell lysate (50 μ L) were incubated with an equal volume of 1 mM *p*-nitrophenyl *N*-acetyl- β -D-glucosaminide dissolved in citrate buffer (pH 4.5) in a 96-well plate at 37 °C for 30 min. Reactions were stopped by adding $\text{Na}_2\text{CO}_3/\text{NaHCO}_3$ buffer (100 μ L; pH 10.4) and the absorbance was measured at 405/655 nm. Percentage degranulation was calculation using the following formula: β -Hexosaminidase release (%) = $\frac{\text{supernatant absorbance}}{\text{supernatant absorbance} + \text{lysate absorbance}} \times 100$.

2.10. Ethidium⁺ influx

Ethidium⁺ influx induced by P2X7 receptor activation was measured by FACS analysis. Cells were collected and washed twice with Ca^{2+} - and Mg^{2+} -free KRH buffer, and stimulated under various conditions with 2.5 μ M ethidium bromide. Reactions were terminated by addition of an equal volume of ice-cold MgCl_2 buffer (KRH buffer containing 20 mM MgCl_2) and centrifuging at $300 \times g$ for 5 min. Ethidium⁺ influx was measured using a FACSCanto II flow cytometer.

2.11. CD23 shedding

Cells were collected and washed twice with calcium- and magnesium-free KRH-BSA buffer. Cells were stimulated with ATP under various conditions at 37 °C for 30 min. Incubation was terminated by addition of an equal volume of ice-cold MgCl_2 buffer and centrifuging at $300 \times g$ for 5 min. Cells were then stained with FITC-conjugated anti-CD23 antibody (clone: M-L233), and FITC fluorescence intensity was measured using a FACSCanto II flow cytometer.

2.12. Cloning and transfection

Full-length human P2X7 receptor cDNA was amplified from RPMI8226 cell cDNA by PCR using *Kpn*I site-introduced sense primer: gtaggtaccGGAGGGAGGCTGTCACCATGC and *Xba*I site-introduced antisense primer: tgctctAGACGTGAGCCACGGTGCTG. In the same way, full-length rat and mouse P2X7 receptor cDNA was amplified from cDNA obtained from rat brain and N18TG2 cells, respectively, using *Eco*RI site-introduced rat sense primer: aggaattcTTGCTGTAGCTAGCCTGGGAA, *Xho*I site-introduced rat antisense primer: gtctcgagTCAGTAGGGATACTTGAAGCCA, *Eco*RI site-introduced mouse sense primer: aggaattcTTGCTGTAGCTAGCCTGGGAA, *Xho*I site-introduced mouse antisense primer: gtctcgagTCAGTAGGGATACTTGAAGCCA. Each cDNA fragment was

cloned into the mammalian expression vector pcDNA3.1 using restriction sites introduced by primers. All clones were verified by DNA sequencing. To obtain stable P2X7 receptor-expressing cells, the human, rat and mouse P2X7 receptor expression vectors were transfected into 1321N1 human astrocytoma cells using the Amaxa system according to the manufacturer's instructions with solution V and programme T16. Stable clones were selected using 0.7 mg/ml G418. Since we could isolate only rat P2X7 receptor-expressing cells, the P2X7 receptor-mediated membrane current was examined in HEK293 cells that transiently expressed P2X7 receptor. HEK293 cells were transfected with the P2X7 receptor plasmid with pEGFP-C1 vector at a ratio of P2X7 receptor:EGFP of 2:1 using FuGENE transfection reagent. After 48–72 h, GFP-positive cells were subjected to patch clamp analysis.

2.13. Patch clamp analysis

P2X7 receptor-mediated membrane current was recorded using the whole-cell patch clamp technique as described previously (Watano et al., 2002). Patch pipettes had a tip resistance of about 6–10 M Ω when filled with an intracellular solution (30 mM CsCl, 110 mM CsOH, 5 mM MgATP, 3 mM MgCl $\cdot$ 6H $_2$ O, 10 mM EGTA, 20 mM HEPES, 50 mM aspartic acid; pH 7.2). The cells were dispersed in a poly-L-lysine-coated experimental chamber perfused with Tyrode solution (140 mM NaCl, 5.4 mM KCl, 1 mM MgCl $_2$, 0.33 mM NaH $_2$ PO $_4$, 5 mM HEPES, 1.8 mM CaCl $_2$, 5.5 mM glucose; pH 7.4). The temperature of the external solution was maintained at approximately 37 °C by a single-line solution heater. Membrane potentials were controlled by a model Axopatch 200B amplifier (Axon Instruments Inc., Foster City, CA, USA). Membrane currents were acquired on-line and subsequently analyzed with a computer using pCLAMP10.4 software (Axon). The current–voltage (*I*–*V*) relationship was obtained using ramp pulses from a holding potential of -10 mV, initially depolarized to 60 mV, then hyperpolarized to -120 mV, and depolarized back to the holding potential with a speed of 1 V s $^{-1}$. The ramp pulse was applied every 3 s.

2.14. Statistics

In each case, experiments were repeated at least three times, with similar results being obtained. Each value indicates a mean $\pm$ S.E.M.. Statistical analyses of the data were performed using the Mann–Whitney *U* test for two sample comparison, and one-way analysis of variance (ANOVA) with the Dunnett two-tailed test for multiple data comparison. *P* values less than 0.05 were considered to be statistically significant.

3. Results

3.1. Effects of anti-allergic agents on P2X7 receptor

The effects of various anti-allergic agents on P2X7 receptor-mediated signalling were measured in terms of the Ca^{2+} response by using fluo-4 AM-treated N18TG2 and J774 cells that expressed functional endogenous P2X7 receptors. We previously showed that N18TG2 cells functionally express the P2Y $_2$ and P2X7 receptors (Kaiho et al., 1998), whereas J774 cells respond to ATP via the P2Y $_2$, P2X $_4$, and P2X7 receptors (Ito and Matsuoka, 2008). In these cells, stimulation with ATP or UTP caused rapid desensitization of P2Y $_2$ receptors allowing P2X7 receptor-mediated Ca^{2+} entry to be assessed by stimulating with 1 mM ATP (Kaiho et al., 1998; Ito and Matsuoka, 2008, Supplement Fig. 1). The role of P2X7 was confirmed by the fact that the increase in $[\text{Ca}^{2+}]_i$ (intracellular Ca^{2+} concentration) that was induced by 1 mM ATP

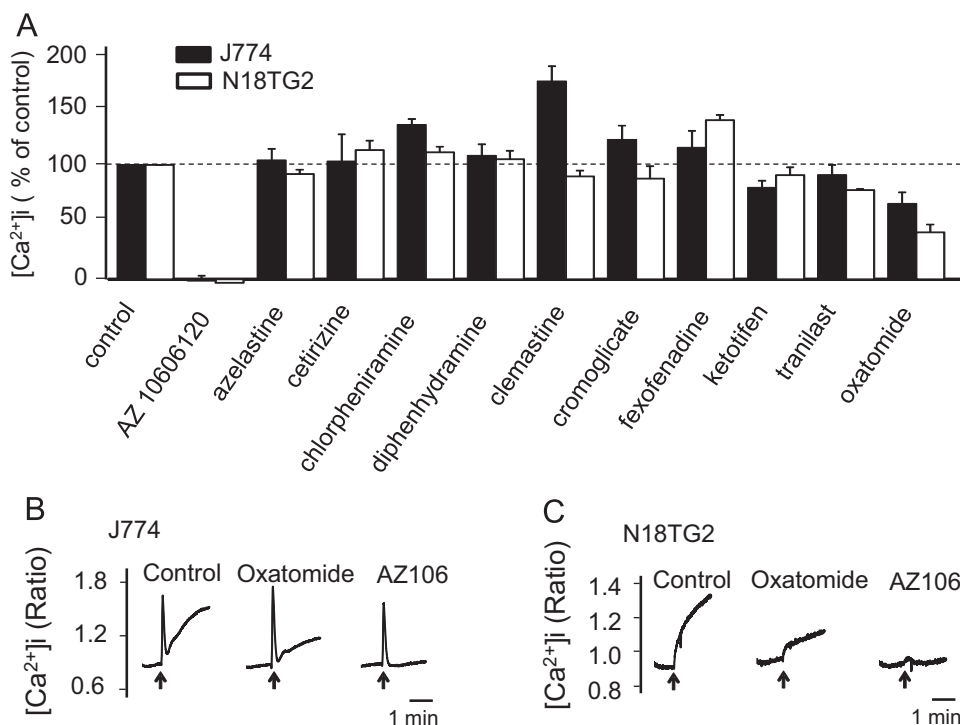


Fig. 1. (A) Effect of the selective P2X7 receptor antagonist AZ10606120 and anti-allergic agents on ATP-induced P2X7 receptor-mediated Ca^{2+} response in J774 (black column) and N18TG2 (open column) cells. Fluo-4 AM-treated cells were pre-treated with 0.1 mM UTP for 3 min (to desensitize the P2Y_2 receptor) in the presence or absence of AZ10606120 or anti-allergic agents at 3 μM . Then, P2X7 receptor-mediated Ca^{2+} influx was triggered by the addition of 1 mM ATP. Data are shown as means $\pm$ S.E.M. ($n=6$). (B and C) Representative P2X7 receptor-mediated ATP-induced $[\text{Ca}^{2+}]_i$ change in J774 (B) and N18TG2 cells (C) with oxatamide or AZ10606120 (AZ106).

was completely inhibited by the selective P2X7 receptor antagonist AZ10606120 (1 μM) in both N18TG-2 and J774 cells (Fig. 1). Based on these results, the following screenings were performed in the presence of 100 μM UTP. The effects of various anti-allergy drugs on P2X7 receptor-mediated Ca^{2+} influx were evaluated at a final concentration of 3 μM (Fig. 1A). Among the drugs tested, oxatamide constantly inhibited the Ca^{2+} influx in both N18TG2 and J774 cells. Clemastine significantly augmented the 1 mM ATP-induced Ca^{2+} influx in N18TG2 cells, a result similar to that reported previously by Nörenberg et al. (2011). Typical P2X7 receptor-mediated increases in $[\text{Ca}^{2+}]_i$ with or without oxatamide or AZ10606124 are shown in Fig. 1A and B. In J774 cells, the addition of 1 mM ATP caused a Ca^{2+} transient, followed by a gradual increase in $[\text{Ca}^{2+}]_i$. The selective P2X7 receptor antagonist AZ10606124 prevented the second phase of the Ca^{2+} response without affecting the initial transient one. Oxatamide also inhibited only the second phase of the Ca^{2+} response (Fig. 1B). In N18TG2 cells, ATP (1 mM) induced a slowly developing Ca^{2+} response, which was inhibited by both AZ10606124 and oxatamide (Fig. 1C).

3.2. Effects of oxatamide on ATP-induced Ca^{2+} response and membrane current in N18TG2 cells

The inhibitory effects of oxatamide on P2X7 receptor-mediated responses were further investigated in N18TG2 cells with respect to $[\text{Ca}^{2+}]_i$ and ATP-induced membrane current. Pretreatment with different concentrations of oxatamide (1–30 μM) inhibited the 1 mM ATP-induced Ca^{2+} response but not the 100 μM ATP-induced Ca^{2+} response (Fig. 2A). Stimulation of 100 μM ATP-treated cells with higher concentrations of ATP (0.3–3 mM) caused a concentration-dependent $[\text{Ca}^{2+}]_i$ elevation. In the presence of 10 μM oxatamide, ATP-induced Ca^{2+} responses were decreased at all tested concentrations of ATP (Fig. 2B). Oxatamide-mediated inhibition of the 1 mM ATP-induced $[\text{Ca}^{2+}]_i$ increase was

concentration-dependent with an IC_{50} value of $3.3 \pm 1.0 \mu\text{M}$ ($n=4$) (Fig. 2C).

ATP-induced membrane current was measured using the whole-cell patch clamp technique. ATP (1 mM) induced membrane current, and it was inhibited by AZ10606120, suggesting that ATP induces a current via P2X7 receptors (Fig. 2D). Oxatamide inhibited the ATP-induced membrane current in a concentration-dependent manner with an IC_{50} value of $3.2 \pm 0.7 \mu\text{M}$ ($n=3$) (Fig. 2E and F).

3.3. Oxatamide inhibited P2X7 receptor-mediated functions in N18TG2 and J774 cell lines

We next investigated whether oxatamide affects the downstream signalling events triggered by P2X7 receptor activation. Mitogen-activated protein kinase signalling is known to be involved in P2X7 receptor-induced responses, such as membrane blebbing and pore formation (Donnelly-Roberts et al., 2004; Pfeiffer et al., 2004). Stimulation of N18TG2 cells with 1 mM ATP for 5 min induced a marked increase in the phosphorylation of ERK and p38 MAPK. Over this time period, P2Y receptor activation by ATP (100 μM) induced only weak phosphorylation of these kinases (data not shown). Pre-treatment of cells with 10 μM oxatamide inhibited the phosphorylation of ERK and p38 MAPK (Fig. 3A). Stimulation of P2X7 receptors with high concentrations of ATP is known to induce formation of large pores on the plasma membrane, leading to the release of cytosolic enzymes such as LDH. In N18TG2 cells, ATP caused LDH release at concentrations higher than 3 mM. Pretreatment of N18TG2 cells with oxatamide (10 μM) significantly inhibited ATP-induced LDH release (Fig. 3B).

In macrophages, activation of P2X7 receptors induces the expression of several inflammatory genes, including cyclooxygenase (COX)-2 (Chiao et al., 2013) and MIP-2 (Kawamura et al., 2012). Stimulation of J774 macrophages with 0.1 mM BzATP, a selective P2X7 receptor agonist, increased mRNA levels for COX-2 and MIP-

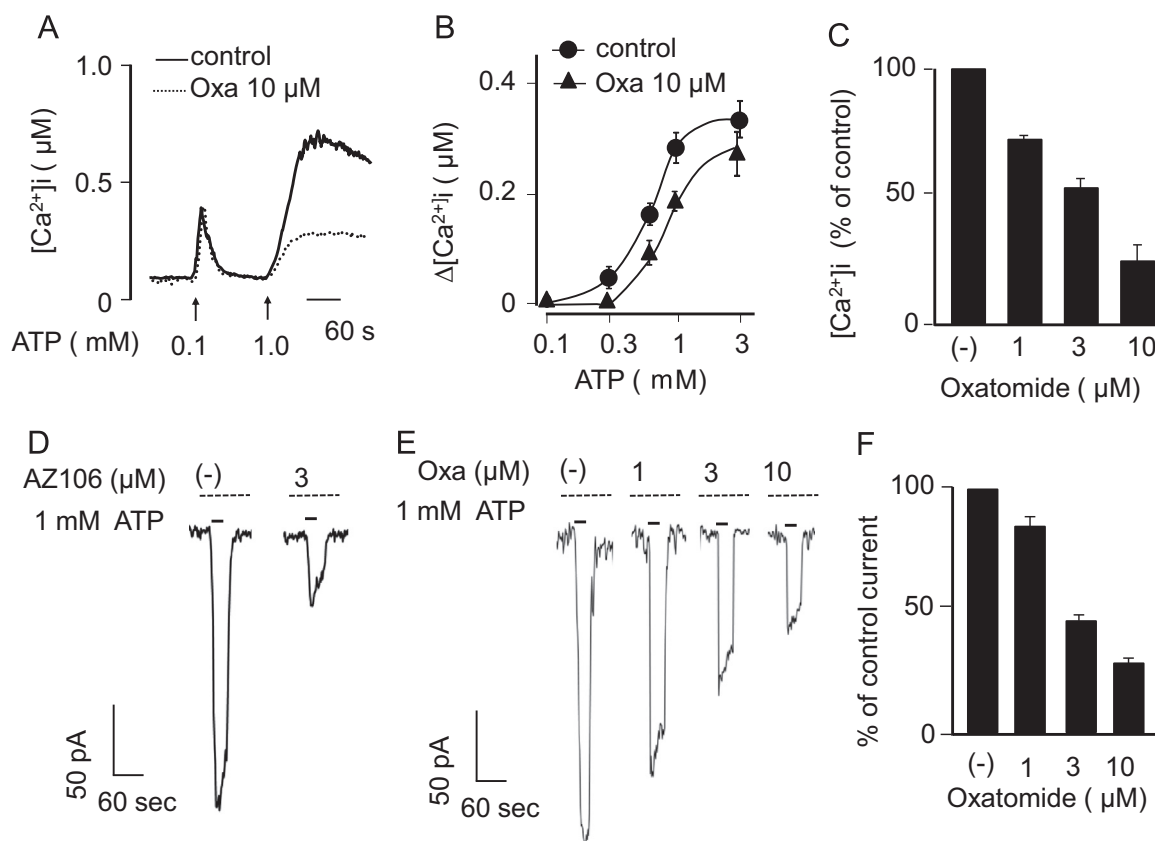


Fig. 2. Effect of oxatomide (Oxa) on P2X7-mediated increase of $[Ca^{2+}]_i$ and membrane current in N18TG2 cells. (A) Representative Ca^{2+} response of N18TG2 cells to 0.1 and 1 mM ATP in the presence or absence of 10 μ M Oxa. (B) $[Ca^{2+}]_i$ response curve at different concentrations of ATP with or without 10 μ M Oxa. Data points represent means $\pm$ S.E.M. ($n=3-6$). (C) Effect of different concentrations of Oxa on 1 mM ATP-induced $[Ca^{2+}]_i$ elevation. Results show means $\pm$ S.E.M. ($n=3-6$) expressed as percentage of the control response. (D and E) Representative whole cell current induced by 1 mM ATP with or without AZ10606120 (AZ106, D) or Oxa (E). Whole-cell recordings were performed in N18TG2 cells at a holding potential of -60 mV. (F) Effect of different concentrations of Oxa on 1 mM ATP-induced current. Results are expressed as percentage of control response. Data show means $\pm$ S.E.M. ($n=3-6$).

2. Oxatomide prevented BzATP-induced up-regulation of COX-2 and MIP-2 expression (Fig. 3C and D). In addition, oxatomide and AZ10606120 prevented BzATP-induced MIP-2 secretion (Fig. 3E)

3.4. Oxatomide inhibited P2X7 receptor-induced mBMMC degranulation

It has previously been shown that mouse mast cell line MC9 cells express P2X7-like receptors, the stimulation of which causes histamine release (Sudo et al., 1996). We therefore prepared mBMMCs and examined whether ATP induces degranulation by measuring β -hexosaminidase release. As the positive control, anti-DNP IgE-sensitized cells were stimulated with DNP-HSA to trigger antigen-induced degranulation. Stimulation with ATP (0.5 mM) and DNP-HSA (50 ng/ml) resulted in marked degranulation to a similar extent. AZ10606120 inhibited ATP-induced degranulation without affecting the DNP-HSA-induced response (Fig. 4A), suggesting that ATP-induced degranulation is mediated by P2X7 receptors. Since oxatomide is known to inhibit the release of chemical mediators at higher concentrations (Awouters et al., 1977), we evaluated its effects on ATP-induced degranulation at concentration that had little effect on DNP-HSA-induced response. Oxatomide at 3 μ M slightly but significantly inhibited ATP-induced degranulation without affecting DNP-HSA-induced response (Fig. 4B).

3.5. Effect of oxatomide on ATP-induced membrane currents in

HEK293 cells expressing human, mouse or rat P2X7 receptors

Since P2X7 receptor antagonists are known to exhibit large interspecies differences in their pharmacological effects (Donnelly-Roberts et al., 2009), we next compared the effect of oxatomide on the membrane current carried by human, mouse, and rat P2X7 receptors expressed in HEK293 cells. In naive HEK293 cells, ATP did not induce membrane currents. In cells expressing human, mouse or rat P2X7 receptors, stimulation with 1 mM ATP induced a rapid and sustained inward current (Fig. 5). After the maximal current was reached, application of AZ10606120 together with ATP resulted in complete blocking of the inward current; additionally, the decreased current was slowly but fully recovered upon removal of AZ10606120, with little difference in cells expressing human, mouse or rat P2X7 receptors (Fig. 5A–C). In contrast to AZ10606120, the effect of oxatomide on ATP-induced current was largely different for human, mouse, and rat P2X7 receptors. The ATP-induced current in cells expressing the human P2X7 receptor was completely blocked by oxatomide (3 μ M), and fully recovered following removal of oxatomide (Fig. 5D); however, the same treatment in cells expressing the mouse P2X7 receptor resulted in only a partial blockade (Fig. 5E). In cells expressing the rat P2X7 receptor, oxatomide had no effect on ATP-induced currents even at a concentration of 10 μ M. Fig. 6 shows the effects of different concentrations of oxatomide on ATP-induced current in HEK293 cells expressing human and mouse P2X7 receptors. Pre-incubation with oxatomide at 1, 3, and 10 μ M for 1 min inhibited the 1 mM ATP-induced inward current in a concentration-dependent manner in cells expressing human or mouse P2X7 receptors.

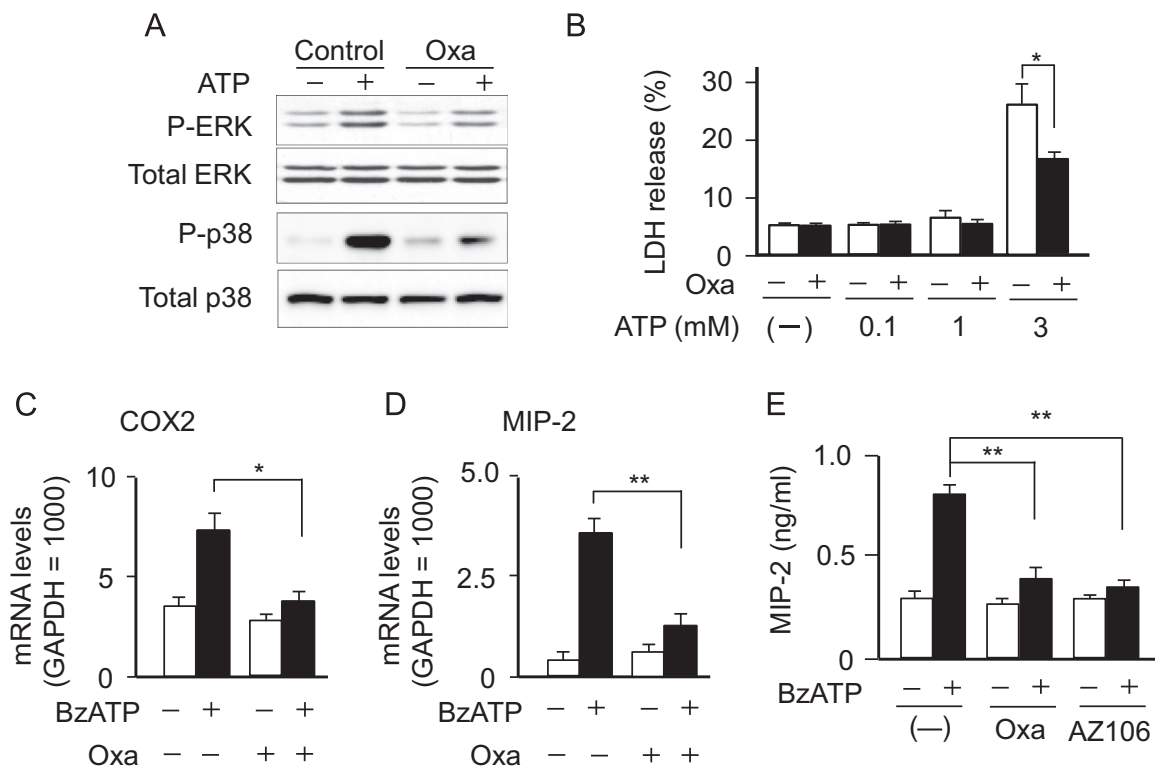


Fig. 3. Effect of oxatomide (Oxa) on P2X7 receptor-mediated responses in N18TG2 and J774 cells. (A) N18TG2 cells were stimulated with 1 mM ATP for 5 min in the presence or absence of 10 μ M Oxa. Cell lysates were subjected to western blot analysis for phospho-ERK, total ERK, phospho-p38MAPK and total p38MAPK. The data presented are from a single experiment that is representative of the two performed. (B) N18TG2 Cells were stimulated with 0.1, 1, and 3 mM ATP for 30 min in the presence or absence of 10 μ M Oxa. Extracellular LDH activity was shown as % of total cellular LDH content ($n=3$). Data are shown as means $\pm$ S.E.M., * $P < 0.05$ (C, D) J774 cells were stimulated with 0.1 mM BzATP for 1 h in the presence or absence of 10 μ M Oxa, and then COX-2 (C) and MIP-2 (D) mRNA levels were analyzed using qPCR. Data show means $\pm$ S.E.M. ($n=3-5$). * $P < 0.05$ (E) J774 cells were stimulated with 0.1 mM BzATP for 3 h in the presence or absence of 10 μ M Oxa or 3 μ M AZ10606120. The content of MIP-2 in the culture supernatant was measured using ELISA. Data show means $\pm$ S.E.M. ($n=3$). * $P < 0.05$, ** $P < 0.01$.

Although oxatomide almost completely blocked the ATP-induced current in human P2X7 receptors (IC_{50} value $0.95 \pm 0.15 \mu$ M ($n=5$)), it only decreased the current to 40% of control in mouse P2X7 receptors (IC_{50} value $4.1 \pm 1.3 \mu$ M ($n=6$)).

3.6. Effect of oxatomide on ATP-induced Ca^{2+} response in RPMI8226 human myeloma cells

We next confirmed the antagonist effect of oxatomide on the human P2X7 receptor using RPMI8226 human myeloma cells, which express functional endogenous P2X7 receptors (Farrell et al., 2010). Stimulation of RPMI8226 cells with 0.1 mM ATP

induced a transient increase in $[Ca^{2+}]_i$ via activation of P2Y receptors, causing a loss of response to subsequent stimulation with 0.1–0.3 mM ATP, whereas addition of 0.75 mM ATP resulted in a large and sustained increase in $[Ca^{2+}]_i$ via P2X7 receptor activation in a manner sensitive to AZ10606120 (Supplement Fig. 2). ATP-induced Ca^{2+} responses in the presence of 0.1 and 1 μ M oxatomide are superimposed in Fig. 7A. Oxatomide inhibited P2X7 receptor-mediated Ca^{2+} responses in a concentration-dependent manner with an IC_{50} value of $0.43 \pm 0.08 \mu$ M ($n=4$) (Fig. 7A and C), without affecting the P2Y receptor-mediated Ca^{2+} response (Fig. 7A and B). Fig. 7D shows that 0.1 μ M oxatomide caused a rightward shift of the concentration response curve of ATP-

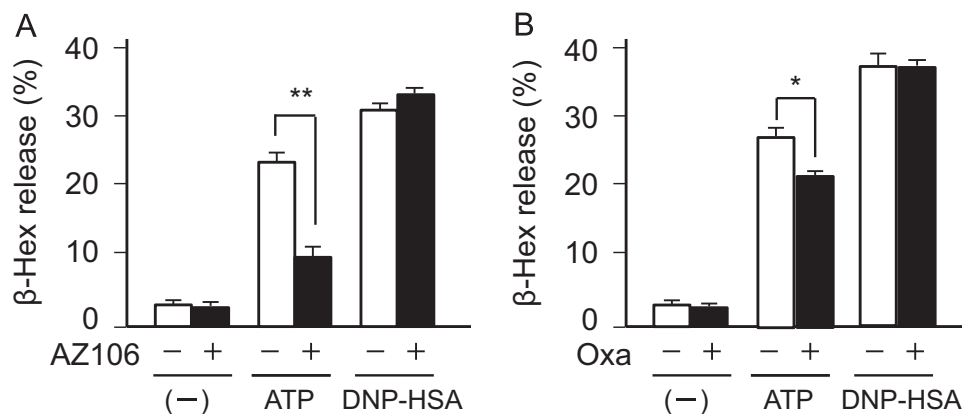


Fig. 4. Effects of oxatomide (Oxa) on ATP-induced degranulation in mBMMCs. mBMMCs were sensitized with 250 ng/ml anti-DNP IgE overnight. (A) BMMCs were stimulated with 0.5 mM ATP or 50 ng/ml DNP-HSA in the presence or absence of 3 μ M AZ106 for 5 min ($n=3$). (B) BMMCs were stimulated by 0.5 mM ATP or 50 ng/ml DNP-HSA with or without 3 μ M Oxa for 5 min ($n=3$). Data are shown as means $\pm$ S.E.M. ($n=3$), * $P < 0.05$, ** $P < 0.01$.

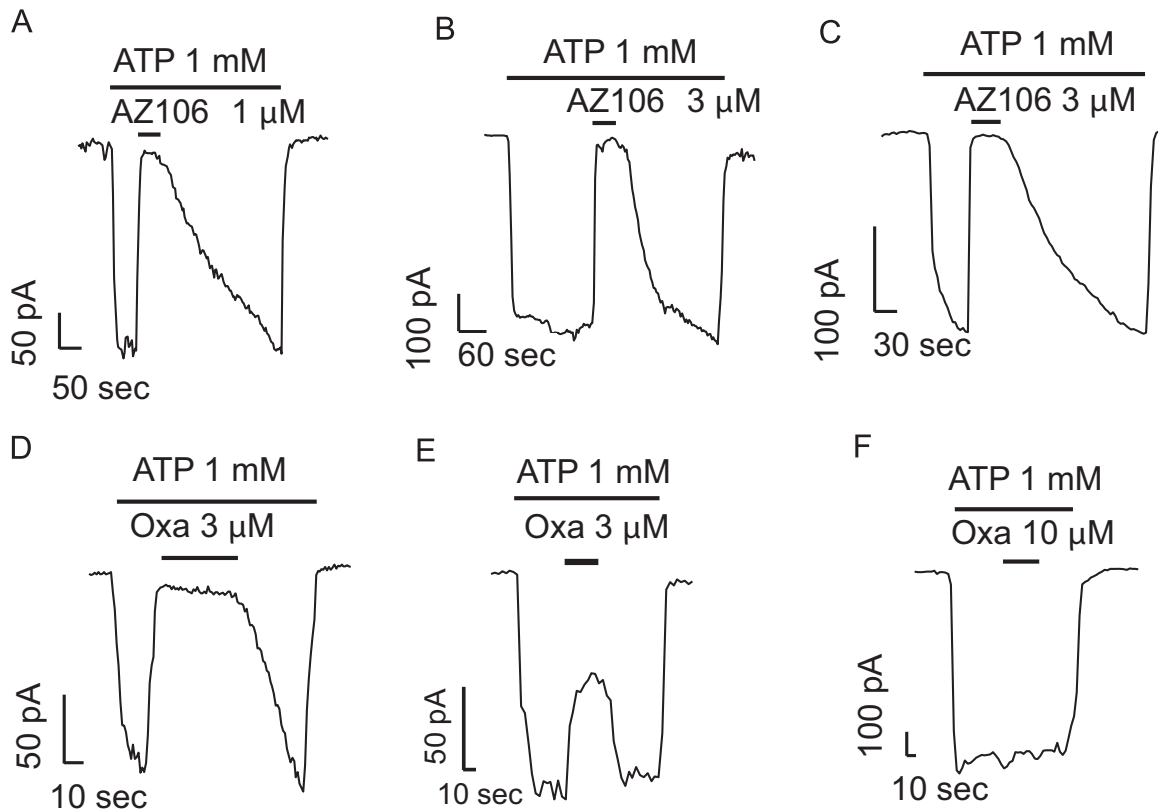


Fig. 5. Representative results showing the effects of oxatamide (Oxa) and AZ10606120 (AZ106) on the whole-cell current induced by human, mouse and rat P2X7 receptors. Human (A and D), mouse (B and E) and rat (C and F) P2X7 receptor-expressing HEK293 cells were stimulated by 1 mM ATP as indicated by grey line. Once the currents had reached a steady state, AZ106 (A–C) or Oxa (D–F) was co-applied with 1 mM ATP as indicated by black line. The currents shown were obtained at a membrane potential of -60 mV.

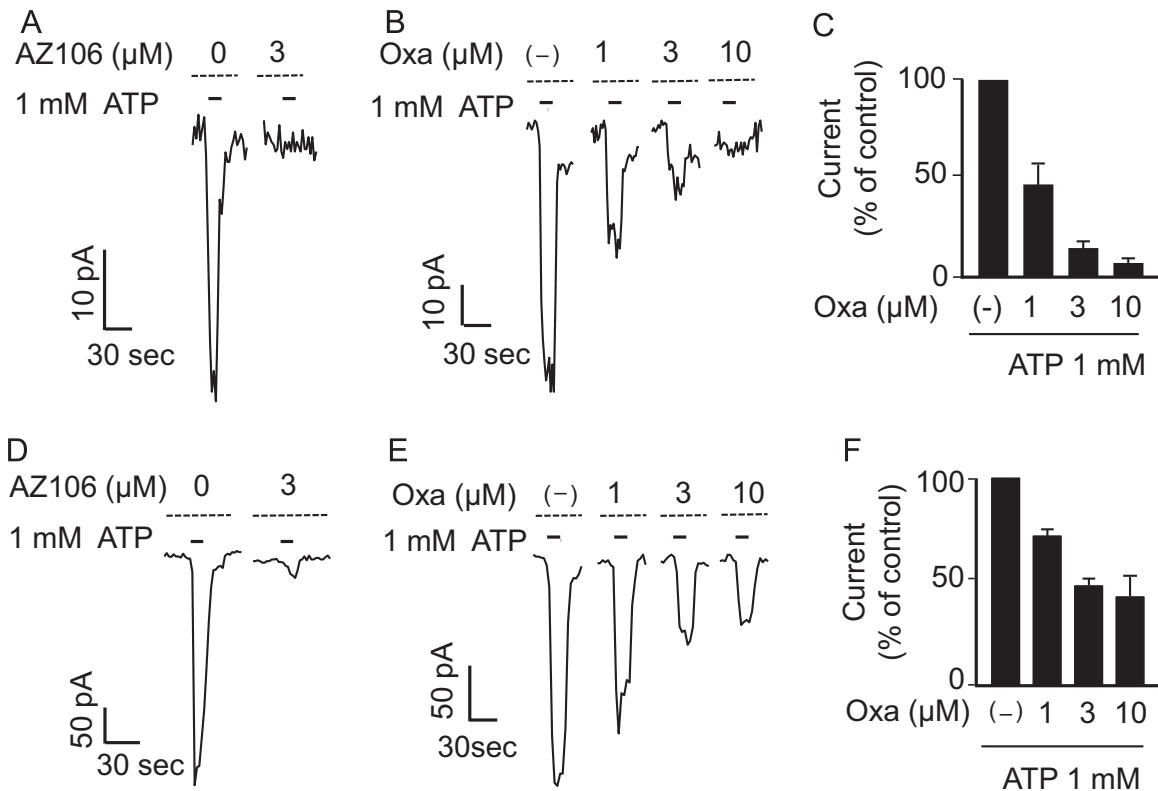


Fig. 6. Effect of different concentrations of oxatamide (Oxa) on whole cell current mediated by human and mouse P2X7 receptors. Human (A–C) or mouse (D–F) P2X7 receptor expressing HEK293 cells were stimulated with 1 mM ATP as indicated by black line in the presence of AZ10606120 (AZ106, 3 μM) or different concentrations of Oxa. Trace shown are representative of those obtained in human (A and B) and mouse (D and E) P2X7 receptor-expressing cells. Results obtained in human and mouse P2X7 expressing cells are summarized in C and F, respectively. Data show means $\pm$ S.E.M. ($n=3-5$).

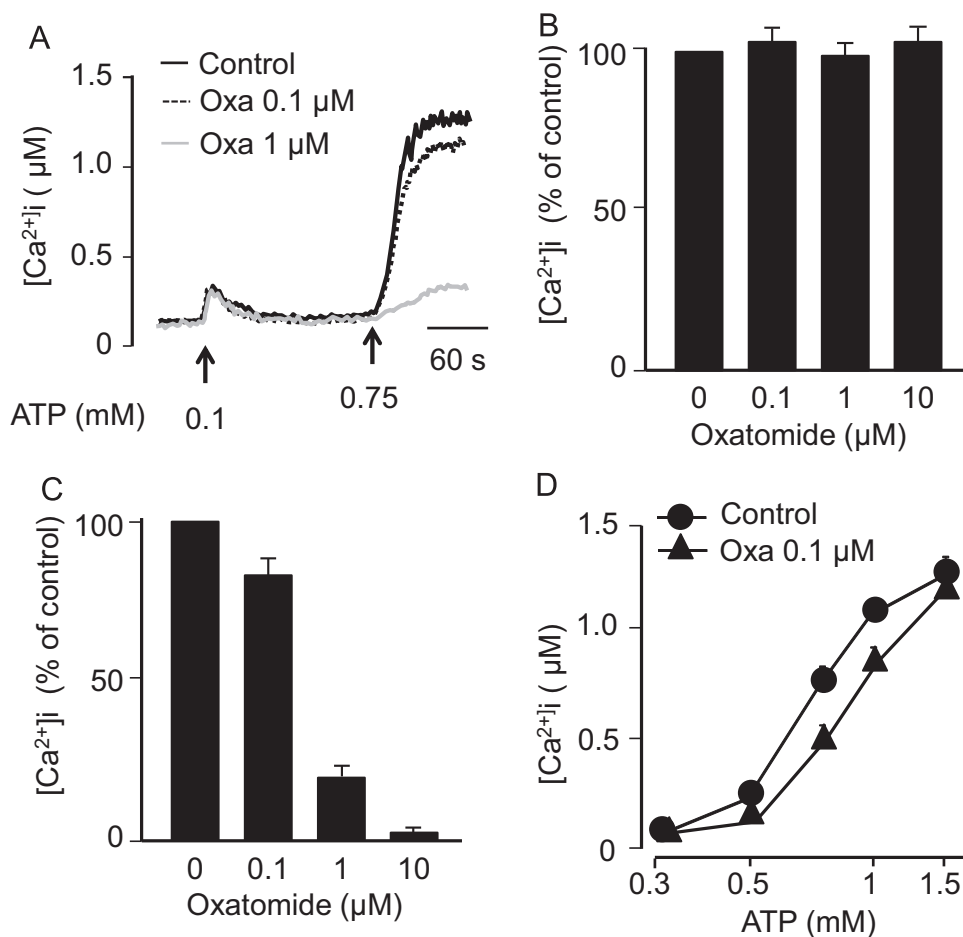


Fig. 7. Effect of oxatomide (Oxa) on human P2X7 receptor-mediated Ca^{2+} response in RPMI8226 cells. (A) Representative Ca^{2+} response to 0.1 and 0.75 mM ATP in the presence or absence of 0.1 and 1 μM Oxa. (B, C) Effect of different concentrations of Oxa on the Ca^{2+} response induced by 0.1 mM ATP (B) or 1 mM ATP (C). Results are expressed as a percentage of the control response. Data show mean $\pm$ S.E.M. ($n=4$). (D) Effects of 0.1 μM Oxa on the concentration- Ca^{2+} response curve for ATP in RPMI8226 cells. Cells were pre-incubated with 0.1 μM Oxa for 1 min, followed by treatment with different concentrations of ATP. Data show mean $\pm$ S.E.M. ($n=3-12$).

induced Ca^{2+} elevation. We also observed that a sustained increase in $[\text{Ca}^{2+}]_i$ induced by the P2X7-receptor agonist BzATP was inhibited by oxatomide as well as by AZ10606120 in RPMI8226 cells (Supplement Fig. 3).

3.7. Oxatomide inhibited P2X7 receptor-induced pore formation and membrane protein CD23 shedding in RPMI8226 cells

We further examined whether oxatomide could inhibit the human P2X7 receptor-mediated downstream events reported in RPMI8226 cells, such as pore formation and membrane protein shedding (Farrell et al., 2010; Pupovac et al., 2015). To observe these responses, it was necessary to remove extracellular Mg^{2+} and Ca^{2+} to increase the affinity of agonists to the P2X7 receptor (Surprenant et al., 1996). Stimulation of RPMI8226 cells with BzATP (10 μM) induced pore formation, which was evaluated by ethidium influx according to FACS analysis (Fig. 8A). This effect was totally inhibited by AZ10606120, suggesting that it was mediated by P2X7 receptor activation. Pretreatment with oxatomide inhibited BzATP-induced ethidium accumulation in a concentration-dependent manner (Fig. 8B). In addition to pore formation, P2X7 receptor activation has been shown to induce rapid shedding of the membrane surface protein CD23, resulting in a loss of CD23 on the cell surface, accompanied by an increase in soluble CD23 in cell-free supernatants (Farrell et al., 2010; Pupovac et al., 2015). We confirmed that ATP (0.75 mM) decreased surface immunoreactivity for CD23, and this response was inhibited by

AZ10606120 (Fig. 8C). Oxatomide inhibited the ATP-induced CD23 shedding in a concentration-dependent manner (Fig. 8D).

4. Discussion

The present study demonstrated that the anti-allergic agent oxatomide has an inhibitory effect on purinergic P2X7 receptors. There are many reports that P2X7 receptor function is inhibited by several chemicals, not only by the non-selective P2 receptor antagonists suramin and pyridoxalphosphate-6-azophenyl-2',4'-disulfonic acid (PPADS), but also by chemicals with other purposes such as KN-62 and brilliant blue (Donnelly-Roberts et al., 2009). Anti-allergic agents not only act as antihistamines or block chemical mediator secretion but also have multi-targeting inhibitory actions, such as anti-cholinergic, anti-PAF, and anti-leukotriene actions. We therefore screened several anti-allergic agents for their ability to affect P2X7 receptor function. To evaluate anti-P2X7 receptor activity, we used two mouse cell lines, J774 macrophages and N18TG2 neuroblastoma cells, in both of which we had previously characterized the functional expression of endogenous P2X7 receptors (Ito and Matsuoka, 2008; Kaiho et al., 1998). Treatment of these cells with lower concentrations of ATP or UTP rapidly desensitizes high-affinity purinoceptors, such as P2Y₁, P2Y₂ and P2X₄, thus allowing us to selectively investigate P2X7 receptor function by subsequently treating cells with higher concentrations (> 500 μM) of ATP. Using this system, we found

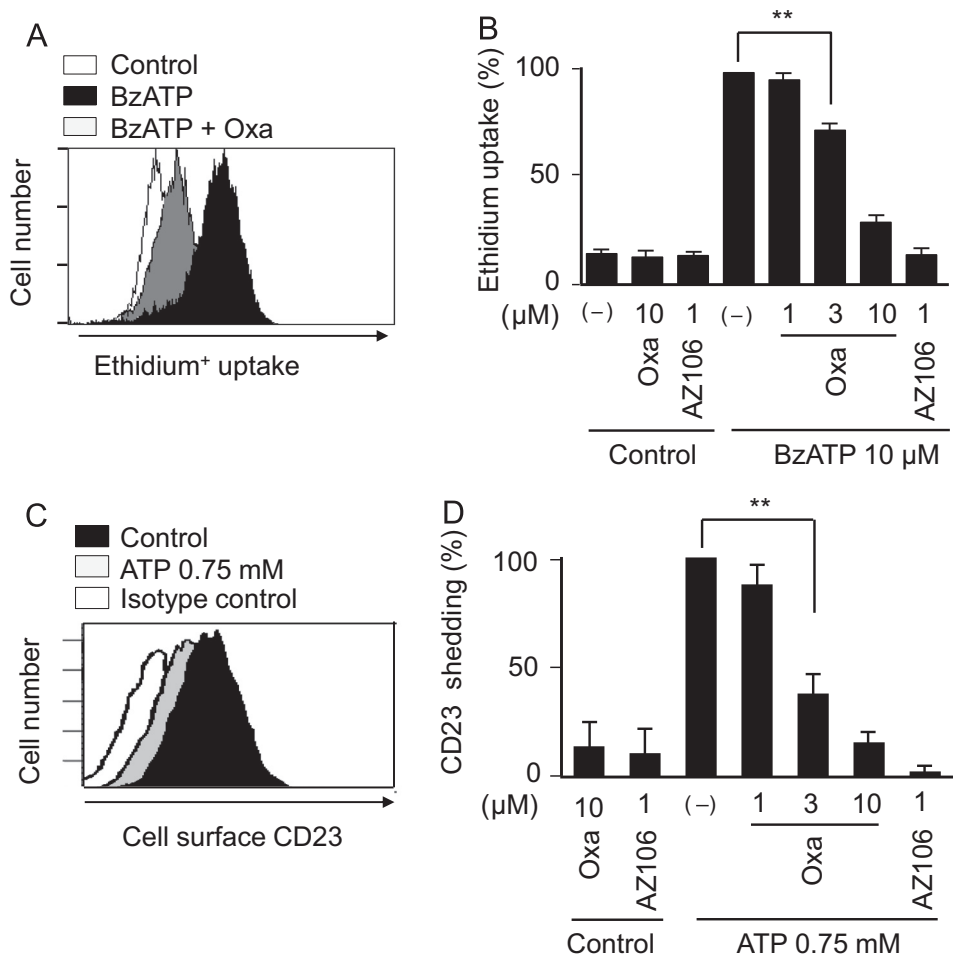


Fig. 8. Effects of oxatomide (Oxa) on human P2X7 receptor-mediated responses in RPMI8226 cells. (A) Representative FACS analysis of ethidium accumulation induced by 10 μ M BzATP for 5 min. (B) Cells were pre-incubated with AZ10606120 (AZ106, 1 μ M) or various concentration of Oxa for 5 min, followed by treatment with 10 μ M BzATP for 5 min in the presence of 2.5 μ M ethidium bromide. Data show mean $\pm$ S.E.M. ($n=3$) expressed as a percentage of the response induced by BzATP alone ($n=3$). * $P < 0.05$ (C) Representative FACS analysis of membrane CD23 shedding induced by 0.75 mM ATP for 30 min. The membrane surface CD23 was labelled with FITC-conjugated anti-CD23 monoclonal antibody or isotype control (mouse IgG2a), and analyzed using FACS. (D) Cells were pre-incubated with AZ106 (1 μ M) or various concentrations of Oxa for 5 min, followed by treatment with 0.75 mM ATP for 30 min. The membrane surface CD23 levels were analyzed using FACS. Data show mean $\pm$ S.E.M. ($n=5$) expressed as the percentage of control response induced by ATP alone ($n=5$). ** $P < 0.01$.

that oxatomide constantly inhibited P2X7 receptor mediated Ca^{2+} influx both in J774 and N18TG2 cells. The inhibitory effect of oxatomide on P2X7 receptor function was confirmed with respect to various downstream effects mediated by P2X7 receptor activation; oxatomide inhibited ATP-induced membrane current, p42/44 ERK and p38MAPK phosphorylation, and LDH release in N18TG2 cells. Oxatomide also inhibited P2X7 receptor-mediated inflammatory responses, such as induction of COX-2 and MIP-2 mRNA, and MIP-2 secretion in J774 macrophages. It is noteworthy that in mBMMCs, oxatomide inhibited ATP-induced degranulation – a response mediated by P2X7 receptors – at a concentration that did not affect the degranulation induced by antigen-induced cross-linking of Fc receptor. These findings suggest that the blockade of P2X7 receptors may partly contribute to the anti-allergy effects of oxatomide.

P2X7 receptor antagonists are known to exhibit a large inter-species variation in potency. For example, the Ca^{2+} /calmodulin-dependent protein kinase inhibitor KN-62 is a potent antagonist of human P2X7 receptors but has little effect on mouse or rat P2X7 receptors (Gargett and Wiley, 1997), whereas brilliant blue G displays marked selectivity for rat P2X7 receptors compared to mouse and human P2X7 receptors (Jiang et al., 2000). Although we initially identified the inhibitory effects of oxatomide on P2X7

receptor function in mouse cell lines, the electrophysiological studies in HEK293 cells, which express recombinant P2X7 receptors, revealed that oxatomide is the most potent antagonist of the human P2X7 receptor. Indeed, oxatomide inhibited the human P2X7 receptor current in a concentration-dependent manner with IC_{50} values of 0.95 ± 0.15 μ M, whereas it only partly inhibited (40% of the control) mouse P2X7 with an IC_{50} value of 4.1 ± 1.3 μ M. In addition, we did not observe any effect of oxatomide on rat P2X7 receptors at concentrations up to 10 μ M. The potent antagonist effect of oxatomide on the native human P2X7 receptor was confirmed in human B cell-like myeloma RPMI8226 cells; oxatomide inhibited ATP-induced Ca^{2+} influx in a concentration-dependent manner with an IC_{50} value of 0.43 ± 0.08 μ M, i.e., with nearly 8 times the potency observed in mouse N18TG2 cells ($\text{IC}_{50}=3.3 \pm 1.0$ μ M).

The mode of action of oxatomide is quite different from those reported for other P2X7 receptor-affecting chemicals including KN-62, brilliant blue 2 and PPADS, which have been shown to have a slow onset in terms of P2X7 receptor inhibition and are only partially reversible by washing (Donnelly-Roberts et al., 2009; Gargett and Wiley, 1997; Jiang et al., 2000). Electrophysiological studies in both human and mouse P2X7 receptor-expressing cells demonstrated that the effects of oxatomide had a rapid onset and

could be reversed by washing out the compound. When ATP-induced current reached a steady-state, co-application of oxatomide and ATP immediately decreased the current; after removal of oxatomide from ATP solution, the current recovered within few minutes. These results suggest that the site of action of oxatomide may be located in the extracellular domain of the receptor and inhibit receptor-ligand interaction in a competitive manner. These interpretations are consistent with the results obtained in the Ca^{2+} influx assay in RPMI8226 cells that showed that oxatomide at a concentration of 0.1 μM resulted in a parallel shift of the ATP concentration-response curve to the right without any change in the maximal response. However, AZ10606120, which had similar effects to oxatomide, has been shown to act as an allosteric P2X7 receptor antagonist (Michel et al., 2007). Therefore, further investigations are needed to determine the complete mode of action of oxatomide.

Oxatomide was originally developed as antihistamine and found to inhibit allergic and other hypersensitive reactions (Awouters et al., 1977). Clinically, oxatomide is indicated for the treatment of allergic rhinitis, chronic urticarial, atopic dermatitis and child bronchial asthma (Marone et al., 1999). In addition to its antihistamine effect, it has been reported to attenuate various pathophysiological responses induced by serotonin, cysteinyl-leukotrienes and PAF (Nijkamp et al., 1989). The present study is the first to show that oxatomide has an antagonistic action on P2X7 receptors. Accumulating evidence from recent studies has indicated that P2X7 receptor-mediated signalling is implicated in allergic airway inflammation (Müller et al., 2011), allergic contact dermatitis (Weber et al., 2010) and food allergy-related bowel disease (Kurashima et al., 2012). It is therefore important to consider whether the inhibition of P2X7 receptor could contribute to the clinical effects of oxatomide. The peak plasma concentration of oxatomide at a clinical dose (30 mg, twice a day) was reported to be 40 ng/ml (Davies and Rocchiccioli, 1983), corresponding to around 0.1 μM . Because the results of the present study showed that oxatomide at 0.1 μM had only a slight inhibitory effect on human P2X7 receptor-mediated responses *in vitro*, it is unlikely that orally administered oxatomide exerts its clinical effects systemically through P2X7 receptor antagonism. However, earlier reports concerning the pharmacodynamics of oxatomide stated that "some patients responding to oxatomide were said to be unresponsive to previously administered antihistamines", and "somewhat more encouraging results have been reported in children with bronchial asthma when higher than presently recommended dosages were employed" (Richards et al., 1989). These unique clinical features of oxatomide might be explained by its effect on P2X7 receptors. Because oxatomide has been used safely as an anti-allergic drug for a long time, future studies should determine whether P2X7 receptor antagonism is involved in the clinical effect of oxatomide with local administration, such as inhalation or transdermal absorption, that can increase the drug concentrations at the target inflamed site.

In conclusion, the present study demonstrates that oxatomide has an inhibitory effect on the purinergic P2X7 receptors in a reversible manner. The effect of oxatomide exhibited large inter-species variation in potency; however, oxatomide was most potent towards human P2X7 receptors. Further studies should evaluate whether the inhibitory effects of oxatomide on the P2X7 receptor can translate into clinical effects.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found in the online version at <http://dx.doi.org/10.1016/j.ejphar.2015.10.002>.

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