P2X7 ionotropic receptor is functionally expressed in rabbit articular chondrocytes and mediates extracellular ATP cytotoxicity

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Abstract

Extracellular ATP regulates various cellular functions by engaging multiple subtypes of P2 purinergic receptors. In many cell types, the ionotropic P2X7 receptor mediates pathological events such as inflammation and cell death. However, the importance of this receptor in chondrocytes remains largely unexplored. Here, we report the functional identification of P2X7 receptor in articular chondrocytes and investigate the involvement of P2X7 receptors in ATP-induced cytotoxicity. Chondrocytes were isolated from rabbit articular cartilage, and P2X7 receptor currents were examined using the whole-cell patch-clamp technique. ATP-induced cytotoxicity was evaluated by measuring caspase-3/7 activity, lactate dehydrogenase (LDH) leakage, and prostaglandin E₂ (PGE₂) release using microscopic and fluorimetric/colorimetric evaluation. Extracellular ATP readily evoked a cationic current without obvious desensitization. This ATP-activated current was dose related, but required millimolar concentrations. A more potent P2X7 receptor agonist, BzATP, also activated this current but at 100-fold lower concentrations. ATP-induced currents were largely abolished by selective P2X7 antagonists, suggesting a predominant role for the P2X7 receptor. RT-PCR confirmed the presence of P2X7 in chondrocytes. Heterologous expression of a rabbit P2X7 clone successfully reproduced the ATP-induced current. Exposure of chondrocytes to ATP increased caspase-3/7 activities, an effect that was totally abrogated by P2X7 receptor antagonists. Extracellular ATP also enhanced LDH release, which was partially attenuated by the P2X7 inhibitor. The P2X7 receptor-mediated elevation in apoptotic caspase signaling was accompanied by increased PGE₂ release and was attenuated by inhibition of either phospholipase A₂ or cyclooxygenase-2. This study provides direct evidence for the presence of functional P2X7 receptors in articular chondrocytes. Our results suggest that the P2X7 receptor is a potential therapeutic target in chondrocyte death associated with cartilage injury and disorders including osteoarthritis.

Keywords Purinergic receptors · P2X7 · ATP · Articular chondrocyte · Cytotoxicity

Introduction

Extracellular ATP is an important signaling molecule that regulates a broad range of cellular functions [1]. In the nervous system, ATP is released in concert with other neurotransmitters from nerve terminals to modulate synaptic transmission [2–4]. In addition, ATP is secreted by most cell types and, once secreted, it serves as a signaling molecule via

autocrine/paracrine pathways. In articular cartilage, chondrocytes release ATP in response to physiological and pathological stimuli. Previous studies have shown that mechanical loading of chondrocytes promotes ATP release which in turn regulates cellular metabolism and differentiation [5]. On the other hand, excessive ATP leakage from injured chondrocytes can occur during trauma and inflammation and has been implicated in the pathogenesis of joint diseases. Indeed, synovial fluid ATP levels are higher in patients with osteoarthritis (OA) [6] or rheumatoid arthritis (RA) [7]. In addition, there is a clinical link between OA-associated pain and ATP levels in synovial fluid [6]. Pericellular ATP accumulation causes articular calcium crystal deposition by increasing levels of its metabolite pyrophosphate [8]. It is also reported that extracellular ATP interacts with cytokines to stimulate the release of prostaglandin E₂ (PGE₂) from chondrocytes [9].

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A number of purinergic receptor subtypes have been implicated in the ATP-induced pro-inflammatory responses [10]. Two distinct families of purinergic receptors, P1 receptors for adenosine and P2 receptors for ATP, are widely distributed. P2 receptors are further divided into ligand-gated ion channel receptors, P2X (P2X1–7), and G protein-coupled P2Y (P2Y₁, 2, 4, 6, 11–14) subfamilies. The P2X7 receptor belongs to the P2X ionotropic receptor family, but it was originally identified as a unique ATP receptor termed the P2Z receptor due to its peculiar functional features which are distinct from P2X and P2Y receptors [11]. Notably, the P2X7 receptor requires an unusually high concentration (millimolar range) of ATP for full activation, suggesting a specific role of P2X7 under pathological conditions. In line with this, there is increasing experimental evidence supporting the concept that extracellular ATP stimulates P2X7 receptors as a ‘danger signal’ triggering the inflammatory response and subsequent cell death of immune cells as well as other cell types [12]. A previous immunocytochemical study demonstrated that articular chondrocytes express abundant P2X7 receptors [13]; yet, little is known about its ionotropic properties or its functional significance.

In the present study, we investigated the electrophysiological properties of the P2X7 receptor channel in rabbit chondrocytes using the whole-cell patch-clamp technique. In addition, we studied the P2X7-dependent cytotoxic effects of high concentrations of extracellular ATP. In this manuscript, we report the first experimental evidence that the chondrocyte P2X7 receptor serves as a ‘death receptor’ for extracellular ATP-induced release of pro-inflammatory PGE₂ and subsequent apoptotic cell death.

Methods

Isolation of rabbit articular chondrocytes

Adult male Japanese White rabbits (body weight 2.5 to 3.5 kg, *N* = 32) were obtained from Shimizu Laboratory Supplies (Kyoto, Japan), housed 1 per cage (FRP bracket cages, 350 × 527 × 350 mm³), and given access to food and water ad libitum. All animals were given at least 3 days to acclimate to the housing facility under SPF conditions at a temperature of 23 ± 5 °C and a humidity of 40–60% in a 12-h light/12-h dark cycle with light on at 0800 and off at 2000. During the acclimation period, the health status of animals was checked every day and no adverse events were observed. Articular chondrocytes were isolated using the enzymatic dissociation procedure described previously [14]. In brief, rabbits were

deeply anesthetized with an i.m. injection of ketamine (70 mg/kg) and xylazine (5 mg/kg) and then killed by an i.v. injection of sodium pentobarbital (70 mg/kg). Articular cartilage was removed from bilateral knee, hip, and shoulder joints as previously described by our group [15]. We carefully shaved off the cartilage to include full depth cartilage but no bone tissue. Cartilage was washed with PBS (ICN Biomedicals Inc., Aurora, OH, USA) and incubated in plastic culture dishes containing serum-free Dulbecco’s modified Eagle’s medium (DMEM; Gibco, Carlsbad, CA, USA) supplemented with antibiotics in a humidified atmosphere of 95% air plus 5% CO₂ at 37 °C for up to a week. On the day of experiments, cartilage was minced into small pieces (~1 mm³) and digested with 0.5% collagenase (type 2; Worthington Biochemical Corp, Lakewood, NJ, USA) for 3.5 h. Dispersed chondrocytes were washed three times and re-suspended in DMEM supplemented with 40 mM mannitol (~360 mosmol/L; close to the physiological osmolality for chondrocytes) [15].

Drugs

ATP (disodium salt, Sigma-Aldrich, St Louis, MO, USA) was directly added to the external solution. After the addition of ATP, the pH of the experimental medium was appropriately adjusted with NaOH. A concentrated stock solution of BzATP (2’(3’)-O-(4-Benzoylbenzoyl)adenosine 5’-triphosphate triethylammonium salt, Sigma-Aldrich) was prepared in dimethyl sulfoxide (50 mM) and diluted into the experimental solution at the final concentrations (0.1–100 μM). These concentrations are lower than those (> 300 μM) reported to induce the receptor-independent effects of triethylammonium on cytosolic pH [16]. A438079 (Abcam PLC, Cambridge, UK) and A740003 (Sigma-Aldrich) were dissolved in water (5 mM), KN-62 (Abcam) in DMSO (10 mM), AACOCF₃ (Abcam), celecoxib (Tocris, Ellisville, MO, USA), and etodolac (Wako Pure Chemical Industries, Osaka, Japan) in ethanol (10 mM). These were appropriately diluted in the extracellular solution or stored in aliquots at –20 °C before use.

Whole-cell patch clamp technique

Whole-cell membrane currents [17] were recorded from isolated rabbit chondrocytes using an EPC-10 patch-clamp amplifier (HEKA, Lambrecht, Germany). Fire-polished pipettes, once pulled from borosilicate glass capillaries (Narishige Scientific Instrument Lab., Tokyo, Japan), had a resistance of 2.5 to 4.0 MΩ when filled with the standard pipette solution. Since the current through P2X7 receptors is partially suppressed in the

presence of extracellular divalent cations [18, 19], recording of the current was performed in a nominally Ca^{2+} -free standard external solution (in mM): mannitol 160, NaCl 100, MgCl_2 0.5, glucose 5.5, HEPES 10 (pH adjusted to 7.4 with NaOH), with 5 μM nisoldipine. The standard pipette solution contained the following (in mM): L-aspartic acid 120, CsOH 100, CsCl 30, tetraethylammonium chloride 20, MgCl_2 2.0, EGTA 5.0, HEPES 5.0, ATP- Na_2 5.0, GTP- Li_2 0.1 (pH adjusted to 7.2 with CsOH). Either square step or voltage ramp protocols were used to record whole-cell currents. Voltage ramps were used to monitor the time course of changes in membrane currents during various interventions. The voltage ramp protocol was repeated every 6 s and consisted of three phases: an initial +50 mV depolarizing phase from a holding potential of −30 mV, a second hyperpolarizing phase of 150 mV (from +50 mV to −100 mV) and then a third phase returning to the holding potential. The current–voltage (I – V) relationship was measured during the second hyperpolarizing phase. Voltage-clamp protocols and data acquisition were controlled with Patchmaster software (v2x90, HEKA), and current records were digitized at 5 kHz and filtered at 1 kHz.

YO-PRO-1 dye uptake

The pore formation of P2X7 receptors was examined by microscopic observations of chondrocyte uptake of the large cationic dye YO-PRO-1 Iodide (Invitrogen, San Diego, CA, USA). Chondrocytes were isolated and divided into three groups on black 96-well plates with glass bottoms. The experimental external solution was the same as used in patch-clamp experiments except that MgCl_2 was omitted to avoid the inhibition of P2X7 pore formation by external divalent cations [20]. YO-PRO-1 (2 μM) was added to all cell samples. ATP (3 mM) was applied with and without A438079 (1 μM). After 1-h treatment with ATP (37 °C, 5% CO_2), cellular uptake of YO-PRO-1 was observed using a FV1000 confocal laser scanning microscope (Olympus, Tokyo, Japan), and fluorescent cells were counted using image analysis software (Image J, NIH, Bethesda, MD, USA) to quantify the percentage of cells exhibiting the dye uptake.

RT-PCR cloning and heterologous expression

Total RNA was extracted and purified from freshly isolated chondrocytes using a NucleoSpin RNA kit (Machery-Nagel, Düren, Germany), and first-strand cDNA synthesis was subsequently carried out on 2 μg of total RNA using SuperScript IV reverse transcriptase (Invitrogen) with an oligo (dT) 20 primer. PCR for rabbit P2X7 was performed using Tks Gflex DNA polymerase (Takara Bio, Otsu, Japan) with the

following primer set: 5'-GGG GAG TGG CTC ACC ATG-3' (sense; position 117–134, accession no. XM_002719745) and 5'-ATC CCC ATG CTG TCA GTA A-3' (antisense; position 1931–1913, accession no. XM_002719745). PCR products were identified in an ethidium bromide-stained 1.5% agarose gel by electrophoresis, purified using the Wizard SV Gel and PCR Clean-Up System (Promega, Madison, WI, USA), and then inserted into the pCR3.1 mammalian expression vector (Invitrogen). Sequencing of the cloned plasmid DNA was performed using the Big Dye Terminator v3.1 kit on an ABI 3500 sequencer (Applied Biosystems, Darmstadt, Germany).

HEK293 cells were maintained in DMEM supplemented with 10% fetal calf serum and antibiotics (100 IU/mL penicillin and 100 mg/mL streptomycin) in a humidified incubator gassed with 5% CO_2 and 95% air at 37 °C. Before transfection, cells were seeded onto 35-mm plastic culture dishes with 7–8 glass coverslips (5 mm × 3 mm) in the bottom and incubated for 24–48 h. Transient transfection was performed using Lipofectamine 3000 (Invitrogen). The amounts of each cDNA used for transfection were as follows ($\mu\text{g}/\text{dish}$): 1.0 P2X7 and 0.5 GFP. Only GFP-positive cells were used for the patch-clamp study 48 h after transfection.

Caspase3/7 assay

Activity of caspases 3 and 7 (caspase-3/7) as an indicator of chondrocyte apoptosis was fluorometrically evaluated using a CellEvent® caspase-3/7 Green Detection Reagent (Invitrogen) according to the manufacturer's instructions. Isolated chondrocytes were seeded onto black 96-well plates with glass bottoms and pre-incubated in serum-free DMEM for 3 h (37 °C, 5% CO_2). Then CellEvent® Reagent was added to the cell culture medium at a final concentration of 5 μM . ATP (3 mM) was applied alone or in combination with A438079 (1 μM), AACOCF3 (10 μM), celecoxib (10 μM), or etodolac (10 μM). After chondrocytes had been exposed to 3 mM ATP for 3 h, fluorescent images were captured using an LSM 510 META (Carl Zeiss, Jena, Germany) confocal laser scanning microscope with FITC/Alexa Fluor 488 filter settings. Fluorescence intensity of each well was measured using an Infinite 200 microplate reader (Tecan Trading AG, Switzerland) with an excitation wavelength of 502 nm and emission wavelength of 530 nm.

Lactate dehydrogenase assay

LDH activity as a measure of necrotic chondrocyte death was assessed using a Cytotoxicity LDH Assay Kit-WST (Dojindo Laboratories, Kumamoto, Japan) according to the manufacturer's protocol. Isolated chondrocytes were seeded onto black 96-well plates with glass bottoms and pre-incubated in serum-free DMEM for 3 h (37 °C, 5% CO_2). ATP (3 mM) was added with or without A438079 (1 μM). After a 3-h incubation,

LDH activity in the medium was quantified by colorimetric measurements of absorbance at 490 nm using an Infinite 200 microplate reader (Tecan Trading AG, Switzerland). The LDH activity was normalized to a positive control value obtained by adding cell lysis buffer to calculate relative LDH release (%).

PGE₂ assay

The PGE₂ release from chondrocytes during exposure to ATP was evaluated by measuring PGE₂ levels using a commercially available competitive enzyme immunoassay (Enzo Life Sciences, Farmingdale, NY, USA). Isolated rabbit chondrocytes were divided into four groups and seeded into wells of a black 96-well plate in serum-free DMEM and pre-incubated for 24 h (37 °C, 5% CO₂). ATP (3 mM) was added alone or in combination with A438079 (1 μM), ZnCl₂ (300 μM), AACOCF₃ (10 μM), celecoxib (10 μM), or etodolac (10 μM). In some experiments, the nominally Ca²⁺-free PBS was used as the external medium. After a 1-h incubation, supernatants (100 μL) were collected and immediately used for measurement of PGE₂ levels.

Statistical analysis

All data are expressed as means ± SEM, with the number of animals and experiments indicated by *N* and *n*, respectively. Statistical comparisons were evaluated using either Student's *t*

test or ANOVA (as appropriate) followed by a post hoc Tukey's test, and differences were considered significant at *P* < 0.05.

Results

Extracellular ATP-induced currents in rabbit articular chondrocytes

The current response to extracellular ATP was examined in freshly isolated single chondrocytes using the whole-cell patch-clamp technique (Fig. 1). ATP was added to the external solution at a concentration of 300 μM, which can potentially activate all known subtypes of P2X receptor channels including P2X₇ [21]. In addition, extracellular ATP might evoke Ca²⁺-activated currents indirectly as a consequence of P2Y receptor stimulation, causing an increase in intracellular Ca²⁺ [22]. In order to minimize contamination of the Ca²⁺-activated currents, recording was performed using a pipette solution containing 5 mM EGTA and the nominally Ca²⁺-free external solution. Figure 1A shows typical current traces recorded before and during application of 300 μM ATP. In response to the ATP application, both inward and outward currents increased in the test potential range between −100 and +50 mV (Fig. 1A, a and b). The ATP-induced “difference” current obtained by digitally subtracting the control current from the current in the

Fig. 1 Whole-cell membrane currents activated by extracellular ATP in rabbit articular chondrocytes. **A a** Representative superimposed current traces in response to 200-ms square-steps applied from a holding potential of −30 mV to test potentials between +50 mV and −100 mV in 10-mV steps before (○) and during (●) exposure to 300 μM ATP. Membrane currents induced by ATP (□), obtained by digital subtraction of current traces (● − ○). Dotted line indicates the zero-current level. **b** Corresponding *I*–*V* relationships of whole-cell currents recorded before (○) and during (●) exposure to 300 μM ATP. **c** *I*–*V* relationship for the ATP-induced “difference” currents, obtained by digital subtraction of

currents traces (● − ○). **B a** Representative continuous chart recording of the whole-cell membrane current during exposure to 300 μM ATP (*left panel*). Vertical deflections of the current trace reflect the imposition of the voltage ramp pulse applied at 6 s intervals. The *right panel* indicates the expanded sample traces in response to the voltage ramp. **b** Superimposed *I*–*V* relationships obtained from current recordings in response to the descending limb of the voltage ramp from +50 to −100 mV before (Cont) and during 2-min exposure to ATP. **c** *I*–*V* relationships of the ATP-induced current obtained by subtraction of the same control trace from the ramp currents recorded during exposure to ATP

presence of ATP exhibited no obvious time dependence and the corresponding I – V relationship showed weak inward rectification with a reversal potential of -7.5 mV (close to the predicted value of -6.7 mV for the nonselective cation conductance under our experimental conditions) (Fig. 1A, a and c).

Previous studies reported differential gating properties among P2X subtypes. Particularly, currents through the P2X7 receptor show little desensitization and reduced rectification compared to those of other P2X subtypes [20]. In the experiment shown in Fig. 1B, changes in the membrane current during a 2-min exposure to ATP were observed by applying a voltage ramp pulse (187.5 mV/s, $+50 \sim -100$ mV) every 6 s to quickly obtain the I – V relationship of the ATP-induced current. Extracellular ATP readily and reversibly activated inward and outward currents without exhibiting apparent desensitization (Fig. 1B, a and b). In addition, the I – V relationship acquired during exposure to ATP showed no obvious changes in either the reversal potential or the rectification property, suggesting that the ATP-induced current is attributable to the activation of a single subtype rather than multiple subtypes of P2X receptor channels.

Functional identification of P2X7 receptors in rabbit articular chondrocytes

We next investigated the current response to the ATP analog BzATP, a more potent agonist of P2X7 receptors than ATP itself [11, 20]. As shown in Fig. 2, exposure of chondrocytes to BzATP activated the membrane current in a similar manner, both quantitatively and qualitatively, to the ATP-induced

current (see also Fig. 1). The average current density of the current activated by $3 \mu\text{M}$ BzATP was 10.2 ± 1.4 pA/pF at -100 mV ($n = 4$, $N = 3$), comparable to that of the ATP-induced current at a concentration of $300 \mu\text{M}$ (8.3 ± 1.1 pA/pF, $n = 10$, $N = 5$). Furthermore, the BzATP-induced current exhibited no obvious desensitization and weak inward rectification (Fig. 2B), again overlapping with properties of the ATP-induced current. It should be noted that qualitatively similar responses to ATP or BzATP were observed in all tested cells ($n = 28$).

In Fig. 3, the concentration–response relationships for current activation by ATP and BzATP are shown. Both ATP and BzATP increased the membrane current in a concentration-dependent manner, but exhibited distinct thresholds for current activation (ATP $\sim 100 \mu\text{M}$; BzATP, $\sim 1 \mu\text{M}$). We failed to observe their maximal efficacy even at the highest concentrations of both agonists. Nevertheless, it is apparent that BzATP showed much greater potency than ATP, as expected for the P2X7 receptor [21]. In addition, very small responses to ATP were observed at concentrations lower than $30 \mu\text{M}$, suggesting that the obtained data largely represent the P2X7 current density.

To determine the relative contribution of P2X7 receptors to current activation by extracellular ATP in chondrocytes, we examined effects of the P2X7 receptor selective inhibitors, A438079 and A740003, on the current evoked by $300 \mu\text{M}$ ATP. As demonstrated in Fig. 4A, B, competitive blocking of ATP binding to P2X7 receptors by either A438079 or A740003 at $1 \mu\text{M}$ readily inhibited a large fraction of the ATP-induced current (A438079, $63.5 \pm 3.6\%$ inhibition, $n =$

Fig. 2 Whole-cell membrane currents activated by extracellular BzATP in rabbit articular chondrocytes. **A** *a* Representative superimposed current traces recorded using the same ramp protocol as in Fig. 1A before ($\circ$) and during ($\bullet$) exposure to $3 \mu\text{M}$ BzATP. The BzATP-induced currents ($\square$) were obtained by digital subtraction of current traces ($\bullet - \circ$). *b* Corresponding I – V relationships of whole-cell currents recorded before ($\circ$) and during ($\bullet$) exposure to $3 \mu\text{M}$ BzATP. *c* I – V relationship for the

BzATP-induced currents, obtained by digital subtraction of currents traces ($\bullet - \circ$). **B** *a* Representative chart recording of the whole-cell membrane current during exposure to $3 \mu\text{M}$ BzATP. Sample traces and panels are labeled as in Fig. 1B. *b* Superimposed I – V relationships before (Cont) and during exposure to BzATP. *c* Corresponding I – V relationships of the BzATP-induced currents

Fig. 3 Concentration-dependent activation of whole-cell membrane currents by extracellular ATP and BzATP. Concentration–response relationships of absolute values of current densities induced by ATP (●) and BzATP (○) at various concentrations. Current amplitudes measured at -95 mV were normalized with respect to cell membrane capacitance and plotted as a function of applied concentrations of ATP or BzATP. Data points are shown as mean $\pm$ SEM ($n=9$ for ATP and $n=5$ for BzATP)

4, $N=2$; A740003, $56.3 \pm 9.6\%$ inhibition, $n=4$, $N=2$). More complete inhibition was obtained at higher concentrations of 10 – 100 μM (Fig. 4C), albeit somewhat less potency than expected from previously reported pIC_{50} values (6.5 – 7.8) for the inhibition of Ca^{2+} influx in recombinant P2X7 receptors [23]. However, these inhibitors are highly selective without affecting other P2 receptors even at 100 μM [23]. We also examined the sensitivity to blocking by KN-62, an allosteric non-competitive antagonist of human P2X7 receptors but inactive on rat P2X7 receptors [24]. As shown in Fig. 4D, a 3-min pretreatment of chondrocytes with KN-62 (1 μM) almost totally abolished the current activation by extracellular ATP, providing evidence for the sensitivity of rabbit P2X7 receptors to KN-62. In addition, blocking of cation-conducting pores of the P2X receptor by external addition of 300 μM ZnCl_2 completely abolished the ATP-induced current (Fig. 4E). It should be noted that ZnCl_2 also reduced the background conductance in the absence of ATP, probably due to inhibition of cation channels such as TRPV5 in chondrocytes [25]. Taken together, these pharmacological assessments clearly indicate that the P2X7 receptor is predominantly responsible for the ATP-induced current in articular chondrocytes.

Since it is known that prolonged stimulation of P2X7 receptors by high (millimolar) concentrations of ATP results in

Fig. 4 Inhibition of ATP-induced current by P2X7 receptor blockers. **A**, **B** Effects of A438079 and A740003 on the ATP-induced current. Superimposed I – V relationships of whole-cell currents recorded using the same ramp protocol as in Fig. 1B before (*a*) and during exposure to ATP (300 μM , *b*), and after addition of either A438079 (**A**, *c*) or A740003 (**B**, *c*) at a concentration of 1 μM . The inset indicates a representative original chart recording of the membrane current during the experiment. **C** Percent inhibition of the ATP-induced current by A438079 and A740003 at the indicated concentrations. Bars represent mean $\pm$ SEM

($n=4$). **D** Effects of KN-62 on the ATP-induced current. Currents were repeatedly activated by 300 μM ATP with a resting interval of >3 min in the absence (*upper trace*) and presence (*lower trace*) of 1 μM KN-62. **E** Inhibition of the ATP-induced current by ZnCl_2 . Superimposed I – V relationships of whole-cell currents before (*a*) and during exposure to ATP (300 μM , *b*), and after addition of ZnCl_2 (300 μM , *c*). The inset indicates an original chart recording of the membrane current during the experiment

Fig. 5 YO-PRO-1 dye uptake induced by extracellular ATP in chondrocytes. **A** Representative confocal microscopic images of chondrocytes exposed for an hour to a nominally divalent cation-free external solution containing 2 μ M YO-PRO-1 iodide in the absence (*left*) and the presence of 3 mM ATP (*middle*) or 3 mM ATP plus 1 μ M A438079 (*right*). **B** Percentage of cells exhibiting YO-PRO-1 uptake. Bars represent means $\pm$ SEM ($n = 3$). $^{\S}P < 0.05$

formation of a pore permeable to large molecules of up to 900 Da [26], we also tested the YO-PRO-1 dye (629 Da) uptake in chondrocytes continuously exposed to 3 mM ATP. As is evident from the microscopic observations shown in Fig. 5, 1-h exposure of chondrocytes to 3 mM ATP successfully induced the dye uptake in $20.5 \pm 3.5\%$ of cells, which was significantly attenuated by P2X7 receptor inhibition by A438079 (1 μ M) ($4.7 \pm 0.8\%$, $P < 0.001$, $n = 5$, $N = 3$). In addition to our electrophysiological and pharmacological characterization (no obvious desensitization, weak inward rectification, low affinity for ATP, preferential activation by BzATP, and sensitivity to blocking by various inhibitors), the observation of the large-pore formation provided strong evidence for the presence of functional P2X7 receptors in rabbit articular chondrocytes.

P2X7 expression in rabbit articular chondrocytes was further validated by RT-PCR cloning and sequence analysis. The designated primers successfully amplified the expected P2X7 fragment with the entire coding sequence of 1785 bp (Fig. 6A). Nucleotide sequence analysis of the PCR product indicated 99.7% identity with the predicted sequence of rabbit P2X7 mRNA in GenBank (accession number: XM_002719745). The deduced amino acid sequence of 595 residues shares 78.3 and 86.1% identity with rat and human P2X7, respectively

(Fig. 6B). Interestingly, a higher level (93.5%) of sequence identity between rabbit and human was found in the extracellular domain (a linker between TM-1 and TM-2), including Phe 95 which is known to determine the species-specific sensitivity of P2X7 to KN-62 [27, 28]. We then inserted the P2X7 coding sequence into the pCR3.1 mammalian expression vector to investigate the ionotropic property in a heterologous expression system. Figure 6C shows an example of an ATP-activated current through rabbit P2X7 receptors transiently expressed in HEK293 cells, recapitulating the conductive and kinetic features of the ATP-induced current in chondrocytes. It was notable that current activation by extracellular ATP was negligibly small in non-transfected cells, indicating minimum contamination of endogenous ATP-induced currents in HEK293 cells. As expected, reconstituted rabbit P2X7 receptors exhibited sensitivity to blocking by KN-62 (Fig. 6D).

ATP-induced chondrocyte apoptosis and necrosis via P2X7 receptor activation

We next explored the potential cytotoxic effect of P2X7 receptors in chondrocytes. For this purpose, caspase-3/7 activation was evaluated as a conventional apoptotic signature with a fluorogenic caspase-cleavable substrate (a four amino acid

Fig. 6 RT-PCR cloning, sequencing, and functional characterization of recombinant rabbit P2X7 receptor. **A** P2X7 mRNA expression in rabbit articular chondrocytes. Ethidium bromide-stained agarose gel showing the ~1800 bp RT-PCR product of P2X7 transcripts. **B** Amino acid sequences of rat, rabbit, and human P2X7. Consensus residues are shaded gray. The residue at 95 (indicated by a box) is known to be responsible for the difference between human and rat P2X7 in the sensitivity to KN-62. **C**

Functional reconstitution of rabbit P2X7 in HEK293 cells. Representative current traces during exposure to 300 μ M ATP recorded from HEK cells transfected with GFP alone (*a*) or GFP plus P2X7 (*b*). The *I*-*V* relationship of the ATP-induced P2X7 current is shown in *c*. **D** Inhibition of rabbit P2X7 current by KN-62. Currents were repeatedly activated by 300 μ M ATP with a resting interval of > 3 min in the absence (*upper trace*) and presence (*lower trace*) of 1 μ M KN-62

DEVD peptide conjugated to a nucleic acid dye). As demonstrated in Fig. 7A, microscopic observations identified a large number of caspase-positive cells (green fluorescent cells) after 3 h of treatment with 3 mM ATP. Fluorometric quantification revealed that the ATP treatment significantly increased caspase-3/7 activities compared with time-matched control ($n = 6$, $P < 0.05$, Fig. 7B). On the other hand, extracellular ATP failed to increase caspase-3/7 signals in the presence of 1 μ M A438079 ($P = 0.97$ vs. control), suggesting that P2X7 receptors are responsible for ATP-induced apoptotic caspase activation.

ATP-induced cytotoxicity was also evaluated by LDH release as an indicator of a loss of membrane integrity in necrotic cells (Fig. 7C, D). In this assay, LDH activity present in cell culture medium was colorimetrically measured and then normalized as a percentage ratio compared with maximum LDH activity determined upon complete cell lysis to quantify fractional LDH release. The fractional LDH

release was significantly increased by cell exposure to 3 mM ATP ($n = 3$, $P < 0.05$, Fig. 7C), which was observed even when ATP was applied in combination with 1 μ M A438079 ($P < 0.05$, Fig. 7C). However, the degree of the ATP-induced increase in LDH release was apparently attenuated by A438079, suggesting that P2X7 receptors are at least partially involved in the necrotic response of chondrocytes to extracellular ATP.

ATP-induced PGE₂ release via P2X7 receptor activation

It has been reported in rabbit articular chondrocytes that PGE₂ is released by ATP stimulation [9]. In accordance, our measurement also demonstrated a significant increase in PGE₂ concentration in the medium during 1-h exposure to 3 mM ATP (Fig. 8, $P < 0.001$, $n = 6$, $N = 3$). A438079 (1 μ M) inhibited the increase in PGE₂ release in ATP-treated cells,

Fig. 7 Extracellular ATP-induced chondrocyte cytotoxicity via P2X7 receptors. **A** Representative bright-field and fluorescence images of active caspase 3/7 signals in chondrocytes. Cells were incubated for 3 h with no treatment (control), ATP (3 mM), A438079 (1 μ M), or ATP (3 mM) plus A438079 (1 μ M). **B** Average fluorescence intensity of caspase 3/7 signals obtained with each treatment, normalized to the control values. Bars

represent means $\pm$ SEM ($n = 6$). * $P < 0.05$ vs. control. **C** ATP-induced LDH release. Cells were incubated for 3 h with no treatment (untreated), A438079 (1 μ M), with/without ATP (3 mM). The LDH activity in the medium was quantified by colorimetric assay and normalized to the maximum LDH activity after complete cell lysis to calculate relative LDH release (%). Bars represent means $\pm$ SEM ($n = 3$). * $P < 0.05$ vs. control

indicating the involvement of P2X7 receptors. Also, the ATP-induced PGE₂ release was attenuated in cells treated with either nominally Ca²⁺-free medium or ZnCl₂ (300 μ M), suggesting a crucial role of Ca²⁺ influx via P2X7 receptors.

To understand the signaling pathway underlying the P2X7-mediated PGE₂ release, we also investigated the involvement of two key enzymes in the PGE₂ synthetic pathway,

phospholipase A₂ (PLA₂) that mediates release of arachidonic acid from phospholipids in the cell membrane and cyclooxygenase-2 (COX-2) that catalyzes conversion of arachidonic acid to different prostaglandins including PGE₂. Inhibition of PLA₂ by arachidonyl trifluoromethyl ketone AACOCF₃ (10 μ M) resulted in a significant decrease in ATP-induced PGE₂ release ($n = 6$, $N = 3$, $P < 0.001$).

Fig. 8 ATP-induced PGE₂ release via P2X7 activation. Extracellular ATP-induced PGE₂ release from articular chondrocytes. Cells were incubated for 1 h with no treatment (untreated), A438079 (1 μ M), nominally Ca²⁺-free medium, ZnCl₂ (300 μ M), AACOCF₃ (10 μ M), celecoxib (10 μ M), etodolac (10 μ M), with/without ATP (3 mM). Cell culture

supernatants were collected and PGE₂ concentration was measured. The data were normalized to the untreated control value to obtain relative PGE₂ release (%). Bars represent means $\pm$ SEM ($n = 6$). * $P < 0.05$ vs. control, † $P < 0.05$ vs. untreated (ATP)

Fig. 9 COX-2 inhibitors reduced the P2X7-induced apoptotic signal without current suppression. **A** Cells were incubated for 3 h with no treatment (untreated), AACOCF3 (10 μ M), celecoxib (10 μ M), etodolac (10 μ M), with/without ATP (3 mM). The fluorescence intensity of caspase-3/7 signals was measured and normalized to the untreated control value. Bars represent means $\pm$ SEM ($n = 6$). * $P < 0.05$ vs. control. **B** Effects of celecoxib and etodolac on the ATP-induced current. Celecoxib and etodolac were added to the external solution during ATP (300 μ M) exposure. Representative original chart recordings of the membrane current during the experiments are shown

Similarly, the COX-2 inhibitors, celecoxib and etodolac, effectively suppressed the increase in PGE₂ release in chondrocytes exposed to ATP (Fig. 8, $P < 0.05$ for celecoxib, $P < 0.001$ for etodolac, $n = 6$, $N = 3$). These results suggest that P2X7 receptor stimulation results in enhanced PGE₂ synthesis through the activation of PLA₂ and subsequent the cyclooxygenase pathway.

Finally, we addressed the question of whether increased PGE₂ release is associated with the ATP-induced chondrocyte apoptotic signals. The caspase assay, illustrated in Fig. 9A, revealed that direct abrogation of PGE₂ production by inhibition of either PLA₂ or COX-2 virtually abolished the ATP-induced caspase-3/7 activation ($P < 0.001$ for AACOCF3, $P < 0.001$ for celecoxib, $P < 0.001$ for etodolac, $n = 6$, $N = 3$). It should be noted that neither celecoxib nor etodolac affected current activation through the P2X7 receptors (Fig. 9B, $n = 4$, $N = 2$).

Discussion

In the present study, we have provided, for the first time, definitive evidence for the presence of functional P2X7 receptors in rabbit articular chondrocytes. This is based on whole-cell patch-clamp recordings of membrane current responses to

extracellular ATP. Our findings include (1) that the current activation requires high ATP concentrations and that BzATP is a more potent agonist, (2) that the current exhibits little or no desensitization, (3) that the current is inhibited by several selective inhibitors of P2X7 receptors, and (4) that prolonged exposure to high ATP concentrations leads to the formation of large pores permeable to YO-PRO-1 dye. All these properties are very similar to those described for P2X7 receptors in other cell types [29, 30] as well as in heterologous expression systems [28]. Thus, our electrophysiological and pharmacological assessments clearly identify a specific function of P2X7 receptors in the ATP-induced current evoked from chondrocytes. Importantly, our study also uncovered the pivotal role of P2X7 receptors in the induction of inflammatory and apoptotic signals leading to ATP-induced chondrocyte death. This supports the concept that the P2X7 receptor serves as a “death receptor” mediating deleterious effects of extracellular ATP, as suggested in previous studies using various cell types [31]. The present results therefore provide important insights into the pathological aspect of the functional P2X7 receptors in articular chondrocytes.

Previous immunostaining studies have revealed protein expression of multiple P2X receptor subtypes, including P2X1, P2X2, P2X3, P2X4, and P2X7 in chondrocytes [13, 32]. A key issue in understanding the cellular responses to

extracellular ATP is the relative contribution and potentially distinct roles of these P2X subtypes. Our patch-clamp experiments showed that the P2X7 receptor predominantly mediates the ATP-activated membrane current with minimum participation of other P2X receptors. However, this does not necessarily indicate an exclusive function of P2X7 receptors in chondrocytes, since other subtypes have a much higher affinity for ATP than P2X7 [21]. In fact, Varani et al. reported a substantial contribution of P2X1 and P2X3 to NO production and PGE₂ release in chondrocytes at ATP concentrations (~10 μ M) [32] much less than those required for activation of P2X7 receptors in the present study. Both P2X1 and P2X2 receptors desensitize quite rapidly in the presence of ATP [20], whereas P2X7 receptors exhibit a uniquely persistent current activation. It is thus suggested that their slow desensitization endows P2X7 receptors with specific roles under pathological conditions in which extracellular ATP is chronically elevated.

A requirement of very high ATP concentrations for current activation through P2X7 receptors raises questions about the functional relevance and importance of this receptor in vivo. In cartilage, chondrocytes are restricted to small spaces called lacunae [33, 34], providing an environment where extracellular ATP is progressively accumulated during its release from chondrocytes themselves. Given that intracellular ATP concentrations in chondrocytes are in the millimolar range, considerable amounts of ATP will be released upon membrane rupture after cell injury and the pericellular ATP concentrations may reach the threshold levels for the activation of P2X7 receptors. In addition, P2X7 receptor activation by extracellular ATP could be amplified through autocrine positive-feedback mechanisms in which ATP release from cells is further enhanced by the interaction between purinergic signaling and pannexin hemichannels [35]. Alternatively, it is possible that ATP sensitivity of P2X7 receptor is augmented by micro-environmental factors. For instance, Moura et al. recently reported that the affinity of ATP binding to P2X7 receptors is increased > 2.5-fold by the allosteric regulation of CD44 proteoglycans through cell-surface interaction with glycosaminoglycan chains [36].

Activation of P2X7 receptors rapidly increases membrane permeability to Na⁺, K⁺, and Ca²⁺, and later induces the formation of membrane pores permeable to large molecules [21]. This increased membrane permeability can dramatically influence the equilibrium potential for transmembrane gradients for multiple ions and organic constituents, thereby causing osmotic flux of water. It is plausible that a perturbed regulatory mechanism of cell volume is involved in P2X7 receptor-mediated chondrocyte death. In fact, it is known that P2X7 receptor activation results in altered cell volume, which has been implicated in either apoptotic cell shrinkage or necrotic swelling [37, 38]. On the other hand, Taylor et al. reported a mixed apoptotic/necrotic phenotype of P2X7-stimulated

lymphocytes, which was characterized by a sequential cell shrinkage, swelling, and collapse [39]. In line with this, the present study showed that ATP-induced chondrocyte toxicity was associated with both apoptotic and necrotic signatures, although involvement of the P2X7 receptor was much more evident in caspase activation than LDH release. Chondrocytes uniquely maintain lower negative resting potentials than many cell types, which likely confers resistance to cell swelling [25]. Instead, chondrocytes readily shrink in response to activation of Cl[−] currents [15, 40, 41], if any, with a concomitant increase in K⁺ efflux. It is known that P2X7 receptors promote K⁺ efflux via activation of the Ca²⁺-activated K⁺ channel, K_{Ca} [42]. Which isoform of K_{Ca} is involved has not yet been established, but previous studies revealed the presence of large-conductance (BK) and small-conductance of K_{Ca} channels in articular chondrocytes [43].

Consistent with earlier work, the present study showed that extracellular ATP induced PGE₂ release in chondrocytes. This was largely abrogated by inhibition of P2X7 receptors in our experiments, although the involvement of other P2X subtypes and P2Y receptors has been suggested in previous studies [32, 44]. Hypothetical pathways suggested by our observations are as follows: (1) ATP-binding to P2X7 receptors rapidly and continuously induces Ca²⁺ influx and (2) concurrently or sequentially activates PLA₂ that then releases arachidonic acid from the plasma membrane. (3) Subsequently, the released arachidonic acid is metabolized by COX-2 to generate PGE₂. These are in line with prior findings of the association between P2X7 receptors and PLA₂-dependent lipid signaling [45–48], albeit with different end-products and consequences. Our results showed that PLA₂ inhibition by AACOCF₃ largely reduced P2X7-mediated PGE₂ production, suggesting that PLA₂ is an obligatory intermediate. Previous reports indicated that the P2X7 receptor could activate both Ca²⁺-dependent and Ca²⁺-independent PLA₂ in submandibular gland cells [45]. In this regard, we showed a pivotal role of Ca²⁺-influx through P2X7 receptors, suggesting a primary or predominant involvement of Ca²⁺-dependent PLA₂ in chondrocytes.

Importantly, our study also reveals that ATP-induced caspase activation is efficiently reduced by inhibition of the PLA₂/COX-2 pathways, consistent with the view that PGE₂ release is associated with the P2X7 receptor-mediated apoptotic pathway [49]. Further, it is reported that PGE₂ causes or sensitizes chondrocyte apoptosis [50, 51]. However, we realize that other contributing factors would be present in the ATP-induced apoptotic pathway, since numerous intracellular signaling molecules are potentially activated by P2X7 receptor stimulation [52]. It should be noted that our previous study demonstrated that a COX-2 inhibitor prevents TNF α -induced apoptosis by blocking Cl[−] current activation, rather than by suppressing PGE₂ synthesis [41]. It is known that purinergic stimulation can activate Cl[−] conductance in cardiomyocytes [53], although the involvement of Cl[−] currents in ATP-

induced chondrocyte apoptosis remains unclear. In addition, our patch-clamp data showed no obvious effects of COX-2 inhibition on ATP-evoked P2X7 receptor currents.

In conclusion, our results suggest that P2X7 receptor stimulation leads to chondrocyte apoptosis through activation of the PLA₂/COX-2 pathway. Caution should be exercised when extrapolating our findings to human chondrocytes, because differences between animal species were observed in the sensitivity of P2X7 receptors to drugs [54]. Apoptotic cell death is commonly associated with the hypocellularity of OA cartilage [34]. The present results therefore implicate the P2X7 receptor as a potential therapeutic target for the treatment of human OA and other joint disorders.

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Authors' contributions

H.T., F.T., K.K., and H.M. designed the experiments. H.T. performed the experiments and analyzed the data. All authors participated in data interpretation. H.T., F.T., K.K., and H.M. wrote the manuscript. All authors revised the manuscript and approved the final manuscript.

Compliance with ethical standards

Ethics This study adheres to the ARRIVE guidelines for reporting animal research [55]. All the animal experiments were conducted in compliance with the protocol that was reviewed by the Institutional Animal Care and Use Committee and approved by the President of Shiga University of Medical Science (Permit Number: 2015-2-7).

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Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval All procedures performed in studies involving animals were in accordance with the ethical standards of the institution or practice at which the studies were conducted.

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