Extracellular ATP signaling via P2X4 receptor and cAMP/PKA signaling mediate ATP oscillations essential for prechondrogenic condensation

Hyuck Joon Kwon

Research Center for Cooperative Projects, Hokkaido University Graduate School of Medicine, Sapporo 060-8638, Japan

Abstract

Prechondrogenic condensation is the most critical process in skeletal patterning. A previous study demonstrated that ATP oscillations driven by Ca$^{2+}$ oscillations play a critical role in prechondrogenic condensation by inducing oscillatory secretion. However, it remains unknown what mechanisms initiate the Ca$^{2+}$-driven ATP oscillations, mediate the link between Ca$^{2+}$ and ATP oscillations, and then result in oscillatory secretion in chondrogenesis. This study has shown that extracellular ATP signaling was required for both ATP oscillations and prechondrogenic condensation. Among P2 receptors, the P2X4 receptor revealed the strongest expression level and mediated ATP oscillations in chondrogenesis. Moreover, blockage of P2X4 activity abrogated not only chondrogenic differentiation but also prechondrogenic condensation. In addition, both ATP oscillations and secretion activity depended on cAMP/PKA signaling but not on K$_{ATP}$ channel activity and PKC or PKG signaling. This study proposes that Ca$^{2+}$-driven ATP oscillations essential for prechondrogenic condensation is initiated by extracellular ATP signaling via P2X4 receptor and is mediated by cAMP/PKA signaling and that cAMP/PKA signaling induces oscillatory secretion to underlie prechondrogenic condensation, in cooperation with Ca$^{2+}$ and ATP oscillations.


Introduction

Cartilage formation in the developing vertebrate embryonic limb consists of a highly coordinated and orchestrated series of events such as prechondrogenic condensation, chondrogenic differentiation, and the production of the cartilaginous matrix, each of which is under the specific regulation of various biological factors such as members of the transforming growth factor-$\beta$ superfamily, fibroblast growth factors, and the Wnt family of secreted glycoproteins (DeLise et al. 2000). Prechondrogenic condensation is necessary, but not sufficient, to trigger chondrogenic differentiation (Goldring et al. 2006). Moreover, prechondrogenic condensations prefigure skeletal elements during limb formation by forming the templates for subsequently formed bones (Solursh 1989, Olsen et al. 2000, Mariani & Martin 2003). This fact means that prechondrogenic condensation is the most critical process in skeletal patterning. However, surprisingly little is known concerning how prechondrogenic condensations are organized. The previous work showed that ATP oscillations driven by Ca$^{2+}$ oscillations play an essential role in prechondrogenic condensation by inducing oscillatory secretion during chondrogenesis (Kwon et al. 2012). However, it remains unclear what mechanism is responsible for initiating the Ca$^{2+}$-driven ATP oscillations, mediating the link between ATP and Ca$^{2+}$ oscillations and driving oscillatory secretion in chondrogenesis.

A previous study reported that ATP oscillations in chondrogenesis are synchronized among cells, leading to intercellular ATP waves (Kwon et al. 2012). This result indicates the involvement of the release of autocrine and paracrine factors in ATP oscillations. Extracellular ATP functions as an autocrine/paracrine signaling molecule that regulates many physiological functions (Gordon 1986). Moreover, it has been reported that extracellular ATP signaling induces Ca$^{2+}$ oscillations in a number of cell types (Mahoney et al. 1992, D’Andrea & Vittur 1996, Evans & Sanderson 1999, Hanley et al. 2004). These facts raise the possibility that extracellular ATP plays an active role in generating the Ca$^{2+}$-driven ATP oscillations during chondrogenesis.

It has been known that protein kinases such as cAMP-dependent kinase (PKA), Ca$^{2+}$-dependent kinase (PKC), and cGMP-dependent kinase (PKG) mediate essential signal pathways for chondrogenesis (Chang et al. 1998, Yoon et al. 2000, Yuasa et al. 2010). PKA, PKC, and PKG signaling can interact with Ca$^{2+}$ dynamics (Stojilkovic et al. 1991, Siso-Nadal et al. 2009, Sharron et al. 2010) and regulate mitochondrial oxidative phosphorylation (Boneh 2006). Moreover, PKA, PKC, and PKG signaling is also involved in secretion processes (Evans & Morgan 2003, Li et al. 2004, Morgan et al. 2005). These facts suggest that PKA, PKC, or PKG signaling may be linked to the coupling between Ca$^{2+}$ and ATP oscillations and the regulation of the secretion process during chondrogenesis.
In addition, it has been known that ATP-sensitive potassium channels (K<sub>ATP</sub> channels) mediate Ca<sup>2+</sup> and metabolic oscillations to drive oscillatory insulin secretion in pancreatic β-cells: namely, glucose stimulates a rise in intracellular ATP levels that closes K<sub>ATP</sub> channels, thus depolarizing the cell membrane and allowing Ca<sup>2+</sup> influx through voltage-dependent calcium channels (VDCCs), which reduces the intracellular ATP levels and allows reopening of the K<sub>ATP</sub> channels (Ashcroft et al. 1984). Furthermore, as the K<sub>ATP</sub> channel was identified in chondrocytes (Mobasher et al. 2007), K<sub>ATP</sub> channels may mediate the coupled oscillations of Ca<sup>2+</sup> and ATP and may regulate the secretion process in chondrogenesis.

This study tested the above hypotheses to elucidate the molecular mechanism underlying ATP oscillations in chondrogenesis. In most experiments, the prechondrogenic cell line ATDC5 that differentiates into chondrocyte to form cartilage nodules via prechondrogenic condensation in the presence of insulin was used as a model of in vitro chondrogenesis (Shukunami et al. 1996). Real-time monitoring of intracellular ATP level was performed using a reporter gene of <i>Phrixothrix hirtus</i> luciferase-emitting red light (PxRe) fused to an ACTIN promoter (P<sub>ACTIN-PxRe</sub> (Kwon et al. 2012). Here, it is reported that extracellular ATP signaling is critical for both ATP oscillations and prechondrogenic condensation. The P2X<sub>4</sub> receptor showed the strongest expression level among P2 receptors and mediated the actions of extracellular ATP on both ATP oscillations and chondrogenesis. In addition, ATP oscillations in chondrogenesis depended on cAMP activity but not on K<sub>ATP</sub> channel activity. Importantly, not only inhibition but also enhancement of cAMP production abolished ATP oscillations in chondrogenesis. Moreover, cAMP and cAMP/PKA, but not the K<sub>ATP</sub> channel, were also shown to be required for the secretion process in prechondrogenic cells. This study proposes that extracellular ATP signaling via P2X<sub>4</sub> receptor plays a key role in generating the Ca<sup>2+</sup>-driven ATP oscillations and that cAMP/PKA signaling mediates the coupling between Ca<sup>2+</sup> and ATP oscillations and regulates the secretion process during chondrogenesis, in cooperation with Ca<sup>2+</sup> and ATP.

**Materials and Methods**

**Cell culture and light microscopic observation**

The ATDC5 cell line was obtained from the RIKEN cell bank (Tsukuba). The cells were cultured in maintenance medium consisting of a 1:1 mixture of DMEM and Ham’s F-12 medium (DMEM-F12) (Invitrogen) supplemented with 5% fetal bovine serum (FBS), 10 mg/ml human transferrin (Roche Molecular Biochemicals), and 3×10<sup>-8</sup> M sodium selenite (Sigma–Aldrich) in polystyrene dishes at 37°C (Shukunami et al. 2007). Mouse mesenchymal stem cells (mMSCs) (Invitrogen), which are produced from bone marrow isolated from C57BL/6 mice, were maintained in DMEM-F12 with GlutaMAX-I supplemented with 10% MSC-qualified FBS (Invitrogen). For chondrogenesis, mMSCs were cultured in a high-density micromass (8×10<sup>6</sup> cells/ml) with the chondrogenic medium (Invitrogen). The chemical compounds were used at the following final concentrations: 3 unit/ml apyrase (Sigma), 100 μM ARL67156 (Sigma–Aldrich), 1 μg/ml brefeldin A (BFA; Sigma–Aldrich), 250 μM diazoxide (Sigma–Aldrich), 10 μM glibenclamide (Sigma–Aldrich), 10 μM A740003, 10 μM A438079 (Sigma–Aldrich), 100 μM pyridoxal phosphate–6–azophenyl–2,4–disulfonic acid tetrasodium salt (PPADS; Tocris Bio, Bristol, United Kingdom), 10 μM 5-BDBD (Tocris Bio), 10 μM H89 (Sigma–Aldrich), 4 μM GF109203X (Sigma–Aldrich), 10 μM MDL12330A (Sigma–Aldrich), 10 μM forskolin (Sigma–Aldrich), and 3 μM 1H–(1,2,4)oxadiazole(4,3–a)quinozaline-1-one (ODQ; Sigma–Aldrich) respectively. Cytotoxicity assays using Cell Counting Kit-8 (Dojindo, Kumamoto, Japan) showed that all these compounds showed little toxicity, with >80% of cells surviving after incubation for 24 h in the indicated concentrations. Microscopic observation was performed with a phase-contrast microscope (Nikon).

**Construction of reporter genes and transfection**

The human actin promoter (−500/+101, donated by Toyobo, Osaka, Japan) was inserted into multiple cloning sites of vectors containing a P<sub>P</sub> hirtus luciferase gene (Toyobo). Then, the promoter region and the luciferase gene were inserted into retrovirus vectors (Clontech) respectively. ATDC5 cells were transfected using retrovirus infection and then were selected by puromycin. mMSCs were transiently transfected using Lipofectamine LTX (Invitrogen).

**Bioluminescence monitoring**

The cells transfected stably with P<sub>ACTIN-PxRe</sub> were seeded in 35 mm dishes. After replacing with the medium including luciferin (0-1 mM) and chemicals, bioluminescence (relative light unit) was monitored using a dish-type luminescencer, Kronos (ATTO, Osaka, Japan), for 1 min at 1–30 min intervals (time = 0 h).

**Real-time PCR analysis**

The total RNA was isolated from the ATDC5 cells cultured under various conditions for 7 days using the RNeasy Mini Kit (Qiagen) following the manufacturer’s protocol. The RNA concentration was determined using a NanoDrop spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA), and the reverse transcription reactions were performed from 0-2 μg of total RNA using a cDNA synthesis kit (Takara, Otsu, Shiga, Japan). The real-time PCRs for GAPDH, collagen II, aggrecan, and P2 receptors were conducted using the SYBR green system. The primer sequences were...
described in Tables 1 and 2. The real-time PCRs were performed using a thermal cycler dice real-time system (Takara). The samples were held at 95 °C for 10 min followed by 40 amplification cycles consisting of a denaturation step at 95 °C for 15 s and an extension step at 60 °C for 1 min. The expression level of the gene was normalized to Gapdh. Aliquots (8 μl) of the PCR products were size separated by electrophoresis on a 1.5% agarose gel. Any DNA fragments were not amplified in the negative controls by performing the negative control reactions by using the solution in which cDNA was omitted. The identity of the PCR-amplified fragments was verified by sequencing the fragments with an Applied Biosystems 377A DNA sequencer (Perkin Elmer).

**Table 1** The primer sequences for gene expression analysis of chondrogenic marker molecules and GAPDH

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward primers</th>
<th>Reverse primers</th>
<th>Accession no.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Col2a1</td>
<td>AGGGCAACACAGGTTCCATAC</td>
<td>TGTCACACAAATCTCTTGTTCA</td>
<td>NM_031163</td>
</tr>
<tr>
<td>Agc</td>
<td>AGTGGATCGTGGCTAAGACAGG</td>
<td>AGAATGTCAGGCTTGTTGGA</td>
<td>NM_007424</td>
</tr>
<tr>
<td>Sox9</td>
<td>GAGCCCAAGGAGACACTCA</td>
<td>CTTCAATCACTTTCGACCTT</td>
<td>NM_011448</td>
</tr>
<tr>
<td>Gapdh</td>
<td>TGTGCTCCTCGTGATCTGA</td>
<td>TGCTGTGAAGTGCAAGGAG</td>
<td>NM_001001303</td>
</tr>
</tbody>
</table>

**Table 2** The primer sequences for gene expression analysis of P2 receptor subtypes

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward primers</th>
<th>Reverse primers</th>
<th>Accession no.</th>
</tr>
</thead>
<tbody>
<tr>
<td>P2X1</td>
<td>CAGAAGGAAAGCCCAAGGTATT</td>
<td>CACGTTCCTACAGTGCATCCAT</td>
<td>NM_008771</td>
</tr>
<tr>
<td>P2X2</td>
<td>CGTGCCTCCGTCTTTCCTTCCG</td>
<td>TCCCACTTCTGTGCTTCCA</td>
<td>NM_153400</td>
</tr>
<tr>
<td>P2X3</td>
<td>AAGGTTTGCCATGTCCGATGC</td>
<td>CATGACAAAGACACAGAAGTGCACC</td>
<td>NM_145526</td>
</tr>
<tr>
<td>P2X4</td>
<td>AGACGGCACAGCAGTGGCTAAC</td>
<td>TGGAGACACGACAGTGGAGA</td>
<td>NM_011026</td>
</tr>
<tr>
<td>P2X5</td>
<td>GATTGTCAGCTACATTTTGATC</td>
<td>CTTCCAAGGGTCAGCTGATG</td>
<td>NM_033221</td>
</tr>
<tr>
<td>P2X6</td>
<td>ACAGTGCTCCGTGGATACACT</td>
<td>TGGACATCCTCCGGACTT</td>
<td>NM_011028</td>
</tr>
<tr>
<td>P2X7</td>
<td>ACAAATGGAAAACGGAGCAG</td>
<td>CATACACCTCAGTTGACCTA</td>
<td>NM_011027</td>
</tr>
<tr>
<td>P2Y1</td>
<td>AGACAGAGGACAGAGTGTGTGG</td>
<td>GGGAGTGCTGTGGACAGATAC</td>
<td>NM_008772</td>
</tr>
<tr>
<td>P2Y2</td>
<td>GAAGAATGGACAGGAGGCCT</td>
<td>CACATTTCCTTGAGCTGGCAT</td>
<td>NM_008773</td>
</tr>
<tr>
<td>P2Y4</td>
<td>CTGCAGGCTGGCTGGTCTTC</td>
<td>GATTTCCGCCGATGGATG</td>
<td>NM_028621</td>
</tr>
<tr>
<td>P2Y6</td>
<td>TGAATACAGAGGGAAACACCAA</td>
<td>CAGGGTTCCTTGTGAGGACA</td>
<td>NM_183168</td>
</tr>
<tr>
<td>P2Y12</td>
<td>CAGACAGGACGCTGGGAGCTA</td>
<td>TGGTCTCCTGTGGCTGAATC</td>
<td>NM_027571</td>
</tr>
<tr>
<td>P2Y13</td>
<td>CAGCTGAGCTGTGGCCGTCAC</td>
<td>TGATCAGGGATGGTGATG</td>
<td>NM_028808</td>
</tr>
<tr>
<td>P2Y14</td>
<td>CACACAGACCCCTCACCAC</td>
<td>CAACACAGGATGGTGGCTT</td>
<td>NM_132000</td>
</tr>
</tbody>
</table>

The amount of released ATP was measured using an ATP measurement kit (Wako, Osaka, Japan). When ATDC5 cells maintained in the maintenance medium reached confluency, the medium was replaced with the maintenance medium or the differentiation medium containing DMSO (control, 1 μl) or BFA (1 μg/ml). After 1 day of culture in each medium, 50 μl of the culture medium was mixed with 50 μl luciferin–luciferase reagent solution, and the luminescence was immediately measured by a luminometer (ATTO).

**Results**

**Exocytotic activity measurement**

ATDC5 cells transfected with PACTIN-CLuc were seeded in 96-well cell culture plates. At a period of 1 h after the addition of chemical reagents, DMSO (control, 1 μl), oligomycin (10 μg/ml), nifedipine (50 μM), 2-aminoethoxydiphenyl borate (2-APB; 100 μM), nifedipine (50 μM) + 2-APB (100 μM), glibenclamide (10 μM), diazoxide (250 μM), H89 (50 μM), GF109203X (4 μM), MDL12330A (10 μM), and ODQ (3 μM) aliquots with a volume of 50 μl were collected from each medium, dispensed into a white 96-well plate, and reacted with an equivalent amount of Cypridina luciferin solution (1 μM Cypridina luciferin, 60 mM phosphate buffer (pH 6.4), 300 mM sodium ascorbate, and 20 mM Na2SO3). The CLuc activity of the collected fraction was measured using a multiplate-type luminometer (ATTO).

**Measurement of released ATP from cells**

The amount of released ATP was measured using an ATP measurement kit (Wako, Osaka, Japan). When ATDC5 cells maintained in the maintenance medium reached confluency, the medium was replaced with the maintenance medium or the differentiation medium containing DMSO (control, 1 μl) or BFA (1 μg/ml). After 1 day of culture in each medium, 50 μl of the culture medium was mixed with 50 μl luciferin–luciferase reagent solution, and the luminescence was immediately measured by a luminometer (ATTO).

**Signaling for ATP oscillations in chondrogenesis**

It was known that extracellular ATP signaling is involved in chondrogenic differentiation (Fodor et al. 2009). Consistent with the previous report, it was shown that the removal of extracellular ATP by apyrase treatment suppressed the gene expression level of chondrogenic markers such as type II collagen (COL2A1), aggrecan (AGC), and SOX9 even in the insulin-supplemented chondrogenic medium (Fig. 1A). Moreover, the apyrase treatment also suppressed cellular condensation in the insulin-supplemented medium (Fig. 1B). These results indicate that extracellular ATP signaling mediates prechondrogenic condensation and chondrogenic differentiation. However, only administration of ATP-γS, a stable ATP derivative, did not induce both prechondrogenic...
Figure 1 Extracellular ATP mediates both prechondrogenic condensation and ATP oscillations. (A) Effect of extracellular ATP on gene expression of COL2A1 (white bars), AGC (gray bars), and SOX9 (black bars). After 1 week culture of ATDC5 cells in the maintenance medium (control), the chondrogenic medium supplemented with insulin, the chondrogenic medium plus apyrase, and the maintenance medium supplemented with ATP-γS, gene expression was analyzed by real-time PCR. Data show mean ± S.D. (n = 4). Dunnett’s test, **P < 0.01. (B) Effect of extracellular ATP on prechondrogenic condensation. After culture for 1 week in the maintenance medium supplemented with insulin, insulin plus apyrase, or ATP-γS, ATDC5 cells were observed with phase-contrast images. Scale bar, 100 μm. (C) Effect of apyrase and ARL67156 on ATP oscillations in chondrogenesis. Bioluminescence monitoring of PACTIN-PxRe activity in ATDC5 cells was performed after chondrogenic induction with the chondrogenic medium (time = 0 h). After 72 h of chondrogenic induction, the cells were treated with apyrase or ARL67156. (D) The amount of ATP released from ATDC5 cells. After 1 day of culture in the maintenance medium and the chondrogenic medium containing DMSO (white bars) or BFA (black bars), the amounts of ATP released from the cells were measured by mixing the culture medium with luciferin–luciferase reagents. Data show mean ± S.D. (n = 4). Non-paired t-test, **P < 0.01.
condensation and chondrogenic differentiation (Fig. 1A and B), indicating that extracellular ATP signaling is not sufficient for prechondrogenic condensation and chondrogenic differentiation. The involvement of extracellular ATP in prechondrogenic condensation suggests that extracellular ATP signaling mediates ATP oscillations during chondrogenesis as the ATP oscillations are essential for prechondrogenic condensation (Kwon et al. 2012). In agreement with this hypothesis, it was found that apyrase treatment abrogated insulin-induced PACTIN-PxRe oscillations (Fig. 1C). This result indicates that extracellular ATP signaling is required for ATP oscillations in chondrogenesis. However, inhibitor for ecto-ATPase ARL67156 has no inhibition effect on PACTIN-PxRe oscillations (Fig. 1C). This result implicates that the ATP oscillations are not dependent on the signaling stimulated by extracellular ADP, AMP, or adenosine, which is produced by hydrolysis of extracellular ATP.

In addition, the involvement of extracellular ATP in ATP oscillations and chondrogenesis suggests that ATP is released during chondrogenesis. It was found that chondrogenic induction increased the amount of ATP released during 1 day of culture by about fourfold (Fig. 1D). In addition, inhibitor of vesicular secretion, BFA, reduced the amount of released ATP substantially (Fig. 1D), indicating that significant amounts of ATP are secreted via secretory vesicles during chondrogenesis.

**Extracellular ATP mediates ATP oscillations via P2X4 receptor**

Extracellular ATP activates P2 receptors that are divided into two classes, ionotropic P2X receptors and metabotropic P2Y receptors (Ralevic & Burnstock 1998). Thus, we examined which subtypes of P2 receptors mediate the extracellular ATP-induced ATP oscillation. We first examined the expression level of P2X and P2Y receptor subtypes in prechondrogenic cells by performing RT-PCRs. Expression of P2X1 and P2X2 receptor subtypes were not detected, and P2X3, P2X5, and P2X6 receptor subtypes showed only very weak signals (Fig. 2A and B). However, P2X4 exhibited the strongest expression levels and P2X7 was moderately expressed (Fig. 3A and B). In addition, the expression of P2Y1, P2Y2, P2Y4, P2Y6, P2Y12, P2Y13, and P2Y14 in prechondrogenic cells was examined because P2Y3 and P2Y5 receptors are not regarded as functional members of metabotropic receptors (Ralevic & Burnstock 1998). P2Y14 was moderately expressed but other P2Y receptor subtypes examined in this experiment exhibited no detectable signals.

![Figure 2](image_url)

Figure 2 The P2X4 receptor mediates ATP oscillations. (A) RT-PCR products show gene expression levels of P2 receptor subtypes in ATDC5 cells. (B) Bar graph shows relative gene expression levels of P2 receptor subtypes normalized to GAPDH. Data show mean ± S.D. (n=4). P2X4 was chosen to be the calibrator. (C) Effect of A740003, A438079, PPADS, and 5-BDBD on ATP oscillations in chondrogenesis of ATDC5 cells. Bioluminescence monitoring of PACTIN-PxRe activity in ATDC5 cells was performed after chondrogenic induction with the insulin-supplemented medium (time = 0 h). After about 72 h of chondrogenic induction, the cells were treated with A740003, A438079, PPADS, and 5-BDBD. (D) Effect of 5-BDBD on ATP oscillations during chondrogenesis in micromass culture of mMSCs. After about 16 h of chondrogenic induction, the mMSCs were treated with 5-BDBD.
P2Y\textsubscript{14} is a receptor for UDP-glucose, whereas P2X\textsubscript{4} and P2X\textsubscript{7} are receptors for ATP. Therefore, P2X\textsubscript{4} and P2X\textsubscript{7} are the possible candidates to mediate ATP oscillations in chondrogenesis.

We then investigated the effects of antagonists of P2X receptors on ATP oscillations during chondrogenesis. As expected, PPADS, the non-selective antagonist for P2X\textsubscript{1}, P2X\textsubscript{2}, P2X\textsubscript{3}, and P2X\textsubscript{5} revealed no inhibition effect on insulin-induced $\text{P}_{\text{ACTIN}}$-PxRe oscillations (Fig. 2C). Selective P2X\textsubscript{7} receptor antagonists, A740003 as well as A-438079, also did not inhibit the $\text{P}_{\text{ACTIN}}$-PxRe oscillations (Fig. 2C). However, the selective P2X\textsubscript{4} receptor antagonist 5-BDBD (Casati et al. 2011) abolished the $\text{P}_{\text{ACTIN}}$-PxRe oscillations (Fig. 2C). It was also demonstrated in the micromass culture of mMSCs that 5-BDBD blocked $\text{P}_{\text{ACTIN}}$-PxRe oscillations during chondrogenesis (Fig. 2D). This result indicates that extracellular ATP signaling via P2X\textsubscript{4} receptor mediates ATP oscillations during chondrogenesis.

**Role of P2X\textsubscript{4} receptor in chondrogenesis**

It was investigated whether the action of P2X\textsubscript{4} receptor on ATP oscillations influences chondrogenesis. Although ATDC5 cells differentiated into chondrocytes, forming condensations within 1 week in the insulin-supplemented medium, the inhibition of P2X\textsubscript{4} receptor activity by
5-BDBD prevented both cellular condensation and chondrogenic differentiation even in the chondrogenic medium (Fig. 3A and B). However, PPADS, A740003, and A438079 did not suppress cellular condensation and chondrogenic differentiation during insulin-induced chondrogenesis (Fig. 3A and B). Rather, PPADS appeared to stimulate expression of type II collagen. It is known that PPADS is not only relatively selective for P2X receptors but can also antagonize P2Y_{1} receptor activity (Ralevic & Burnstock 1998). As P2Y_{1} activity inhibits chondrogenic differentiation (Meyer et al. 2001), PPADS may stimulate type II collagen synthesis by suppressing the negative effect of P2Y_{1} activity on chondrogenic differentiation.

In addition, it was also shown in the micromass culture of mMSCs that 5-BDBD prevented both cellular condensation and chondrogenic differentiation even in the chondrogenic medium (Fig. 3C and D). These results indicate that extracellular ATP signaling via the P2X_{4} receptor is crucial in chondrogenic differentiation and cellular condensation. This suggests that the P2X_{4} receptor plays a key role in prechondrogenic condensation by mediating ATP oscillations.

**ATP oscillations in chondrogenesis are independent of K_{ATP} channels**

It is known that extracellular ATP signaling stimulates the transient increase in intracellular Ca^{2+} level by stimulating Ca^{2+} influx via VDCC and Ca^{2+} release from intracellular stores and then induces Ca^{2+} oscillations in a number of cell types (Mahoney et al. 1992, D’Andrea & Vittur 1996, Evans & Sanderson 1999). As ATP oscillations in chondrogenesis were induced by Ca^{2+} oscillations (Kwon et al. 2012), it is highly likely that the stimulation of the P2X_{4} receptor by extracellular ATP leads to ATP oscillations by inducing Ca^{2+} oscillations. However, the molecular pathways that mediate the link between Ca^{2+} and ATP oscillations remain to be explored. As K_{ATP} channels mediate ATP oscillations to induce oscillatory insulin secretion by modulating Ca^{2+} influx via VDCC in pancreatic cells (Kennedy et al. 2002), it was examined whether K_{ATP} channels also mediate ATP oscillations in chondrogenesis. However, it was shown that neither the K_{ATP} channel opener diazoxide nor the K_{ATP} channel blocker glibenclamide revealed any inhibition effect on PACTIN-PxRe oscillations in chondrogenesis (Fig. 4), indicating that K_{ATP} channels are not linked to ATP oscillations in chondrogenesis. This result suggests that K_{ATP} channels do not mediate Ca^{2+}-induced ATP oscillations in chondrogenesis.

**cAMP/PKA signaling mediates ATP oscillations in chondrogenesis**

As PKA, PKC, and PKG signaling can interact with Ca^{2+} dynamics, regulate mitochondrial oxidative phosphorylation, and use ATP as a substrates for signal pathways (Boneh 2006, Siso-Nadal et al. 2009, Sharron et al. 2010), these protein kinases can mediate link between Ca^{2+} and ATP oscillations in chondrogenesis. It was found that the PKA inhibitor H89 eliminated PACTIN-PxRe oscillations, whereas the PKC inhibitor GF109203X and the PKG inhibitor ODQ did not (Fig. 5A). This result implicates that PKA signaling, but not PKC or PKG signaling, mediates Ca^{2+}-driven ATP oscillations in chondrogenesis. Consistent with this result, it was found that inhibition of cAMP synthesis with MDL12330A, an adenylyl cyclase inhibitor, eliminated the PACTIN-PxRe oscillations (Fig. 5B). Importantly, the activation of cAMP synthesis by forskolin also inhibited the PACTIN-PxRe oscillations (Fig. 5B). These results indicate that ATP oscillations in chondrogenesis depend on the homeostatic balance of cAMP/PKA signaling.

**Ca^{2+}, ATP, and cAMP/PKA signaling mediate the secretion process in chondrogenesis**

A previous report showed that ATP oscillations drive oscillatory secretion during chondrogenesis (Kwon et al. 2012).
To elucidate how oscillatory secretion is generated during chondrogenesis, it was examined which factors determine secretion activity in prechondrogenic cells. The secretory activity was monitored using a reporter carrying the secreted Cypridina luciferase (CLuc) gene fused to ACTIN promoter (Nakajima et al. 2004). It was shown that an inhibitor of mitochondrial oxidative phosphorylation oligomycin suppressed CLuc secretion in prechondrogenic cells (Fig. 6A), indicating that the secretion process depends strongly on ATP. In addition, either nifedipine that blocks Ca\(^{2+}\) influx through VDCC or 2-APB that blocks Ca\(^{2+}\) influx through store-operated channels (SOC) suppressed CLuc secretion and the treatment with both nifedipine and 2-APB showed the synergistic effect on inhibition of the secretion activity (Fig. 6A). This result indicates that secretion activity depends on Ca\(^{2+}\) influx through both VDCC and SOC. Then, it was investigated whether cAMP/PKA signaling that mediates the Ca\(^{2+}\)-induced ATP oscillations also determines the secretion activity. In parallel to the action on ATP oscillations in chondrogenesis, both inhibition of PKA activity by H89 and inhibition of cAMP production by MDL12330A markedly inhibited CLuc secretion, whereas inhibition of PKG activity by ODQ or inhibition of PKC activity by GF109203X exhibited no inhibition effect on CLuc secretion (Fig. 6B). Neither the K\(_{\text{ATP}}\) channel opener diazoxide nor the K\(_{\text{ATP}}\) channel blocker glibenclamide showed any influence on secretion activity in prechondrogenic cells (Fig. 6B). This result indicates that cAMP/PKA signaling, but not K\(_{\text{ATP}}\) channel or PKC or PKG signaling, mediates secretion the process during chondrogenesis. Thus, it is suggested that Ca\(^{2+}\), ATP, cAMP, and PKA are major intracellular signals that underlie oscillatory secretion during chondrogenesis.

**Discussion**

This study demonstrated the involvement of extracellular ATP signaling via the P2X\(_4\) receptor in ATP oscillations during chondrogenesis. This result indicates that ATP is released during chondrogenesis. It has been reported that ATP can be released via vesicle-dependent mechanisms (Coco et al. 2003, Pascual et al. 2005), and many intracellular vesicles and secretory granules operate in chondrocytes (Schuman et al. 1995). Consistent with these previous reports, the present work demonstrated that ATP secretion significantly depends on vesicle-dependent exocytosis during chondrogenesis. ATP secretion via exocytosis can form a positive feedback loop as follows: the released ATP activates the P2X\(_4\) receptor and then leads to membrane depolarization by inducing the influx of cations such as Ca\(^{2+}\) and Na\(^{+}\) and subsequently opens VDCC (North 2002), which stimulates ATP release via exocytosis by enhancing Ca\(^{2+}\) influx. This positive feedback may initiate Ca\(^{2+}\) oscillations and subsequent ATP oscillations in chondrogenesis. Moreover, the previous result that ATP oscillations in chondrogenesis lead to oscillatory secretion of secreted molecules (Kwon et al. 2012) suggests that ATP can be released periodically through
oscillatory secretion during chondrogenesis. The oscillatory release of ATP would amplify the oscillations of intracellular ATP levels directly by reducing periodically intracellular ATP levels and indirectly by activating periodically the P2X4 receptor to stimulate Ca^{2+} influx. Therefore, the release of ATP via exocytosis may play a crucial role in both initiation and subsequent amplification of ATP oscillations during chondrogenesis (Fig. 7).

In addition, it has been proposed that ATP is released through hemichannels (Cotrina et al. 1998), the large pore of the P2X7 purinergic receptor (Suadicani et al. 2006) and ion channels (Declèves et al. 2000, Darby et al. 2003). Our previous report showed that the antagonist for connexin hemichannels blocks ATP oscillations in chondrogenesis (Kwon et al. 2012). Another report also demonstrated that the gap junction protein pannexin 3 promotes the release of ATP to switch the cell fate from proliferation to differentiation in chondrogenic cells (Iwamoto et al. 2010). Thus, hemichannels can play a key role in ATP oscillations during chondrogenesis by inducing the release of ATP. In addition, as calcium-activated chloride channels were reported to stimulate ATP release in chondrocytes (Kono et al. 2006), ion channels may contribute to the release of ATP during chondrogenesis. However, as the present data showed that P2X7 activity has no effect on ATP oscillations despite the moderate expression in prechondrogenic cells, P2X7 is not likely to contribute largely to the release of ATP during chondrogenesis.

Intercellular communication plays a central role for prechondrogenic condensation and subsequent cartilage patterning (Coelho & Kosher 1991). Our recent data also showed that ATP oscillations in chondrogenesis are synchronized among cells via quorum sensing-like transition and induce propagation of ATP waves (Kwon et al. 2012), which implicates that intercellular communication is essential for ATP oscillations in chondrogenesis. However, as the extracellular matrix increases with chondrogenesis (Hall & Miyake 1995), intercellular communication via direct cell-to-cell contact is probably not a major pathway in chondrogenesis. Moreover, it has been known that most of the quorum-sensing systems use secreted molecules for intercellular communication (Waters & Bassler 2005, Gregor et al. 2010). Therefore, extracellular ATP can function as the intercellular signaling molecule for quorum sensing-like synchronization of ATP oscillations during chondrogenesis.

Although extracellular ATP signaling via the P2X4 receptor promotes chondrogenic differentiation by inducing Ca^{2+} influx (Fodor et al. 2009), the action of extracellular ATP on prechondrogenic condensation has remained unclear. This study showed that deletion of extracellular ATP and inhibition of P2X4 signaling prevented both prechondrogenic condensation and ATP oscillations. These results suggest that extracellular ATP signaling via the P2X4 receptor stimulates prechondrogenic condensation by inducing ATP oscillations. In addition, it has been known that extracellular ATP is involved not only in chemotaxis (Honda et al. 2001) but also in the morphological change from spread shape to spherical shape, which results in platelet aggregation (Kahener et al. 2006). Therefore, in addition to the action via ATP oscillations, extracellular ATP signaling may promote the formation of tightly packed condensations by regulating cellular migration and inducing a spherical shape in prechondrogenic cells during chondrogenesis. It remains to be elucidated whether extracellular ATP signaling regulates prechondrogenic condensation via ATP oscillation-independent processes.

This study demonstrated that cAMP/PKA signaling mediates ATP oscillations in chondrogenesis. Importantly, the result that not only suppression but also activation of cAMP synthesis prevented the ATP oscillations suggests the
oscillations of cAMP/PKA signaling in chondrogenesis. Ca\(^{2+}\) can regulate the activity of some isoforms of adenylyl cyclases (Houslay & Milligan 1997). This fact suggests that Ca\(^{2+}\) oscillations drive the oscillations of cAMP/PKA signaling by controlling cAMP production. Indeed, cAMP oscillations arising from Ca\(^{2+}\) oscillations have been reported (Gorbunova & Spitzer 2002, Willoughby & Cooper 2006).

In addition, as ATP is converted to cAMP by adenylyl cyclase and cAMP/PKA signaling regulates energy metabolism (Boneh 2006), the oscillations of cAMP/PKA signaling can drive ATP oscillations. These facts suggest that cAMP/PKA signaling can mediate the coupling between Ca\(^{2+}\) and ATP oscillations in chondrogenesis. As suggested in the previous study (Kwon et al. 2012), Ca\(^{2+}\) can drive ATP oscillations in chondrogenesis through modulation of metabolic pathways. Therefore, Ca\(^{2+}\) oscillations can drive ATP oscillations, depending on both metabolic pathways and cAMP/PKA signaling. Early studies have demonstrated that PKA signaling promotes chondrogenic differentiation by activating PKC and enhancing SOX9 transcriptional activity at the precondensation stage (Huang et al. 2000, Yoon et al. 2000), but it has remained unclear how PKA affects prechondrogenic condensation. The involvement of cAMP/PKA signaling in ATP oscillations, as shown in this study, indicates that cAMP/PKA signaling regulates prechondrogenic condensation by mediating ATP oscillations. Collectively, the present results propose that ATP, Ca\(^{2+}\), cAMP, and PKA activity oscillates in synchronization during chondrogenesis, forming a highly integrated oscillatory circuit (Fig. 7).

It has been known that ATP is essential for vesicle transport using motor proteins to the plasma membrane, the priming of secretory vesicles for membrane fusion, and activation of protein kinases required for exocytosis (Martin 1997) and that Ca\(^{2+}\) triggers the formation of the SNARE complex to drive exocytosis and activates accessory proteins to enhance the spatial and temporal precision of SNARE-driven exocytosis (Hu et al. 2002, Barclay et al. 2005). In agreement with these facts, the present result showed that secretion activity in prechondrogenic cells depends strongly on intracellular ATP and Ca\(^{2+}\) levels. In addition, the present data showed that secretion activity in prechondrogenic cells depends on cAMP/PKA signaling. PKA is known to regulate secretion activity by phosphorylation of essential proteins for exocytosis (Evans & Morgan 2003). Therefore, the Ca\(^{2+}\)-ATP-cAMP-PKA oscillatory circuit drives the oscillatory secretion to underlie prechondrogenic condensation by regulating the multiple steps in secretion process (Fig. 7).

On the other hand, although Ca\(^{2+}\) dynamics regulate both ATP oscillations and the secretion process in chondrogenesis, PKC signaling is not involved in the ATP oscillations or the secretion process. This result is consistent with the previous report that PKC activity is not directly related to prechondrogenic condensation despite promoting chondrogenic differentiation by reducing the expression of N-cadherin, integrin α5β1, and fibronectin (Chang et al. 1998). This implicates that Ca\(^{2+}\) regulates ATP oscillations and secretion activity via a PKC-independent pathway in chondrogenesis.

In summary, it has been found that extracellular ATP signaling via the P2X\(_4\) receptor and intracellular cAMP/PKA signaling mediate ATP oscillations and the subsequent regulation of secretion process to underlie prechondrogenic condensation in chondrogenesis. These results suggest that extracellular ATP signaling via the P2X\(_4\) receptor and cAMP/PKA signaling can play a significant role in skeletal development by mediating ATP oscillations. Therefore, our study would provide clinical insight into treatment for diseases and disorders of skeletal systems, but in vivo study will be needed to confirm the involvement of P2X\(_4\) receptor and cAMP/PKA signaling in ATP oscillations during skeletal development.

Declaration of interest

The author declares that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

Funding

This work was supported by grants from a Grant-in-Aid for Scientific Research (No. 21790195) from the Ministry of Education, Science and Culture, Japan.

References


Received in final form 13 May 2012
Accepted 7 June 2012
Made available online as an Accepted Preprint 8 June 2012