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ATP induces Ca^{2+} signaling in human chondrons cultured in three-dimensional agarose films

M. K. Elfervig*†, R. D. Graff‡, G. M. Lee†‡, S. S. Kelley†‡, A. Sood† and A. J. Banes*†‡§
**Department of Biomedical Engineering, †Department of Orthopaedics, ‡Thurston Arthritis Research Center, §Curriculum in Applied and Material Sciences, University of North Carolina, Chapel Hill, North Carolina 27599-7055, U.S.A.*

Summary

Objective: In vivo, chondrocytes are surrounded by an extracellular matrix, preventing direct cell-to-cell contact. Consequently, intercellular communication through gap junctions is unlikely. However, signaling at a distance is possible through extracellular messengers such as nitric oxide (NO) and nucleotides and nucleosides, adenosine triphosphate (ATP), uridine triphosphate (UTP), or adenosine diphosphate (ADP). We hypothesized that chondrons, chondrocytes surrounded by their native pericellular matrix, increase their intracellular calcium concentration ($[Ca^{2+}]_{ic}$) in response to ATP and other signaling molecules and that the source of Ca^{2+} is from intracellular stores. The objectives of this study were to determine if chondrons in a 3-D gel respond to ATP by increasing $[Ca^{2+}]_{ic}$ through a purinoceptor mechanism and to test whether chondrons in whole tissue samples would respond to ATP in a similar fashion.

Design: Human chondrons, cultured in a three-dimensional agarose gel or in whole cartilage loaded with Fura-2AM, a calcium sensitive dye, were stimulated with 1, 5 and 10 μM ATP. A ratio-imaging fluorescence technique was used to quantitate the $[Ca^{2+}]_{ic}$.

Results: ATP-stimulated chondrons increased their $[Ca^{2+}]_{ic}$ from a basal level of 60 nM to over 1000 nM. Chondrons incubated in calcium-free medium also increased their $[Ca^{2+}]_{ic}$ in response to ATP, indicating the source of Ca^{2+} was not extracellular. ATP-induced calcium signaling was inhibited in chondrons pre-treated with suramin, a generic purinoceptor blocker. In addition, UTP and adenosine 5′-O-(3-thiotriphosphate) (ATPγs) induced a calcium response, but 2-methylthio-ATP (2-MeSATP), ADP, and adenosine did not induce a significant increase in $[Ca^{2+}]_{ic}$, substantiating that the $P2Y_2$ purinoceptor was dominant. Chondrons in whole cartilage increased $[Ca^{2+}]_{ic}$ in response to ATP.

Conclusions: We conclude that chondrons in 3-D culture respond to ATP by increasing $[Ca^{2+}]_{ic}$ via $P2Y_2$ receptor activation. Thus, ATP can pass through the agarose gel and the pericellular matrix, bind purinoceptors and increase intracellular Ca^{2+} in a signaling response. © 2001 OsteoArthritis Research Society International

Key words: Chondrocytes, Intracellular Calcium, Signal Transduction, ATP.

Introduction

Articular cartilage acts as a load-bearing surface within synovial joints, protecting bones from excessive forces and distributing load across the epiphyseal ends. During exercise and heavy lifting, forces greater than the normal body weight can be imposed upon the joint. Cartilage absorbs shock by providing a large contact area thus reducing the applied stresses. Repetitive motion during joint movement, particularly with excessive force, can lead to cartilage damage, resulting in osteoarthritis (OA) and joint inflammation[1,2].

Cartilage tissue is a viscoelastic material consisting of extracellular or pericellular, matrix, water and chondrocytes. The pericellular matrix is comprised primarily of types II and VI collagens and proteoglycans[3,4]. The matrix components of articular cartilage in combination with the fluid components are designed to withstand compressive loading. Compressive loading of chondrocytes results in the release of ATP and NO[5,6]. A consequence of compressive load is fluid flow[7] to which bovine chondrocytes in monolayer respond by releasing NO or by increasing $[Ca^{2+}]_{ic}$[8–10]. The fluid components of articular cartilage also provide nutrition to the chondrocytes by diffusion through the matrix because in the mature tissue, blood vessels do not penetrate articular cartilage[11,12]. Hence, the fluid in the cartilage matrix serves three purposes: (1) a reservoir of hydraulic fluid to dampen compressive loads, (2) a nutrifying medium transporting glucose and other molecules to cells and metabolites from cells[2,11] and (3) a carrier for signaling molecules, such as NO or ATP, secreted by chondrocytes in response to chemical or physical stimuli[5,8].

Human tendon, ligament (both ACL and MCL), and intervertebral disc annulus cells signal by an increase in intracellular calcium in response to a mechanical stimulus[13–16]. Chondrocytes in monolayer also signal in response to mechanical stimuli and to ATP-stimulation by increasing their $[Ca^{2+}]_{ic}$[17,18]. Graff and colleagues have shown that human and porcine chondrons in pellet cultures release ATP at a basal level and that the amount of released ATP increased with cyclic compressive loading[5]. However, the mechanism by which chondrocytes in their

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Address correspondence to: Albert J. Banes, Department of Orthopaedics, 253 Burnett-Womack Bldg, CB#7055, University of North Carolina, Chapel Hill, NC 27599-7055, U.S.A. Tel: (919) 966-2566; Fax: (919) 966-6730; E-mail: ajbvault@med.unc.edu

native pericellular matrix respond to extracellular ATP has not been shown.

Chondrocytes lack direct cell-to-cell contact. Several studies indicate that chondrocytes communicate through extracellular signaling pathways of which the ATP-purinoceptor pathway is prominent[19,20]. Articular chondrocytes express the messenger RNA (mRNA) for $P2Y_2$ purinoceptors[21]. Extracellular ATP binds to $P2Y_2$ purinoceptors (formerly designated as P_{2U}) in the cell membrane[22], activating these receptors and causing an increase in free intracellular calcium[23–25]. Hence, after a mechanical stimulus, extracellular nucleotides, such as ATP, are secreted[5] and most probably initiate signal transduction involving an increase in $[Ca^{2+}]_{ic}$ within chondrocytes[19]. $P2Y_2$ receptors can also interact with uridine triphosphate (UTP) and adenosine 5′-O-(3-thiotriphosphate) (ATPγs), but they have a lower affinity for adenosine diphosphate (ADP) and 2-methylthio-ATP (2-MeSATP), a $P2Y_1$ receptor (formerly designated as P_{2Y}) agonist[24,26,27]. Furthermore, ATP is actively secreted by chondrocytes in a response to stimuli such as mechanical load[5]. UTP is secreted by astrocytoma cells in response to shear stress[28].

Several studies show that chondrocytes in monolayer culture respond to ATP with an increase in $[Ca^{2+}]_{ic}$[19,20,29]. The question remains, however, whether ATP can pass through the matrix and activate chondrocytes prior to ATP hydrolysis. Chondrocytes in three-dimensional (3-D) cultures do not dedifferentiate; therefore, culturing chondrocytes in 3-D films simulates natural conditions found in cartilage where the differentiated state is maintained[30]. Chondrons, either in cultures or in whole tissue, have not been tested for a response to ATP. We hypothesized that chondrons in three-dimensional agarose gels would respond to ATP by increasing their intracellular calcium concentration. Furthermore, we also hypothesized that the response of the chondrons to ATP will be similar to the response of cartilage explants.

Methods and materials

CHONDRON ISOLATION AND CULTURE

Human articular cartilage was obtained from osteoarthritic hip and knee joints at the time of total joint arthroplasty from three patients. The cartilage was isolated and minced within one hour of removal from the body, transferred to Opti-MEM plus GlutaMax (Life Technologies, Grand Island, NY), 2% fetal bovine serum, 25 μg/ml ascorbate-2-PO_4, 2.7 mM calcium, penicillin (100 units/ml), streptomycin (100 μg/ml) and a low concentration of phenol red[31] and incubated overnight at 37°C in a CO_2 incubator. To obtain chondrons, the minced cartilage was digested with 0.4% dispase plus 0.2% collagenase in phosphate buffered saline (PBS) solution for five hours at 37°C with gentle agitation[28]. Freshly isolated chondrons were cast in 0.2 mm thick agarose films using 3% agarose gels (SeaPlaque, FMC) and a casting chamber composed of two microscope slides separated by thin strips of No. 2 coverglass. The films were cut into squares and then maintained in a humidified CO_2 incubator at 37°C in the same culture medium as above.

For explant experiments, full thickness human articular cartilage (obtained as above) and porcine articular cartilage, obtained from two- to three month-old pigs, were kept in culture medium as above. Within four hours of surgical removal, the explants were incubated with 1 or 5 μM Fura-2 acetoxy methylester (Fura-2AM).

QUANTITATION OF $[CA^{2+}]_{IC}$ IN RESPONSE TO ATP

At days 9–12 post-isolation, chondrons in agarose gels were incubated at room temperature (25°C) for 60–90 min in 1 or 5 μM Fura-2AM with 0.1% pluronic-127 and Earl's Balanced Salt Solution (EBSS) with 20 mM Hepes pH 7.2, 1.80 mM calcium, and 0.8 mM magnesium (Sigma Cell Culture Catalogue). After incubation, the preparation was washed in EBSS to remove residual Fura-2AM. Explants were also labeled with Fura-2AM as above. In the first set of experiments, the chondron-agarose film was mounted in an 83 mm diameter plastic culture plate on a microscope stage. The chondron film was covered with a dialysis membrane (Spectra/Pro, 6–8 kd cut-off) followed by a rubber membrane (20×20×0.5 mm) with a square opening (5×5 mm) sealed at the periphery with silicone grease to the culture plate substratum [see Fig. 1(a)]. In later experiments, chondron-agarose gels were potted in 1.5% agarose in EBSS in a 35 by 10 mm culture plate [see Fig. 1(b)]. These two conditions immobilized the chondron-agarose film during observation, addition of ATP, and shielded the cells from fluid-induced shear stresses. After the agarose films were immobilized and placed on the microscope stage, they were allowed to stabilize for fifteen to thirty minutes before making $[Ca^{2+}]_{ic}$ observations.

Chondrons and explants were imaged with an Olympus BH-2 upright microscope using a 40× long working distance, water immersion objective (Olympus WPlanTL). Internal calcium concentration was determined using Image-1 calcium fluorescence imaging software (Universal Imaging Corporation, West Chester, PA). Five to 40 cells were outlined to collect pixel intensities from individual cells. The area encompassing the cells was also outlined to collect pixel values from the entire visual field (see Fig. 2). Changes in $[Ca^{2+}]_{ic}$ after addition of ATP were calculated using the free/bound Ca^{2+} ratio method[32,33]. The Image-1 data analysis program is designed to quantitate Fura-2 emission with dual wavelength excitation (340/380 nm, with a Metaltek (Raleigh, NC) alternating filter wheel and detection measurements at 510 nm and above). Images were collected every 1.5 s post-ATP stimulation. Background images were obtained from an area with no fluorescent cells at the beginning of each experiment. Background pixel values were subtracted from each measured image to correct for non-uniformity due to variation in the fluorescence excitation beam across the field. Calcium ion concentration was quantified by comparison to known calcium standards. Fluorescence emission intensity at 510 nm (the ratio of excitation at 340/380 nm) was converted to $[Ca^{2+}]_{ic}$ based on the calcium standard and plotted over time.

ATP TREATMENT OF CHONDRON-AGAROSE GELS

Chondrons were stimulated by the addition of 1, 5, and 10 μM ATP final concentrations. To ensure exact concentrations of ATP, the chondron-agarose film was initially bathed in 1 ml of EBSS with 20 mM Hepes, pH 7.2. Half of the initial volume of EBSS was aspirated and discarded, then 500 μL of 2 μM ATP were added to the chondron-agarose film resulting in a 1 μM ATP final concentration.

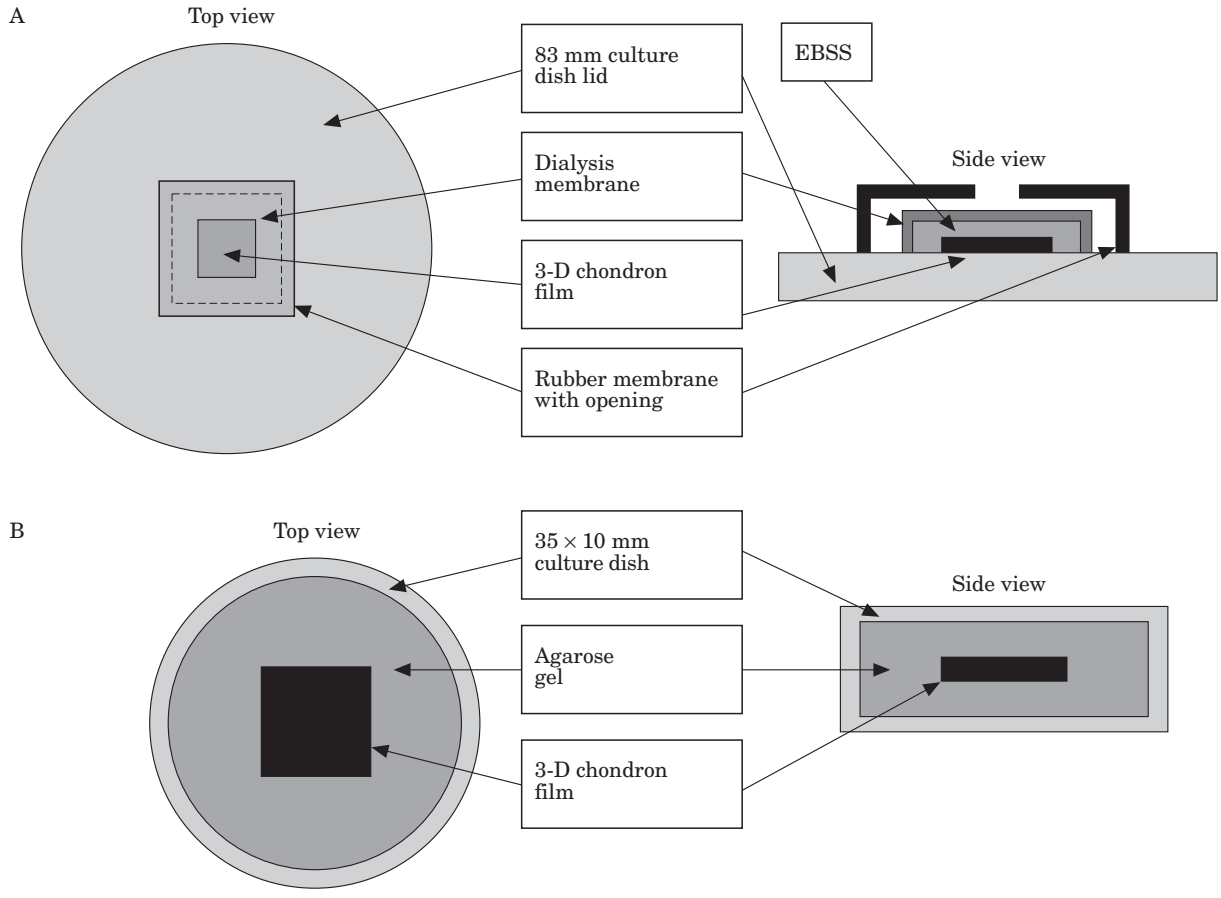

Fig. 1. Schematic of the experimental setups of the chondron-agarose film immobilization methods: (a) incorporates a dialysis membrane and a rubber membrane and (b) potting in an agarose gel.

The same approach was used to obtain final ATP concentrations of 5 and 10 μM. The cells could be repeatedly stimulated with ATP; therefore, the same group of chondrons were stimulated with all three concentrations of ATP (0, 1, 5, 10 μM) with a double wash with EBSS and an incubation at room temperature for 15 min following each stimulation. The appropriate concentration of ATP for stimulation was determined from an ATP dose response experiment with concentrations starting at 0.1 μM and increasing to 100 μM. The procedure described above was repeated for chondrons pre-treated for 1 h with 50 μM suramin, a purinoceptor blocker, and stimulated with 1 and 5 μM ATP. In order to test the capacity to signal via $P2Y_2$ receptors, chondrons in agarose gels were treated with 100 μM ATPγs, a $P2Y_2$ receptor agonist, and 100 μM 2-MeSATP, a $P2Y_1$ receptor agonist[24]. Cross desensitization of the receptor was tested by treating the cells with 10 μM ATP or UTP followed by a treatment of 10 μM UTP or ATP 60–90 s after the Ca^{2+} response returned to baseline. In addition, the cells were treated with 100 μM adenosine and 100 μM ADP to determine if the breakdown products of ATP hydrolysis could produce a calcium signal. Chondrons in agarose gels were tested for a $[Ca^{2+}]_{ic}$ response to ATP in EBSS with and without exogenous Ca^{2+} (calcium-free EBSS) to determine the source of Ca^{2+} in the cellular response. In order to compare the results in cultured cells *in vitro* with cells in whole living tissue, full thickness articular cartilage explants from human and pig were mounted in agarose and stimulated with ATP.

Human annulus (hAN) cells, which are known to release intracellular calcium stores in the presence of ATP[16], were used as a positive control to test for ATP diffusibility through the dialysis membrane. hAN cells were mounted on the microscope stage with and without an overlaying dialysis membrane and were stimulated with 5 and 10 μM ATP, which resulted in an increase in $[Ca^{2+}]_{ic}$ (data not shown).

DATA ANALYSIS

All data were normalized to the mean basal level of $[Ca^{2+}]_{ic}$ to account for the variations from daily set-up errors in light intensity and in the amount of Fura-2AM uptake. Significance between groups was determined using a one way analysis of variance with a Tukey post-hoc test with $P<0.05$. The mean peak $[Ca^{2+}]_{ic}$ and the mean average response were also calculated. Mean peak $[Ca^{2+}]_{ic}$ refers to the average of the maximum $[Ca^{2+}]_{ic}$ post-ATP stimulation for all responding cells. Mean average response for each cell refers to the $[Ca^{2+}]_{ic}$ measured every 1.5 s and averaged from the time of initial rise until the return to basal level post-ATP stimulation. Signal strength of the mean average response that was two standard deviations above baseline for an individual cell was the measure for a significant cellular response.

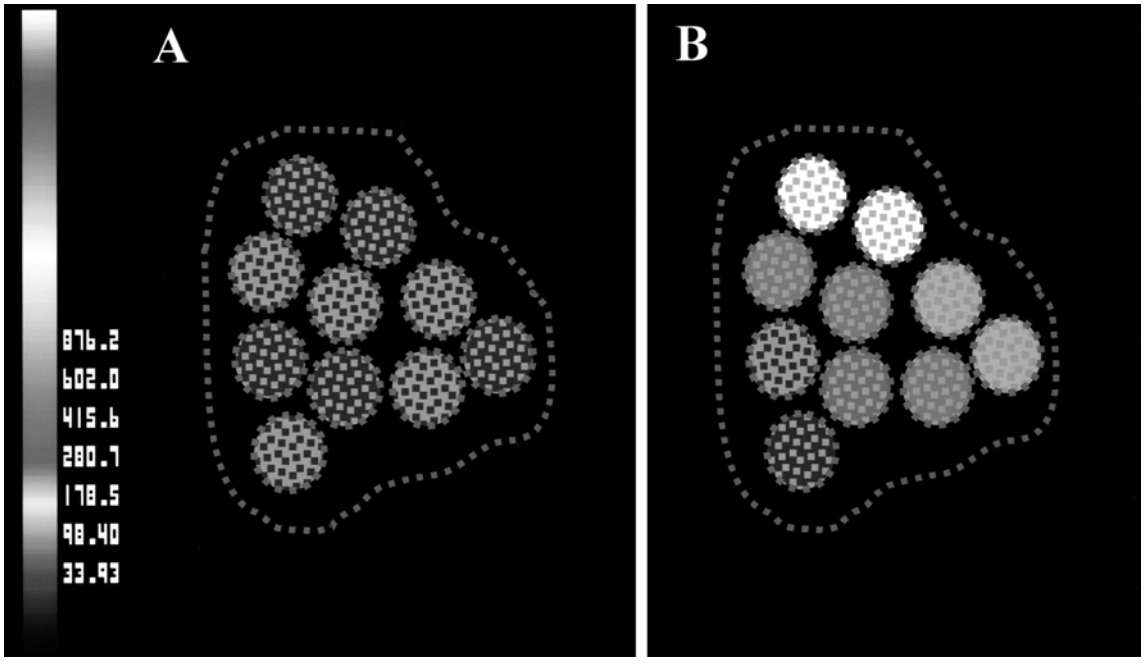

Fig. 2. Schematic of the ratio images of chondrons (a) at basal level and (b) post-ATP. In both images the individual chondrons are outlined as well as the entire group of cells.

Results

The dose response data of ATP-stimulated chondrons are given in Table I and Fig. 3. All concentrations of ATP induced a significant increase in $[Ca^{2+}]_{ic}$ ($P<0.05$) when compared with basal values. The greatest increase was observed between 1 and 12.5 μM ATP; therefore, 1, 5, and 10 μM ATP were chosen for testing the response of chondrons to ATP stimulation, spanning concentrations that are low, half-maximal and maximal.

The responses of the chondrons to ATP treatments of 0, 1, 5, 10 μM ATP in calcium-containing EBSS and calcium-free EBSS are given in Table II. The difference in the response magnitude of the cells in the Ca^{2+}-containing EBSS and the cells used in the dose response experiments is due to specimen variability. Chondrons in Ca^{2+}-free medium responded to ATP with an increase in $[Ca^{2+}]_{ic}$ as in Ca^{2+}-containing medium (Fig. 4). The response kinetics to ATP of chondrocytes in Ca^{2+}-containing and Ca^{2+}-free media were also similar. The majority of responding cells immediately increased their $[Ca^{2+}]_{ic}$ after ATP addition and continued to respond for 100–250 s [Fig. 5(a)]. A few chondrocytes had a 50–100 s delay in signal initiation but continued to signal for 200–300 s. For the chondrocytes in Ca^{2+}-free medium, all concentrations of ATP induced a significant increase in $[Ca^{2+}]_{ic}$ ($P<0.05$) when compared with the basal values.

To demonstrate a role for purinoceptors in the Ca^{2+} response to ATP, chondrons were incubated with 50 μM suramin prior to the addition of ATP. The data in Fig. 5(b) indicate that signaling was completely inhibited in chondrons pre-treated with suramin and subjected to a bolus of 5 μM ATP. ATP-induced signaling was also inhibited in chondrocytes subjected to 1 μM ATP. Chondrocytes were

Table I
ATP dose response results

μM ATP	Mean peak $[Ca^{2+}]_{ic}$ (nM)	Average response $[Ca^{2+}]_{ic}$ (nM)	% Cells responding
0 (*N*=296)	—	98± 79	NA
0.1 (*N*=55)	470±319	208±122	44
1 (*N*=45)	540±108	271± 36	51
12.5 (*N*=19)	445±150	235± 75	100
25 (*N*=34)	420±165	248± 86	94
50 (*N*=47)	330±115	215± 71	28
100 (*N*=22)	325± 55	210± 36	100

0 μM ATP corresponds to baseline intracellular calcium concentration in human chondrons. '*N*' is the number of cells analysed. Mean peak $[Ca^{2+}]_{ic}$ refers to the average of the maximum increase in $[Ca^{2+}]_{ic}$ for all responding cells. Mean average response refers to the $[Ca^{2+}]_{ic}$ measured every 1.5 s and averaged from the time of initial rise until the return to basal level for responding cells.

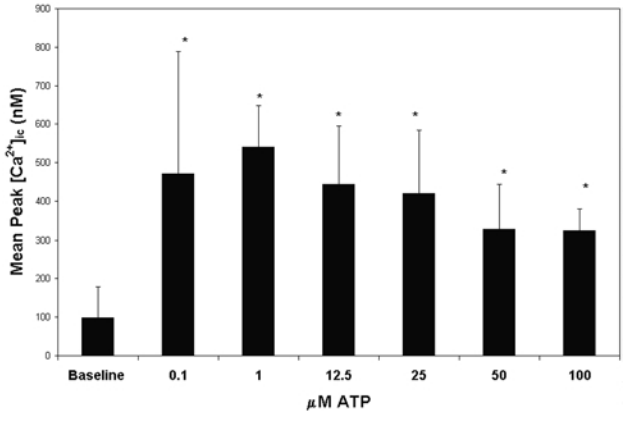

Fig. 3. ATP dose response of mean peak $[Ca^{2+}]_{ic}$ (nM)±S.D. in human chondrocytes. The highest response was seen between 1 and 12.5 μM ATP; therefore, 1, 5, and 10 μM ATP were chosen for experimental purposes. All ATP dose treatments had a significant increase over baseline ($P<0.05$).

Table II
ATP response of human chondrons in Ca^{2+}-containing medium and Ca^{2+}-free medium

μM ATP	Mean peak $[Ca^{2+}]_{ic}$ (nM)	Average response $[Ca^{2+}]_{ic}$ (nM)	% Cells responding
0 (*N*=365)	—	65±24	NA
Ca-containing EBSS			
1 (*N*=159)	177±167	103±68	40
5 (*N*=240)	165±190	89±61	40
10 (*N*=115)	383±400	163±97	60
Ca-free EBSS			
1 (*N*=40)	145±68	95±43	40
5 (*N*=33)	265±211	151±91	84
10 (*N*=32)	415±134	285±74	13

0 μM ATP corresponds to baseline intracellular calcium concentration in chondrons. '*N*' is the number of cells analysed. Mean peak $[Ca^{2+}]_{ic}$ refers to the average of the maximum increase in $[Ca^{2+}]_{ic}$ for all responding cells. Mean average response refers to the $[Ca^{2+}]_{ic}$ measured every 1.5 s and averaged from the time of initial rise until the return to basal level for responding cells.

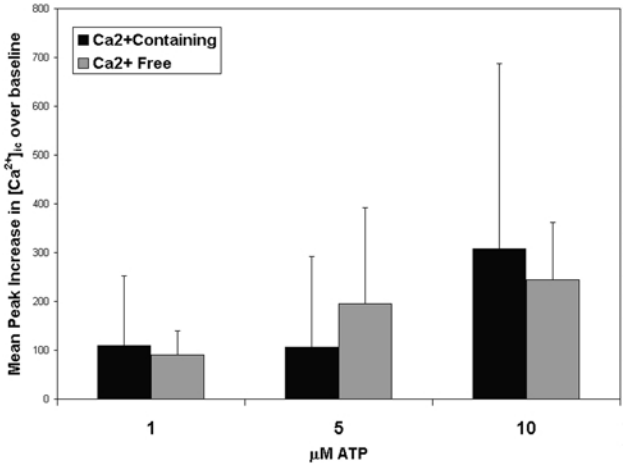

Fig. 4. The mean peak increase in $[Ca^{2+}]_{ic}$ over baseline of human chondrocytes to ATP (1, 5, and 10 μM) in calcium-free medium was not decreased compared to the total response in calcium-containing medium for responding cells. There was no statistically significant difference in results between the calcium-free and the calcium-containing media conditions. For the calcium-free medium group, *N*=16, 28, and 4 for the 1, 5, and 10 μM stimuli, respectively; for the calcium-containing medium group, *N*=65, 98, and 72 for the 1, 5, and 10 μM stimuli, respectively, where '*N*' is the number of cells responding.

also stimulated with both UTP and ATP, which both have an equal affinity for $P2Y_2$ receptors. Thus a primary challenge with either UTP or ATP should desensitize the $P2Y_2$ receptor to a secondary challenge with these nucleotides as illustrated in Fig. 6. ATP completely inhibited a subsequent challenge by either ATP or UTP. UTP also completely inhibited a subsequent challenge with UTP but not with ATP. To further demonstrate the activation of $P2Y_2$ receptors, chondrocytes were stimulated with purinoceptor agonists ATPγs, a $P2Y_2$ receptor agonist, and 2-MeSATP, a $P2Y_1$ receptor agonist. Data in Fig. 7 show the Ca^{2+} responses induced by treatments with UTP and ATPγs as compared to an ATP-induced Ca^{2+} response. The other nucleotides and nucleosides at 100 μM, 2-MeSATP, ADP, and adenosine, did not induce a response in the chondrocytes under our conditions for a responding cell. Only 10 μM UTP and 100 μM ATPγs induced a significant increase in $[Ca^{2+}]_{ic}$ over basal values; 10 μM ATPγs did not induce a significant increase in $[Ca^{2+}]_{ic}$ (data not shown).

The overall responses of porcine and human chondrocytes in cartilage explants to 0, 1, 5, and 10 μM ATP are shown in Fig. 8. The data in Fig. 8 represent the mean peak $[Ca^{2+}]_{ic}$ for all cells analysed. All concentrations of ATP induced a significant increase in $[Ca^{2+}]_{ic}$ ($P<0.05$) compared with basal values for porcine and human cartilage. Thapsigargin (1 μM) was also added to the porcine explants as a positive control to test if chondrocytes could release their Ca^{2+} stores. The Thapsigargin treated cells increased their $[Ca^{2+}]_{ic}$ three-fold over basal levels (data not shown).

Discussion

Chondrons cultured in 3-D agarose gels increased their intracellular calcium concentration in response to ATP. Results of previous studies showed that chondrocytes cultured in monolayer and subjected to mechanical stimulation by shear stress or by membrane indentation delivered by a micropipette, also caused an increase in $[Ca^{2+}]_{ic}$[8–10,34]. Results of these studies showed that NO, G-proteins, phospholipase C, inositol-1,4,5-triphosphate (IP_3), and calcium were involved in the response of chondrocytes to fluid-induced shear stress[8–10]. Stimulation of chondrocytes with a micropipette involved ATP-mediated signaling and gap junctions[34]. Accordingly, these results indicate that a mechanical stimulus induces numerous signal transduction events in chondrocytes in monolayer culture. However, further investigation was required to determine whether a pericellular matrix affects activation of or the kinetics of signaling pathways. Moreover, if the pathway involves an extracellular messenger, can that messenger diffuse through the pericellular matrix of the chondron and at what rate? We hypothesized that chondrons in three-dimensional agarose gels would respond to ATP by increasing their intracellular calcium concentration and that the magnitude and rate of the response would be comparable to that of cartilage explants.

Human chondrocytes express the mRNA for $P2Y_2$ receptors[21] and respond to as little as 0.1 μM ATP, despite the presence of extracellular ATPases, which degrade ATP into ADP and AMP[5,35]. ADP significantly increases $[Ca^{2+}]_{ic}$ in chondrocytes in monolayer[29]; therefore, a secondary response to ATP could result from ATP hydrolysis to ADP. However, ADP has a lower affinity for $P2Y_2$ receptors than ATP[24]. 100 μM ADP did not induce a significant increase in $[Ca^{2+}]_{ic}$ in chondrons in a 3-D agarose film. Therefore, it is unlikely that ADP produced by hydrolysis of ATP is inducing the Ca^{2+} response.

In articular chondrocytes, extracellular ATP activates $P2Y_2$ receptors through an IP_3-dependent pathway[26] resulting in the release of calcium from intracellular stores[19]. The inhibition of increased $[Ca^{2+}]_{ic}$ in response to ATP seen with suramin-treated chondrons indicates the involvement of purinoceptors in the signaling pathway, consistent with similar results on rabbit and sheep articular cartilage chondrocytes[29,34]. The involvement of $P2Y_2$ purinoceptors is indicated by the significant increases in $[Ca^{2+}]_{ic}$ in response to ATP, UTP, and ATPγs and by the lack of a significant response to 2-MeSATP, a $P2Y_1$ receptor agonist, and ADP. Similar responses to these

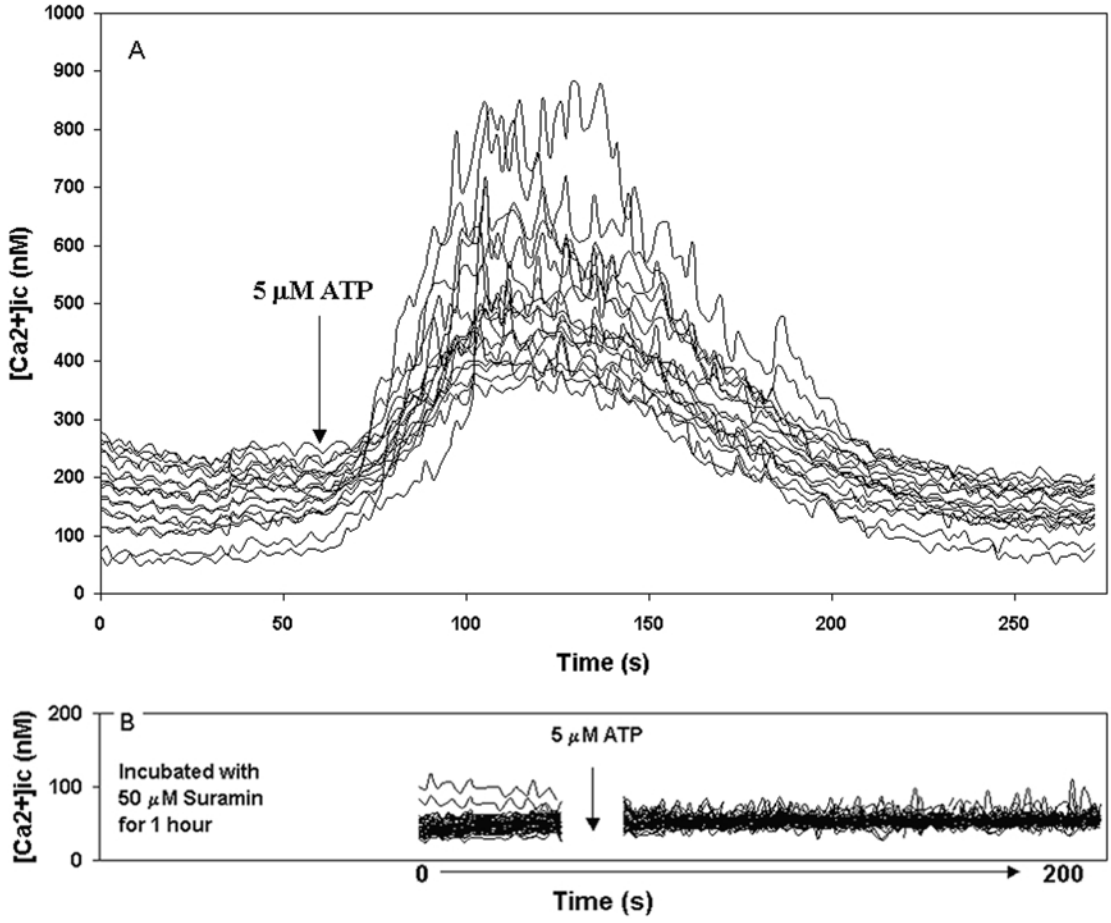

Fig. 5. (a) The response for individual cells (N=18) to 5 M ATP in Ca^{2+}-containing medium. The cells were from one experiment. (b) Pre-incubation with 50 μM suramin, a generic purinoceptor blocker, inhibits the response of human chondrocytes (N=47) to 5 μM ATP. For both graphs, each line represents the response to ATP for an individual cell.

nucleotides have been observed in chondrocytes in monolayer[27]. Furthermore, the desensitization of the receptors after a primary challenge of ATP or UTP indicates that both ATP and UTP can interact with the same receptors. The desensitization of purinoceptors with UTP and ATP has also been observed in human rheumatoid synovial cells and sheep chondrocytes[27,29].

The $P2Y_2$ purinoceptor-mediated increase in free intracellular calcium is dependent upon intracellular calcium stores[26]. Chondrons utilized intracellular calcium stores in the response to extracellular ATP. ATP-stimulation of chondrons in calcium-free medium caused a rise in the free intracellular calcium concentration of the cells suggesting that the ATP signal transduction pathway is independent of extracellular calcium. There was no significant difference in response to ATP between the chondrons in calcium-free and calcium-containing medium. Therefore, data indicate the involvement of a purinoceptor pathway in the response of chondrocytes to ATP-stimulation.

Chondrocytes in cartilage do not physically contact adjacent cells with pseudopods, but are surrounded by a pericellular matrix consisting of collagen types II and VI, proteoglycans and other matrix molecules[3,4,31]. Chondrocytes must rely principally on diffusible substances to communicate with nearby cells. When a signal such as compressive stimulus occurs, a chondrocyte releases ATP into the matrix[5]. The ATP could diffuse through the matrix and bind to receptors on either another chondrocyte or the same chondrocyte that released the ATP resulting in a paracrine or autocrine signaling response. Rapid diffusion of ATP was observed in the two different experimental setups: (1) chondron-agarose films covered with a dialysis membrane, and (2) chondron-agarose films potted in agarose. In addition, human annulus cells treated in the same manner responded to ATP with the same kinetics and magnitude as did cells exposed directly to the fluid medium (data not shown). The result of this positive control indicates that shielding the cells with agarose or a dialysis membrane did not block ATP diffusion. Furthermore, cell response was not confounded by fluid-induced shear stress as a result of ATP addition to the preparation. Between these two experimental procedures, there was an overall response to ATP and the total $[Ca^{2+}]_{ic}$ did not vary between experimental setups. The increase in $[Ca^{2+}]_{ic}$ illustrates that ATP can readily diffuse through different matrices without appreciable degradation and stimulate target cells.

Cells in whole cartilage samples increased $[Ca^{2+}]_{ic}$ in response to ATP. These data indicate that ATP diffused through native cartilaginous matrix and activated cells. However, chondrocytes in whole cartilage from either human or porcine sources displayed more limited responses and kinetics than did chondrons *in vitro*. Chondrocytes in whole porcine tissue had fewer cells

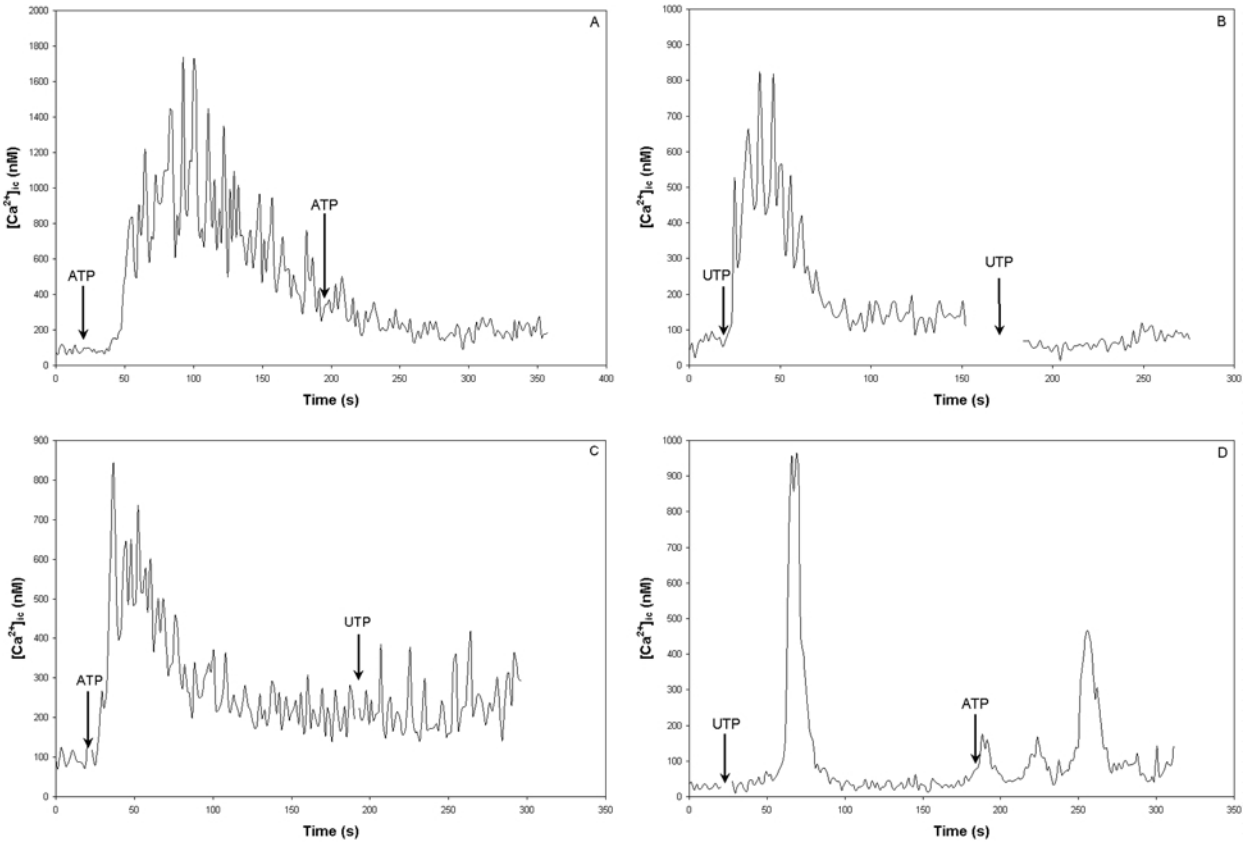

Fig. 6. Desensitization experiments. (a) Primary challenge with 10 μM ATP followed by a secondary challenge of 10 μM ATP. (b) Primary challenge with 10 μM UTP followed by a secondary challenge of 10 μM UTP. (c) Primary challenge with 10 μM ATP followed by a secondary challenge of 10 μM UTP. (d) Primary challenge with 10 μM UTP followed by a secondary challenge of 10 μM ATP. Each line represents an individual chondrocyte response to the corresponding nucleotide stimulus. Each experiment was performed twice with replicates of four agarose gels with similar results.

responding and the observed response was slow and of low magnitude compared to that for chondrons in culture. The observed response in chondrocytes in whole human tissue was slower than for chondrons in culture. This difference in chondrocyte responsiveness may be due to increased levels of extracellular ATPases present in whole tissue or differential expression of purinoceptors on chondrocytes. The lack of availability of antibodies to purinoceptors hampers an investigation of the latter question. In addition, the human cartilage has low cellularity. Therefore, only a few cells can be viewed in any given field. This low cellularity, the 3-D nature of the tissue, and the autofluorescence of the extracellular matrix contribute to high background fluorescence. Due to the lack of uniformity in sample surface architecture for a given field of view, background subtraction did not adequately correct for the 'spurious' fluorescence resulting in high basal levels in the human cartilage relative to the porcine cartilage. This elevated background was not observed in the agarose gels because polysaccharide is not fluorescent.

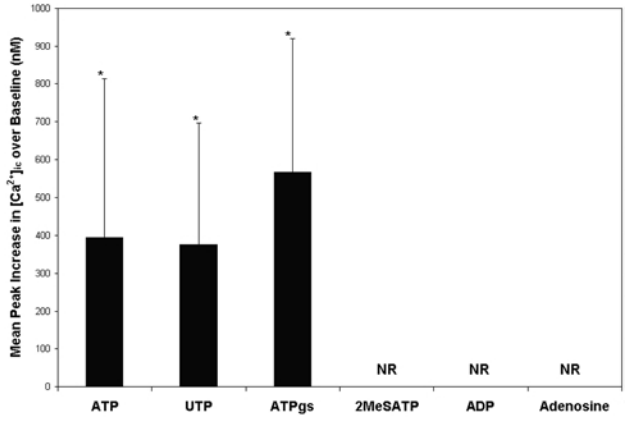

Fig. 7. The mean peak response over baseline of human chondrocytes to 10 μM ATP (N=72), 10 μM UTP (N=18), 100 μM ATPγs (N=30). 100 μM 2-MeSATP, 100 μM ADP, and 100 μM Adenosine did not induce a response in chondrocytes which is represented by 'NR' on the graph. ATP, UTP and ATPγs induced a significant (P<0.05*) increase in $[Ca^{2+}]_{ic}$ when compared to baseline. However, there was no was significant (P<0.05) different between ATP, UTP and ATPγs.

In conclusion, the cell-to-cell communication pathway in cartilage includes extracellular ATP-mediated signaling, which involves $P2Y_2$ purinoceptors. This is illustrated by the fact that the chondrons were inhibited by pre-incubation with 50 μM suramin, a purinoceptor blocker, and that chondrons responded to UTP, ATP and ATPγs. Furthermore, chondrons increased cytostolic free calcium in the absence of extracellular calcium, which is consistent with activation of $P2Y_2$ receptors on chondrocytes[29]. We propose that ATP may act as an external signaling molecule released by reactive chondrocytes to stimulate adjacent cells in a recruitment response to a stimulus such as a compressive load. Our data indicate that extra-

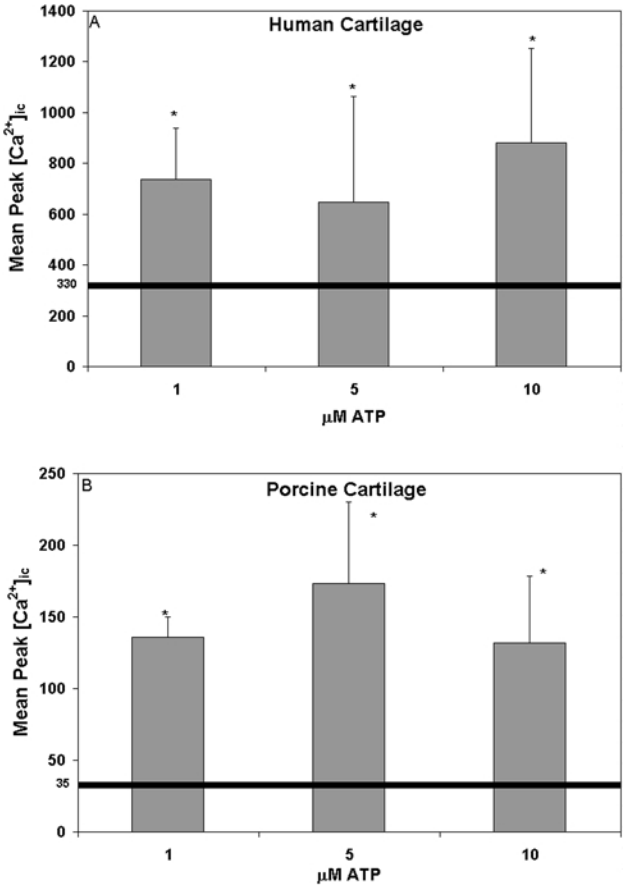

Fig. 8. Cartilage tissue explants from both human (a) and porcine (b) sources respond to ATP by increasing intracellular calcium concentration. The columns represent the mean peak $[Ca^{2+}]_{ic}$ for all cells analysed. All concentrations of ATP induced a significant ($P<0.05$*) increase in $[Ca^{2+}]_{ic}$ over the basal level in both human and porcine cartilage. The dark black line represents the average baseline $[Ca^{2+}]_{ic}$ level for each tissue explant. The high basal level for the human cartilage is due to background fluorescence (see Discussion). For the human cartilage, $N=42$, 34, and 37 cells that were analysed for the 1, 5, and 10 μM stimuli, respectively. For the porcine cartilage, $N=60$, 57, and 49 cells that were analysed for the 1, 5, and 10 μM stimuli, respectively.

cellular ATP can diffuse through the pericellular matrix of chondrons and induce a response in chondrocytes in the differentiated state.

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Appendix
Abbreviations

ACL	anterior cruciate ligament
ATP	adenosine triphosphate
ADP	adenosine diphosphate
ATPγs	adenosine 5′-O-(3-thiotriphosphate)
2-MeSATP	2-methylthio-ATP
$[Ca^{2+}]_{ic}$	intracellular calcium concentration
EBSS	Earl's Balanced Salt Solution
Fura-2AM	Fura – 2 acetoxy methylester
hAN	human annulus
IP_3	inositol–1,4,5-triphosphate
MCL	medial cruciate ligament
mRNA	messenger ribose nucleic acid
NO	nitric oxide
PBS	phosphate buffered saline
UTP	uridine triphosphate