Ionotropic Purinergic Receptor $P2X_4$ is Involved in the Regulation of Chondrogenesis in Chicken Micromass Cell Cultures

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SUMMARY

We have previously demonstrated that elevation of free cytosolic Ca^{2+} concentration at the time of differentiation of chondroblasts was mainly due to a Ca^{2+}-influx and it was indispensable to cartilage formation in chicken high density mesenchymal cell cultures (HDC) [1]. Here, we report that chondrogenic cells secreted ATP and administration of ATP to the culture medium evoked Ca^{2+} transients exclusively in the presence of extracellular Ca^{2+} and only on day 3 of culturing, when the final commitment of chondroblasts occurs. Moreover, ATP caused elevated protein expression of the chondrogenic transcription factor Sox9 and stimulated cartilage matrix production. Expression pattern of different types of both ionotropic and metabotropic purinergic receptors was detected. Agonists of metabotropic receptors, ADP and UDP did not evoke any Ca^{2+}-transients and had no influence on cartilage formation, while UTP caused transient elevation of cytosolic Ca^{2+} concentration in 3-day-old HDC without stimulating matrix production. Suramin, which blocks all P2X receptors but not $P2X_4$ did not impede the effects of ATP, furthermore, $P2X_4$ appeared in the plasma membrane fraction and gave signals with immunocytochemistry only from day 3. In summary, we suggest a role of ionotropic purinergic signalling of $P2X_4$ in the generation of ATP-dependent Ca^{2+}-transients of differentiating chondroblasts.

Key words: *in vitro* cartilage formation; high density cell culture; Fura-2; single cell Ca-measurement; P2X receptors; P2Y receptors; ATP secretion; immunocytochemistry

1. INTRODUCTION

Complex regulatory and signalling networks involving cell–matrix and cell–cell interactions, including tightly regulated gene expression, mediate the successive stages of proliferation, nodule formation and differentiation that produce hyaline cartilage [2]. One of the key regulators of these signalling processes in chondrogenic cells is Sox9. Since the expression of collagen type II and the core protein of aggrecan are controlled by this transcription factor, Sox9 is often referred to as the master gene of chondrogenesis [3,4]. Another important factor in the regulation of molecular steps leading to chondrogenic differentiation is the transient elevation of the intracellular Ca^{2+} concentration [1]. The tightly regulated level of cytosolic Ca^{2+} is involved in a number of signalling processes in a variety of cell types. Among non-excitable cells, the role of intracellular Ca^{2+} in the differentiation process of keratinocytes [5] and osteoblasts [6] has been established. Cytosolic free Ca^{2+} concentration changes are characterised by long-term, high amplitude changes, and by short-term, spontaneous, periodic Ca^{2+} concentration changes, so called oscillations in differentiating mesenchymal stem cells [7]. Moreover, the two different types of Ca^{2+} concentration changes influence the activity of different transcription factors: oscillations activate CREB, while long-time sustained Ca^{2+} concentration elevations activate NFAT [8,9]. Both transcription factors have important functions during chondrogenesis [10,11].

High density cell culture (HDC) established from chondrogenic mesenchymal cells isolated from distal limb buds of 4-day-old chicken embryos is a widely accepted model of *in vitro* cartilage differentiation [12,13], providing data on the molecular regulation of the differentiation of chondroprogenitor mesenchymal cells to chondroblasts. In this model a spontaneous cartilage formation occurs; the initial appearance of chondroblasts and cartilage specific extracellular matrix molecules takes place on day 3 of culturing, and the majority of

cells differentiate into chondrocytes by day 6, when a high amount of cartilage matrix can be detected.

In our previous work [1] we reported that a tight regulation of free cytosolic Ca^{2+} levels (between 80 and 140 nM) is needed for proper chondrogenesis in cells of HDC. We also described a characteristic temporal pattern of the changes of cytosolic Ca^{2+} levels during chondrogenic differentiation with a definitive peak on day 3 of culturing, the day on which chondroprogenitor cells differentiate to chondroblasts. Although intracellular elements of Ca^{2+}-homeostasis (*e.g.* RyR, IP_3 receptor and SERCA) were detected in chondrogenic cells, we failed to show any evidence concerning their contribution in evoking Ca^{2+}-transients in differentiating chondroblasts. Therefore, the extracellular space has been proved to be the source of the elevated cytosolic Ca^{2+} concentration.

In the present work, we aimed to determine transmembrane protein candidates responsible for the Ca^{2+} influx into chondrogenic cells. The family of purinergic receptors is ubiquitously present in a number of cell types and provides receptors for extracellular nucleotides acting as paracrine or autocrine mediators. Purinergic receptors have two major types: P1 receptor families are sensitive to adenosine, while P2 receptor families are sensitive to ATP, ADP, and UTP. The latter is further divided into two major receptor subtypes: P2Y and P2X. Members of the metabotropic P2Y subtype are 7 transmembrane domain-containing receptors coupled to G proteins and linked to PLC signalling transduction pathways that lead to the release of intracellular Ca^{2+} from inositol-1,4,5-trisphosphate (IP_3)-sensitive Ca^{2+} stores. The ionotropic P2X receptors are ATP-gated ion channels allowing Ca-influx. Seven P2X subunits ($P2X_1$–$P2X_7$) have been described and cloned so far [14,15]. On the other hand, eight P2Y isoforms have been described in human tissues: $P2Y_1$, $P2Y_2$, $P2Y_4$, $P2Y_6$, $P2Y_{11}$, $P2Y_{12}$, $P2Y_{13}$ and $P2Y_{14}$ receptors. Although, based on sequence analysis $P2Y_3$ and $P2Y_5$ were supposed to be members of the P2Y subfamily, further studies revealed that they have

different phamacology and are involved in non-purinergic signalling pathways as reviewed in [16]. The fact that P2X receptors are sequentially expressed in embryonic rat and mouse skeletal muscle cells and osteoblasts [17-19] raised the possibility of the involvement of these channels in the Ca^{2+} homeostasis of differentiating chondrogenic mesenchymal cells.

In this study, we report that cells of HDC responded to extracellular ATP by elevating their intracellular Ca^{2+} levels mainly at the time of chondroblast formation and administration of ATP to the culture medium stimulated chondrogenesis. Receptors responsible for the elevation of Ca^{2+} concentration seemed to be members of the P2X family, and based on our data, we propose that $P2X_4$ receptors contribute to the elevation of cytosolic Ca^{2+} levels of chondrogenic cells on day 3 of culturing. Moreover, cells of HDC secreted ATP into the culturing medium, which supports our theory that a purinergic autocrine regulation is involved in the proper control of chondrogenesis.

2. MATERIALS AND METHODS

2.1. Cell culture

High density cell cultures were prepared as described in [1]. Briefly, distal parts of the limb buds of 4-day-old Ross hybrid chicken embryos (Hamburger–Hamilton stages 22–24; [20]) were removed and chondrifying micromass cultures of mesenchymal cells were established. 15 or 30 μL droplets of the suspension containing 1.5×10^7 cells/mL were inoculated on round coverglasses (diameter: 30 or 10 mm; Menzel-Gläser, Menzel GmbH, Braunschweig, Germany) placed into plastic Petri dishes (Nunc, Naperville, IL, USA). Cells were allowed to attach to the surface for 2 hrs at 37 °C. Day of inoculation is considered as day 0. Colonies were grown in Ham's F12 medium (Sigma, Budapest, Hungary) supplemented with 10% foetal calf serum (Gibco, Gaithersburg, MD, USA), antibiotics and antimycotics, and were kept at 37 °C in an atmosphere of 95% air and 5% CO_2 and 80% humidity. The medium was

changed on every second day.

2.2. Single cell Ca^{2+} measurements

Measurements were performed on different days of culturing using the calcium dependent fluorescent dye Fura-2 as described previously [1]. Fura-2-loaded cells were placed on the stage of an inverted fluorescent microscope (Diaphot, Nikon, Kowasaki, Japan) and viewed using a 40× oil immersion objective. Measurements were performed in normal (137 mM NaCl, 5.4 mM KCl, 0.5 mM $MgCl_2$, 1.8 mM $CaCl_2$, 11.8 mM HEPES, 1g/L glucose, pH 7.4) or calcium-free (containing 5 mM EGTA, without $CaCl_2$) Tyrode's solution. ATP solution was prepared from normal and Ca^{2+}-free Tyrode's at 180 μM final concentration. ADP, UDP, UTP (180 μM) and bradykinin (20 μM) were prepared from Ca^{2+}-free Tyrode's. Before application of Ca^{2+}-free ATP, ADP, UDP and UTP, cells were treated in Ca^{2+}-free Tyrode's for 150 sec. Suramin solution was prepared in Tyrode's and used at a final concentration of 10 μM. Excitation wavelength was altered between 340 and 380 nm and fluorescence intensities (F_{340} and F_{380}) were measured as described previously [1]. Test solutions were directly applied to the cells through a perfusion capillary tube (Perfusion Pencil™; AutoMate Scientific, San Francisco, CA, USA) with an internal diameter of 250 μm at a 1.5 μL/s rate, using a local perfusion system (Valve Bank™ 8 version 2.0, AutoMate Scientific). All measurements were performed at room temperature. Data were statistically analysed by Student's *t*-test.

2.3. Preparation of cell extracts

Cell cultures were harvested on each day of culturing. Cell pellets were suspended in 100 μL of homogenization buffer containing 50 mM Tris-HCl buffer (pH 7.0), 10 μg/mL Gordox, 10 μg/mL leupeptin, 1 mM phenylmethylsulphonyl (PMSF), 5 mM benzamidine, 10 μg/mL

trypsin inhibitor and 0.5% Triton X-100. Samples were snap-frozen in liquid nitrogen, and were stored at –70 °C. Samples were sonicated for four times 30 sec by 50 cycles (Branson Sonifier, Danbury, USA). For Western blot analyses, total cell lysates and plasma membrane fractions were used. For isolation of plasma membrane fraction of HDC, sonicated samples were centrifuged at 50,000 ×*g* for 90 min at 4 °C. Pellet was triturated continuously in 50 μL homogenization buffer supplemented with 1 % Triton X-100 at 4 °C. After 1 h of trituration samples were centrifuged again at 50,000 ×*g* for 55 min at 4 °C, and supernatant containing plasma membrane fraction was used for Western blot analyses.

2.4. RT-PCR analysis

For RT-PCR analysis, cartilage colonies were washed three times with RNase-free physiological sodium chloride, snap-frozen in liquid nitrogen and stored at –70 °C. Total RNA was isolated from cells of HDC of various ages using Quiagen RNeasy® Micro Kit according to the instructions of the manufacturer (Quiagen, Budapest, Hungary). The assay mixture (20 μL) for reverse transcriptase reaction (Omniscript, Quiagen) contained 500 ng RNA, 0.25 uL RNase inhibitor, 0.25 μL oligo(dT), 1 μL dNTP (200 μM), 1μl M-MLV RT in 1× RT buffer. Amplifications of specific cDNA sequences were performed with specific primers (Integrated DNA Technologies, Coralville, IA, USA) that were designed based on published chicken nucleotide sequences (for sequences of primer pairs, see Supplementary Material, Table 1). PCR reactions were allowed to proceed in a final volume of 50 μL (containing 2 μL forward and reverse primers, 1 μL dNTP [200 μM], and 5 units Promega GoTaq® DNA polymerase in 1× reaction buffer) in a programmable thermocycler (Eppendorf Mastercycle, Netheler, Hinz GmbH, Hamburg, Germany) with the following settings: 2 min at 95 °C for initial denaturation followed by repeated cycles of denaturation at 94 °C for 1 min, primer annealing for 60 sec at an optimized temperature, and extension at 72 °C for

1 min 30 sec. After the final cycle, further extension was allowed to proceed for another 10 min at 72 °C. PCR products were analysed using a 1.5% ethidium bromide-stained agarose gel.

2.5. Western-blot analysis

Total cell lysates and plasma membrane fractions were examined by Western blot. Samples for SDS–PAGE were prepared by the addition of 1/5 volume of 5-fold concentrated electrophoresis sample buffer (310 mM Tris–HCl, pH 6.8; 10 % SDS, 50% glycerol, 100 mM DTT, 0.01% bromophenol blue) to cell lysates and boiled for 5 min. About 50 μg of protein was separated by 7.5% SDS–PAGE gel for immunological detection of P2X-receptors. Proteins were transferred electrophoretically to nitrocellulose membranes. After blocking in 5 % non-fat dry milk in PBS, membranes were incubated with primary antibodies raised against the carboxy termini of P2X-receptors (Alomone Labs, Jerusalem, Israel) and $P2Y_4$ receptor (Sigma, Budapest, Hungary), amino terminus of $P2Y_1$ receptor (Sigma, Budapest, Hungary) and 3rd intracellular loop of $P2Y_2$ receptor (Alomone Labs, Jerusalem, Israel) overnight at 4°C in 1:200 dilution. After washing three times for 10 min with PBST (PBS supplemented with 0.1% Tween 20), membranes were incubated with a secondary antibody, anti-rabbit IgG (Sigma, Budapest, Hungary) in 1:1000 dilution in PBS containing 5% non-fat dry milk for 1 h. Signals were detected by enhanced chemiluminescence reaction (Amersham Biosciences, Budapest, Hungary).

2.6. Immunocytochemical staining of P2X receptors

3-day-old cultures were washed twice with PBS and fixed in 4% paraformaldehyde for 15 min at 4 °C. After washing in PBS, cells were permeabilized with 0.1% Triton X-100 in PBS for 30 min. Non-specific binding sites were blocked by 30 min preincubation in 1% bovine

serum albumin (BSA) in PBS, followed by incubation with the primary antibodies diluted in 1:100 at 4 °C overnight. Subsequently, the cultures were washed three times with PBS for 10 min, and were incubated with a FITC-conjugated anti-rabbit IgG (Vector Laboratories, Burlingame, CA, USA) diluted 1:500 in PBS for 1 h. Cultures were washed three times with PBS and mounted with Vectashield® mounting medium containing DAPI (Vector Laboratories, Burlingame, CA, USA). Control experiments were carried out with primary antibodies incubated with their control peptides according to the instructions of the manufacturer (data not shown).

2.7. Administration of extracellular ATP, ADP, UDP, UTP and suramin

ATP (100 μM), ADP, UDP and UTP (180 μM), and suramin (10 μM) were administered to cells of HDC on various days of culturing. The nucleotides and suramin were diluted in the culture medium. Effects on metachromatic cartilage matrix formation were examined by metachromatic staining with dimethylmethylene blue and toluidine blue as described previously [1].

2.8. Determination of extracellular ATP in the culture medium

Concentration of extracellular ATP secreted by cells of high density cell cultures was determined using Adenosine 5'-triphosphate (ATP) Bioluminescent Assay Kit (Sigma, Budapest, Hungary). Measurements were carried out according to the instructions of the manufacturer, with minor modifications. Briefly, 20 droplets of the cell suspension (100 μL each) were inoculated into Petri dishes (diameter: 200 mm, Orange Scientifique, Braine-l'Alleud, Belgium) and were fed with 20 mL culture medium. Concentration of ATP secreted by cells of HDC into the culture medium was determined at approximately the same period of each culturing day. The medium was changed every day following measurements. 50 μL of

the culture medium (pH adjusted to 7.8) was used to determine the amount of ATP in the culture medium in 2 parallel experiments. Background light emission was determined using blanks (both sterile water and Ham's F12 culture medium). Since the ATP Assay Mix is not stable for a long period, a gradual decrease in the sensitivity may occur. Therefore, a new standard curve was prepared each day prior to measurements (concentrations of ATP standard solutions were as follows: 10^{-6} M, 10^{-7} M, 10^{-8} M, 10^{-9} M and 10^{-10} M). Luminescence of samples was determined using a microwell plate reader (Chameleon, Hidex, Turku, Finland).

3. RESULTS

3.1. Cells of HDC respond to extracellular ATP by elevating intracellular Ca^{2+} concentration

ATP at a constant concentration of 180 μM was administered to the close proximity of cells of HDC on various days of culturing. Figure 1 (A–E) shows that administration of ATP could induce a transient increase in intracellular Ca^{2+} levels in cells of a Ca^{2+}-containing bathing solution. Note that both the amplitude of the average response (maximal increase in intracellular Ca^{2+} concentration) and the time of exposure to ATP needed to induce the transient exhibited a differentiation-dependent pattern (see also Fig. 1F–G). The shortest exposure of ATP needed to evoke a Ca^{2+}-transient was characteristic to culturing days 3 and 4, and at the same time Ca^{2+}-transients with the highest amplitude (179 and 165 nM, respectively) were also recorded on these days. On the first culturing day ATP, even though administered for a long period (120 sec), could not evoke any changes in the intracellular Ca^{2+} concentration (Fig. 1A), furthermore, we could hardly detect characteristic peaks in 2-day-old cells either (Fig. 1B). By day 6, the amplitude of the transients decreased and only a prolonged application of ATP could evoke such responses (Fig. 1E).

Striking differences were also observed regarding the number of cells responding to ATP (Fig. 1F–G). While most of the cells (90%) responded to ATP in 3-day-old cultures,

essentially none did at day 1 of culturing. Administration of ATP on other days could also induce calcium transients, but the proportion of cells that responded was hardly comparable with that on day 3 (Fig. 1F).

To establish whether metabotropic or ionotropic purinergic receptors were responsible for these effects, ATP was administered to cells in a Tyrode's solution lacking free Ca^{2+} (Fig. 2A–B). In the 30 cells examined, no response was detected on either days of culturing in the absence of extracellular Ca^{2+}. This observation firmly supported our theory that influx of extracellular Ca^{2+} was needed to evoke the effect of extracellular ATP and the receptor of ATP could be a member of the ionotropic purinergic receptor family (P2X), but did not exclude the role of metabotropic purinergic receptors and intracellular Ca^{2+} stores.

To find candidates among P2X receptors, their non-specific antagonist suramin was tested on cells of HDC. It is known that suramin inhibits all P2X receptors except $P2X_4$ and $P2X_6$ [16]. Cells treated with suramin (10 μM) showed no significant alteration in the intracellular Ca^{2+} level following the administration of ATP (Fig. 2C). Repetitive administration of ATP could induce repetitive and transient elevations in intracellular Ca^{2+} concentration in 3-day-old HDC (Fig. 2D). Relatively short periods of washing (approximately 100 sec) were required to allow the cells to recover from the 30-sec-long exposures of ATP. This observation, together with the lack of significant desensitisation (Fig. 2D) raised the possibility of the presence and function of $P2X_4$ and/or $P2X_6$ receptor subtypes.

Although the above results clearly suggested that P2X receptors play the decisive role, we also carried out experiments to obtain data on the function of metabotropic P2Y receptors. First we intended to examine whether intracellular Ca^{2+} stores are present and contain releasable Ca^{2+} by the activation of IP_3 pathway. Since bradykinin receptors are known to activate this pathway and are described as being expressed by chondrocytes [21], therefore

bradykinin was administered to 3-day-old cells at a concentration of 20 μM. A slight elevation (30 nM) of free cytosolic Ca^{2+} concentration was observed in 60% of cells proving the presence and active functioning of IP_3 signalling (Fig. 3A).

ADP, UDP and UTP are non-specific agonists of metabotropic purinergic receptors (P2Y). These compounds were administered to cells of HDC at a concentration of 180 μM on day 3 of culturing (Fig 3B-D). Slight elevation of cytosolic Ca^{2+} was detected only in 50% of cells measured during the administration of UTP. The average amplitude of UTP-evoked Ca^{2+} transients was 57 nM. On the other hand, administration of ADP and UDP did not result in any significant Ca^{2+} transients.

3.2. Chondrogenic mesenchymal cells express various P2X and P2Y receptor subtypes during differentiation

To identify the presence and expression pattern of various purinergic receptors during chondrogenic differentiation of chicken mesenchymal cells, RT-PCR reactions were performed. mRNA sequences of chicken P2X receptors, but not of $P2X_6$ (not yet published) as well as $P2Y_1$, $P2Y_3$, and $P2Y_5$ receptors were downloaded from GenBank and specific primer pairs for each mRNA sequence were designed for amplification (see Supplementary Material). Amplimers of expected sizes were identified for all the available mRNAs, except for $P2X_2$, where only very weak signals were detected (Fig. 4A). mRNA expression of $P2X_1$ and $P2X_7$ receptor subtypes followed a peak-like pattern during differentiation with the highest expression levels on days 3 or 4, respectively. $P2X_3$ receptor subtype mRNA exhibited a rather variable expression profile: the strongest bands were detected between days 1 and 3 of culturing. $P2X_4$ and $P2X_5$ receptor subtypes showed the strongest expression levels. Both receptors expressed markedly on day 1 then the signal became gradually weaker.

mRNAs of $P2Y_1$, $P2Y_3$ and $P2Y_5$ showed constant expression levels throughout the culturing period (Fig. 6B).

Western blot analysis showed a different expression profile for the different P2X receptor subtypes (Fig. 4B). We could not detect the $P2X_2$ receptor subtype, and no signals were visible for $P2X_3$ and $P2X_6$, either in total lysates or in isolated plasma membrane fractions (data not shown). Protein expression of $P2X_1$ subtype in total lysates followed a similar profile to the mRNA expression, but in the plasma membrane fractions strong bands were detected on day 1 to 3, thereafter the protein levels markedly decreased. In contrast with the results of RT-PCR reactions, protein levels of $P2X_5$ receptor subtype were hardly detectable in total cell lysates, but in plasma membrane fractions of day 4 was characterised by a stronger signal.

For $P2X_7$, a profile showing a variable expression pattern was observed in total lysates with the strongest bands on days 2 and 4, respectively, however, in the plasma membrane fractions a peak-like pattern with strongest bands on days 2, 3 and 4 was observed.

Nevertheless, protein expression of $P2X_4$ receptor subtype proved to be the most interesting (Fig. 4B). While in total cell lysates it showed a rather variable profile, in isolated plasma membrane fractions it first appeared on day 3 with a strong band, and by days 4 and 6 its expression rapidly diminished. It is important to note that the vast majority of chondrogenic mesenchymal cells responded to ATP on this day of culturing, which also coincides with the day of differentiation characterised by elevated cytosolic Ca^{2+} levels reported earlier [1]. Presence of $P2X_1$, $P2X_4$ and $P2X_7$ receptors was also proved by immunocytochemical staining of HDC. A membrane-bound localisation was clearly visible for $P2X_4$ receptors (Fig. 4C). Furthermore, presence of $P2X_4$ in cartilagineous primordia in developing limbs of chicken embryos was also demonstrated at a developmental stage (8-day-old embryo) corresponding to approximately 4-day-old HDC (data not shown).

We also detected the expression of metabotropic purinergic receptors in HDC. As $P2Y_3$ and $P2Y_5$ receptors are not regarded as functional members of this family of receptors [16], we investigated $P2Y_1$, $P2Y_2$, and $P2Y_4$ protein in total cell lysates and plasma membrane fractions. On day 1, the protein of $P2Y_1$ was not expressed by cells of HDC, then it was present at a constant level both in total cell lysates and in plasma membrane fractions until day 6, when it showed a small decline. The $P2Y_2$ receptor protein was found to be expressed in a peak like pattern in total lysates with strongest signals on days 2-4. However, we detected a constant level of expression in the plasma membrane fraction with the exception of day 6, when the signal became weaker. We could only detect specific signals for $P2Y_4$ receptor in the plasma membrane fraction with a stronger band on day 2, but no immunopositivity was observed in total lysates (Fig. 6C).

3.3. Administration of extracellular ATP on day of differentiation increases matrix production

In order to support our hypothesis that the entrance of extracellular Ca^{2+} into chondrogenic mesenchymal cells is via P2X receptors, especially via $P2X_4$ subtype, further experiments were performed. ATP was administered at various concentrations to cells of high density cultures on day 3 of culturing. At the concentration of 100 μM, extensive matrix production occurred by day 6 (Fig. 5A) demonstrated by both DMMB and TB stainings. mRNA levels of collagen II and the core protein of aggrecan also reflected the slightly higher rate of matrix production under the effect of ATP (Fig. 5B). Although mRNA expression level of Sox9 did not change (Fig. 5B), protein expression of this transcription factor became higher as a result of the administration of ATP (Fig. 5C). Treatment of HDC with ATP on days 2 and 4 of culturing did not alter the cartilage matrix production (data not shown). These results indicate that ATP has a positive effect on both cartilage matrix production and chondroblast

differentiation when it is applied at the time of final commitment of chondroprogenitor cells and ATP does not exert any effect on premature or mature chondroblasts.

Administration of the non-specific P2X receptor antagonist suramin alone or combination with ATP did not cause any significant alteration in the amount of cartilage matrix produced by the end of the 6-day-old culturing period (Fig. 5A). Moreover, the ATP-stimulated Sox9 expression was not affected by suramin treatment, further supporting our theory that $P2X_4$ receptor could be involved in the transmission of the chondrogenesis promoting effect of extracellular ATP (Fig. 5B–C).

3.4. Administration of ADP, UDP and UTP to the culture medium has no effect on cartilage formation

We examined the administration of the nucleotides on cartilage matrix production of HDC to elucidate a putative role of metabotropic purinergic receptors. The nucleotides applied at a concentration of 180 μM into the culturing medium on day 3 did not exert any effect on the amount of cartilage matrix produced by the end of the 6-day-long culturing period as revealed by metachromatic staining (Fig 6A).

3.5. Cells of high density cultures secrete ATP into the culture medium

The demonstration of the effectiveness of administration of ATP on matrix production raises the question whether the chondrogenic mesenchymal cells secrete ATP into the culture medium as an autocrine mediator to promote and facilitate their own differentiation. To investigate this, the culture medium was removed from the cells of high density cultures on each day of culturing and ATP assays were performed. We found that on each day of culturing a small amount of ATP was detectable in the culture medium in the range of 2–10

nM, which is comparable to data measured in culture medium of other non-excitable cells [22].

4. DISCUSSION

In vitro chondrogenesis is a dynamic, multistep process regulated by a variety of molecular processes, many of which involve activation and deactivation of protein kinases and phosphatases sensitive to changes of intracellular Ca^{2+} levels. In chicken high density mesenchymal cell cultures, chondrogenic mesenchymal cells differentiate into chondroblasts and then to chondrocytes during a 6-day-long culturing period. The majority of chondroblasts, characterised by the ability of production of a cartilage specific ECM, appear from culturing day 3.

We have previously demonstrated that cytoplasmic free Ca^{2+} concentration of chondrogenic cells exhibited a characteristic transient elevation on day 3 of culturing. This has been found to be indispensible to proper differentiation and the essential role of the influx of extracellular Ca^{2+} has been documented [1]. Intracellular Ca^{2+}-stores have been shown to contain releasable Ca^{2+}, but the rate of leak was low and free cytoplasmic Ca^{2+} concentration became only slightly higher in the absence of extracellular Ca^{2+}. Moreover, RyR and IP_3 receptors have been found to be expressed weakly and stimulation of RyR did not result in the elevation of cytoplasmic Ca^{2+}. Our data have underlined the role of Ca^{2+} influx from extracellular space in the generation of the cytoplasmic Ca^{2+} -peak. The intracellular stores seemed to be contributing to the maintenance of cytosolic basal Ca^{2+}-concentration [1].

In the present study, we report the possible involvement of P2X and P2Y, ligand-gated purinergic receptors, in the regulation of the Ca^{2+} homeostasis of chondrogenic cells particularly during their differentiation. Purinoreceptors are known to be expressed in embryonic tissues [23] and are probably involved in the differentiation process of excitable

[24] and non-excitable [25] cells. However, no data are available concerning the possible involvement of such processes in the differentiation of chondroblasts from mesenchymal cells. During single cell measurements using Fura-2-loaded cells, ATP, an agonist of purinergic receptors, was administered on various days of culturing. We found that the cells of HDC responded to ATP by characteristic Ca^{2+} transients. We also found that most of the cells only responded to ATP on day 3 of culturing, at the time of differentiation of chondroblasts. The phenomenon that some cells showed response on other days than day 3 can be explained by considering the fact that the cells of HDC exhibit some heterogeneity in their stage of differentiation, and though the vast majority differentiates on day 3 of culturing, there are some cells, which could reach this stage of development somewhat earlier or later. We also showed that probably the members of the ionotropic P2X receptor subfamily can be accounted for the influx of extracellular Ca^{2+}.

We also tested the effect of P2Y receptor agonists on Ca^{2+} transients in cells of 3-day-old HDC. When ATP was administered to the cells in a Tyrode's solution lacking free Ca^{2+}, ATP failed to elevate the intracellular Ca^{2+} concentration. ADP, the agonist of $P2Y_1$, and UDP, the ligand of $P2Y_6$ receptor, neither evoked any significant elevation in the free cytoplasmic Ca^{2+} concentration, nor did they influence cartilage formation of HDC. However, the agonist of $P2Y_2$ and $P2Y_4$ receptors, UTP caused a transient elevation of cytosolic free Ca^{2+} in 50% of cells investigated. RT-PCR and Western blot analyses proved the presence of $P2Y_1$, $P2Y_2$, $P2Y_3$, $P2Y_4$ and $P2Y_5$ receptors in cells of HDC.

All the P2X receptor mRNAs investigated, except that of $P2X_2$, were expressed by cells of HDC showing variable expression profiles. The phenomenon of age-dependent expression suggests the involvement of purinergic signalling in the mediation of chondrogenic differentiation. Our findings that P2X receptor subtypes are expressed by differentiating chondrogenic cells in a differentiation stage-dependent manner are comparable to studies

conducted on hematopoietic cell lines [26]. At the protein level, cells of HDC expressed only receptor subtypes $P2X_1$, $P2X_4$, $P2X_5$ and $P2X_7$ and the expression profiles in total cell lysates and plasma membrane fractions were different. This difference was exceptionally interesting in the case of $P2X_4$: this receptor started to appear on day 3 in the plasma membrane fraction with a very strong signal, whereas weaker signals were detected on days 4 and 6. The characteristic expression profile of $P2X_4$ raised the possibility that this ligand-gated receptor could be an important channel through which extracellular Ca^{2+} enter the cytosol and contribute to the elevated Ca^{2+} level needed for the chondrogenic differentiation process. Although other P2X receptors were also present in plasma membrane fractions, ATP-evoked Ca^{2+} transients were not eliminated when suramin and ATP were applied simultaneously. Since suramin is not a $P2X_4$ antagonist but inhibits other P2X type purinergic receptors expressed by cells of HDC, our data suggest that these receptors may contribute to the maintenance of the basal cytosolic Ca^{2+} concentration.

The ATP-mediated function of $P2X_4$ receptor during the differentiation process was proved by the addition of extracellular ATP to the culture medium. It resulted in an increase of the expression of Sox9, the master transcription factor of chondrogenesis. We detected higher amount of metachromatic cartilage matrix produced in ATP-treated HDC by the end of the 6-day-long culturing period. This effect was achieved exclusively when ATP was administered on day 3 of culturing. When ATP was added prior to (day 3) or after the differentiation period of chondrogenic cells (day 4 of culturing), it did not result in any significant effect on the matrix formation of HDC. This observation underlines the importance of ATP in the facilitation of cartilage differentiation and may rule out its role in the stimulation of matrix production of mature cartilage. Although application of metabotropic P2Y receptor agonists to the culture medium on day 3 caused Ca^{2+} transients similar to those generated by ATP, but did not alter the amount of cartilage matrix produced by the end of the

6-day-long culturing period. Therefore we suggest that metabotropic P2Y receptors rather contribute to the maintenance of basal cytosolic Ca^{2+} concentration in cells of HDC.

Although suramin did not eliminate the ATP-evoked Ca^{2+}-transients of chondrogenic cells, the increased metachromatic cartilage matrix production caused by ATP was completely diminished. This effect does not seem to be exerted via the inhibition of chondrogenesis, since the expression of Sox9, was not reduced by suramin, and the mRNA expression levels of neither collagen type II nor aggrecan were affected. Suramin has been reported to inhibit hyaluronic acid synthesis of fibroblasts [27], and hyaluronic acid is responsible for holding aggregates of aggrecan together in cartilage matrix. The reduced amount of hyaluronic acid may cause increased loss of aggrecan during metachromatic staining procedures of HDC, causing virtual reduction of the detected amount of cartilage matrix.

We also showed that cells of HDC secreted ATP into the culture medium. This ATP may act as an autocrine mediator to facilitate and promote their own differentiation. Chondrogenic cells secreted ATP throughout the culturing period, and they responded to the extracellularly administred ATP with a peak-like elevation of ic. Ca^{2+} concentration only at the time of differentiation. This fact further supports the purinergic concept in the control of chondrogenesis. Our data on ATP concentrations secreted into the culture medium seem significantly less than the concentrations applied for matrix production assays. It is important to emphasize that ATP assays were performed in 20 mL of culture medium. Furthermore, the volume of the cell culture itself and the volume of the culture medium differ by a factor of approximately 1,000. Therefore, the ATP secreted by the cells could have reached much higher concentrations at the close proximity of cells. Thus the detected concentrations of ATP secreted by chondrogenic cells should be in the range in which P2X receptors respond to this ligand [28,29].

In summary, our observations provide the first evidence on the possibility of a purinergic autoregulation of chondrogenesis. Purinergic receptors, members of the Ca2+ tool kit used by cells of HDC can be one of the key elements in the regulation of the elevated cytosolic Ca^{2+} levels during cartilage differentiation *in vitro* and *in vivo*.

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FIGURES

Figure 1. (Fodor *et al.*)

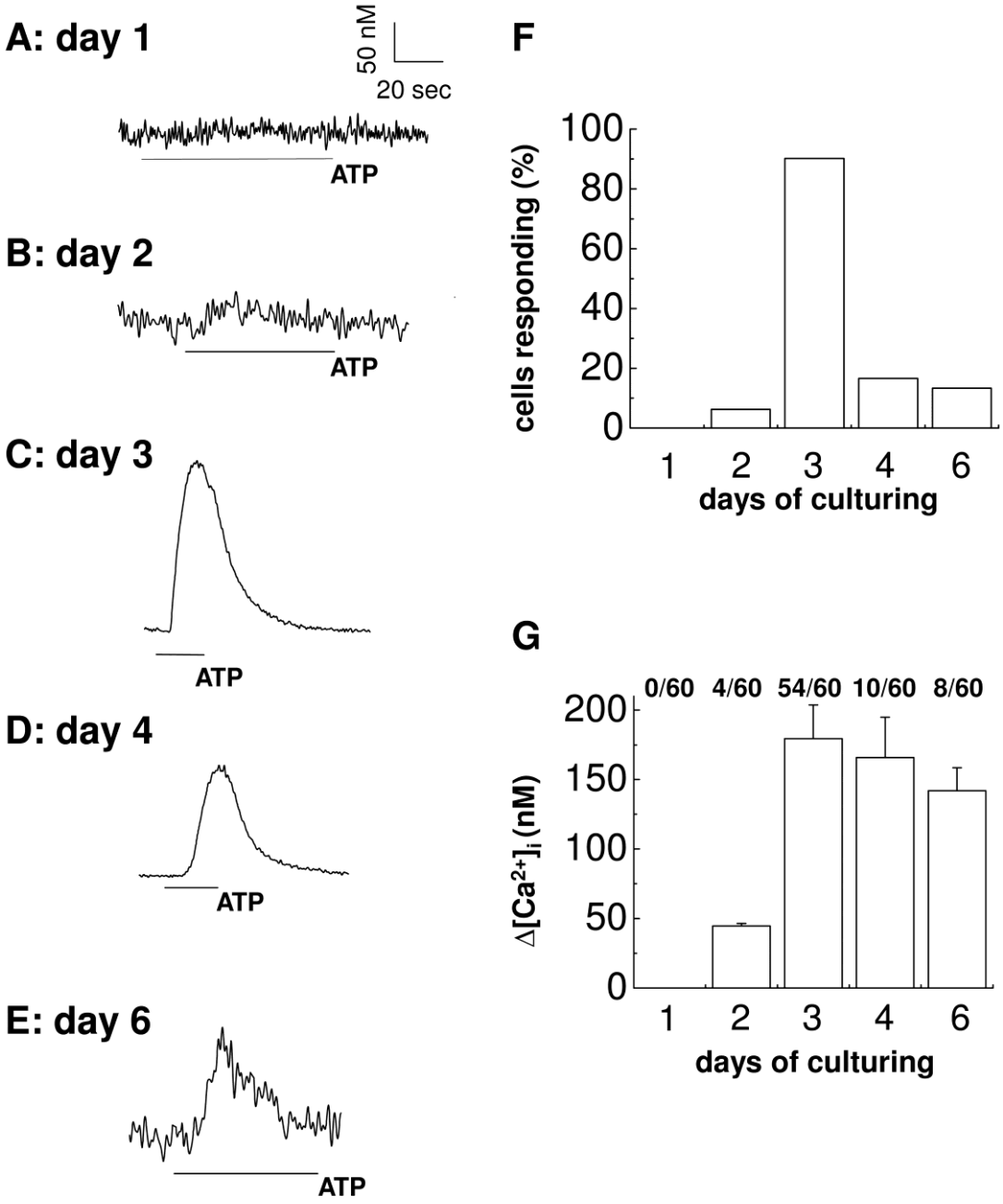

Figure 1. Effect of 180 μM ATP on the cytosolic Ca^{2+} levels of Fura-2-loaded cells of HDC. **(A–E)** Ca^{2+} transients evoked by administration of ATP in cells on different days of culturing. Representative records of 5 independent experiments. Lines indicate the application of ATP. **(F)** Ca^{2+} transients were measured in the presence of 1.8 mM external calcium. Ratio of cells responding to ATP on each day of culturing. Representative data of 5 independent

experiments. **(G)** Changes in the peak amplitude of calcium transients detected on different days of culturing. Numbers indicate the proportion of cells responding to ATP. Representative data of 5 independent measurements.

Figure 2. (Fodor *et al.*)

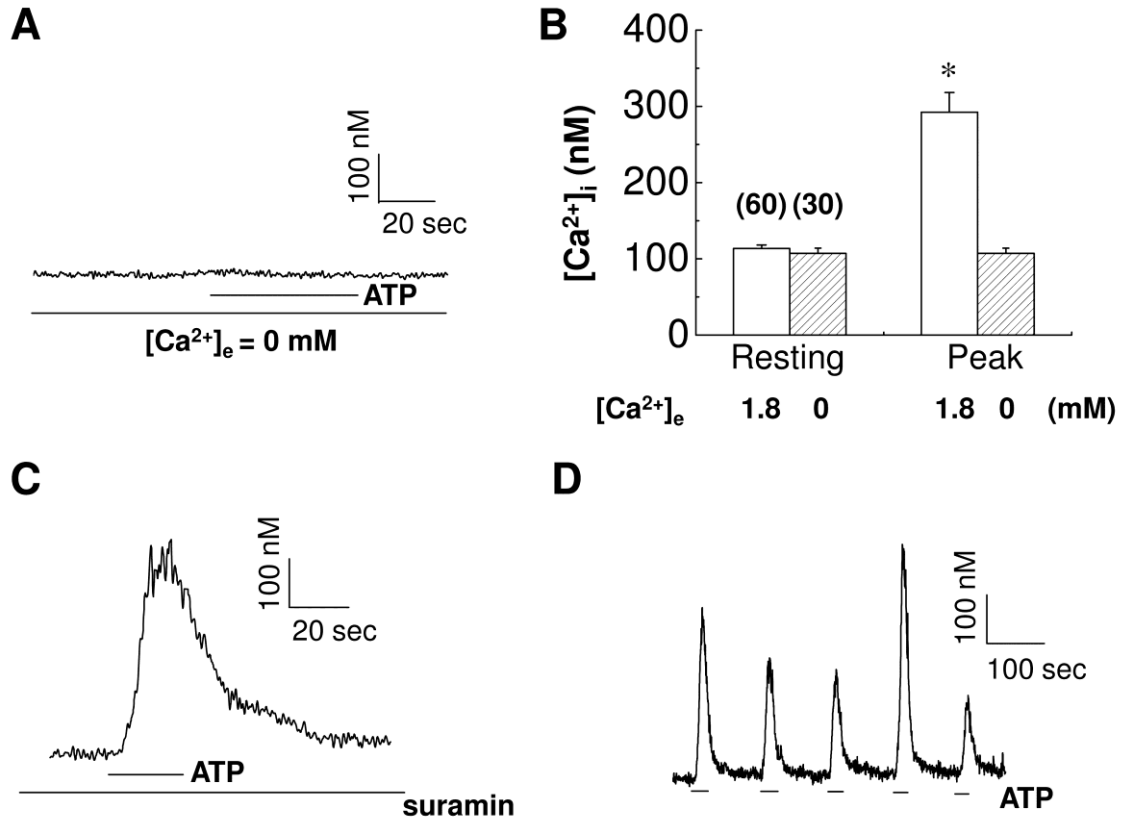

Figure 2. Responses of cells of HDC to administration of ATP on day 3 of culturing. **(A)** Record showing the lack of ATP-evoked Ca^{2+} transients in the absence of external calcium. **(B)** Basal intracellular Ca^{2+} levels and the peak amplitude of ATP-evoked Ca^{2+} transients in the presence and absence of external calcium. Numbers in parentheses show the number of cells measured. Data represent mean ± standard error of the mean of intracellular Ca^{2+} levels of cells assayed in 5 independent experiments. Asterisk indicates significant (*$P < 0.01$) increase in peak amplitude of ATP-evoked Ca^{2+} transients as compared to the respective control. **(C)** Effect of the P2X antagonist suramin (10 μM) on ATP-evoked calcium transients in the presence of external calcium. **(D)** Calcium transients evoked by repeated administration of ATP in the presence of external calcium showing the lack of desensitisation of P2X receptors in differentiating chondrocytes. Representative record of 5 independent experiments

is presented in panels A, C or D. Lines in panels A, C and D indicate the application of ATP. Preceding the application of Ca-free ATP cells were treated in Ca-free Tyrode's for 150 s.

Figure 3. (Fodor *et al.*)

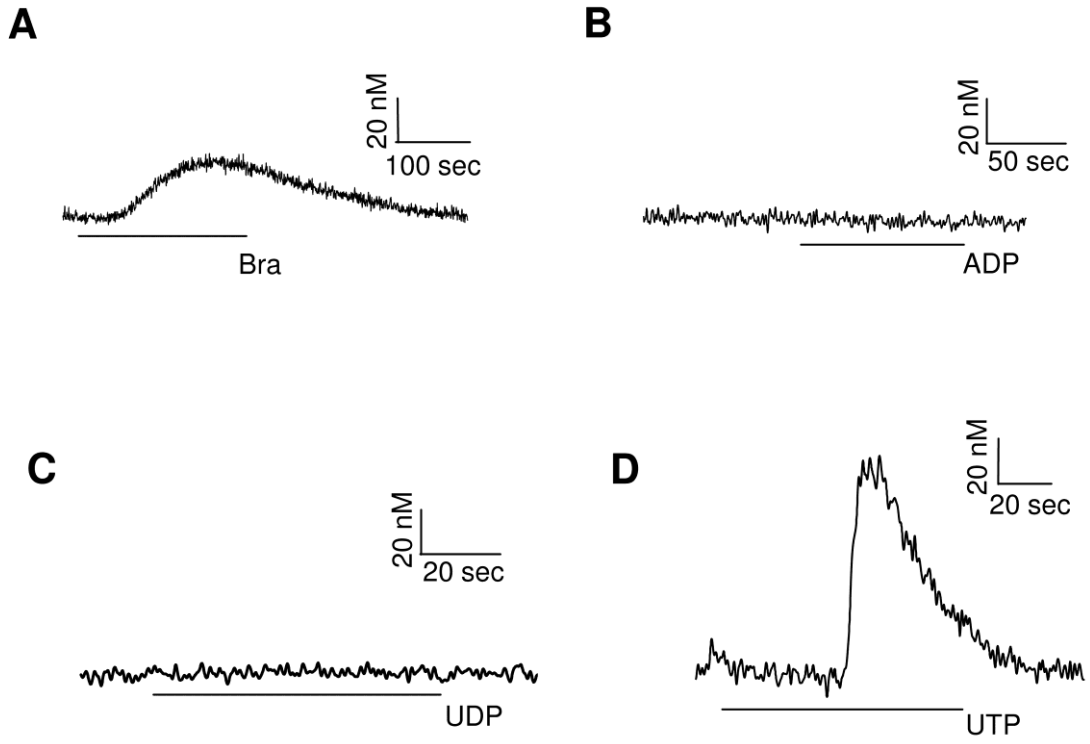

Figure 3. Functional characterisation of P2Y receptors in cells of HDC on day 3 of culturing. **(A)** 20 μM bradykinin-evoked Ca^{2+} transients measured in the absence of external calcium. Line indicates the application of bradykinin. **(B)** Effect of 180 μM ADP on Ca^{2+} transients measured in the absence of external calcium. Line indicates the application of ADP. **(C)** Effect of 180 μM UDP on Ca^{2+} transients measured in the absence of external calcium. Line indicates the application of UDP. **(D)** 180 μM UTP-evoked Ca^{2+} transients in the absence of external calcium. Line indicates the application of UTP. Representative records of 3 independent experiments.

Figure 4. (Fodor *et al.*)

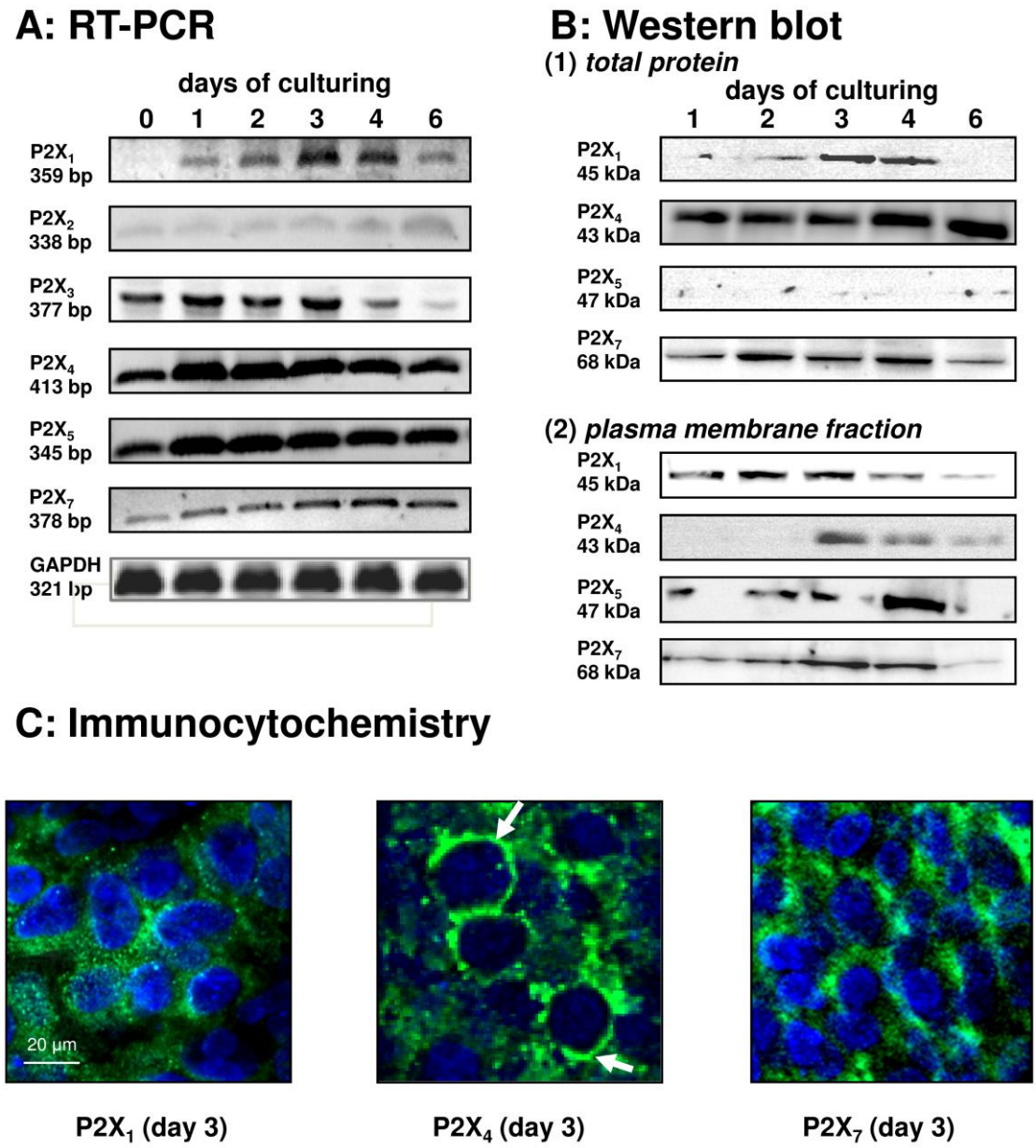

Figure 4. Expression pattern of P2X receptor subtypes in cells of HDC on different days of culturing. **(A)** mRNA expression pattern of various P2X receptors was detected by RT-PCR reactions. P2X receptor subtypes (but not $P2X_6$) were amplified using specific primers and detected at expected sizes. GAPDH was used as a control. **(B)** Western blot analysis of P2X receptor proteins in cells of HDC. Total protein and membrane fraction samples were used (50 µg in each lane) to examine the protein expression level. Representative data each of 3 independent experiments, performed in triplicates. **(C)** Immunocytochemical staining of 3-

day-old HDC demonstrating presence of $P2X_1$, $P2X_4$ and $P2X_7$ receptors. Arrows indicate the accumulation of immunopositive signal in the plasma membrane of chondrogenic cells. Images were recorded from1 μm thick optical slices. Original magnification was 40×. Representative images of 3 independent experiments, each performed in triplicates.

Figure 5. (Fodor *et al.*)

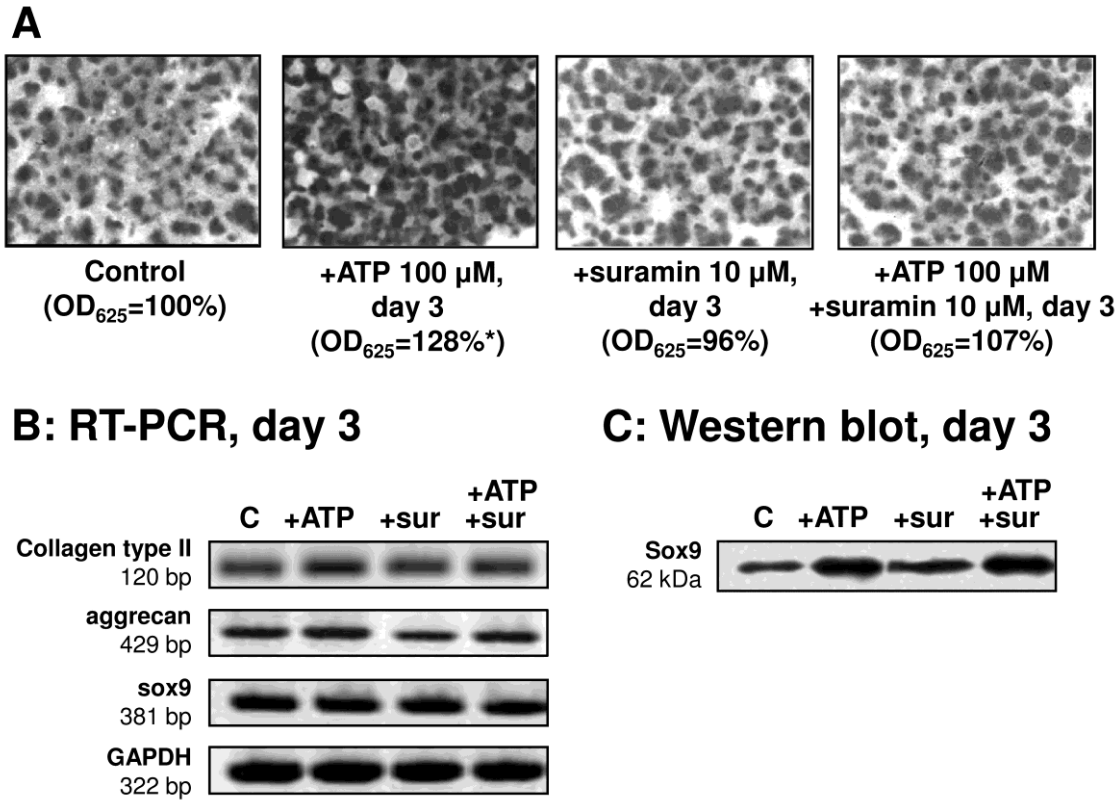

Figure 5. Effects of ATP (100 µM) and suramin (10 µM) on cartilage development of chondrifying micromass cultures. Both chemicals were administered on day 3 of culturing. **(A)** Metachromatic cartilage areas in 6-day-old high density colonies visualized with DMMB dissolved in 3 % acetic acid. Optical density (OD_{625}) of samples containing toluidine blue extracted with 8 % HCl dissolved in absolute ethanol. Data are mean values of each experimental group out of 5 measurements. Standard errors of the means were within ± 8 %. Asterisks indicate significant (*$P < 0.01$) increase in optical density of extracted toluidine blue as compared to the respective control. **(B)** mRNA expression of collagen type II, core protein of aggrecan and sox9 after treatment with ATP and/or suramin. GAPDH was used as a control. Representative data of 3 independent experiments, each performed in triplicates **(C)** Effect of ATP and/or suramin on the protein expression of Sox9. Representative data of 3 independent experiments, each performed in triplicates.

Figure 6. (Fodor *et al.*)

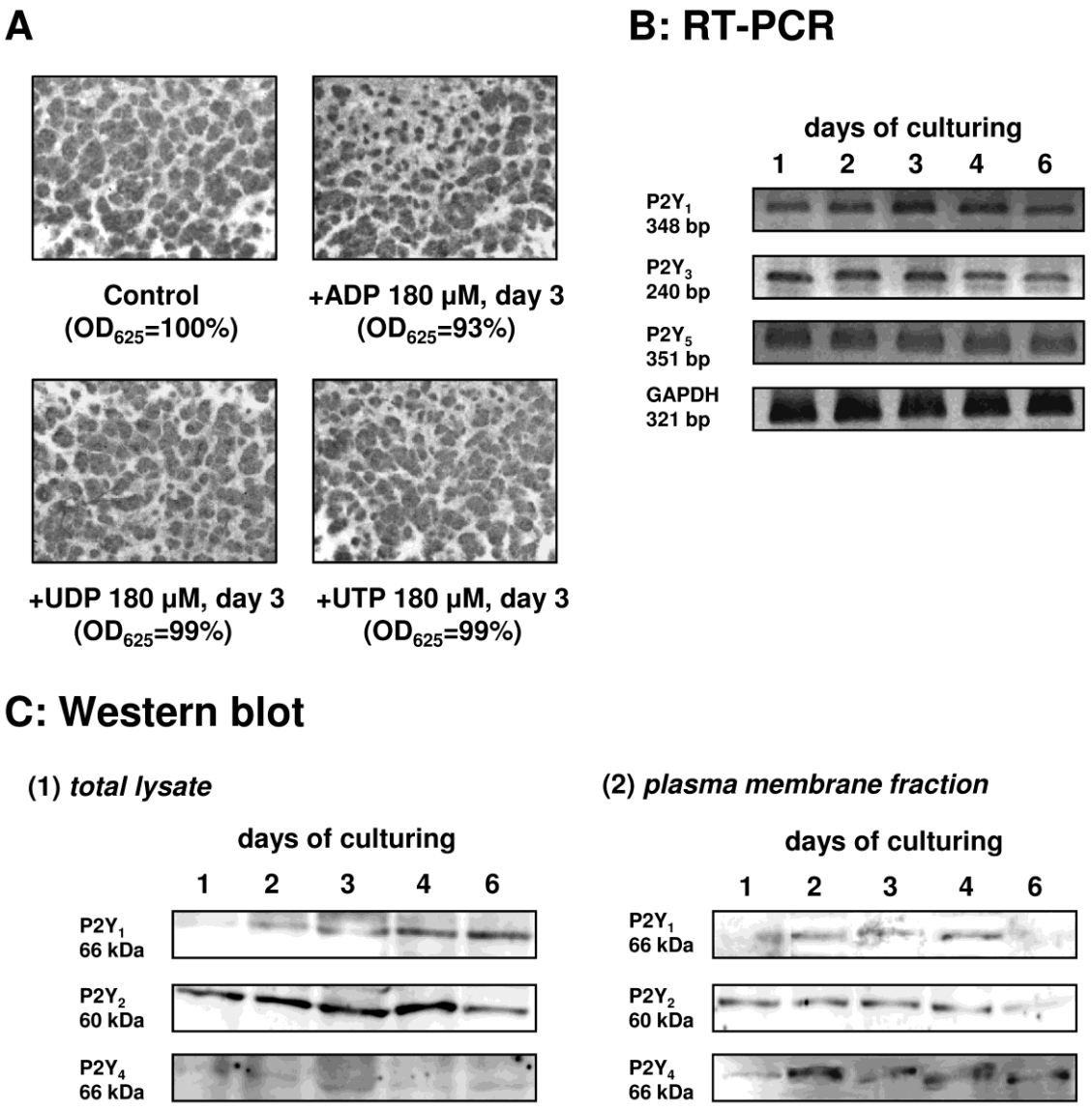

Figure 6. **(A)** Metachromatic cartilage areas in 6-day-old HDC visualized with DMMB dissolved in 3 % acetic acid. Optical density (OD_{625}) of samples containing toluidine blue extracted from HDC with 8 % HCl dissolved in absolute ethanol. Data are mean values of each experimental group out of 4–4 parallel samples of 3 independent measurements. Standard errors of the means were within ± 8 %. **(B)** mRNA expression pattern of various P2Y receptors detected by RT-PCR reactions. P2Y receptor subtypes were amplified using specific primers and detected at expected sizes. GAPDH was used as a control. Representative data of 3 independent experiments, each performed in triplicates. **(C)** Western blot analysis of

$P2Y_1$ $P2Y_2$ and $P2Y_4$ receptor proteins in cells of HDC. Total protein and membrane fraction samples were used (50 µg in each lane) to examine the protein expression level. Representative data of 3 independent experiments, each performed in triplicates.