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Glutamate signaling in chondrocytes and the potential involvement of NMDA receptors in cell proliferation and inflammatory gene expression

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Summary

Objective: Increased levels of glutamate, the main excitatory neurotransmitter, are found in the synovial fluid of osteoarthritis (OA) patients. Our aim was to study glutamate signaling in chondrocytes, focusing on the composition, pharmacology, and functional role of *N*-methyl-D-aspartate (NMDA) glutamate receptors.

Methods: We used the human chondrocyte cell line SW1353 and, in parallel, primary rat articular chondrocytes. Glutamate release and uptake were measured by fluorimetric and radiometric methods, respectively. Gene expression was analyzed by quantitative polymerase chain reaction. NMDA receptor pharmacology was studied in binding experiments with [^{3}H]MK-801, a specific NMDA receptor antagonist. RNA interference was used to knock-down the expression of NR1, a subunit of NMDA receptors.

Results: Glutamate release, sodium- and calcium-dependent glutamate uptake, and the expression of a glutamate transporter were observed in chondrocytes. NR2D was the most abundant NMDA receptor subunit in these cells. Consistent with this observation, the binding affinity of [^{3}H]MK-801 was much lower in chondrocytes than in rat brain membranes (mean K_d values of 700 and 2.6 nM, respectively). NR1 knock-down, as well as NMDA receptor blockade with MK-801, reduced chondrocyte proliferation. Interleukin (IL)-1β significantly altered glutamate release and uptake (about 90% increase and 50% decrease, respectively, in SW1353 cells). Moreover, IL-1β induced the gene expression of cytokines and enzymes involved in cartilage degradation, and MK-801 significantly inhibited this response.

Conclusions: Our findings suggest that chondrocytes express a self-sufficient machinery for glutamate signaling, including a peripheral NMDA receptor with unique properties. This receptor may have a role in the inflammatory process associated with cartilage degradation, thus emerging as a potential pharmacological target in OA.

Key words: Osteoarthritis, Glutamate, NMDA receptors, Chondrocytes, Inflammation.

Introduction

Glutamate is the main excitatory neurotransmitter in the central nervous system (CNS). However, the discovery that glutamate signaling is functional in peripheral tissues (neuronal and non-neuronal) indicates that it may also act as an autocrine and/or paracrine mediator to influence many cellular activities[1–3]. Some studies in the late nineties have suggested that glutamate is one of the endogenous factors used for intercellular communications in bone[4,5]. Both ionotropic and metabotropic glutamate receptors are expressed in bone cells, and the activity of *N*-methyl-D-aspartate (NMDA) receptors, which belong to the ionotropic glutamate receptor family, is modulated *in vitro* by specific antagonists such as MK-801[6–8].

Various components of the glutamate system have been recently detected in cartilage cells. For instance, rat costal chondrocytes express mRNA for NMDA and non-NMDA receptors[9,10]. In addition, these cells express different excitatory amino acid transporters (EAATs), including the glial subtypes glutamate transporter-1 (GLT-1) and glutamate/aspartate transporter (GLAST)[11]. We owe to Salter and his group the first demonstration that the NMDA receptor is present in human articular cartilage *in vivo*, together with the hypothesis that it may be involved in biomechanical responses[12]. In parallel, other authors have reported on increased glutamate and cytokine levels in the synovial fluid of patients with active arthropathies[13,14], consistent with the recent finding that NMDA receptors on human synoviocytes may contribute to joint inflammation and destruction in rheumatoid arthritis (RA)[15]. However, little attention has been paid to the possible involvement of chondrocytes in the inflammatory response mediated by glutamate at the joint level. The contribution of chondrocytes to this process might be relevant to osteoarthritis (OA), a disease in which inflammation plays a role regardless of its classification as a non-inflammatory arthropathy[16].

Thus, an experimental study was undertaken to investigate the role of glutamate signaling in cartilage physiology and pathology. We used the human chondrosarcoma cell line SW1353, a well-established chondrocyte model[17,18], and, in parallel, primary cultures of rat articular chondrocytes. Here we describe a self-sufficient machinery for glutamate signaling, the composition and pharmacology of the NMDA receptor expressed in chondrocytes, and the potential role of this receptor under physiological and pathological conditions.

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Materials and methods

MATERIALS

Leibovitz's Medium + GlutaMAX I, Dulbecco's modified Eagle Medium (DMEM) + GlutaMAX I, Dulbecco's phosphate-buffered saline (PBS), Hanks' balanced salt solution (HBSS), Trypsin/EDTA, and FM1-43 were purchased from Invitrogen (Carlsbad, CA). Hyaluronidase, trypsin, collagenase, recombinant human interleukin (IL)-1β (100.000 U/ml, 2 μg/ml), and FuGENE 6 Transfection Reagent were from Roche (Indianapolis, IN). MK-801 and DL-threo-β-benzyloxyaspartate (TBOA) were from Tocris Cookson (Avonmouth, UK). EDTA, $NADP^+$ and glutamate dehydrogenase (GDH) were obtained from Sigma (St. Louis, MO). [^{3}H]MK-801 (specific activity 17–22 Ci/mmol), [^{3}H]glutamate (specific activity 52 Ci/mmol), and UltimaGold-MV were purchased from PerkinElmer Life and Analytical Sciences (Wellesley, MA).

CELL CULTURES

SW1353 cells (human chondrosarcoma cell line from ATCC, Promochem, UK) were grown adherent at 37°C, without CO_2, in Leibovitz's medium supplemented with 10% fetal bovine serum (FBS) and gentamicin (50 μg/ml). SW1353 cells were plated at a density of 3×10^4 cells/cm^2. For expression analysis, confluent cells were synchronized by incubation in Leibovitz's medium for 16 h, in the presence of 0.4% FBS. All experiments were performed using cells at passages 5 through 18.

Rat articular chondrocytes were obtained from young adult Sprague Dawley male rats (7 weeks; Charles River, Calco, Italy). All studies involving animals were carried out in accordance with the Guide for the Care and Use of Laboratory Animals as adopted and promulgated by the US National Institutes of Health. Articular cartilage from shoulders, femoral heads, and knees was prepared according to Berenbaum and colleagues[19]. The cell suspension was then diluted to 10^4 cells/ml and seeded into 12-well culture plates (1 ml/well) in DMEM supplemented with 10% FBS and gentamicin (50 μg/ml). Cells reached confluence in 12–14 days. For expression analysis, confluent cells were synchronized for 16 h in DMEM supplemented with 0.4% FBS.

To study the effect of NMDA receptor blockade on gene expression, cells were pre-treated with MK-801 for 1 h before stimulation with IL-1β (2 ng/ml) for 6 h. The drug concentrations used for gene expression experiments were selected after toxicity analysis (viability assay using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide [MTT]).

GLUTAMATE DETERMINATION

Levels of glutamate were determined by fluorimetric assay, according to Genever and Skerry[20]. Fluorescence was monitored for 30 min, at 5 min intervals, using excitation and emission wavelengths of 355 nm and 460 nm, respectively (VICTOR fluorescence reader; PerkinElmer, Wellesley, MA). To quantify glutamate levels, a standard curve was constructed for each assay with known concentrations of glutamate (0.8–25 μM). Intracellular concentration of glutamate was assessed after cell permeabilization with 1% Triton X-100.

GLUTAMATE UPTAKE

Cells were washed with *N*-(2-Hydroxyethyl)piperazine-*N*′-(2-ethanesulfonic acid) (HEPES) Krebs-Ringer (HKR) (125 mM NaCl, 3.5 mM KCl, 1.5 mM $CaCl_2$, 1.2 mM $MgSO_4$, 1.25 mM KH_2PO_4, 25 mM $NaHCO_3$, 10 mM HEPES, and 10 mM D-glucose, pH 7.4) buffer. Cells were then incubated at 37°C for 20 min in HKR buffer containing 25 nM [^{3}H]glutamate. The reaction was terminated by removing the buffer and rinsing with ice-cold HKR buffer; washed cells were then solubilized in 0.1 M NaOH. For liquid scintillation spectrometry, 8 ml of UltimaGold-MV were used. Protein concentration was determined using a Bio-Rad Protein Assay Kit (Hercules, CA), and results were normalized to protein content. To further characterize glutamate uptake into chondrocytes, tests were performed in HKR buffer without Ca^{2+} or Na^+, with increasing concentrations of Ca^{2+}, with EDTA, and in HKR buffer containing TBOA.

VESICLE RECYCLING

Vesicle recycling was investigated by using the membrane probe FM1-43, a water-soluble dye, non-toxic to cells and non-fluorescent in aqueous medium. This method is generally used for identifying actively firing neurons and was previously used for the labeling of recycling vesicles in osteoblastic cells[20]. In brief, after 5 min pre-incubation in recycling buffer (HBSS supplemented with $CaCl_2$, 1 mM), cells were exposed to 10 μM FM1-43 for different time periods (up to 20 min). At the end of incubation, cells were washed and fixed in 4% paraformaldehyde for 30 min, then mounted and viewed with a fluorescence microscope (Axioskope-Zeiss, Germany). For destaining experiments, cells were exposed to the dye for 20 min, then washed in FM1-43-free HBSS for 30 min before fixation.

TOTAL RNA PURIFICATION

Total RNA was purified using ABI PrismTM 6100 Nucleic Acid PrepStation, an RNA isolation platform from Applied Biosystems (Foster City, CA).

REVERSE TRANSCRIPTION (RT)

Total RNA was retrotranscribed with the High-Capacity cDNA Archive Kit (Applied Biosystems) by adding 50 μl of total RNA to 50 μl of reaction mix (following the manufacturer's protocol). The reaction (final volume: 100 μl) was carried out in a Bio-Rad (Hercules, CA) "iCycler".

QUANTITATIVE POLYMERASE CHAIN REACTION (PCR)—REAL-TIME PCR (RT-PCR)

All primers and probes were purchased from Applied Biosystems as TaqMan® Gene Expression Assays, with the exception of the primers and probes for the endogenous controls glyceraldehyde-3-phosphate dehydrogenase (GAPDH; human) and 18S (rat), which were purchased as PDARs (Pre-Developed TaqMan® Assay Reagents).

Identification codes for the human primers and probes are as follows: GLAST-1 (Hs00188193_m1), NR1 (Hs00246956_m1), NR2A (Hs00168"-219_m1), NR2B (Hs00168230_m1), NR2C (Hs00168236_m1), NR2D (Hs00181352_m1), postsynaptic density-95 (PSD-95; Hs00176354_m1), IL-1β (Hs00174097_m1), IL-6 (Hs00174131_m1), cyclooxygenase-2 (COX-2; Hs00153133_m1), matrix metalloproteinase-3 (MMP-3; Hs00233962_m1).

Identification codes for the rat primers and probes are as follows: GLAST-1 (Rn00570130_m1), NR1 (Rn00433800_m1), NR2A (Rn00561341_m1), NR2B (Rn00561352_m1), NR2C (Rn00561364_m1), NR2D (Rn005756"-38_m1), PSD-95 (Rn00571479_m1), IL-1β (Rn00580432_m1), IL-6 (Rn00561420_m1), COX-2 (Rn00568225_m1), MMP-3 (Rn00591740_m1).

The reaction was performed using the ABIPRISM 7000 Sequence Detection System, and each sample was analyzed in triplicate. Data were normalized according to the amplified values of GAPDH/18S, with the aid of the Relative Quantification/RQ Software (Applied Biosystems).

WESTERN BLOT

Cells were lysed with the M-PER Eukaryotic Protein extraction kit (Pierce, Rockford, IL) in the presence of Halt™ Protease Inhibitor Cocktail kit (Pierce). Protein extracts were electrophoresed on 8% sodium dodecyl sulfate-polyacrylamide (SDS-polyacrylamide) gels under reducing conditions and transferred onto PVDF membranes. Membranes were probed overnight (o/n) at 4°C with anti-NR1 (1:1000; Upstate, Lake Placid, NY) or anti-β-actin (1:250; Sigma, St. Louis, MO) antibodies. Detection was carried out using ECL reagents (GE Healthcare Europe GmbH, Freiburg, Germany).

RECEPTOR BINDING ASSAY

For binding assays on SW1353 and rat chondrocytes, the medium was removed and cells were washed three times with PBS. In preliminary studies, chondrocyte membranes and intact cells were tested in parallel. All other assays were carried out using intact chondrocytes and were performed in 1 ml binding buffer (20 mM HEPES, 140 mM NaCl, 2.8 mM KCl, 1 mM $CaCl_2$, 10 mM glucose, pH 7.4), without magnesium. [^{3}H]MK-801 (3 nM) was incubated for 90 min at 25°C in the absence (total binding) or presence of test compounds. In selected experiments, magnesium and zinc cations were added at concentrations known to maximally inhibit the specific binding of [^{3}H]MK-801 to the central NMDA receptor. At the end of the incubation period, cells were washed three times with ice-cold binding buffer and hydrolyzed in 0.5 M NaOH, and bound radioactivity was quantified. Specific binding was calculated as the difference between the total binding and the binding in the presence of 50 μM MK-801.

Binding assays to rat brain cortical membranes were carried out using washed crude synaptic membranes (250–300 μg protein), according to Foster and Wong[21] but in the same buffer as above. The membrane suspension was incubated for 60 min at 25°C with [^{3}H]MK-801 (2.5 nM), in the absence or presence of 100 μM MK-801 (non-specific binding). Protein concentration was determined by the Bradford method, using bovine serum albumin (BSA) as a standard.

SMALL INTERFERING RNA (siRNA)

siRNAs expressed from short hairpin RNAs (shRNAs) are a powerful way to mediate gene specific RNA interference (RNAi) in mammalian cells. For our knock-down experiments we used the MISSION product line from Sigma, which uses a viral vector-based RNAi library against human genes. This library consists of sequence-verified shRNA lentiviral plasmid vectors targeting human NR1 (more specifically, a clone set of five individual constructs

targeting different regions of the gene sequence). We obtained the plasmids directly from Sigma.

To permit accurate interpretation of knock-down results and provide assurance of the specificity of the response observed, an empty pLKO.1-puro vector was used as a negative control (mock). Cells were transfected with the five different shRNA vectors for NR1 knock-down (MISSION™ pLKO.1-puro GRIN1 vector) by FuGENE 6 Transfection Reagent (Roche). Transfected cells were selected by puromycin. NR1 subunit knock-down was determined by RT-PCR analysis. The different clones obtained were named with the code pLKO-block letter (e.g., pLKO-A).

Proliferation studies (up to 9 days) were done by colorimetric MTT assay (Roche).

STATISTICAL ANALYSIS

Unless otherwise indicated, results are presented as mean ± standard error (SE). Data were analyzed by one-way analysis of variance (ANOVA) (gene expression experiments), two-way ANOVA for repeated measures (proliferation experiments), or unpaired *t*-test (glutamate release and uptake, and GLAST-1 expression). A *P* value of 0.05 or less was considered statistically significant.

Results

GLUTAMATE RELEASE AND UPTAKE, AND VESICLE RECYCLING

Endogenous glutamate was spontaneously released both from human chondrosarcoma cells and from rat articular chondrocytes. After 30 min of incubation, SW1353 cells released 21.3 ± 4.6 nmol glutamate/mg protein ($n = 8$), and rat articular chondrocytes released 55.7 ± 2.4 nmol glutamate/mg protein ($n = 3$). Cell permeabilization with Triton X-100 allowed estimation of the intracellular concentration of free glutamate, which was lower in SW1353 cells than in rat articular chondrocytes (55.3 ± 9.8 and 112.5 ± 21.4 nmol/mg protein, respectively).

In uptake experiments at 37°C, the accumulation of [^{3}H]glutamate was linear with time up to 1 h, whereas no relevant accumulation was seen when cells were incubated at 2°C (not shown). After 20 min of incubation at 37°C, [^{3}H]glutamate concentrations ranged from 13 to 90 pmol/mg protein in SW1353 cells and from 100 to 500 pmol/mg protein in rat articular chondrocytes. The uptake of [^{3}H]glutamate was Na^+- and Ca^{2+}-dependent in both cell types [Fig. 1(A, B), and Supplementary Fig. S1] and was significantly inhibited by 100 μM TBOA, a specific blocker of glutamate transporters [Fig. 1(C)]. In agreement with this finding, RT-PCR analysis revealed constitutive expression of mRNA for the glutamate transporter GLAST-1 in both cultures [Fig. 1(D)].

Vesicle recycling activity was demonstrated in SW1353 cells by using the styryl dye FM1-43. Cells rapidly accumulated the fluorescent dye over a 20-min incubation period; after removal of FM1-43 from the medium, destaining was complete within 30 min (Fig. 2).

COMPOSITION OF THE NMDA RECEPTOR IN CHONDROCYTES

Messenger RNAs for different subunits of the NMDA receptor, namely NR1, NR2A, NR2C, and NR2D, were

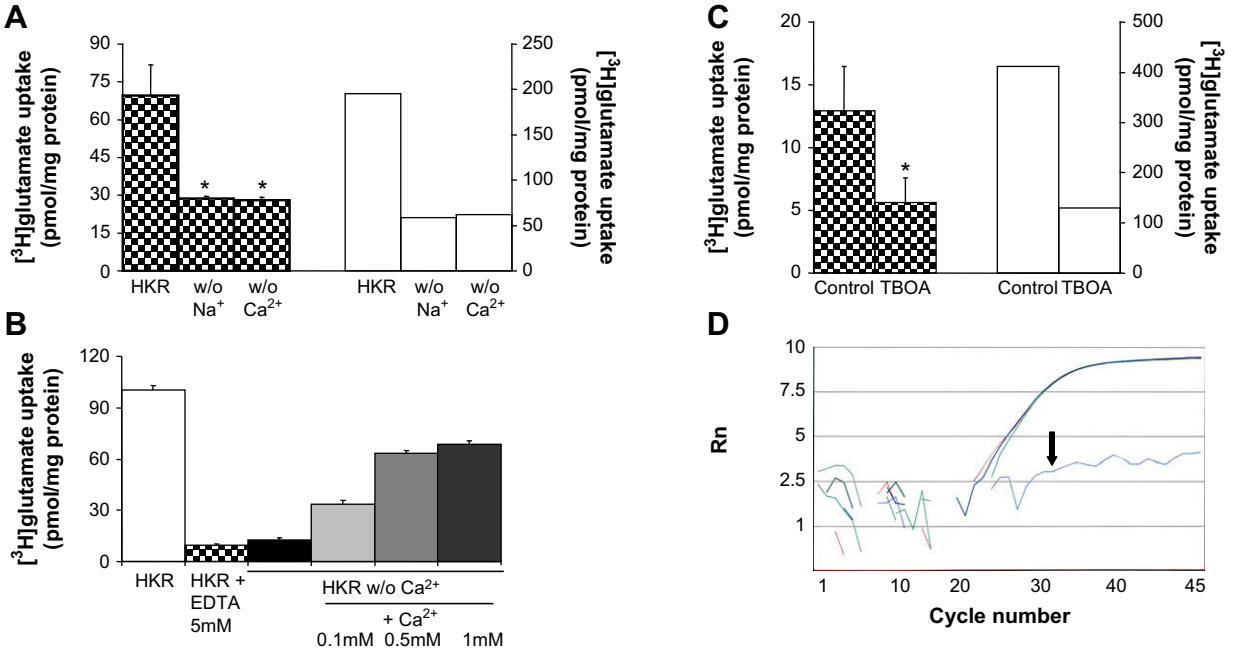

Fig. 1. Glutamate uptake. (A) [^{3}H]glutamate accumulation was measured in SW1353 cells (closed bars) and rat chondrocytes (open bars) after 20 min of incubation in complete HKR buffer or in HKR buffer without Ca^{2+} or Na^+. A representative experiment of three (SW1353) or two (rat chondrocytes) independent tests is shown. Bars represent the mean ± SE of triplicate samples or the mean of duplicate samples. *$P < 0.05$ vs HKR buffer, by *t*-test. (B) Effect of calcium on [^{3}H]glutamate uptake in rat chondrocytes. Bars represent the mean of quadruplicate samples from two independent experiments. Similar results were obtained with SW1353 cells (Supplementary Fig. S1). (C) [^{3}H]glutamate accumulation was measured in SW1353 cells (closed bars) and rat chondrocytes (open bars) after 20 min of incubation in the absence or presence of TBOA 100 μM. A representative experiment of three (SW1353) or two (rat chondrocytes) independent tests is shown. Bars represent the mean ± SE of triplicate samples or the mean of duplicate samples. *$P < 0.05$ vs control, by *t*-test. (D) Representative amplification plot of GLAST-1 in SW1353 cells. The arrow shows that there was no amplification in the blank (H_2O). A similar plot was obtained using rat articular chondrocytes. Rn: normalized reporter (i.e., the level of fluorescence detected during PCR, expressed as the ratio of target mRNA to GAPDH [human] or 18S [rat] mRNA).

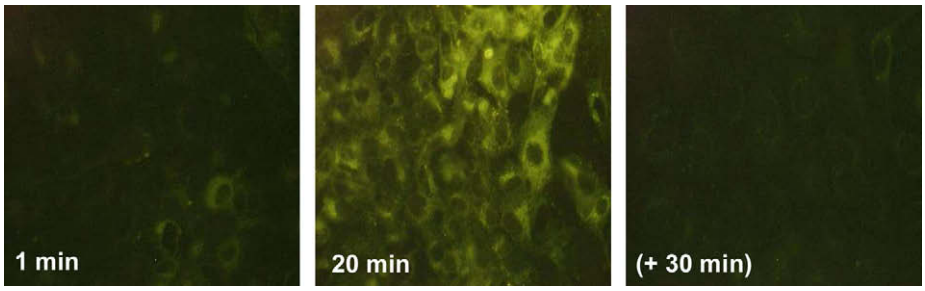

Fig. 2. Vesicular recycling. SW1353 cells were incubated in the presence of 10 μM FM1-43 up to 20 min (first two panels). FM1-43 was then removed from the medium, and cells were followed until destaining was complete (last panel). Results from a representative experiment are shown. Similar results were obtained in three independent experiments.

detected in both cell types. The pattern of expression in SW1353 cells was similar to that seen in rat articular chondrocytes. NR2D was the most abundantly expressed subunit, as determined by the lower number of cycles required to reach the exponential phase of amplification. Conversely, there was little or no expression of NR2B [Fig. 3(A)]. Western Blot analysis demonstrated the presence of NR1 protein [Fig. 3(B)]. In addition, both cell types were found to express mRNA encoding PSD-95 [Fig. 3(A)], a protein required for proper clustering of glutamate receptors[22].

BINDING STUDIES

In preliminary competition experiments, we found that the binding affinity of MK-801 was similar in chondrocyte membranes and intact cells (IC_{50s} around 1 μM). Thus, to characterize pharmacologically this peripheral NMDA receptor, we evaluated the specific binding of [^{3}H]MK-801 to intact chondrocytes. There was no significant difference between the association rate constants in the absence or presence of 100 μM glutamate and 30 μM glycine (not shown), both of which increase [^{3}H]MK-801 binding to brain membranes[21,23]. Moreover, the binding affinity of [^{3}H]MK-801 was much lower in chondrocytes than in rat brain membranes; mean K_d (dissociation constant) values of 700 and 2.6 nM, respectively, were calculated from two independent experiments with similar results.

Competition curves were then generated for the following inhibitors of [^{3}H]MK-801 binding: MK-801, phencyclidine, and memantine. All these compounds displayed micromolar affinity for the NMDA receptor expressed in chondrocytes. The IC_{50} values of MK-801 and phencyclidine were, respectively, about 70 and 40 times higher in chondrocytes than in rat brain membranes. Conversely, the IC_{50} of memantine was about five times lower in chondrocytes (Table I).

The modulatory effects of divalent cations on [^{3}H]MK-801 binding were also investigated. In SW1353 and rat primary cultures, Mg^{2+} (100 mM) inhibited [^{3}H]MK-801 binding by approximately 36 and 61%, respectively, whereas inhibition by Zn^{2+} (0.3 mM) did not exceed 50% in either cell type (Table I).

EFFECT OF GLUTAMATE SIGNALING ON CHONDROCYTE PROLIFERATION

To gain insight into the role of glutamate signaling *via* the NMDA receptor in chondrocytes, we knocked-down the expression of NR1 in SW1353 cells, using RNAi. After transfection, we observed that some pLKO clones proliferated slowly. Thus, clones expressing low levels of NR1 mRNA (i.e., less than 30% of that found in mock-transfected cells) were isolated and tested for proliferation. Consistent with our preliminary observation, SW1353 pLKO clones

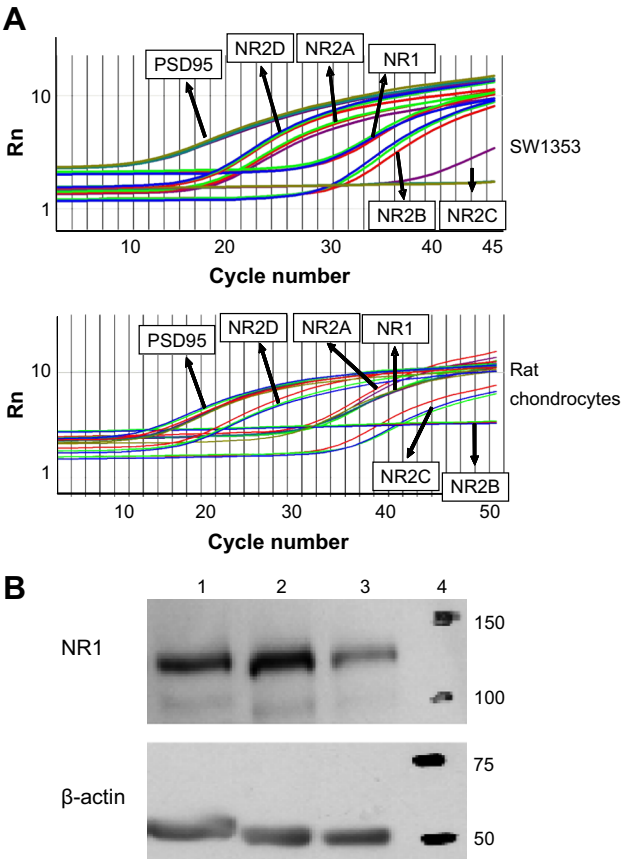

Fig. 3. Molecular composition of the NMDA receptor in chondrocytes. Results from a representative experiment performed in triplicate are shown. Similar results were obtained in three independent experiments. (A) Amplification plot of different NMDA receptor subunits and PSD-95 in SW1353 cells and rat articular chondrocytes: the higher the expression of a transcript, the lower the number of cycles required to reach the exponential phase of amplification. Rn: normalized reporter (i.e., the level of fluorescence detected during PCR, expressed as the ratio of target mRNA to GAPDH [human] or 18S [rat] mRNA). (B) Western Blot analysis of NR1 and β-actin. Lane 1: SW1353 (40 μg of total proteins). Lane 2: rat articular chondrocytes (40 μg of total proteins). Lane 3: rat brain extract (10 μg of total proteins). Lane 4: molecular weight markers (kd).

Table I
Binding affinities of different molecules to the NMDA receptor and the effects of divalent cations on [^{3}H]MK-801 binding

	SW1353	Rat chondrocytes	Rat brain membranes
Binding affinity, IC_{50}, μM			
MK-801	1.1 ± 0.4	1.7 ± 0.5	0.023 ± 0.004
Memantine	1.7 ± 0.2	1.3 ± 0.4	6.9 ± 2.0
Phencyclidine	6.8 ± 1.5	9.3 ± 2.4	0.19 ± 0.02
Inhibition of [^{3}H]MK-801 specific binding, %			
Mg^{2+} (100 mM)	36%	61%	81%
Zn^{2+} (0.3 mM)	47%	31%	84%

proliferated less rapidly than did wild type and mock-transfected cells [$P < 0.05$; Fig. 4(A)], as determined by MTT assay.

To confirm the specificity of this antiproliferative effect, wild type SW1353 cells were cultured in the absence or presence of the NMDA receptor antagonist MK-801 (10−100 μM). As shown in Fig. 4(B), sustained exposure to MK-801 resulted in decreased cell proliferation. This

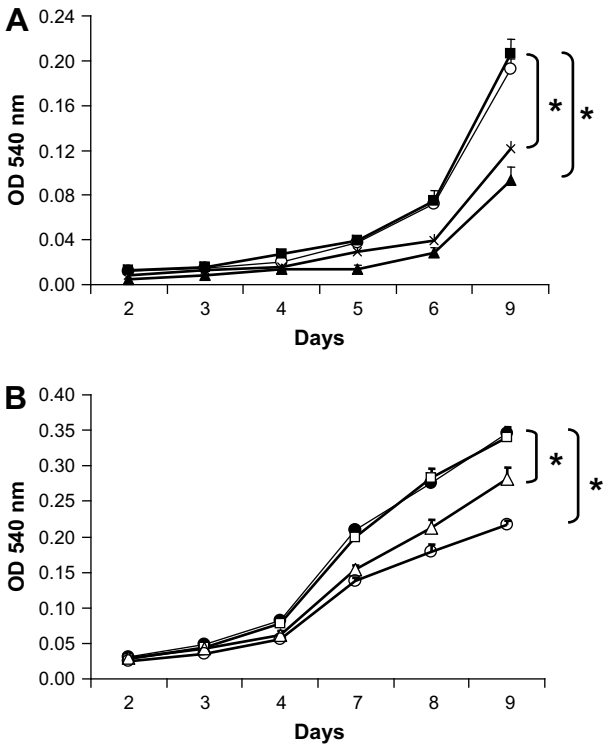

Fig. 4. Effect of glutamate signaling on chondrocyte proliferation. Results (mean ± SE) from representative experiments performed in triplicate are shown. Similar results were obtained in two independent experiments. (A) NR1 subunit was knocked-down in SW1353 cells using RNAi, and cell proliferation was tested by MTT assay. Wild type SW1353 (open circle), mock-transfected cells (closed square), and two different NR1 knock-down clones—pLKO-A (star) and pLKO-B (closed triangle)—were used. *$P < 0.001$ vs mock-transfected cells, by two-way repeated measures ANOVA. (B) Proliferation of SW1353 in the presence of increasing MK-801 concentrations: control (no treatment; closed circle); 10 μM MK-801 (open square); 50 μM MK-801 (open triangle); 100 μM MK-801 (open circle). *$P < 0.001$ vs control, by two-way repeated measures ANOVA.

response was concentration-dependent and was not related to cytotoxicity, given that MK-801 did not influence cell viability during the first 48 h, as assessed by a cell-counting system that distinguishes viable from dead cells (Supplementary Fig. S2). Similar results were obtained in experiments using rat articular chondrocytes ($n = 2$; not shown).

INVOLVEMENT OF GLUTAMATE SIGNALING IN THE INFLAMMATORY RESPONSE INDUCED BY IL-1β

Recent evidence suggests that increased glutamate levels are present in the synovial fluid of patients with active arthropathies, including OA[14]. We thus tested the effects of IL-1β, the main cytokine responsible for cartilage degradation in OA, on glutamate release and uptake. After short-term (15 min) treatment with 2 ng/ml IL-1β, glutamate release from SW1353 cells and rat articular chondrocytes increased significantly over control values [by about 90 and 120%, respectively; Fig. 5(A)]. Short-term incubation with IL-1β did not affect [^{3}H]glutamate uptake (not shown). However, the uptake of [^{3}H]glutamate decreased by about 50% [$P < 0.01$; Fig. 5(B)] in SW1353 cells exposed for 48 h to the cytokine, whereas it was not significantly affected in rat articular chondrocytes under the same conditions. In agreement with this finding, IL-1β significantly inhibited the expression of GLAST-1 mRNA in SW1353 cells only [Fig. 5(C)].

We finally investigated whether agonism/antagonism at the NMDA receptor could modulate the gene expression of cytokines and enzymes involved in cartilage degradation. IL-1β increased the expression of COX-2, IL-1β, IL-6, and MMP-3 mRNA in both cell types. In agreement with this finding, in silico analysis revealed the presence of common binding sites for transcription factors (e.g., NF-κB and AP-1) in the promoter regions of these genes (Supplementary Table S1). Exogenous glutamate and NMDA neither induced the expression of the genes analyzed nor significantly altered the effect of IL-1β (data not shown). Instead, MK-801 inhibited the response to IL-1β in a concentration-dependent manner, although significant effects were generally observed only at the highest concentration tested (100 μM; Fig. 6). No inhibition was seen when IL-8 transcript levels were analyzed (not shown).

Discussion

We report that chondrocytes express a self-sufficient machinery for glutamate signaling, including a peripheral NMDA receptor with unique properties. Depending on whether signaling is physiological or pathological, this peripheral NMDA receptor may be involved in chondrocyte proliferation or in the inflammatory process associated with cartilage destruction. We used the human chondrosarcoma cell line SW1353 because it is a well-established chondrocyte model[17,18]. Parallel experiments with primary chondrocytes allowed to validate the results obtained.

Yoneda and his group have demonstrated that rat costal chondrocytes release endogenous glutamate and accumulate [^{3}H]glutamate[9,11]. In extending these findings to SW1353 cells and rat articular chondrocytes, we noticed a peculiarity that is worth mentioning. That is, in addition to being sodium-dependent as it is in glial cells and neurons[24], [^{3}H]glutamate uptake into chondrocytes was calcium-dependent. To our knowledge, no such effect of calcium has been previously reported in the literature.

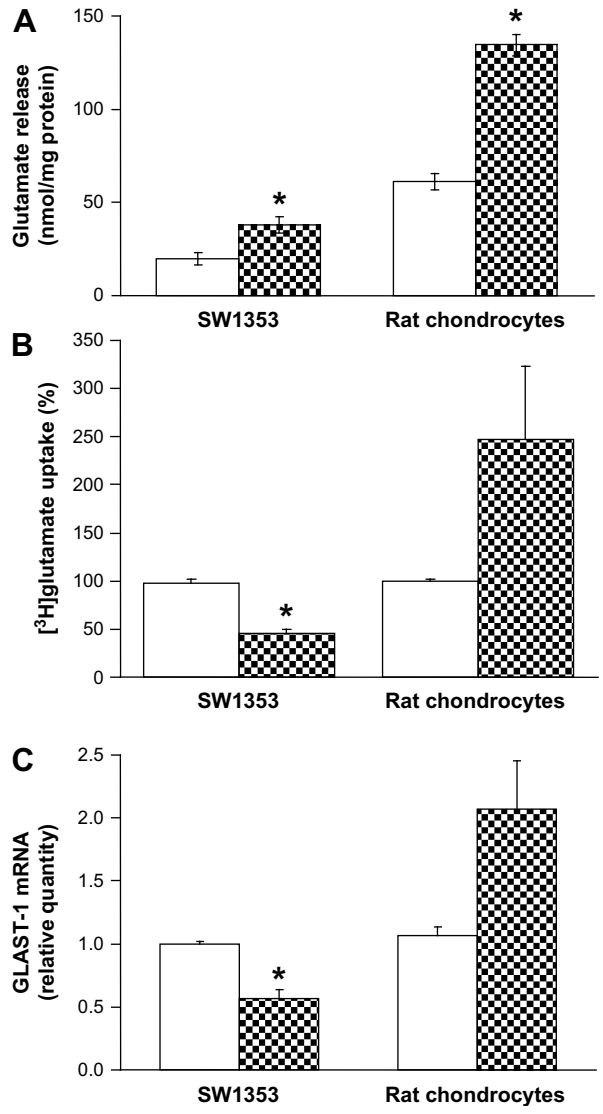

Fig. 5. Effect of IL-1β on glutamate release and uptake. Results (mean ± SE) from representative experiments performed in triplicate are shown. Similar results were obtained in at least three independent experiments. (A) Glutamate release in the absence (control; open bars) or presence of 2 ng/ml IL-1β (closed bars), after 15 min of incubation. *$P < 0.05$ vs respective control, by *t*-test. (B) [^{3}H]glutamate uptake in the absence (control; open bars) or presence of 10 ng/ml IL-1β (closed bars), after 20 min of incubation. *$P < 0.001$ vs control, by *t*-test. (C) The gene expression of the glutamate/aspartate transporter GLAST-1 was analyzed after incubation for 6 h with 2 ng/ml IL-1β. Control (open bars); IL-1β (closed bars). *$P < 0.01$ vs control, by *t*-test.

Although it was not our main goal to study glutamate transport in chondrocytes, we demonstrated the expression of mRNA for the glial transporter GLAST-1. Glutamate release and uptake were accompanied by vesicle recycling activity, as shown by experiments with the fluorescent dye FM1-43, which becomes incorporated into the vesicular membrane during endocytosis. This finding is consistent with previous studies showing that osteoblasts regulate extracellular levels of glutamate by virtue of a self-sufficient machinery[20,25], whereas neurons rely on astrocytes for the bulk of glutamate uptake from the synaptic cleft[24]. Several possibilities of glutamate origin are conceivable in bone, including glutamatergic fibers and blood vessels. Conversely, chondrocytes themselves might be the main source of glutamate within the cartilage matrix because neither innervation nor vascularization is present within normal articular cartilage[10].

Cartilage cells have been shown to express various components of the glutamate system[9–12]. In the present study we found constitutive expression of mRNA for different subunits of the NMDA receptor, with NR2D being the most abundantly expressed. Little or no expression of NR2B was detected in SW1353 cells and rat articular chondrocytes, in contrast with what is generally observed in the CNS[26] but in agreement with the most recent findings in chondrocytes from healthy subjects[27]. Like human primary chondrocytes, both SW1353 cells and rat articular chondrocytes also expressed mRNA encoding PSD-95. PSD-95 promotes anchoring of the NMDA receptor to the postsynaptic density and connects it to downstream signaling molecules[22,27]. Therefore, its expression points to a functional receptor anchored to the cellular membrane.

Binding studies revealed large differences—mainly with regard to the binding affinity of MK-801—between the receptor expressed in chondrocytes and that expressed in brain. This result was not unexpected, as ligand affinities at recombinant NMDA receptors depend on their subunit composition. In support of our finding that NR2D is abundantly expressed in chondrocytes, MK-801 shows the following affinity ranking: NR1−NR2A = NR1−NR2B ≫ NR1−NR2C = NR1−NR2D[28]. In the present study, the affinity of [^{3}H]MK-801 binding in rat brain membranes was in line with published data[21]. Thus, our results argue in favor of a peripheral receptor with distinct pharmacological characteristics.

Another peculiarity of the chondrocyte receptor was the low sensitivity to the divalent cations $Mg^{2+}$and Zn^{2+}. Interestingly, the extent of Mg^{2+} blockade depends on the structural arrangement of the NMDA receptor-channel complex[29], and channels containing NR2D are less sensitive to Mg^{2+} than those containing NR2B[30].

To study the physiological function of NMDA receptors in chondrocytes we chose to knock-down the expression of NR1, the subunit required to form functional channels[29]. Knock-down clones grew slower than wild type cells, pointing to a direct involvement of glutamate signaling in chondrocyte proliferation. Parallel experiments, in which chondrocytes were cultured in the presence of the NMDA receptor antagonist MK-801, substantiated this hypothesis. Glutamate has been shown to suppress the proliferation of mesenchymal stem cells[31], which have the potential to differentiate into chondrocytes, but no study had previously tested the effect of NMDA receptor blockade (or NR1 knock-down) on chondrocyte proliferation. In synoviocytes, the antiproliferative activity of glutamate receptor antagonists is concentration-dependent and similar to that of antirheumatic drugs[32]. In chondrocytes, the response to MK-801 was concentration-dependent as well, and the high concentrations required to produce a significant effect are in keeping with the low affinity observed in binding studies.

Beyond the physiological role of glutamate in joints, growing evidence indicates that glutamate-mediated events may contribute to the pathogenesis of human arthritic conditions. Increased levels of glutamate are seen in the knee joint of rats with early OA[33,34] and, most importantly, in the synovial fluid of patients with active arthropathies[13]. Significant associations between excitatory amino acids and inflammatory mediators have been demonstrated in the synovium of

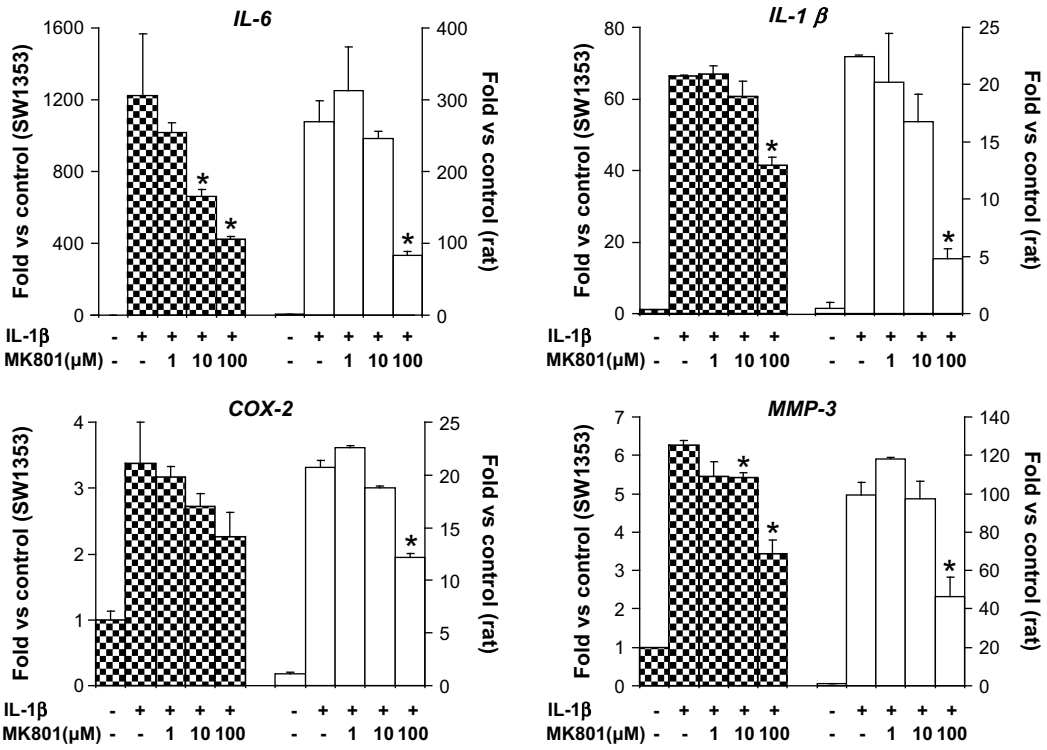

Fig. 6. IL-1β-induced expression of inflammatory mediators and matrix-degradative enzymes: effect of MK-801. Transcript levels of selected genes were analyzed after incubation of SW1353 cells (closed bars) and rat chondrocytes (open bars) for 6 h. Cells were treated with IL-1β in the absence or presence of increasing concentrations of MK-801. Results (mean ± SE) from a representative experiment performed in triplicate are shown. Similar results were obtained in three independent experiments. $^*P < 0.05$ vs IL-1β, by one-way ANOVA.

these patients[14]. Based on this evidence we examined whether IL-1β, the main cytokine responsible for cartilage destruction in OA[16], could affect glutamate signaling in chondrocytes. IL-1β increased glutamate release in both cell types while inhibiting glutamate uptake and GLAST-1 expression in SW1353 cells only. Data from the literature suggest that the effects of inflammatory mediators on glutamate uptake/EAAT expression may depend on the cell type and species studied. For instance, inflammatory cytokines inhibit glutamate uptake in human but not murine astrocytes in culture, whereas lipopolysaccharide increases GLT-1 levels and glutamate uptake in rat microglial cells[35–37]. Other authors have found increased GLAST expression in cartilage specimens from rabbits 30 weeks after anterior cruciate ligament transection (ACLT)[38]. We agree with these authors that EAATs may be involved in the pathogenesis of OA, but their role is far from being understood.

In OA, the balance between cartilage degradation and synthesis is shifted toward catabolism. IL-1β plays a major role in activating this catabolic cascade, which involves many pro-inflammatory mediators and enzymes[16,18,39]. In our experiments, IL-1β increased the gene expression of key cytokines and enzymes involved in cartilage degradation. MK-801 inhibited this response in a concentration-dependent fashion, suggesting the potential involvement of the NMDA receptor in joint inflammation. Again, significant effects were seen only at high concentrations, possibly because of the low ligand-receptor affinity. An alternative explanation, also suggested by Hinoi *et al.* for cultured osteoblasts[8], is that NMDA receptors were constantly stimulated by glutamate in the medium. Tonic stimulation may also have masked an effect of NMDA receptor agonists, whose contribution (if any) to IL-1β-induced gene expression could not be determined. Indeed, chondrocytes started being non-viable after a few hours of incubation in a medium not containing glutamine or glutamate, making it unfeasible to assess gene expression under these control conditions.

In conclusion, this is the first evidence that glutamate may function as an autocrine factor in chondrocytes under both physiological and pathological conditions. Our findings complement and extend the results of a recent study by Salter and his group, who demonstrated altered composition and activity of NMDA receptors in osteoarthritic chondrocytes[27]. In particular, new information is provided in support of the following hypothesis by these authors: "NMDA receptor signaling is likely to have a variety of important functions in cartilage and depending on whether signaling is physiological or pathological, maintains healthy cartilage or contributes to the pathological changes in OA"[27]. Therefore, the chondrocyte NMDA receptor could be a target for new drugs with therapeutic potential in OA.

Conflict of interest

Employment (Rottapharm): all authors.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.joca.2009.02.002.

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