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Abolition of chondral mineralization by group III metabotropic glutamate receptors expressed in rodent cartilage

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1 Previous studies have demonstrated the functional expression by osteoblasts of glutamate (Glu) signaling machineries responsible for the stimulation of cell proliferation and differentiation in bone, while there is no information available on the expression of the Glu signaling system by cartilage to date.

2 In cultured mouse embryonic metatarsals isolated before vascularization, chondral mineralization was almost completely inhibited in the presence of the group III metabotropic Glu receptor (mGluR) agonist L-(1)-2-amino-4-phosphonobutyrate (L-AP4) in a manner sensitive to an antagonist, with the total length being unchanged.

3 A group II mGluR agonist was similarly more effective in inhibiting the mineralization than a group I mGluR agonist, while none of ionotropic GluR agonists drastically affected the mineralization.

4 Both histological and *in situ* hybridization analyses revealed that L-AP4 specifically inhibited chondral mineralization, without apoptotic cell death, in cultured metatarsals.

5 In addition to the constitutive expression of mRNA for particular mGluRs in both cultured mouse metatarsals and rat costal chondrocytes, L-AP4 significantly inhibited the accumulation of cyclic AMP by forskolin and parathyroid hormone in a manner sensitive to a group III mGluR antagonist in cultured chondrocytes.

6 Moreover, L-AP4 drastically inhibited the expression of osteopontin mRNA in both cultured metatarsals and chondrocytes.

7 These results suggest that Glu may at least in part play a role as a signal mediator in mechanisms associated with chondral mineralization through the group III mGluR subtype functionally expressed by chondrocytes in rodent cartilage.
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Abbreviations: AB, Alcian Blue; ALP, alkaline phosphatase; AMPA, DL-α-amino-3-hydroxy-5-methylisoxasole-4-propionate; AR, Alizarin Red; cAMP, cyclic AMP; CNS, central nervous system; CPPG, (RS)-a-cyclopropyl-4-phosphonophenylglycine; DCG-IV, (2S,2′R,3′R)-2-(2′,3′-dicarboxycyclopropyl)glycine; DHPG, 3,5-dihydroxyphenylglycine; DIG, digoxigenin; DMEM, Dulbecco's modified Eagle medium; FBS, fetal bovine serum; Glu, glutamate; HE, hematoxylin and eosin; HKR, HEPES-buffered Krebs–Ringer; IBMX, 3-isobutyl-1-methylxanthine; iGluR, ionotropic glutamate receptor; KA, kainate; L-AP4, L-(1)-2-amino-4-phosphonobutyrate; MEM, minimum essential medium; mGluR, metabotropic glutamate receptor; NBT-BCIP, 4-nitroblue tetrazolium chloride-5-bromo-4-chloro-3-indolyl-phosphate; NMDA, *N*-methyl-D-aspartate; PB, phosphate buffer; PBS, phosphate-buffered saline; PKA, protein kinase A; PPARγ, peroxisome proliferator-activated receptor γ; PTH, parathyroid hormone; PTHrP, parathyroid hormone related peptide; RT–PCR, reverse transcription–polymerase chain reaction; runx2/cbfa1, runt-related gene2/core binding factor α-1; TUNEL, terminal deoxynucleotidyl transferase deoxyuridine triphosphate nick end labeling

Introduction

L-Glutamate (Glu) is one of the most abundant free amino acids with a major excitatory neurotransmitter role in the vertebrate central nervous system (CNS), while recent trends are toward a role in neuronal differentiation, migration and survival in the developing brain (Hollmann *et al*., 1989; Nakanishi *et al*., 1990; Yoneda *et al*., 2001). The actions of extracellular Glu are mediated by membranous receptors, which can be divided into two major groups (Yoneda *et al*., 2001). One is ionotropic Glu-gated ion channels (iGluRs) that are further classified into DL-α-amino-3-hydroxy-5-methylisoxasole-4-propionate (AMPA), kainate (KA) and *N*-methyl-D-aspartate (NMDA) subtypes according to sequential similarities as well as responsiveness to different agonists and antagonists (Wisden & Seeburg, 1993; Hollmann & Heinemann, 1994), whereas the other is G-protein-coupled metabotropic receptors (mGluRs) that are a member of the class 3 G

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protein-coupled receptor family (Masu *et al*., 1991; Tanabe *et al*., 1992). The latter mGluRs also play diverse roles in mechanisms related to the regulation of synaptic neurotransmission and plasticity, in addition to sensory transduction, in the nervous system. In mammals, there are eight mGluR genes (mGluR1–8) that code for receptors categorized into three functional groups on the basis of sequence homology, pharmacology and G protein-coupling profiles. The group I mGluR subtype stimulates the formation of inositol 1,4,5-triphosphate and diacylglycerol, while both group II and III mGluR subtypes similarly induce a reduction of the formation of intracellular cyclic AMP (cAMP) by adenylyl cyclase (Masu *et al*., 1991; Tanabe *et al*., 1992).

Recently, evidence that glutamatergic signaling is also functional in non-neuronal tissues, such as bone, pancreas and skin, is accumulating outside the CNS in the literature (Skerry & Genever, 2001; Hinoi *et al*., 2004). Indeed, Glu may act as a more widespread 'cytokine' rather than a 'neurotransmitter' to influence a variety of cellular activities in different tissues (Skerry & Genever, 2001; Hinoi *et al*., 2004). Recent studies have raised the possibility that Glu may be one of the endogenous paracrine (autocrine) factors used for intercellular communications in bone (Mason *et al*., 1997; Chenu *et al*., 1998). The addition of an NMDA receptor antagonist inhibits cell differentiation in cultured osteoclasts (Peet *et al*., 1999), for example, while Glu induces an elevation of intracellular free Ca^{2+} in a manner sensitive to antagonism by the NMDA receptor antagonist dizocilpine in osteoblasts (Laketic-Ljubojevic *et al*., 1999). We have recently demonstrated marked exacerbation of osteoblastic differentiation by different NMDA receptor antagonists (Hinoi *et al*., 2003). In addition to NMDA receptors, osteoblasts constitutively express mRNA for non-NMDA receptors such as GluR3 subunit of AMPA receptors as well as KA1 and KA2 subunits of KA receptors (Hinoi *et al*., 2002b), whereas AMPA receptors modulate the exocytotic release of endogenous Glu from cultured rat calvarial osteoblasts (Hinoi *et al*., 2002a). In cultured rat costal chondrocytes where constitutive expression is seen with mRNA for GluR3 subunit, the addition of AMPA markedly stimulates the release of endogenous Glu (Wang *et al*., 2005). Constitutive expression is also seen for particular mGluR (mGluR4 and mGluR8) that are negatively coupled to adenylyl cyclase, thus inhibiting the formation of cAMP stimulated by forskolin in cultured osteoblasts during different developmental states (Hinoi *et al*., 2001). In addition to Glu, moreover, recent studies have demonstrated that particular neurotransmitters could affect cell proliferation and/or differentiation in bone (Westbroek *et al*., 2001; Takeda *et al*., 2002).

On the other hand, bone is formed through a well-organized and highly regulated process. During embryogenesis the mesenchymal precursor cells differentiate into skeletal elements by forming a cartilaginous model, which then induces bone formation known as endochondral ossification in the vertebral column and long bones (Karsenty, 2003). Through this endochondral ossification, the cartilaginous rudiment, which is a tightly regulated area of both differentiation and maturation of chondrocytes, undergoes developmental growth. Within the cartilaginous rudiment chondrocytes differentiate, progressing through the resting, proliferation, hypertrophy and calcifying stages, which leads to mineralization of the cartilage matrix around the central region of the rudiment in the area of hypertrophic chondrocytes. Shortly after the mineralization process takes place, most hypertrophic chondrocytes undergo the sustained apoptotic process. Upon death of chondrocytes after mineralization, osteoblasts, osteoclasts and capillaries begin to invade the cartilage matrix to produce new bone, leading to growth of endochondral bones (Kronenberg, 2003). Metatarsals isolated from embryonic mice at 15.5 days after gestation have been used as isolated embryonic pure cartilage rods capable of undergoing a series of cellular developmental events without invasion by osteoblasts, osteoclasts and capillaries, for the long bone normally grows through endochondral ossification *in vivo* (Klement & Spooner, 1993). However, no much attention has been paid to the functional expression by chondrocytes of glutamatergic signaling machineries in cartilage to date.

In the present study, therefore, we have attempted to demonstrate a possible role of glutamatergic signaling in mechanisms related to chondral cellular development with a focus on the signal input machinery GluRs in both mouse embryonic metatarsals and rat costal chondrocytes in culture.

Methods

Embryonic metatarsal rudiment organ culture

The three central metatarsal rudiments were isolated from ddY mice embryos at 15.5 days postgestation. Each of three metatarsals was individually placed in a well of a 24-well plate containing 1 ml of organ culture medium: minimum essential medium (MEM) supplemented with 0.05 mg ml^{-1} ascorbic acid, 1 mM β-glycerophosphate and 0.25% fetal bovine serum (FBS) in either the presence or absence of the iGluR agonists AMPA, KA and NMDA, and the mGluRs agonists 3,5-dihydroxyphenylglycine (DHPG), (2S,2′R,3′R)-2-(2′,3′-dicarboxycyclopropyl)glycine (DCG-IV) and L-(1)-2-amino-4-phosphonobutyrate (L-AP4) at 500 μM. These explants were grown at 37°C in a humidified 5% CO_2 incubator for 5 days. On the day of the experiments, the total length of each bone rudiment and the length of each middle mineralized part determined by Alizarin Red (AR) staining were measured for subsequent calculation of mineralization ratio based on the length under an Olympus IMT-2-21 dissecting microscope. Similar results were invariably obtained on the calculation of mineralization ratio based on the areas in place of the lengths.

Histological analysis

On day 5 cultured metatarsal rudiments were harvested and fixed with 10% formalin neutral buffer solution, followed by decalcification with 20% EDTA and subsequent immersion in 30% sucrose overnight at 4°C, respectively. Metatarsals were then dissected for sections with a thickness of 5 μm in a cryostat for histological analyses. Sections were stained with AR, hematoxylin and eosin (HE) and Alcian Blue (AB) under standard procedures, respectively. The activity of alkaline phosphatase (ALP) was determined by enzymatic histochemistry. In brief, mounted sections were preserved in phosphate-buffered saline (PBS) at room temperature, and then staining was developed by incubation in ALP buffer (0.01 M Tris-HCl (pH 9.5), 0.1 M NaCl, 0.05 M $MgCl_2$) supplemented with 100-fold diluted solution of NBT-BCIP as a substrate for ALP

for 1.5 h at 37°C, followed by several rinses in phosphate buffer (PB). Sections were finally mounted in 50% glycerol, and photographs of sections were taken using an Olympus microscope.

Terminal deoxynucleotidyl transferase deoxyuridine triphosphate nick end labeling (TUNEL) assay

TUNEL staining was performed to detect apoptotic cells based on labeling of DNA strand breaks. Metatarsals cultured for 5 days were fixed with 10% formalin neutral buffer solution, followed by decalcification with 20% EDTA and subsequent immersion in 30% sucrose overnight at 4°C, respectively. Metatarsals were then dissected for frozen sections with a thickness of 5 μm in a cryostat. Mounted sections of metatarsals were then subjected to the TUNEL assay with TUNEL detection kit according to the manufacturer's instructions.

In situ *hybridization analysis*

Sections mounted as described above were fixed with freshly prepared 4% paraformaldehyde in 0.1 M PB (pH 7.4) for 10 min at room temperature, followed by washing three times with 0.1 M PB, treating with 0.2 M HCl for 10 min, washing three times with 0.1 M PB, treating with 10 μg ml^{-1} Proteinase K for 5 min, and washing three times with 0.1 M PB. Sections were then subjected to acetylation in 0.1 M triethanolamine/0.25% acetic anhydride for 10 min, followed by washing with 0.1 M PB and subsequent stepwise dehydration in 70, 80, 90, 95 and 100% ethanol for 5 min each. After being dried, sections were covered with the hybridization buffer (10% dextran sulfate, 5 × SSC, 20 mM Tris-HCl (pH 8.0), 300 mM NaCl, 50% formamide, 1 × Denhard's, and 500 μg ml^{-1} yeast tRNA) containing 25 μg ml^{-1} salmon sperm DNA at 65°C for 1 h, and then incubated with hybridization buffer containing the digoxigenin (DIG)-labeled cRNA probes at 65°C for 16 h. Posthybridization washes were carried out stepwise with 4 × SSC at 65°C for 20 min, 50% formamide in 2 × SSC at 65°C for 30 min, 10 mM Tris-HCl buffer (pH 7.5) containing 1 mM EDTA and 500 mM NaCl (TNE buffer) at 37°C for 10 min three times, 4 μg ml^{-1} RNase A in TNE buffer at 37°C for 30 min, TNE buffer at 37°C for 10 min, 2 × SSC at 65°C for 30 min, 0.2 × SSC at 65°C for 30 min, Buffer1 (100 mM Tris-HCl (pH 7.5), 150 mM NaCl) at room temperature for 10 min, and then 1.5% blocking buffer (1.5% blocking reagent in Buffer1) at room temperature for 1 h. Subsequently, sections were washed with Buffer1 at room temperature, and then incubated with 0.75 U ml^{-1} anti-DIG-AP Fab fragments in 0.5% blocking buffer containing 0.2% Tween at 4°C for 16 h. After being washed four times with Buffer1 containing 0.2% Tween at room temperature for 15 min, sections were treated with Buffer2 (100 mM Tris, 100 mM NaCl, 50 mM $MgCl_2$, pH 9.5), and then with Buffer2 containing 375 μg ml^{-1} nitro blue tetrazolium chloride and 188 μg ml^{-1} 5-bromo-4-chloro-3-indolyl phosphate for different periods to obtain pictures most appropriate for subsequent development. After washing with Buffer2 at room temperature for 5 min, the development was stopped by incubation in 1 × TE (10 mM Tris-HCl (pH 7.5), 1 mM EDTA (pH 8.0)).

Primary culture of costal chondrocytes

Cartilages were isolated from adult female Wistar rat ribs, followed by incubation at 37°C for 10 min in calcium- and magnesium-free PBS containing 0.1% EDTA and subsequent digestion with collagenase in Dulbecco's modified Eagle medium (DMEM) at 37°C for 2.5 h. Cells were collected in DMEM containing 10% FBS and antibiotics and then centrifuged at 500 × g for 5 min. The pellets were suspended in DMEM containing 10% FBS. Cells were plated at a density of 4×10^4 cells (cm^2)$^{-1}$, followed by culturing at 37°C under 5% CO_2 for additional 6 days. Culture medium was exchanged to DMEM supplemented with 10% FBS and 50 μg ml^{-1} ascorbic acid for subsequent culturing for different periods up to 28 days. Medium was changed every 2 to 3 days. Cell viability was determined by Cell Counting Kit-8, according to the manufacturer's instruction.

Determination of ALP activity

Chondrocytes were plated at a density of 4×10^4 cells (cm^2)$^{-1}$ in 24-well dishes and cultured for different days. Cells were washed twice with cold PBS, and then sonicated in 0.1 M Tris-HCl buffer (pH 7.5) containing 0.1% Triton X-100. Assay buffer composed of 0.05 M 2-amino-2-methylpropanol, 2 mM $MgCl_2$ and 10 mM p-nitrophenylphosphoric acid was added at a volume of 200 μl into 10 μl of cell suspensions, followed by a reaction for 30 min at 37°C and subsequent immediate determination of absorbance of p-nitrophenol at 405 nm.

Reverse transcription–polymerase chain reaction (RT–PCR)

Cultured rat costal chondrocytes as well as cultured murine metatarsals were washed with PBS twice, followed by extraction of mRNA using mRNA purification kit and subsequent synthesis of cDNA with 12.5 μM random hexamer primers and first-strand beads. Reverse transcriptase reaction was run at 37°C for 60 min and an aliquot of synthesized cDNA was directly used for PCR. PCR was performed in 10 mM Tris-HCl buffer (pH 8.3) containing 50 mM KCl, 1.5 mM $MgCl_2$, 200 μM each of dNTP (deoxy nucleotide triphosphate), 20 pmol of each primer for the corresponding mGluR subunits, iGluR subunits and chondral markers (Table 1) and 2 U Taq DNA polymerase as described previously (Hinoi *et al*., 2002a). The conditions of 40 PCR cycles for GluR primers were as follows: denaturation at 95°C for 1 min; annealing at 60°C for 1 min and extension at 72°C for 1 min, respectively. The conditions of 32 PCR cycles for chondral marker primers were as follows: denaturation at 95°C for 1 min; annealing at 55°C for 1 min and extension at 72°C for 1 min, respectively. An aliquot of PCR amplification products was run on 2% agarose gel, followed by detection of DNA with ethidium bromide. Appropriate PCR DNA products were extracted from agarose gel using DNA extraction spin columns, followed by sequencing by ABI Prism 310 Genetic Analyzer using cycle sequencing kit. Amplified PCR products were invariably subjected to sequential analysis for confirmation of expression of the corresponding mRNA.

Table 1 Primers used for detection of glutamatergic machineries and chondral markers

Genes	*Upstream (5′-3′)*	*Downstream (5′-3′)*	*Estimated base pair*
iGluR			
NR1	ACGGAATGATGGGCGAGC	GGCATCCTTGTGTCGCTTGTAG	1033
NR2A-C	GGGGTTCTGCATCGACATCC	GACAGCAAAGAAGGCCCACAC	2A: 548, 2B: 546, 2C: 547
NR2D	CGATGGCGTCTGGAATGG	CTGGCAAGAAAGATGACCGC	465
GluR5	GGTTTTTCACCCTTATCATCAT	GCACTTCAGGGACATTCTCAG	692
GluR6	TATGTTCTGCTGGCTTGCTTG	GCACTTCAGGGACATTCTCAG	918
GluR7	GGTTTTTCACCCTTATCATCAT	TGCTCCCGTTCCGCTGTCTTGC	637
KA1	GGTGTAATCTCCTGGTCAAC	GATGCTTCTGAGTGTCTGAG	763
KA2	TCGCCCGTGTCCTCAACTCA	CACCGACACCTCCTCAGACT	398
mGluR			
mGluR1	CCAGTGATGTTCTCCATACC	CACTCTGGGTAGACTTGAGTG	361
mGluR2	TTTAGGTCAGAAGCCAGAGT	CAGTAACCATCCTCTCTATCC	250
mGluR3	TATTCTCAGTCCTCTGCAAG	TTGTAGCACATCACTACATACC	261
mGluR4	TCATTTTCTCTTCTGTTCCC	GACATGCTACACATCAGAGAC	340
mGluR5	CCCCAAACTCTCCAGTCT	ATTTTTCACCTCGGGTTC	210
mGluR6	CAAGTAGCAAGGTTGAGTGT	GGTTGTAGTGTTGGATCAAG	363
mGluR7	GAACTCTGTGAAAATGTAGACC	TTAGGGAGTCCAGAATTACAG	321
mGluR8	CGAGGGTTATAACTACCAGGT	TAGGTGCTGTGACAGATTTCT	440
Markers			
sox9	AGGAAGCTGGCAGACCAGTA	CCCTCTCGCTTCAGATCAAC	407
runx2/cbfa1	CCGCACGACAACCGCACCAT	CGCTCCGGCCCACAAATCTC	289
Type II collagen	GGAAAGTCTGGGGAAAGAGG	CAGTCCCTGGGTTACCAGAA	457
Aggrecan	CTTTCCTGCAGAAGTCAC	ACATCCTCTACTCCAGAG	1872
Osteopontin	TCACCATTCGGATGAGTCTG	ACTTGTGGCTCTGATGTTCC	437
PPARγ	TATGGAGTTCATGCTTGTGA	CGGGAAGGACTTTATGTATG	315
Type I collagen	GCAATCGGGATCAGTACGAA	CTTTCACGCCTTTGAAGCCA	485
GAPDH	GGTGAAGGTCGGTGTCAACGGATT	GATGCCAAAGTTGTCATGGATGACC	502

Quantification of cAMP

Chondrocytes cultured for 7 days were washed twice with PBS and incubated with 1 mM 3-isobutyl-1-methylxanthine (IBMX) in PBS for 20 min at 37°C under 5% CO_2, followed by the addition of 10 μM forskolin or 10 nM parathyroid hormone (PTH) in either the presence or absence of the group III mGluR subtype agonist L-AP4 at 1 mM and the antagonist (RS)-a-cyclopropyl-4-phosphonophenylglycine (CPPG) at 1 mM in PBS containing 1 mM IBMX for 10 min at 37°C under 5% CO_2 as described previously (Hinoi *et al*., 2001). Cells were then sonicated in the lysis regent (0.25% solution of dodecyltrimethylammonium bromide in 0.05 M acetate buffer; 0.02% (w v^{-1}) BSA; 0.01% (w v^{-1}) preservatives). Measurement of cAMP was conducted by enzyme immunoassay according to the manufacturer's instructions.

Data analysis

Results are all expressed as the mean ± s.e. and the statistical significance was determined by the two-tailed and unpaired Student's *t*-test or the one-way analysis of variance ANOVA with Bonferroni/Dunnett *post hoc* test.

Materials

Leica CM 3050s cryostat and a fluorescent microscope (IMT-2-21-RFL; Olympus, Tokyo) were used. Quickprep Micro mRNA Purification Kit, First-strand cDNA Synthesis kit, DYEnamic ET Terminator Cycle Sequencing Kit and cAMP enzyme immunoassay system were purchased from Amersham Pharmacia Biotech (Buckinghamshire, U.K.). *In situ* Cell Death Detection Kit and NBT/BCIP (4-nitroblue tetrazolium chloride-5-bromo-4-chloro-3-indolyl-phosphate, toluidine salt) stock solution were purchased from Roche Diagnostics GmbH and Roche Molecular Biochemical (Mannheim, Germany). Taq DNA polymerase was obtained from Takara (Tokyo, Japan). DHPG, DCG-IV, L-AP4 and CPPG were all supplied by Tocris Cookson (Bristol, U.K.). DMEM and MEM were purchased from Gibco BRL (Gaithersburg, MD, U.S.A.). Cell Counting Kit-8 was obtained from Dojindo (Osaka, Japan). Other chemicals used were all of the highest purity commercially available.

Results

Effects of agonists for iGluRs and mGluRs in cultured mouse metatarsals

In order to at first evaluate the possible glutamatergic signal input into cartilage, metatarsals before vascularization were isolated from embryonic mice at 15.5 days after gestation and cultured in either the presence or absence of an agonist at 500 μM for iGluRs and mGluRs for consecutive 5 days. As shown in Figure 1a, none of agonists for iGluRs, including AMPA, KA, and NMDA, markedly affected the total length of the cartilage rudiment and the length of the middle mineralized part determined by AR staining in cultured metatarsals. Both the group II and group III mGluR agonists DCG-IV and L-AP4 almost completely inhibited the mineralization at the middle part, respectively, without markedly affecting the total length, by contrast, while the group I agonist DHPG induced slight but statistically significant inhibition of

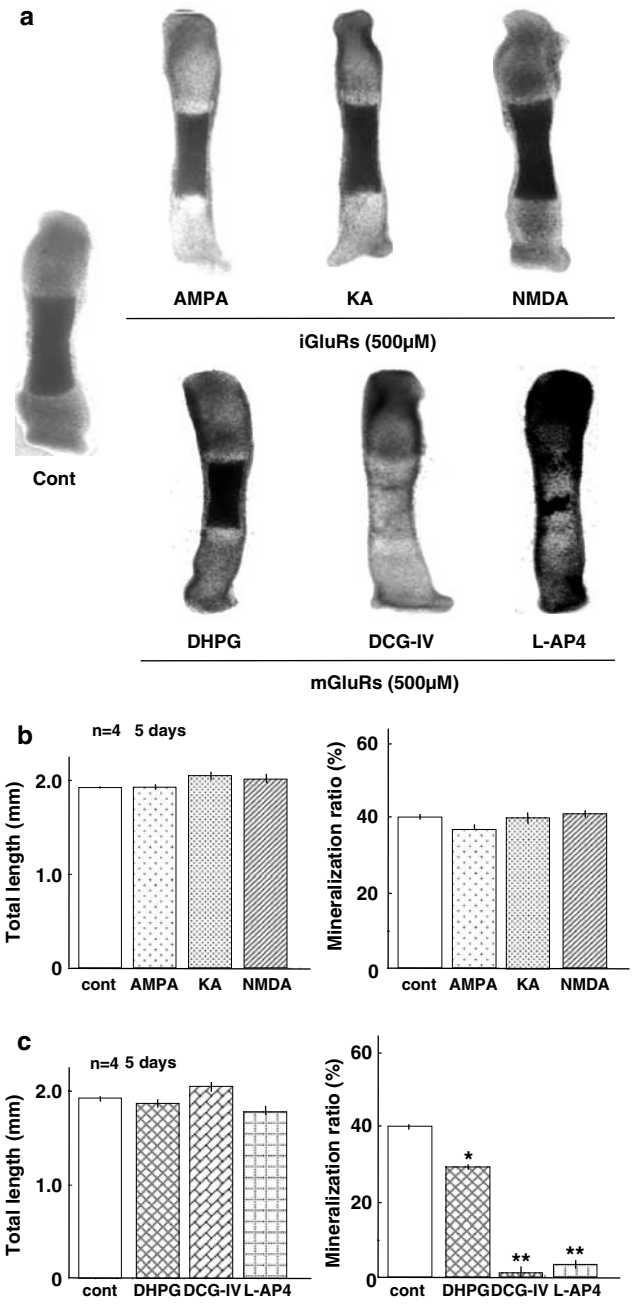


Figure 1 Effects of GluR agonists on mineralization in cultured mouse metatarsals. Metatarsals before vascularization were isolated from embryonic mice, followed by cultivation in MEM in either the presence or absence of a GluR agonist at 500 μM for 5 days and subsequent determination of the total length and mineralization ratio as described in the text. Typical pictures are shown in the upper panel (a), while in the lower panel quantitative data are shown for iGluRs (b) and mGluRs (c) as the mean $\pm$ s.e. in four independent experiments. $^*P<0.05$, $^{**}P<0.01$, significantly different from each control value obtained in metatarsals cultured in the absence of any GluR agonist.

the mineralization with the total length being unchanged. These experiments were repeated four times for quantification. Both DCG-IV and L-AP4 were much more potent than DHPG in abolishing the mineralization without significantly altering the total length of cartilages, while no significant inhibition was seen in both the mineralization ratio and total length in metatarsals cultured in the presence of AMPA, KA, or NMDA (Figure 1b).

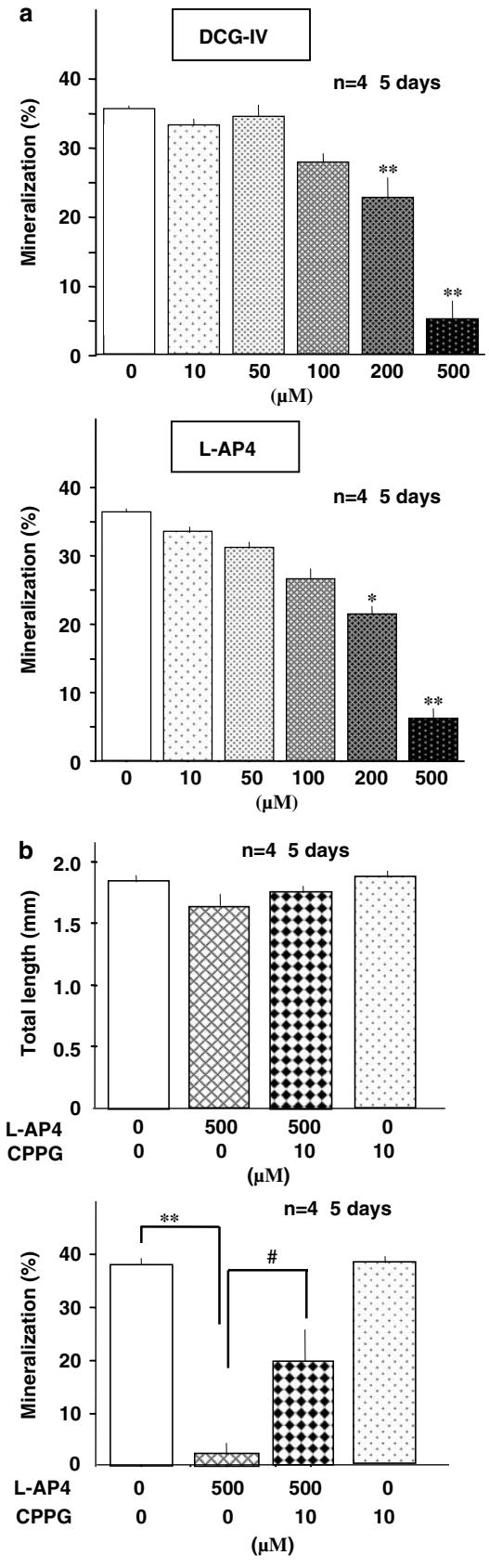
Possible involvement of mGluRs in mineralization of cultured mouse metatarsals

Mouse metatarsals were then cultured in either the presence or absence of the group II mGluR agonist DCG-IV and the group III mGluR agonist L-AP4 at a concentration range of 10–500 μM for 5 days, followed by measurement of the total length and mineralization ratio as described above. Sustained exposure to either DCG-IV or L-AP4 at a concentration of over 200 μM significantly inhibited the mineralization in cultured metatarsals (Figure 2a). Neither DCG-IV nor L-AP4 significantly altered the total length of cultured metatarsals at concentrations used (data not shown). The group III mGluR antagonist CPPG at 10 μM significantly prevented the inhibition of mineralization by L-AP4 at 500 μM in metatarsals cultured for 5 days, while the mineralization was not significantly affected in the presence of CPPG alone at the concentration used (Figure 2b).

Histological analysis in cultured mouse metatarsals

To further investigate the exact site of the inhibition, several histological analyses were conducted on sections dissected from metatarsals cultured in the presence of L-AP4. Mouse metatarsals were cultured in the presence of L-AP4 at 500 μM for 5 days, followed by dissection of frozen sections at 5 μm for histological analysis by staining with AR, HE and AB, in addition to for ALP. Sustained exposure to L-AP4 for 5 days markedly decreased the chondral mineralization in cultured metatarsals as revealed by AR staining (Figure 3a, left panel). No obvious histological abnormality was seen in prehypertrophic to hypertrophic chondrocytes in metatarsals cultured in the presence of L-AP4 at 500 μM for consecutive 5 days on staining with HE and AB (Figure 3a, left middle two panels), while there were no different staining patterns for ALP in metatarsals cultured even in the presence of L-AP4 (Figure 3a, right second panel). In addition, no profound apoptotic cells positive to TUNEL staining were detected in metatarsals cultured for 5 days irrespective of the presence of L-AP4 (Figure 3a, right panel).

An attempt was next made to determine whether L-AP4 indeed affects the expression of mRNA for different markers selectively expressed by chondrocytes at distinct differentiation stages in cultured metatarsals using *in situ* hybridization techniques. Under the experimental conditions used, negligibly little cells were labeled by cRNA probe for type I collagen specifically expressed by osteoblasts in any layers of differentiating chondrocytes irrespective of the presence of L-AP4 (Figure 3b, left panel). Sustained exposure to L-AP4 did not markedly affect the numbers and the patterns of cells labeled by cRNA probe for either type II collagen preferentially expressed by proliferating to prehypertrophic chondrocytes (Figure 3b, second left panel) or type X collagen highly expressed by hypertrophic chondrocytes (Figure 3b, second right panel) when determined in mouse metatarsals cultured for 5 days. However, an almost complete abolition was seen in the expression of mRNA for osteopontin in metatarsals cultured in the presence of L-AP4 at 500 μM for 5 days (Figure 3b, right panel).



Isolation of rat costal chondrocytes and effect of L-AP4 on cell differentiation

An attempt was next made to determine the possible functional expression of mGluRs in chondrocytes. For this purpose, cartilages were isolated from adult female Wistar rat ribs, followed by digestion with collagenase and collection of cells toward subsequent plating for culture. In proportion to increasing culture periods up to 18 days, a linear increase was found in AB staining used for detection of acidic mucopolysaccharide in cultured rat costal chondrocytes (data not shown). Expression of mRNA was seen for several chondral marker genes including sox9, runt-related gene2/core binding factor α-1 (runx2/cbfa1), type II collagen, aggrecan and osteopontin but not for both the adipocyte marker gene peroxisome proliferator-activated receptor γ (PPARγ) and osteoblastic marker gene type I collagen, in costal chondrocytes cultured for 4–28 days (Figure 4a). Semiquantitative RT–PCR revealed that expression of mRNA was drastically increased for runx2/cbfa1 during culturing from 7 to 14 days with a gradual increase thereafter up to 28 days, while a transient increase was seen in mRNA expression for both type II collagen and sox-9 on 14 days in rat costal chondrocytes cultured for a period up to 28 days (data not shown). Expression of mRNA for sox9, runx2/cbfa1, type II collagen, aggrecan and type X collagen was not markedly changed in the presence of 500 μM L-AP4 at any stages from 4 to 28 days, while marked suppression was found in osteopontin mRNA expression in the presence of L-AP4 from 21 to 28 days (Figure 4a). Quantitative analysis on four different experiments clearly showed significant and drastic inhibition of the expression of mRNA for osteopontin (Figure 4b), but not for type X collagen (Figure 4c), in chondrocytes cultured in the presence of 500 μM L-AP4 for 28 days. A marked increase was also seen in the activity of ALP in chondrocytes cultured for a period of 7–28 days with a plateau on 21 days (Figure 4d), while the activity was not significantly altered in chondrocytes cultured in the presence of 500 μM L-AP4 at any stages from 7 to 28 days. No significant change was found in viability of chondrocytes cultured in the presence of L-AP4 at 500 μM for 4 days when cells were at a proliferative stage (Figure 4d).

Expression of mRNA for GluRs in cultured mouse metatarsals and rat chondrocytes

To examine whether mGluRs is indeed expressed in cultured mouse metatarsals and rat costal chondrocytes, RT–PCR

Figure 2 Inhibition by group II and group III mGluR agonists of mineralization in cultured mouse metatarsals. (a) Metatarsals before vascularization were cultured in MEM in the presence of the group II mGluR agonist DCG-IV or the group III mGluR agonist L-AP4 at different concentrations of below 500 μM for 5 days for subsequent determination of mineralization ratios. (b) Metatarsals were also cultured in either the presence or absence of L-AP4 at 500 μM and the group III mGluR antagonist CPPG at 10 μM for subsequent determination of the total length and mineralization ratio. Values are the mean ± s.e. in four separate experiments. $^{*}P<0.05$, $^{**}P<0.01$, significantly different from each control value obtained in metatarsals cultured in the absence of any mGluR agonists and antagonists. $^{\#}P<0.05$, significantly different from the value obtained in metatarsals cultured in the presence of L-AP4 alone.

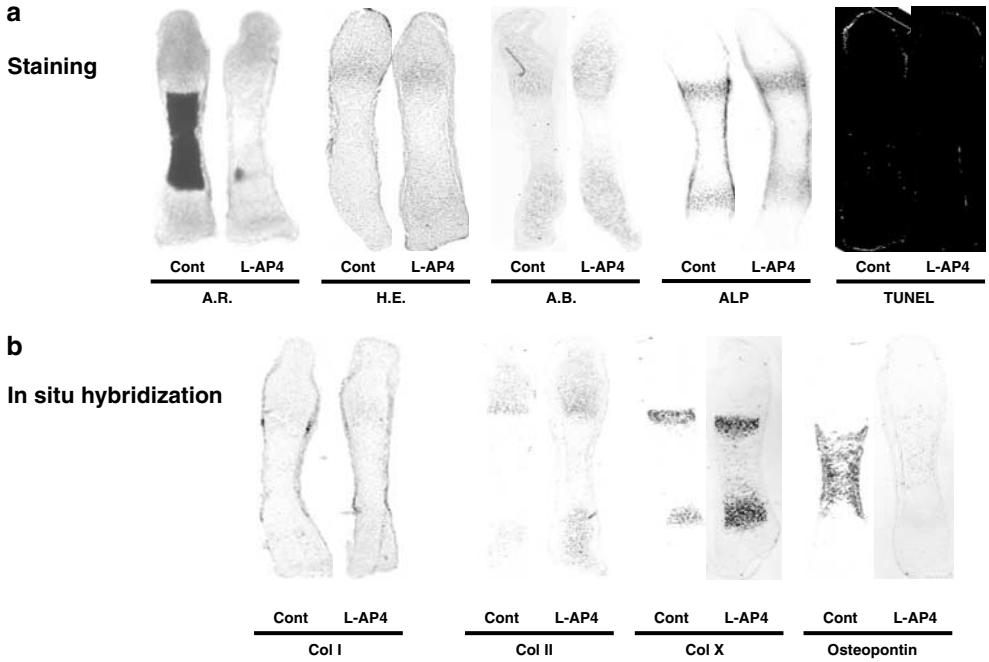


Figure 3 Morphological analysis in cultured mouse metatarsals. Metatarsals before vascularization were cultured in MEM in either the presence or absence of L-AP4 at 500 μM for 5 days, followed by fixation with formalin and subsequent dissection of frozen sections in a cryostat for (a) dye staining and (b) *in situ* hybridization. Typical micrographic pictures are shown in this figure, while similar results were invariably obtained in at least five independent determinations. Abbreviations: AB, Alcian Blue; ALP, alkaline phosphatase; AR, Alizarin Red; Col I, type I collagen; Col II, type II collagen; Col X, type X collagen; Cont, control; HE, hematoxylin and eosin.

analysis was conducted using specific primers for each mGluR. RT–PCR analysis revealed that constitutive expression of mRNA was seen for mGluR1 of the group I subtype, mGluR2 of the group II subtype and all of the group III subtype in cultured mouse metatarsals irrespective of the culture period up to 5 days, in addition to adult mouse whole brain used as a positive control (Figure 5a). Expression was also detected with mRNA for mGluR5 of the group I subtype in metatarsals cultured for particular periods of 3–5 days, however, while mRNA expression was not seen for mGluR3 of the group II subtype in metatarsals throughout the culture period.

In rat costal chondrocytes cultured for 7–21 days, similarly constitutive expression was seen with mRNA for mGluR2 and mGluR8 (Figure 5b) as seen in cultured mouse metatarsals before vascularization. In contrast to cultured mouse metatarsals, however, mRNA expression was seen for mGluR1, mGluR4 and mGluR5 in rat chondrocytes cultured for 14–21 days, but not in those for 7 days. Although mGluR3 mRNA only was absent from cultured metatarsals, expression of mRNA for mGluR3, mGluR6 and mGluR7 was not seen in rat chondrocytes cultured for a period from 7 to 21 days. Moreover, constitutive expression was found with mRNA for both NR1 and NR2D subunits of NMDA receptor channels, but not for NR2A to NR2C subunits, in rat chondrocytes cultured for 7 to 21 days (Figure 5c). Irrespective of the cellular maturity, mRNA was expressed for KA2 subunit of KA receptor channels without expression of mRNA for other subunits including GluR5, GluR6, GluR7 and KA1 in cultured rat chondrocytes.

Accumulation of cAMP in cultured rat chondrocytes

Both group II and group III mGluR subtypes are G protein-coupled receptors that induce the reduction of the formation of cAMP. To assess the biological relevance of expression of the group III mGluR subtype by chondrocytes, cAMP accumulation assay was carried out using isolated chondrocytes. In rat costal chondrocytes cultured for 7 days, the basal level of cAMP was 11.2 ± 5.2 pmol well^{-1} when determined in the presence of IBMX alone. The addition of PTH at 10 nM (Figure 6a) or forskolin at 10 μM (Figure 6b) markedly increased the endogenous level of cAMP (PTH, 653.3 ± 83.2 pmol well^{-1}; forskolin, 125.1 ± 15.6 pmol well^{-1}), while the further addition of the group III agonist L-AP4 at 1 mM significantly inhibited the increase by PTH (412.6 ± 38.1 pmol well^{-1}) or forskolin (75.2 ± 10.1 pmol well^{-1}), respectively. The group III antagonist CPPG at 1 mM was effective in significantly preventing the inhibition by L-AP4 on the PTH-induced or forskolin-induced increase in the level of cAMP in cultured chondrocytes (PTH, 588.9 ± 62.5 pmol well^{-1}; forskolin, 117.5 ± 11.3 pmol well^{-1}).

Discussion

The essential importance of the present findings is that the group III mGluR agonist markedly abolished chondral mineralization in a manner sensitive to an antagonist in cultured mouse embryonic metatarsals isolated before vascularization. In addition, functional expression of particular mGluRs was for the first time shown in both cultured mouse

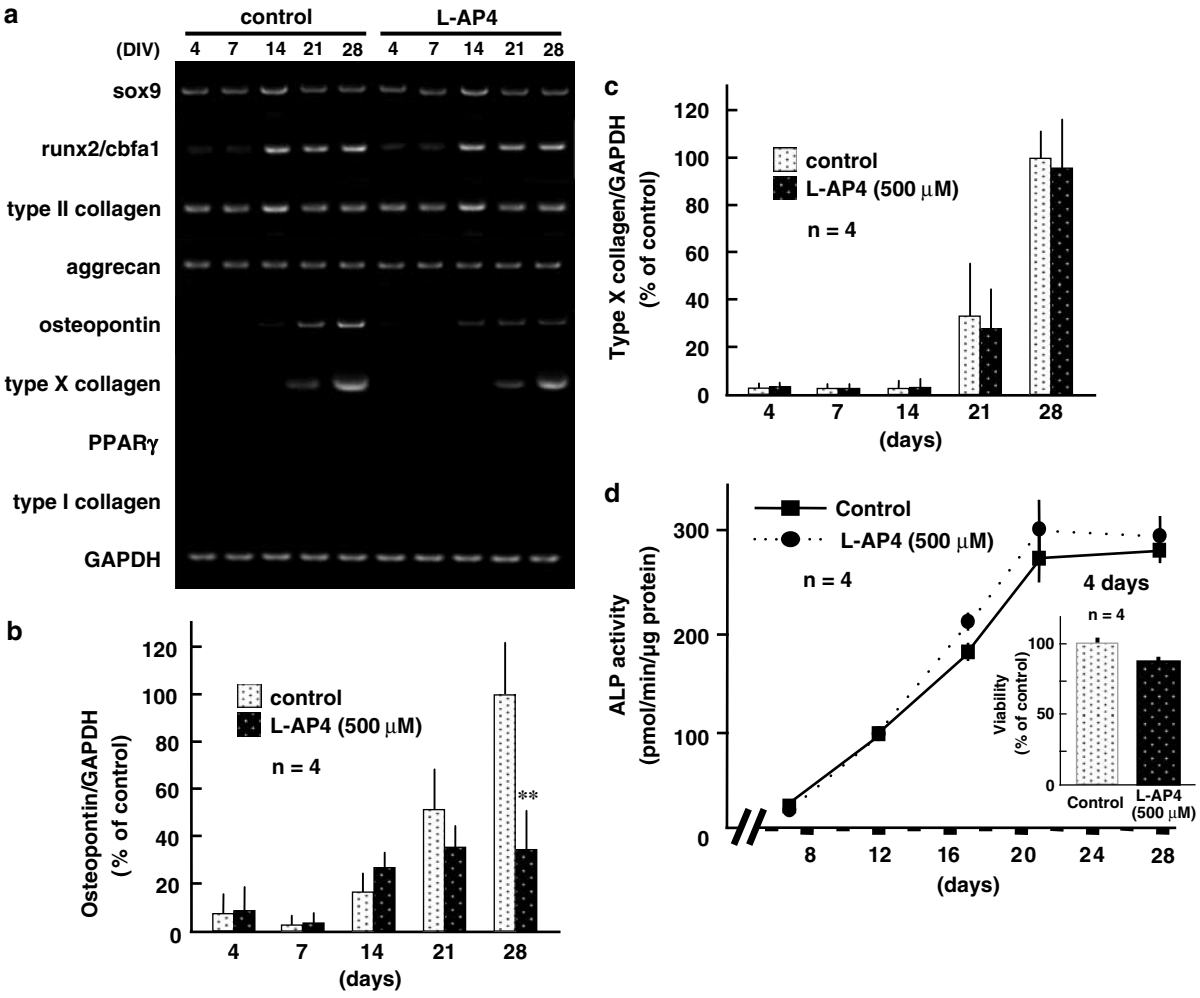


Figure 4 Effect of L-AP4 on the different chondral markers in cultured rat costal chondrocytes. (a) Costicartilage was isolated from ribs of adult female Wistar rats, followed by digestion with collagenase and subsequent cultivation of resultant chondrocytes in DMEM for different days up to 28 days in either the presence or absence of L-AP4 at 500 μM. Cells were harvested on different days for determination of mRNA expression of different chondral markers by semiquantitative RT–PCR. Quantification of mRNA expression for osteopontin (b) and type X collagen (c) was carried out on the basis of expression of the housekeeping gene GAPDH. (d) Costal chondrocytes were also cultured in the presence of L-AP4 at 500 μM for different durations up to 28 days, followed by determination of ALP activity at different days and cell viability on day 4 (inset). Values are the mean $\pm$ s.e. obtained in four independent determinations. $^{**}P<0.01$, significantly different from each control value obtained in the absence of L-AP4.

metatarsals and isolated rat costal chondrocytes. To our knowledge, this paper deals with the first direct demonstration of functional expression of group III mGluR by the rodent cartilage. Although several previous studies have already demonstrated the functional expression of different subtypes of mGluRs in bone (Gu & Publicover, 2000; Hinoi *et al*., 2001), no direct evidence for a role of the group III mGluR subtype in mechanisms underlying the cellular proliferation and differentiation in cartilage is available in the literature to date. In addition to group III mGluR, moreover, the data cited above also raise the possible functionality of the group II mGluR subtype in the proliferation and (or) differentiation of mouse metatarsals before vascularization in organ culture. The absence of mGluR3 means that the group II mGluR agonist DCG-IV may almost completely abolish the mineralization solely through mGluR2 expressed by metatarsals. Since definite discrimination is not done between group II and group III mGluR subtypes so far; however, this study has focused on the group III subtype with more constitutive expression by metatarsals throughout culture periods than the group II subtype, in addition to the easy availability of an agonist and an antagonist.

The metatarsal culture system used in this study is an *ex vivo* model of chondrocyte proliferation and differentiation, which retains normal patterns of proliferation and differentiation for at least 5 days in culture (Klement & Spooner, 1993). This allowed us to investigate the effects of different GluR agonists, in a system where chondrocytes retain their normal architecture of round, columnar, prehypertrophic and hypertrophic zones. From metatarsals isolated before vascularization, osteoblasts, osteoclasts and capillaries are absent even after 5 days in organ culture. Indeed, our data from *in situ* hybridization using a probe for the osteoblastic marker protein type I collagen clearly demonstrated the almost complete absence of osteoblasts from cultured metatarsals employed here. In primary cultured chondrocytes isolated from rat ribs,

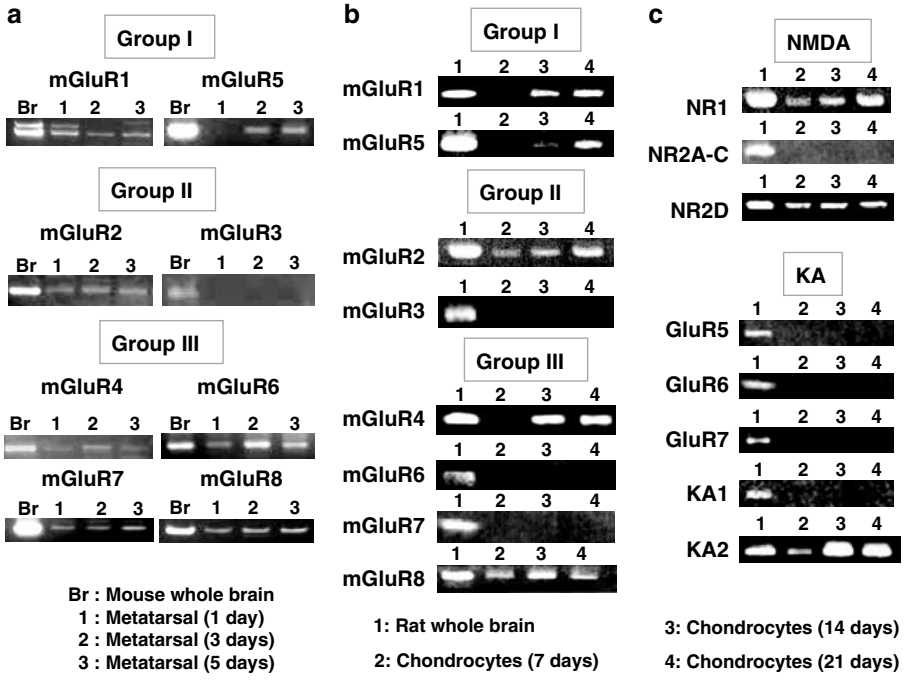


Figure 5 RT–PCR on GluRs in cultured mouse metatarsals and rat costal chondrocytes. Metatarsals before vascularization were cultured in MEM for different days up to 5 days, followed by extraction of mRNA and subsequent RT–PCR using primers specific for each mGluR (a). Chondrocytes were cultured in DMEM for 7–21 days, followed by extraction of mRNA and subsequent RT–PCR using primers specific for each mGluR (b) and iGluR (c). Mouse or rat whole brain was used as a positive control. These experiments were repeated at least four times with similar expression profiles.

furthermore, no contamination of osteoblasts was found by RT–PCR using specific primers for type I collagen. Taken together, the possibility that the effect of mGluR agonists on chondral mineralization is through particular mGluR subtypes expressed by osteoblasts contaminated in cultured metatarsals and chondrocytes is ruled out. Actually, we have already demonstrated the constitutive expression of both mGluR4 and mGluR8 of the group III subtype that is negatively coupled to adenylyl cyclase thus inhibiting the formation of cAMP stimulated by forskolin in cultured rat calvarial osteoblasts at different developmental states (Hinoi *et al*., 2001). In our preliminary experiments where isolated osteoblasts were cultured for 28 days in either the presence or absence of L-AP4 at a concentration range of 100–750 μM for determination of the calcium accumulation as an index of cell maturation; however, L-AP4 failed to significantly affect the calcium accumulation in cultured osteoblasts (unpublished data). These results also give support to the proposal that the group III mGluR subtype expressed by chondrocytes, but not that by osteoblasts, would be predominantly responsible for the modulation of chondral mineralization in cartilage.

The prevailing view is that PTH (or PTH-related protein; PTHrP) receptors are a member of the B superfamily of G protein-coupled receptors with seven transmembrane domains and coupled to at least three different isoforms of the trimeric G protein. These include Gs protein for the activation of adenylyl cyclase-protein kinase A (PKA), Gq (or G_{11}) protein for the activation of phospholipase C-protein kinase C and Gi (or Go) protein for the inhibition of adenylyl cyclase, respectively (Bringhurst *et al*., 1993; Offermanns *et al*., 1996). It is widely accepted that the role of PTH (or PTHrP) would vary according to experimental conditions, such as cell densities and differentiation stages, in regulating proliferation and differentiation of chondrocytes (Koike *et al*., 1990; Schwartz *et al*., 1997). Although the effect of activation of PTH (or PTHrP) receptors on chondrogenesis is still controversial, there is accumulating evidence that chondral mineralization is markedly suppressed through continued proliferation and delayed hypertrophy following activation of PTH (or PTHrP) receptors effective in stimulating adenylyl cyclase-PKA signaling pathways (Guo *et al*., 2002). It should be emphasized that sustained exposure to both group II and group III mGluR agonists invariably led to almost complete abolition of chondral mineralization in cultured mouse metatarsals. As both mGluR subtypes are believed to inhibit the formation of cAMP through Gi protein negatively linked to adenylyl cyclase as described above, the data cited here argue in favor of an idea that a decreased intracellular cAMP level may be at least in part responsible for the suppression of chondral mineralization. The paradox may be accounted for by taking into consideration possible differential expression profiles between PTH (or PTHrP) receptors and mGluRs in chondrocytes at different developmental stages in cartilage. Prehypertrophic chondrocytes are shown to predominantly express PTH (or PTHrP) receptors (Guo *et al*., 2002), in fact, whereas the constitutive expression profile during organ culture from 1 to 5 days gives support to the expression of both group II and III mGluR subtypes by proliferating to calcified chondrocytes. The exact mechanism as well as functional significance of the abolition of chondral mineralization by group III mGluR, however, remains to be elucidated in future studies.

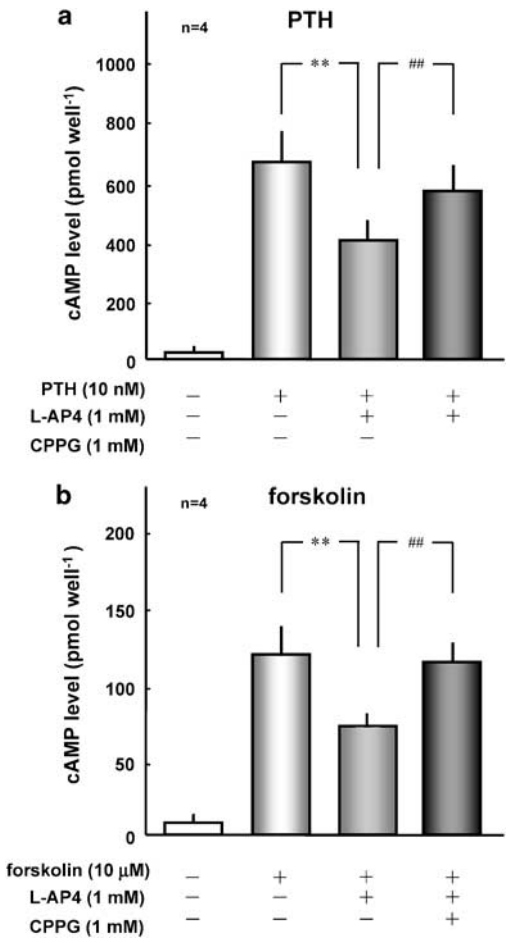


Figure 6 Functionality of group III mGluR expressed by cultured rat costal chondrocytes. Costal chondrocytes were culture for 7 days and incubated with PTH at 10 nM (a) or forskolin at 10 μM (b) in buffer containing 1 mM IBMX in either the presence or absence of 1 mM L-AP4 and 1 mM CPPG for 10 min. Values are the mean $\pm$ s.e. obtained in four independent experiments. $^{**}P<0.01$, significantly different from the value obtained in the presence of PTH or forskolin alone. $^{\#\#}P<0.01$, significantly different from the value obtained in the presence of both PTH or forskolin and L-AP4.

One of the interesting findings obtained in this study is that mRNA for both NR1 and NR2D subunits was expressed by cultured rat costal chondrocytes devoid of contamination with osteoblasts. Although these data are suggestive of a heteromeric assembly between NR1 and NR2D subunits toward functional NMDA receptor channels as shown in osteoblasts (Hinoi *et al*., 2003), whereas the possible functionality and significance would lie on mechanisms other than modulation of chondral mineralization in cartilage. We have also shown that the addition of AMPA markedly evokes the release of endogenous Glu into incubation medium from cultured rat costal chondrocytes with further potentiation by the AMPA receptor desensitization blocker cyclothiazide (Wang *et al*., 2005). These findings are in good agreement with our previous observations on the release of endogenous Glu from cultured rat calvaria osteoblasts (Hinoi *et al*., 2002b), and suggest that endogenous Glu could be used as a paracrine and/or autocrine signal mediator for intercellular communications between chondrocytes as proposed in osteoblasts (Genever & Skerry, 2001; Hinoi *et al*., 2002b). In bone, several possibilities of Glu origin are conceivable. Both sympathetic and sensory nerve fibers innervate into bone, while glutamatergic innervation is distributed even in bone (Serre *et al*., 1999; Takeda *et al*., 2002). In osteoblasts, the endogenous agonist Glu could be supplied by these glutamatergic nerve fibers as well as by osteoblasts themselves, in addition to by blood circulation. In contrast to these osteoblasts, however, either nerve fiber innervation or vascular invasion has never been demonstrated in chondrocytes so far. It is therefore highly conceivable that Glu released by AMPA receptor activation from adjacent chondrocytes could be one possible origin of Glu responsible for activation of particular subtypes of mGluRs and/or iGluRs expressed by chondrocytes for the signal input in cartilage.

Furthermore, our *in situ* hybridization analysis gives rise to a proposal that L-AP4 would inhibit the terminal differentiation from hypertrophic to calcified chondrocytes. Metatarsals isolated before vascularization are shown to undergo a series of cellular maturation processes from resting to proliferating, prehypertrophic, hypertrophic and calcified chondrocytes in a rank order of progressing development during *in vitro* cultivation, culminating in chondrocyte hypertrophy and matrix mineralization, without marked invasion by osteoblasts, osteoclasts and capillaries as described above (Klement & Spooner, 1993; MacLean *et al*., 2004). Type II collagen is widely distributed in proliferating to prehypertrophic chondrocytes with selective expression of type X collagen by hypertrophic chondrocytes, while osteopontin is predominantly expressed by calcified chondrocytes (Guo *et al*., 2002). One possible speculation is that activation of group III mGluR may result in almost complete abolition of chondral mineralization through the inhibition of terminal differentiation from hypertrophic to calcified chondrocytes, which is undoubtedly different from delayed mineralization seen after activation of PTH (or PTHrP) receptors (Koike *et al*., 1990; Schwartz *et al*., 1997). It is conceivable that L-AP4 may markedly increase the number of cells under a differentiation stage after elimination of the hypertrophic chondrocyte marker gene type X collagen but before expression of the calcified chondrocyte marker gene osteopontin. In fact, a recent study has shown the presence of abnormal chondrocytes that cease proliferation but do not differentiate to hypertrophic cells in the Komeda miniature rat Ishikawa (Chikuda *et al*., 2004).

As described above, the metatarsal culture system is an *ex vivo* model of growth plate chondrocytes that undergo well-ordered and controlled phases of cell proliferation, differentiation and maturation (Klement & Spooner, 1993). On the contrary, *in vitro* evaluation of growth plate cell kinetics would be hindered by the spatial arrangement and the heterogeneity of cells within the plate (Oberbauer & Peng, 1995). Not all chondrocyte markers are therefore well correlated with each other between *ex vivo* metatarsal and *in vitro* chondrocyte culture systems. In addition, the former *ex vivo* metatarsal system could allow us to histologically investigate the effects of various reagents on the development of chondrocytes, while the latter *in vitro* study is a powerful tool for the quantitative analysis. We should therefore utilize both *ex vivo* organ and *in vitro* cell culture models to investigate of proliferation, differentiation and maturation of chondrocytes. Furthermore, the fact that sustained exposure to L-AP4 markedly inhibited mRNA expression for osteopontin without affecting that for

other all different markers tested, including type X collagen, does not argue in favor of possible involvement of reduced cell proliferation and viability in a molecular mechanism underlying the inhibition by a group III mGluR agonist of chondral mineralization in *ex vivo* metatarsal and *in vitro* chondrocyte culture studies. In our experiments where isolated chondrocytes were cultured for up to 28 days in the presence of 500 μM L-AP4, this group III mGluR agonist failed to significantly affect the amount of total protein contents in cultured chondrocytes at all days examined (unpublished data).

It thus appears that the group III mGluR subtype is expressed by chondrocytes to inhibit chondral mineralization without affecting viability. Extracellular Glu would play a pivotal role as a signal mediator in mechanisms related to the cellular development and maturation through particular subtypes of mGluRs and iGluRs expressed by chondrocytes in cartilage as well as osteoblasts in bone. Glutamatergic signaling machineries could be thus a target for the development of a drug useful for the treatment and therapy of a variety of bone diseases relevant to abnormal development and maturation of chondrocytes as well as osteoblasts in human beings.

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