Cytosolic free Ca^{2+} concentration exhibits a characteristic temporal pattern during *in vitro* cartilage differentiation: a possible regulatory role of calcineurin in Ca-signalling of chondrogenic cells

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SUMMARY

We measured changes of cytosolic Ca^{2+} concentration during chondrogenesis, which occurs in high density cultures (HDC) of chondrifying chicken mesenchymal cells. A significant, transient elevation was detected in Fura-2-loaded cells on day 3 of culturing, when majority of chondrogenic cells of HDC become differentiated. This 140 nM peak of cytosolic Ca^{2+} concentration is a result of increased Ca-influx and is indispensable to proper chondrogenesis, because addition of 0.8 mM EGTA to culture medium on day 2 or day 3 significantly decreased the intracellular Ca^{2+} concentration abolishing the Ca^{2+}-peak of day 3 and inhibited cartilage formation. Uncontrolled Ca^{2+} influx evoked by a Ca^{2+} ionophore exerted dual effects on chondrogenesis in a concentration dependent manner; 0.1 mg/L A23187 increased, whereas 5 mg/L A23187 almost totally blocked cartilage formation. Intracellular Ca-stores seemed not to have any significant participation in the regulation of changes of cytosolic Ca^{2+} concentration of chondrifying cells. Activity of Ca-calmodulin dependent protein phosphatase, calcineurin responded to changes of intracellular Ca^{2+} concentration induced by EGTA or A23187 in a differentiation stage-dependent manner. Since inhibition of calcineurin with cyclosporine A eliminated the peak in the cytosolic Ca^{2+} concentration, an active regulatory role of calcineurin on Ca^{2+} influx of chondrifying cells can be supposed.

Key words: chondrogenesis; high density culture; intracellular Ca^{2+} concentration; fura-2; cyclosporine A; Sox9

1. INTRODUCTION

Hyaline cartilage is an important element of the vertebrate skeletal system. It provides primordia of bones formed by endochondral ossification and remains the major shock-absorbing structure of the articular surfaces of joints. Chondrogenic mesenchymal cells can be derived from different embryonic structures: the cranial part of neural crest is the source of cartilage primordia of several craniofacial bones; sclerotome of somites differentiates into vertebrae; appendicular bones derive from mesenchymal cells of somatopleura [1].

High density cell culture system (HDC) established from chondrogenic mesenchymal cells isolated from limb buds of 4-day-old chicken embryos is a well-known model of *in vitro* cartilage differentiation [2-4]. This simple model can provide information on the molecular steps leading to differentiation of chondroprogenitor cells to chondroblasts. In HDC, formation of cartilage starts with the recruitment of chondroprogenitor mesenchymal progenitor cells that after condensation and nodule formation, differentiate into chondroblasts and chondrocytes. Condensation and nodule formation take place on the first day of culturing and are partly regulated by transient appearance of Ca^{2+} dependent intercellular junctions like N-CAM (neural cell adhesion molecule) and N-cadherin [5]. Chondroprogenitor cells differentiate into chondroblasts on the second and third days of culturing [4,6], controlled by numerous growth factors and other signal molecules, *e. g.* FGF, BMP, Wnt, IGF and members of Hedgehog and Sox transcription factor families [7]. In parallel to the intracellular changes, extracellular matrix (ECM) surrounding the differentiating chondrogenic cells is also subject to profound changes: differentiating cells start to secrete cartilage-specific matrix components, such as collagen type II and aggrecan on the third day of culturing period [8]. The unique

composition and organization of ECM is crucial for maintenance of the proper morphology and function of these cells [9]. Expression of collagen type II and core protein of aggrecan is controlled by Sox9, a high-mobility-group domain containing transcription factor [10-12]. Detection of the expression level and the phosphorylation status of Sox9, as well as monitoring the expression of the core protein of aggrecan are a reliable markers of chondrogenesis.

Calcium ion is a ubiquitous cellular signal. The concentration of intracellular free Ca^{2+} (~10^{-7} M) is 10^4 times lower than that of the extracellular fluid. This distribution provides the potential for the influx of Ca^{2+} into cells, where it can act as a second messenger. Various stimuli promote the movement of Ca^{2+} either from the extracellular space or from intracellular stores into the cytosol. The elevated level of cytosolic free Ca^{2+} exerts a variety of specific changes in cellular function, such as activation of protein kinases and protein phosphatases, which, in turn, regulate other processes, like proliferation or differentiation [13]. The molecular steps leading to cartilage differentiation, among other factors are regulated by Ca^{2+} sensitive enzymes like one of the Ser/Thr specific protein kinases, PKCalpha [14] or the Ser/Thr-specific protein phosphatase calcineurin [15,16], that is unique among phosphatases for its ability to sense changes of intracellular Ca^{2+} concentration through its activation by its calcium binding subunit and calmodulin. Calcineurin is best known as a regulator of T-lymphocyte activation, since its pharmacological inhibitors, cyclosporine A (CsA), tacrolimus, pimecrolimus and rapamycin are all used in the clinical practice as immunosuppressants [17]. Calcineurin is also known to participate in several differentiation processes, such as development of different muscle tissues and the nervous system [18].

In this study we measured the cytosolic free Ca^{2+} concentration during cartilage differentiation in the chondrogenic cells of HDC. A characteristic temporal pattern in the

changes of cytosolic Ca^{2+} concentration could be observed; there was a significant and transient elevation on the third culturing day, the crucial day of chondrocyte differentiation. Moreover, beside the changes of the basal cytosolic Ca^{2+} level, cells of chondrifying micromass cultures also exhibit spontaneous calcium events, a phenomenon characteristic to several other primary cell cultures [19,20]. We provide evidence that the temporal pattern of the changes of cytosolic free Ca^{2+} concentration in chondrifying cells is indispensible to proper cartilage formation and depends on extracellular Ca^{2+} rather than the availability of intracellular Ca-stores. We also demonstrate that calcineurin can play a dual role in Ca-signalling of chondrogenic cells: its activity is modulated by cytosolic Ca^{2+} concentration and the inhibition of calcineurin with CsA eliminates the Ca^{2+} peak of HDC resulting in a pronounced decrease in cartilage formation. This second observation raises the possibility of the active regulatory effect of this enzyme on the enhancement of Ca^{2+} influx to chondrifying cells.

2. MATERIALS AND METHODS

2.1. Cell culture

Distal parts of the limb buds of 4-day-old Ross hybrid chicken embryos (Hamburger–Hamilton stages 22–24; [21]) were removed and primary micromass cultures of chondrifying mesenchymal cells were established from a cell suspension with a density of 1.5×10^7 cells/mL. 15 μL droplets of the suspension were inoculated on round coverglasses (diameter: 30 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) 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 or after treatments.

2.2. Determination of cytosolic free Ca^{2+} concentration

Measurements were performed on different days of culturing using the calcium dependent fluorescent dye Fura-2. Cultures were transferred to 2 mL fresh Ham's F12 medium containing 10 µL Fura-2-AM (10 µM) and 4 µL neostigmin (0.3 nM), in order to inhibit extracellular choline esterases. After 60 min of incubation at 37 °C in a CO_2 incubator, cultures were washed twice in Tyrode's solution containing 137 mM NaCl, 5.4 mM KCl, 0.5 mM $MgCl_2$, 1.8 mM $CaCl_2$, 11.8 mM Hepes–NaOH, 1 g/L glucose, pH=7.4, in order to remove the Fura-2-AM attached to the extracellular matrix. 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 carried out in the same salt solution in a perfusion chamber using a dual wavelength monochromator (DeltaScan, Photon Technologies International, Lawrenceville, KY, USA) equipment. All measurements were performed at room temperature. Fluorescence of Fura-2-loaded cells was measured using excitation wavelengths of 340 and 380 nm and an emission wavelength of 510 nm. Intracellular Ca^{2+} concentrations were calculated from the ratios of intensities ($R= F_{340}/F_{380}$) as described by Grynkiewicz *et al.* [22]. Intracellular Ca^{2+} levels of HDC were measured 2 hrs after inoculation on round coverglasses then on culturing days 1, 2, 3, 4, 5 and 6 at the same period of each day. Intracellular Ca^{2+} levels of untreated control cultures and cultures treated with EGTA, calcium ionophore A23187,

cyclopiazonic acid (CPA) or CsA were assayed in 5 independent experiments measuring 30 cells in each case. All measurements were carried out directly after treatments with EGTA, calcium ionophore A23187, CPA or CsA. Data were statistically analyzed by Student's t-test.

2.3. Treatments with cyclopiazonic acid and caffeine

To determine the role of intracellular Ca-stores in the changes of the cytosolic Ca^{2+} concentration, the intracellular Ca^{2+} pump inhibitor CPA was administered to HDC. For single cell measurements, CPA was dissolved in DMSO and diluted in a modified, Ca^{2+}-free Tryode's solution (containing 5 mM EGTA, without $CaCl_2$) to a final concentration of 10 µM. The inhibitor was directly perfused in the close proximity (approximately 50 µm) of cells. Prior to administration of CPA, cells were washed with Ca^{2+} free Tyrode's solution to remove all traces of free Ca^{2+} from the medium. To investigate prolonged effect of depletion of intracellular Ca^{2+} source, CPA was administered directly into the culture medium for 12 hrs on culturing days 2 or 3 at a final concentration of 10 µM. Caffeine (15 mM, diluted in Tyrode's solution), an agonist of ryanodine receptor (RyR) was administered at close proximity (about 50 µm) of cells for 100 sec during single cell measurements.

2.4. Measurement and analysis of spontaneous calcium transients

Spontaneous calcium transients were monitored using LSM 510 META Laser Scanning Confocal Microscope (Zeiss, Oberkochen, Germany). Cells of high density micromass cell cultures were incubated for 1 h at 37 °C with 10 µM Fluo-4-AM in Ham's

F12 medium. Calcium imaging was performed in normal Tyrode's solution (see above). x–y analysis and line scan images were taken to monitor the fluorescence intensity during spontaneous activities. 50 images were recorded during a 48.4 sec interval to perform the x–y analysis. Line scan images were recorded at 1.54 ms/line, 512 pixels/line and 4096 lines using a 63× water immersion objective. Fluo-4-loaded cells were excited with a 488 nm argon ion laser and emitted fluorescence was collected at 500–570 nm. Images were analyzed using an automatic event detection program developed in the Department of Physiology.

2.5. Determination of Ca concentration of the culture medium

Ca concentration of the culture medium (F12 supplemented with 10 % FCS) was determined with atomic absorption spectrometry (AAS) on Philips PU9200X equipment in air-acetylene flame. Measurements were performed at the wavelength of 422.7 nm; each sample was measured three times for 4 sec. Determination of Ca concentration was carried out with standard addition method; samples were diluted in 0.1 M nitric acid [23].

2.6. Treatments with EGTA, A23187 calcium ionophore and cyclosporine A

In order to examine the effects of removal of Ca^{2+} from the extracellular fluid, cell cultures were fed a culture medium containing 0.8 mM EGTA (Amresco, Solon, OH, USA; pH adjusted to 7.4) for 12 hrs on day 2 or 3 of culturing. To assess the effect of elevated intracellular Ca^{2+}, cultures were fed with a culture medium containing calcium ionophore A23187 (Sigma, Budapest, Hungary) at concentrations of 0.1 and 5 mg/L for 1 h on culturing days 2 and 3. Calcium ionophore A23187 was dissolved in DMSO then

diluted in culture medium. Activity of calcineurin was inhibited with the continuous application of 2 μM cyclosporine A (Sigma, Budapest, Hungary) started on day 1.

2.7. Light microscopical analysis of cartilage differentiation

Cartilage matrix was visualized by staining with dimethylmethylene blue (DMMB, Aldrich, Germany) as described previously [2]. The amount of sulphated matrix components was determined with a semi-quantitative method, by measuring the optical density of extracted toluidine blue (Reanal, Budapest, Hungary) bound to glycosaminoglycans in mature HDC. 6-day-old cell cultures were fixed in a solution containing 28 % ethanol, 4 % formalin and 2 % acetic acid, stained with 0.1 % toluidine blue dissolved in glycine-HCl buffer (pH 1.8) for 15 min, the unbound toluidine blue was washed in glycine-HCl buffer for 1 h. The dye bound to highly sulphated proteoglycans and glycosaminoglycans was extracted in 8 % HCl dissolved in absolute ethanol. Absorbance of samples containing extracted toluidine blue was measured at the wavelength of 625 nm on a microplate reader (Chameleon, Hidex, Turku, Finland). Samples from 10 cultures of each experimental group were determined in 5 independent experiments. Data were statistically analyzed with Student's t-test.

2.8. Measurement of cell proliferation with ^{3}H-thymidine labelling and mitochondrial activity with MTT assay

For measurement of proliferation rate of cells in HDC 15 μL droplets of cell suspension were inoculated into wells of special, opaque 96-well microtiter plates (Wallac, PerkinElmer Life and Analytical Sciences, Shelton, CT, USA). Ham's F12 medium

containing 1 µCi/mL (185 GBq/mM) ^{3}H-thymidine (diluted from methyl-^{3}H-thymidine solution, Amersham Biosciences, Budapest, Hungary) was added to the wells for 16 hrs on different days of culturing. After washing twice with PBS (phosphate buffered saline), proteins were precipitated with ice-cold 5 % trichloroacetic acid, washed with PBS again, and placed in an exsiccator containing phosphorous pentoxide in order to absorb moisture. Prior to measurements, 50 µL scintillation solution (MaxiLight; Hidex, Finland) was added to each well, and radioactivity was counted by a liquid scintillation counter (Chameleon, Hidex, Turku, Finland). 10 samples of each experimental group from 5 independent experiments were statistically analyzed with Student's t-test.

Cellular metabolic activity was determined by MTT assay, a means of measuring the activity of living cells via mitochondrial dehydrogenases. Cells cultured in wells of 96-well microtiter plates were used and 10 µL MTT reagent [3-(4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide; 5 mg MTT/1 mL PBS] was added into each well. Cells were incubated for 2 hrs at 37 °C in MTT-containing Ham's F12 medium. Following addition of 100 µL MTT solubilizing solution (10 % Triton X-100 and 0.1 M HCl dissolved in anhydrous isopropanol) optical density was measured at 570 nm on a microplate reader (Chameleon, Hidex, Turku, Finland). 10 samples of each experimental group from 4 separate experiments were statistically analyzed with Student's t-test.

2.9. Determination of cell survival by FACS analysis

100 µL droplets of cell suspension were inoculated into plastic Petri dishes. Following treatments of either A23187 or EGTA as given in *2.6*, cultures were washed twice with CMF-PBS (calcium and magnesium free PBS), incubated with 250 µL annexin-V DY647 reagent (Central European Biosystems, Budapest, Hungary) and/or 10 µg/mL

propidium-iodide for 10 min at room temperature, washed again with CMF-PBS, and trypsinized for 15 min to obtain cell suspension. Cells were collected by centrifugation at 800 *g* for 10 min, resuspended in 1 mL FACS buffer (PBS supplemented with 1 % BSA and 0.05 % NaN_3) and rate of cell survival was determined using a CyFlow® space Flow Cytometer (Partec GmbH, Münster, Germany). Annexin-V DY647 was monitored at 670 nm, propidium-iodide was measured at 620 nm. Measurement lower threshold were set on cell-size particles. Analysis was performed with WinMDI 2.8 software.

2.10. RT-PCR analysis

Total RNA was isolated from cells using RNA Isolation Kit according to the manufacturer's instruction (Gentra Systems Inc., Minneapolis, MN, USA). The assay mixture for reverse transcriptase reaction contained 2 µg RNA, 0.112 µM oligo(dT), 0.5 mM dNTP, 200 units M-MLV RT in 1× RT buffer. The sequences of primer pairs used for PCR reactions were as follows: for chicken aggrecan 5'–CAA TGC AGA GTA CAG AGA–3' and 5'–TCT GTC TCA CGG ACA CCG–3', for chicken Sox9 5'–CCC CAA CGC CAT CTT CAA–3' and 5'–CTG CTG ATG CCG TAG GTA–3', for chicken calcineurin 5'–CTG CTC TGA TGA ACC AAC AGT T–3' and 5'–ACG GCA AGG ACC AGG TAA ACA–3', for chicken GAPDH 5'–GAG AAC GGG AAA CTT GTC AT–3' and 5'–GGC AGG TCA GGT CAA CAA–3', for chicken inositol-1,4,5-trisphosphate receptor (IP_3R) type-1 5'–CGG CTG TGG TCT GAG ATA C–3' and 5'–GGT AAT AGG GAA GAT GGT AGT G–3', for chicken IP_3R type-2 5'–AAG CCT ACC TTA TGA CCT CC–3' and 5'–CAT TGT TTC CTC CAT CCT G–3', and for chicken IP_3R type-3 5'–TGT GGG TGG ACA AGA AAG G–3' and 5'–GCA GGA ACT GAT GGG TGA A–3'. Amplifications were performed in a thermocycler (PCR Express Temperature Cycling

System, Hybaid, UK) as follows: 94 °C, 1 min, followed by 30 cycles (94 °C, 30 s, 54 °C, 30 s, 72 °C, 30 s) and then 72 °C, 5 min. 35 cycles were used at 57 °C for IP_3-receptors. PCR products were analyzed by electrophoresis in 1.2 % agarose gel containing ethidium bromide.

2.11. Western-blot analysis

Total cell lysates or endoplasmic reticulum (ER) fraction of HDC were examined by Western blot. Cell cultures were harvested immediately after treatments on respective days of culturing. Cell pellets were suspended in 100 µL of homogenization buffer [containing 50 mM Tris–HCl buffer (pH 7.0), 0.5 mM dithiothreitol, 10 µg/mL Gordox, 10 µg/mL leupeptin, 1 mM phenylmethylsulphonyl (PMSF), 5 mM benzamidine and 10 µg/mL trypsin inhibitor]. After storing them at –70 °C, suspensions were sonicated by pulsing burst for four times 30 sec by 50 cycles (Branson Sonifier, Danbury, USA). ER fraction of HDC was prepared from 3-day-old cultures grown in Petri dishes. Cells were homogenized using a Dounce homogeniser in a buffer containing 5 mM HEPES, 320 mM sucrose and protease inhibitors [3.4 µg/mL Gordox, 3.4 µg/mL leupeptin, 1 mM phenylmethylsulphonyl (PMSF), 1.6 mM benzamidine and 3.4 µg/mL trypsin inhibitor, pH 7.4]. After centrifugation at 4,500 *g* for 20 min, supernatant was collected and centrifuged at 10,000 *g* for 15 min. Supernatant was centrifuged at 150,000 *g* for 120 min. Pellet containing endoplasmic reticulum vesicles (microsome fraction) was collected in lysis buffer (50 mM Tris–HCl, pH 7.2 containing protease inhibitors, see above), snap-frozen in liquid nitrogen and stored at –70 °C. 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 10 min. About 40 µg of protein was separated by 7.5 % SDS–PAGE gel for immunological detection of Sox9, phospho-Sox9, RyR, IP_3-receptor and calcineurin. Proteins were transferred electrophoretically to nitrocellulose membrane. After blocking in 5 % non-fat dry milk in PBS, membranes were incubated with the following primary antibodies overnight at 4 °C: polyclonal anti-Sox9 antibody (Abcam Ltd, Cambridge, UK) in 1:200 dilution, polyclonal anti-phospho-Sox9 antibody (Sigma, Budapest, Hungary) in 1:200 dilution, monoclonal anti-RyR antibody (Affinity BioReagents, Golden, CO, USA) in 1:1000 dilution, polyclonal anti-IP_3R type 1 antibody (Sigma, Budapest, Hungary) in 1:250 dilution and polyclonal anti-calcineurin (α subunit) antibody (Upstate, Dundee, Scotland, UK) 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-mouse IgG (Sigma, Budapest, Hungary) in 1:1000 dilution for RyR and anti-rabbit IgG (Sigma, Budapest, Hungary) in 1:1000 dilution for detection of IP_3 receptor, Sox9, phospho-Sox9 and calcineurin in PBS containing 5 % non-fat dry milk for 1 h. Signals were detected by enhanced chemiluminescence (Amersham Biosciences, Budapest, Hungary).

2.12. Enzyme activity assay of calcineurin

Activity of calcineurin was measured by the release of $^{32}P_i$ from ^{32}P-labelled protein phosphatase inhibitor-1 (780 cpm/pmol) as described by Yang *et al.* [24] with some modifications [25]. 30 µL of the assay mixture (50 mM Tris–HCl buffer pH 7.0) containing 0.16 mM dithiothreitol, 3.4 µg/mL Gordox, 3.4 µg/mL leupeptin, 1 mM phenylmethylsulphonyl (PMSF), 1.6 mM benzamidine, 3.4 µg/mL trypsin inhibitor, 40 µg/mL calmodulin, 0.2 mM $CaCl_2$, 100 nM okadaic acid (OA), 2 nM protein phosphatase

inhibitor-2, extract containing about 80 µg protein and ^{32}P-labelled protein phosphatase inhibitor-1 (20–30 000 cpm/reaction mixture) was incubated at 30 °C for 20 min. The reaction was terminated by the addition of 100 µL of 20 % trichloroacetic acid and 100 µL of 6 mg/mL bovine serum albumin. After centrifugation, $^{32}P_i$-content of 180 µL of the supernatant fraction was determined in a liquid scintillation counter.

3. RESULTS

3.1. Cytosolic free Ca^{2+} concentration of untreated cell cultures shows a characteristic age-dependent pattern

Cytosolic free Ca^{2+} concentration was determined in Fura-2-loaded cells on different days of culturing. Basal level of intracellular Ca^{2+} concentration of chondroblasts was found to have an age-dependent pattern (Fig. 1A). Initially, Ca^{2+} level is low, with a starting concentration of about 75 nM on day 0, then it slightly increases in parallel with the progression of differentiation. A 140 nM peak of the cytosolic free Ca^{2+} concentration was observed on day 3 of culturing in cells of untreated control cultures. It should be noted that chondrogenic cells of HDC differentiate into chondroblasts on this day of culturing [3]. From day 4, Ca^{2+} level drops, however, it retains a slightly elevated concentration (about 100 nM) as compared to days 0-2.

Differentiating chicken chondroprogenitor cells exhibit periodical increases in cytosolic free Ca^{2+} (Fig. 1B, 1C, 1D). These oscillations were detectable mostly on culturing day 3. Frequencies of oscillations were similar in all the cells observed: the period was 4 ± 1.2 sec (mean ± standard error of the mean; n = 20), maximum amplitudes were 15–20 % higher than the mean basal fluorescence intensity.

3.2. Changes of cytosolic Ca^{2+} concentration of HDC hardly depend on intracellular sources

We aimed to determine whether the extracellular Ca^{2+} or the intracellular Ca-stores could be the source of elevated cytosolic Ca^{2+} levels. The Ca concentration of F12 medium containing 10 % FCS proved to be approximately 0.78 mM according to AAS measurements (data not shown). In order to reduce the concentration of free Ca^{2+} in the culture medium, EGTA was applied in equimolar (0.8 mM) concentration. EGTA treatment significantly decreased the cytosolic Ca^{2+} level to approximately 60 % of that of untreated control cells (Fig. 2A). 12 hrs of EGTA treatment proved to be effective; when maintained in 0.8 mM EGTA throughout the culturing period, cultures detached from the glass or plastic surface and died. To investigate the role of intracellular Ca-stores, cyclopiazonic acid (CPA), an inhibitor of the Ca^{2+} pump of smooth endoplasmic reticulum was administered at a concentration of 10 µM for 8 min. To remove free Ca^{2+} from the medium, cells were washed with Ca^{2+} free Tyrode's prior to administration of CPA. After addition of Ca^{2+}-free Tyrode's the basal cytosolic Ca^{2+} level decreased from 140 nM to 120 nM showing the dependence of this parameter on the extracellular Ca^{2+} concentration. Approximately 60 sec after the administration of 10 µM CPA, cytosolic Ca^{2+} level started to increase very slowly (Fig. 2B). The slight elevation in cytosolic Ca^{2+} clearly shows that the intracellular Ca^{2+}-stores are not empty, however, the amount of stored Ca^{2+} is either low or the rate of leak is small. When administration of both CPA and Ca-free Tyrode's ceased and were washed out with normal Ca^{2+} containing solution, a well-defined peak in cytosolic Ca^{2+} level could be observed owing to the entry of extracellular Ca^{2+} into the

cytosol, reflecting on a possible activation of store-operated Ca^{2+} entry (SOCE) channels [26]. The exact nature of this phenomenon, however, remains to be further investigated.

To investigate the possible role of internal Ca-stores in the regulation of Ca-homeostasis of differentiating chondroprogenitor cells, further experiments were performed. 10 µM CPA was administered to the culture medium of HDC on days 2 or 3 for 12 hrs. This prolonged inhibition of the Ca-pump of smooth endoplasmic reticulum must have resulted in a complete abolishment of intracellular stores, however, no detrimental effect on chondrogenesis could be observed (Fig. 3A). CPA-treatment slightly elevated cytosolic Ca^{2+} level (Fig. 3B), which could be related to the function of SOCE channels. Combined treatments with EGTA and CPA for 12 hrs (*i. e*., inhibition of Ca^{2+} entry from both extracellular and intracellular sources) resulted in a complete loss of metachromatically stained cartilage matrix demonstrating the Ca^{2+} dependence of *in vitro* chondrocyte differentiation (data not shown). This phenomenon may implicate the insufficient capacity of intracellular Ca-stores to replenish the function of Ca^{2+} entry pathways in chondrogenic cells.

The importance of Ca^{2+}-influx from extracellular space is further supported by investigating the endoplasmic reticulum ryanodine receptor (RyR) and the inositol-1,4,5-trisphosphate (IP_3) receptors. RyR was not detectable by Western blot analyses performed on total cell lysates of HDC and only a weak band was observed in samples containing separated endoplasmic reticulum fraction of HDC (Fig. 4A). Furthermore, no response was detected when caffeine, an agonist of RyR was administered during single cell measurements (Fig. 4B). These results indicate that though present, the low amount of RyR located in the endoplasmic reticulum of cells of HDC may not be functioning and probably does not significantly contribute to the elevation of basal cytosolic Ca^{2+}. Amplification of IP_3 receptor isoforms by RT-PCR shows that only the mRNA of IP_3R type 1 is expressed

by cells of HDC (Fig. 4C). The IP_3 receptor protein could be hardly detected in samples prepared from the endoplasmic reticulum fraction of chondrogenic cells by Western blotting (Fig. 4D).

3.3. Decreased extracellular Ca^{2+} concentration inhibits cartilage formation and reduces the expression of chondrogenic master transcription factor Sox9

Cultures treated with EGTA for 12 hrs on day 2 or 3 of culturing showed a profound decrease in metachromatic staining performed on day 6 of culturing (9 % or 17 % of untreated control cultures, respectively, Fig. 5A), demonstrating a significant reduction of cartilage formation. Administration of EGTA after day 5 did not have any significant effect on matrix production (data not shown).

Inhibition of chondrogenesis was monitored by detection of mRNAs of aggrecan core protein and Sox9, the major cartilage-specific transcription factor. A significant decrease in the mRNA levels of both aggrecan core protein and Sox9 was observed under the effect of EGTA on each day of treatments, demonstrating that reduced intracellular Ca^{2+} level decreases cartilage formation, at least partly, via inhibition of cartilage differentiation (Fig. 5B). These findings were further supported by Western blot analyses showing that treatment with EGTA slightly reduced the protein level of Sox9 on day 2, and a significant decrease was observed in the phosphorylation level of Sox9 on day 3 (Fig. 5C).

3.4. Ca^{2+} ionophore has dual concentration-dependent effects on cartilage formation

0.1 mg/L concentration of the Ca^{2+} ionophore A23187 raised the intracellular Ca^{2+} levels to approximately 125 % of untreated control cells, and 5 mg/L concentration of Ca^{2+} ionophore resulted in an even higher increase (about 150 %) in cytosolic Ca^{2+} levels (Fig. 6A). On the other hand, the two concentrations of ionophore applied had opposite effects on cartilage formation. Following treatment with the lower concentration (0.1 mg/L) of A23187 on both days 2 and 3 for one hour, an extensive cartilage formation occurred by day 6; when treatment was performed on day 3 only, the amount of metachromatically stained cartilage matrix increased to 153 % of control cultures (Fig. 6B). On the contrary, higher concentration (5 mg/L) of A23187 caused a marked inhibition of cartilage formation, especially when administered on day 2, reducing the amount of metachromatically stained cartilage matrix to approximately 36 % of untreated control cultures (Fig. 6B). Since mRNA levels of cartilage differentiation markers Sox9 and aggrecan core protein, and both protein level and phosphorylation of Sox9 showed a slight increase rather than inhibition under the effect of 5 mg/L ionophore (Figs. 6C and 6D), the mechanism of the decrease of cartilage formation should be further investigated. Administration of A23187 after day 5 did not have any significant effect on matrix production (data not shown).

3.5. Effects of EGTA, A23187 Ca^{2+} ionophore and CPA on rate of proliferation, mitochondrial activity and cellular viability of cells of HDC

Cell proliferation, mitochondrial activity and cellular viability assays were performed following treatments with EGTA, the Ca^{2+} ionophore A23187 and CPA, since decreased cartilage matrix production could be accounted for either by the inhibition of the differentiation of chondrogenic mesenchymal cells to chondroblasts or by the decrease in

cell numbers. Although the proliferation rate of cells in micromass cultures was significantly reduced under the effect of 0.8 mM EGTA (Fig. 7A), the mitochondrial activity of cells was not affected (Fig. 7B) as revealed by ^{3}H-thymidine incorporation and MTT assay, respectively. Cell proliferation was slightly stimulated by low concentration of the Ca^{2+} ionophore A23187 on day 3 (Fig. 7A). High concentration (5 mg/L) of A23187 reduced the rate of cell proliferation on both days, but it did not cause any significant decrease in mitochondrial activity (Fig. 7B). Treatment with CPA did not influence the proliferation rate of cells (Fig. 7A) and it did not prove to be cytotoxic according to MTT assays (Figs. 7B).

Cellular viability following treatments with EGTA or the Ca^{2+} ionophore A23187 was also analyzed by FACS (Figs. 7C and 7D). The ionophore did not influence the distribution of cells compared to the control. The percentage increase of dead cells were as follows: 3.2 % and 0.7 % (in the presence of 0.1 mg/L) and 1.8 % and 3.5 % (in the presence of 5 mg/L) on days 2 and 3, respectively. Treatment with 0.8 mM EGTA led to a higher rate of cell death, thus the percentage increase of dead cells proved to be 10 % and 9.1 % on days 2 and 3, respectively. Apoptosis was not detected in the ionophore-treated HDC (data not shown).

3.6. Calcineurin plays a dual role in Ca-signalling of HDC

Calcineurin is one of the target molecules regulated by the changes of intracellular Ca^{2+} level. Therefore we measured the enzymatic activity of calcineurin in samples prepared from untreated control cultures and from HDC treated with EGTA or A23187. When cytosolic Ca^{2+} level became lower as a consequence of reducing the extracellular Ca^{2+} concentration with EGTA, the activity of calcineurin decreased

significantly on both days of treatments. Elevation of cytosolic Ca^{2+} level with A23187 had partly unexpected effects. On day 2 the elevation of Ca^{2+} concentration induced by the ionophore increased the activity of calcineurin (the change was significant in case of 5 mg/L A23187), while on day 3, the ionophore-induced elevation of cytosolic free Ca^{2+} level resulted in a suppression of calcineurin activity. This decrease was significant when 5 mg/L A23187 was applied (Fig. 8A). RT-PCR and Western blot analyses demonstrated that expression of calcineurin was only slightly modified by any kind of change of cytosolic Ca^{2+} concentration (Fig. 8B). When the activity of calcineurin was inhibited by its pharmacological inhibitor CsA, the cytosolic Ca-peak observed in control HDC on day 3 of culturing was eliminated, although cells had a slightly higher basal Ca^{2+} level than those of the untreated controls (Fig. 8C). It is worth to mention that 2 μM of CsA causes about 40 % inhibition of the enzymatic activity of calcineurin measured in samples of HDC as we have reported it previously [16].

4. DISCUSSION

Changes of intracellular Ca^{2+} concentration are important signalling events in different cellular processes, including cell and tissue differentiation. The Ca^{2+} sensitive PKCalpha is reported to influence proliferation and differentiation of chondrifying cells, via modulation of MAPK-signalling [14] and we have described a positive regulatory role of calcineurin in the *in vitro* chondrogenesis occurring in chicken HDC either under physiological conditions or under the effect of oxidative stress [16]. Our present investigations were based on the prediction that intracellular Ca^{2+} concentration of cells in HDC may show a correlation with the onset of chondrogenic differentiation. We found that during the differentiation of chicken limb bud-derived chondrogenic cells to chondroblasts

and chondrocytes, the cytosolic free Ca^{2+} concentration exhibits characteristic temporal changes. Starting from lower (about 75 nM) Ca^{2+} levels, it reaches its maximum on day 3 of culturing with a peak of 140 nM and remains at a higher concentration (about 100 nM) until the end of the investigated 6-day-long culturing period. It is important to emphasize that no enzymatic digestions were performed on cells of HDC prior to Ca^{2+} assays, since the composition and organization of ECM surrounding chondroblasts and chondrocytes is crucial to maintain the adequate function and morphology of these cells [9]. Basal Ca^{2+} concentrations of differentiating mesenchymal cells are comparable with that of other non-excitable cells, *e. g*. basal cytosolic Ca^{2+} level of HaCaT keratinocytes is approximately 80–90 nM [27], that of ROS and UMR osteoblastic cell lines is 90–100 nM [28]. These findings are all based on measurements in Fura-2-loaded cells *in situ*.

Beside the long-term changes of basal cytosolic Ca^{2+} level, cells of HDC exhibit short-term, spontaneous periodical increases in cytosolic Ca^{2+} concentration. These oscillations were detectable mostly on culturing day 3 raising the possibility of a correlation between periodical changes of cytosolic free Ca^{2+} concentration and chondrogenic differentiation. Presence of calcium oscillations in differentiating cells is characteristic to other non-excitable cells as well [29] and it is reported that intracellular Ca^{2+} oscillations promote the activation of Ca^{2+} dependent transcription factors needed for the differentiation of human mesenchymal stem cells [30]. In the present work only the phenomenon is presented, the mechanism of the generation of these oscillations and their role in the process of chondrogenesis remains to be further elucidated.

To determine whether the elevated cytosolic free Ca^{2+} level on day 3 of culturing is derived from extracellular sources or is released from intracellular stores, *e. g*. smooth endoplasmic reticulum, further experiments were performed. Free Ca^{2+} of the culturing medium was bound by the Ca^{2+} chelator EGTA. This treatment significantly lowered

cytosolic Ca^{2+} levels and cartilage formation was also significantly reduced by the end of the 6-day-long culturing period. Furthermore, a significant decrease in the mRNA levels of both aggrecan core protein and Sox9 was also observed underlying the decreased cartilage differentiation. Lower cytosolic Ca^{2+} level can be a secondary effect of the abolishment of intracellular Ca-stores, but our experiments carried out with the application of CPA, an inhibitor of the Ca^{2+} pump of endoplasmic reticulum, demonstrated that intracellular Ca^{2+} stores of chondrogenic cells did not play a significant role in the changes of cytosolic free Ca^{2+} concentration and cartilage formation. However, somewhat higher cytosolic Ca^{2+} levels could be observed following the treatment with CPA, probably owing to store-operated Ca^{2+} entry processes. In addition, RyR was found weakly expressed by cells of HDC, and caffeine treatments did not cause any significant change in the cytosolic Ca^{2+} level. Taken together, we conclude that intracellular Ca-stores could play a less significant role in the modulation of the peak in cytosolic Ca^{2+} of HDC during their differentiation. Nonetheless, the well-known elements of the Ca^{2+} toolkit of non-excitable cells [31], from which we have demonstrated the presence of store-operated Ca^{2+} entry pathways, PMCAs, SERCAs and IP_3 receptors are expressed by cells of HDC, and work together to play a role in the modulation of intracellular Ca^{2+} concentration. The detailed characterisation of the Ca^{2+} toolkit is well beyond the scope of the present work.

Our data, nevertheless, suggest that the Ca^{2+} homeostasis of chondrogenic cells investigated here depends largely on extracellular Ca^{2+} sources; in the absence of extracellular free Ca^{2+} ions, differentiating cells failed to elevate their cytosolic Ca^{2+} level, which, in turn, blocked the differentiation process leading to a decrease in metachromatically stained cartilage areas on day 6 of culturing. Since chondrogenesis is very sensitive to cell density, one can argue that a decrease in metachromatic staining could be due simply to a decrease in cell number via affecting cell proliferation and

cellular viability. Although administration of EGTA significantly reduced the proliferation rate of cells in micromass cultures, the viability of cells was not altered. These findings suggest that elevated Ca^{2+} levels regulate proliferation of chondrogenic mesenchymal cells during *in vitro* chondrogenesis. Decrease of intracellular Ca^{2+} concentration can alter the proliferation of chondrogenic cells via modulation of the activity of classical PKC izoenzymes, particularly via PKCalpha [32].

To investigate the effects of opposite changes of intracellular Ca^{2+} level, we have generated uncontrolled Ca-influx with the application of a Ca^{2+} ionophore. Low (0.1 mg/L) concentrations of A23187 raised intracellular Ca^{2+} levels in parallel with increased cartilage differentiation and matrix production. Although high (5 mg/L) concentration of Ca^{2+} ionophore resulted in higher (about 150 % of untreated control cells) intracellular Ca^{2+} levels, this led to a dramatic decrease in cartilage formation. Interestingly, the cartilage specific mRNA markers of Sox9 and core protein of aggrecan failed to show lowered expression, while the protein level of Sox9 was reduced implying the possibility of a translational regulation of this protein via Ca^{2+} sensitive pathways [33,34]. Since none of the applied concentrations of A23187 caused any significant decrease in cellular viability or cell proliferation, one can conclude that proper chondrogenesis requires a tightly controlled concentration range of intracellular Ca^{2+} and the proliferation ability of chondrogenic cells is more sensitive to the decrease than to the increase of cytosolic free Ca^{2+} concentration. Moreover, our data indicate that a slight elevation of cytosolic Ca^{2+} levels of chondrifying mesenchymal cells promotes differentiation, but further elevation (about 150 % of untreated control cells) inhibits chondrogenesis.

Calcineurin plays an important role in numerous cellular processes including activation of T-lymphocytes [35], apoptosis of cardiomyocytes and cardiac hypertrophy [36], regulation of blood vessel formation and myogenesis [37] and is a sensitive target of

the changes of the cytosolic Ca^{2+} concentration. In our previous paper [16] we showed the presence and active function of calcineurin in micromass cultures. In the present work we demonstrate that activity of calcineurin responds to the manipulation of intracellular Ca^{2+} concentration in a cell-differentiation-stage dependent manner. On day 2, when a rapid proliferation and initiation of the differentiation of chondroblasts occurs, calcineurin responded to the changes of cytosolic Ca^{2+} concentration as it was expected, *i. e.* EGTA decreased, while the ionophore increased its activity. On the contrary, any kind of alteration of cytosolic Ca^{2+} level did result in a reduction its activity on day 3 of culturing, when the differentiation process of chondrogenic cells was already accomplished. Since calcineurin has a positive role in the regulation of *in vitro* chondrogenesis of chicken micromass cultures [15,16], we suppose that the maintenance of its enzymatic activity requires a precisely set regulation of intracellular Ca^{2+} concentration when the commitment of the chondrogenic cells is definitely determined (on day 3). It is remarkable to notice that under the effect of CsA, when the activity of calcineurin is lowered, differentiating cells failed to produce the peak-like increase in cytosolic free Ca^{2+} concentration on culturing day 3, although the basal Ca^{2+} levels were higher in CsA-treated cultures than in the untreated ones. This observation may imply an active regulatory role of calcineurin in the enhancement of Ca-influx responsible for the rapid transient elevation of cytosolic Ca^{2+} concentration of chondrogenic cells on day 3. This idea is supported by numerous data gained by different experimental models, in which calcineurin regulated the activity of various components of Ca^{2+} toolkits. Recently calcineurin was described to dephosphorylate, and in this way desensitizing/inactivating several types of Ca-channels located either in the cell membrane [38] or in the endoplasmic reticulum [39]. This function of calcineurin was described both in excitable and non-excitable cell types. These

target Ca-channels include store-operated Ca-channels [40], high-threshold voltage-activated Ca-channels [41] and TRPV1 receptors [42].

Among non-excitable cell types, the Ca-conductance of non-committed embryonic mesenchymal cells or bone marrow derived mesenchymal stem cells are extensively investigated due to their high clinical practical impact [30,43,44]; however, we have to emphasize that our experimental model contains committed chondroprogenitor cells having mesenchymal cell-like morphology prior to differentiation into chondroblasts, but their developmental faith has been determined when isolated from chicken limb buds. Thus data achieved on observations of these cells are not fully comparable to the aforementioned results. There are only sporadic reports on the existence of subtypes of Ca-channels expressed by chondrogenic cells describing the presence of an L-type voltage sensitive Ca-channel [45] and a new mechanosensitive Ca^{2+} channel polycystin-2 [46]. Recently TRPV4 receptor was reported to contribute to the regulation of Sox9 expression of chondrogenic cells in a Ca-calmodulin-dependent manner [47]. Activity of the TRPV receptor family is regulated by phosphorylation and it is known that phosphorylation/dephosphorylation of these cation-channels is PKA and calcineurin-dependent, at least in neuronal elements [42].

Taken together, calcineurin plays a pivotal role in the Ca^{2+} homeostasis of many different cell types, its activity is regulated by changes of intracellular Ca^{2+} concentration and this phosphatase is also implicated in the regulation of function of several components of Ca-entry, as well as endoplasmic reticulum IP_3 receptors.

In summary, our data provide evidence that the extracellular Ca^{2+} is required to initiate differentiation of chondrogenic mesenchymal cells to chondroblasts and chondrocytes. This process can be inhibited by decreasing the concentration of extracellular Ca^{2+} and can be stimulated by elevating the intracellular level of this crucial

cation. Nonetheless, elevation of cytosolic free Ca^{2+} above a threshold level is detrimental to chondrogenesis. Under normal conditions a transient elevation of basal cytosolic Ca^{2+} precedes differentiation of chondrogenic cells and manipulation of the intracellular concentration of calcium alters chondrogenesis. In conclusion, the regulation of *in vitro* chondrogenesis is directly related to changes of cytosolic free Ca^{2+} concentration and calcineurin is an important signal molecule in these events. The precise mechanism by which extracellular Ca^{2+} enters the cytoplasm of chondroprogenitor cells remains to be further elucidated.

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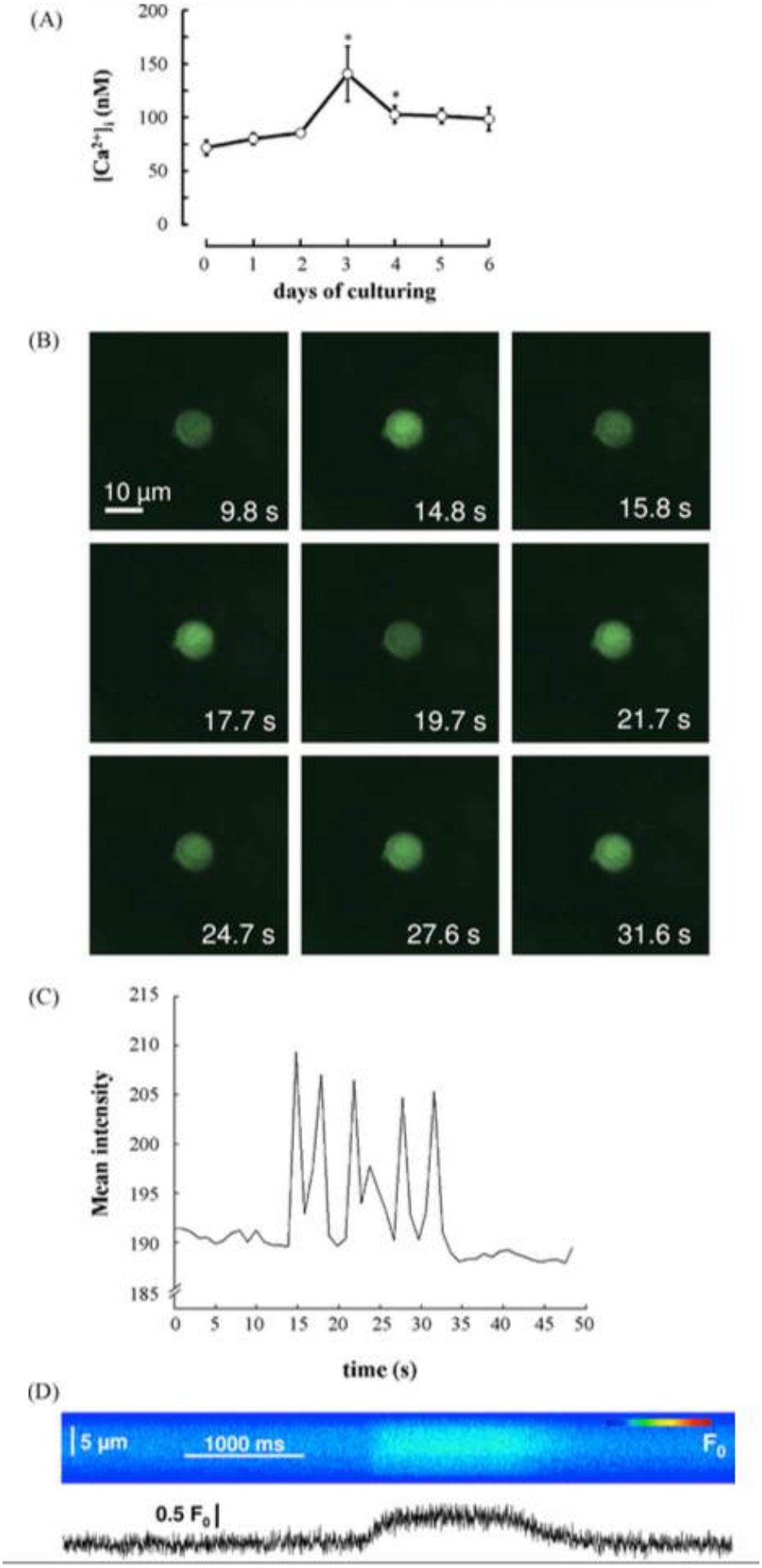
FIGURES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215
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Mean intensity
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FIGURE 1. Day by day variation of basal intracellular Ca^{2+} levels in chondrifying cells of untreated control HDC (**A**). Ca^{2+} concentrations were determined in Fura-2-loaded cells as described in Materials and Methods. Representative data of five independent experiments showing mean values of basal intracellular Ca^{2+} levels of 30 cells ± standard error of the mean. Statistical analysis by Student's t-test comparing the respective data to the previous culturing day, $*P < 0.01$. Spontaneous calcium events in cells of chondrifying high density micromass cell cultures on culturing day 3 (**B, C, D**). Calcium transients were determined in Fluo 4-AM loaded cells as given in Materials and Methods. Changes of fluorescence intensity were recorded in a 50 sec interval. x–y analysis of a representative cell is shown in panel **B**, time course of mean intensity is shown in panel **C**. A representative calcium event measurement performed by line scan imaging is shown in panel **D**.

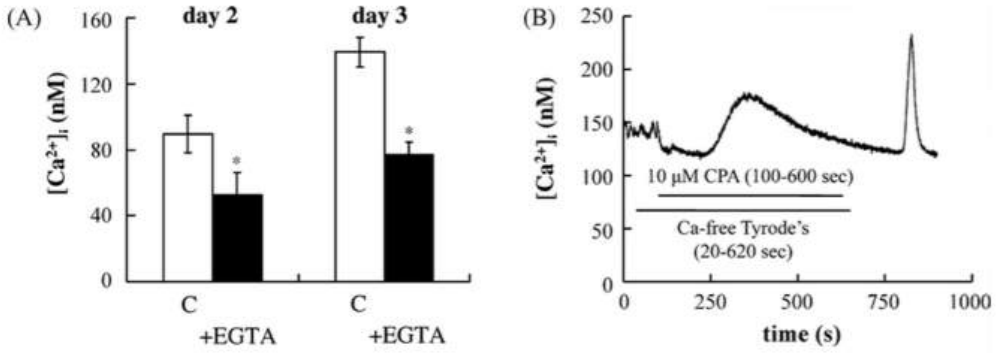

FIGURE 2. Basal intracellular Ca^{2+} levels of 0.8 mM EGTA-treated cultures on days 2 and 3 (**A**). Measurements were carried out following 12 hrs of EGTA treatments. Data represent mean ± standard error of the mean of intracellular Ca^{2+} levels of 30 cells measured in 5 representative experiments. Effect of cyclopiazonic acid (CPA) on the release of Ca^{2+} from intracellular stores (**B**). Representative record out of 30 cells in 5 independent experiments. Asterisks indicate significant (*$P < 0.01$) decrease in basal Ca^{2+} concentration as compared to the respective control.

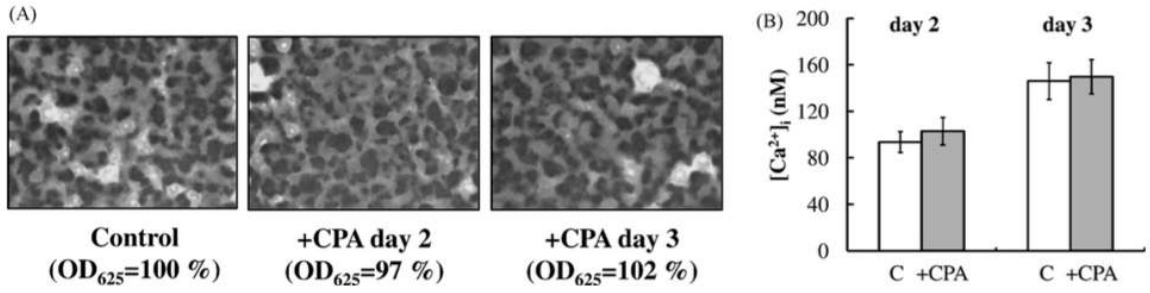

FIGURE 3. Effect of 10 µM CPA on cartilage formation in chondrifying micromass cultures (**A**). Metachromatic cartilage areas in 6-day-old high-density colonies visualized with DMMB dissolved in 3 % acetic acid. Optical density (OD_{625}) of samples containing toluidine blue extracted with 8 % HCl dissolved in absolute ethanol. Data are mean values ± standard error of the mean (± 7 %) of each experimental group out of 10 measurements. Basal intracellular Ca^{2+} levels of 10 µM CPA-treated cultures on day 2 and 3 (**B**). Measurements were carried out directly after the 12-hrs treatment with CPA. Data represent mean ± standard error of the mean of intracellular Ca^{2+} levels of 30 cells measured in 5 independent experiments.

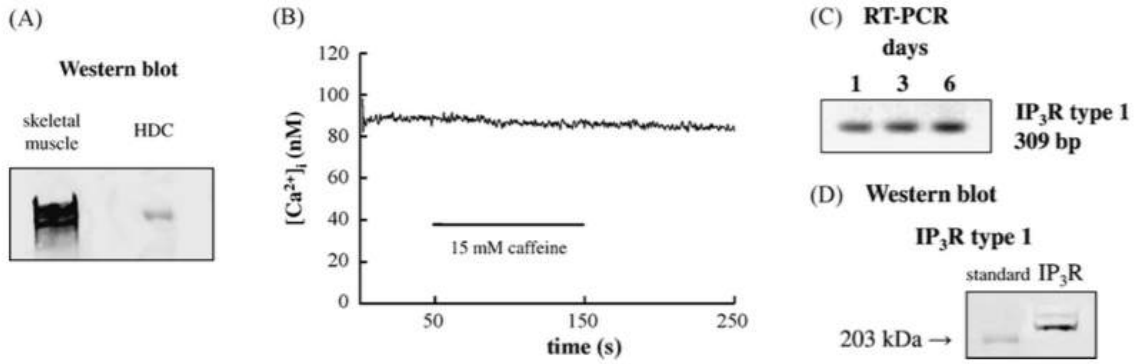

FIGURE 4. Detection of the expression and fuction of RyR and IP_3R in HDC. Western blot analysis of RyR in ER fraction of cells of HDC on culturing day 3 (**A**). Representative data of 5 independent experiments. Effect of the RyR-agonist caffeine (15 mM) on basal cytosolic Ca^{2+} level of cells of HDC on culturing day 3 (**B**). Representative record out of 30 cells in 5 independent experiments. Amplification of chicken IP_3R type 1 receptor (**C**). Representative result out of 3 independent experiments. Western blot analysis of IP_3R in ER fraction of cells in HDC (**D**). Representative analysis out of 3 independent experiments.

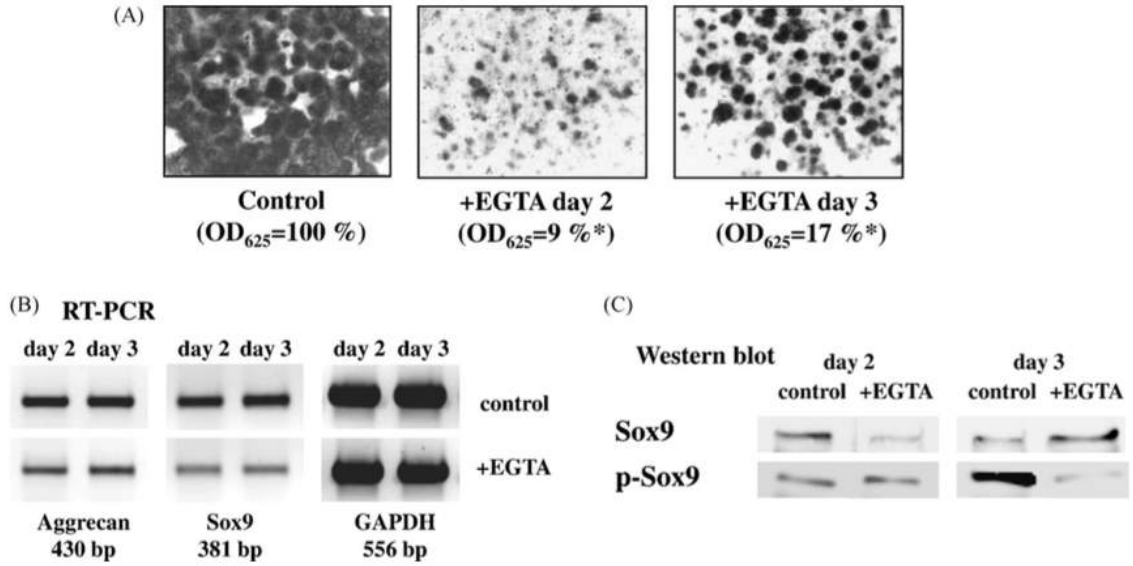

FIGURE 5. Effect of 0.8 mM EGTA on cartilage development of chondrifying micromass cultures (**A**). Metachromatic cartilage areas in 6-day-old high-density colonies visualized with DMMB dissolved in 3 % acetic acid. Optical density (OD_{625}) of samples containing toluidine blue extracted with 8 % HCl dissolved in absolute ethanol. Data are mean values ± standard error of the mean (± 5 %) of each experimental group out of 10 measurements. Effect of EGTA on the expression of aggrecan and the expression and phosphorylation of Sox9 transcription factor in HDC of various ages (**B, C**). For RT-PCR reactions GAPDH was used as a control. Representative data of 5 independent experiments. Asterisks indicate significant (*$P < 0.01$) decrease in optical density of extracted toluidine blue as compared to the respective control.

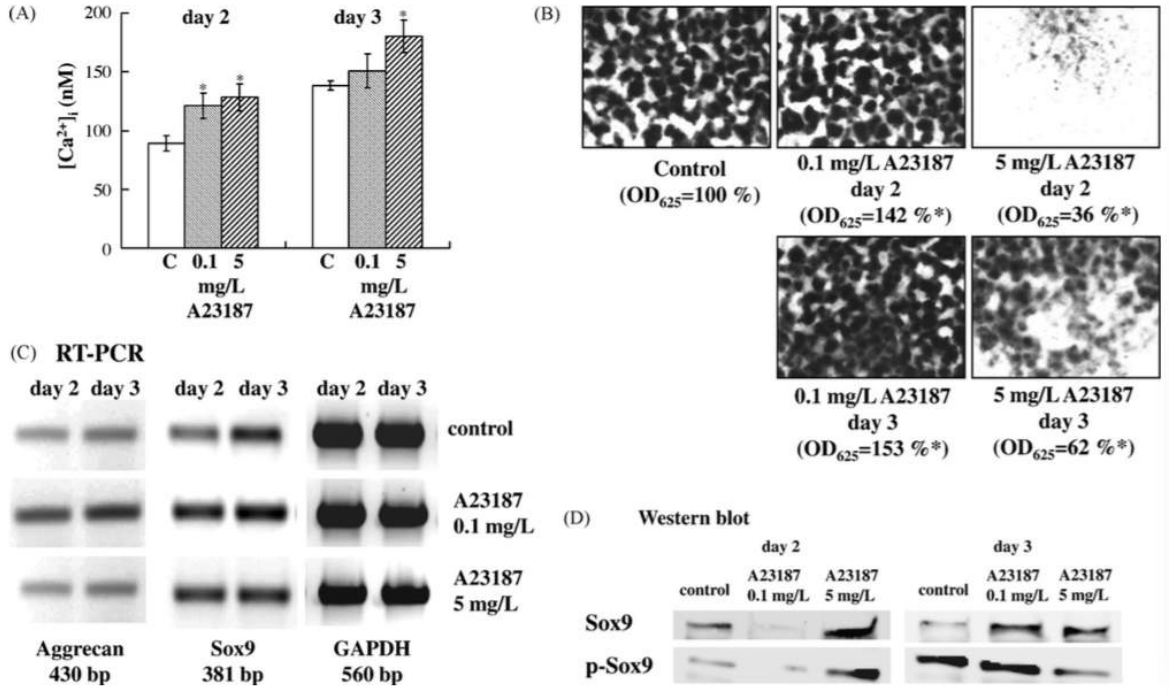

FIGURE 6. Effects of the Ca^{2+} ionophore A23187 on cartilage development of chondrifying micromass cultures. Basal intracellular Ca^{2+} levels of 0.1 mg/L or 5 mg/L A23187-treated cultures on day 2 and 3 (**A**). Measurements were carried out directly after the 30-min treatment with A23187. Data represent mean ± standard error of the mean of intracellular Ca^{2+} levels of 30 cells measured in 5 independent experiments. Metachromatic cartilage areas in 6-day-old high-density colonies visualized with DMMB dissolved in 3 % acetic acid (**B**). Optical density (OD_{625}) of samples containing toluidine blue extracted with 8 % HCl dissolved in absolute ethanol. Data shown are mean values ± standard error of the mean (± 6 %) of each experimental group out of 10 measurements. Effect of A23187 on the mRNA expression of aggrecan and Sox9 transcription factor (**C**) and protein expression and phosphorylation of Sox9 (**D**) in HDC of various ages. For RT-PCR reactions GAPDH was used as a control. Representative data of 5 independent experiments. Asterisks indicate significant (*$P < 0.01$) increase in basal Ca^{2+} concentrations or significant (*$P < 0.01$) change in optical density of extracted toluidine blue as compared to the respective control.

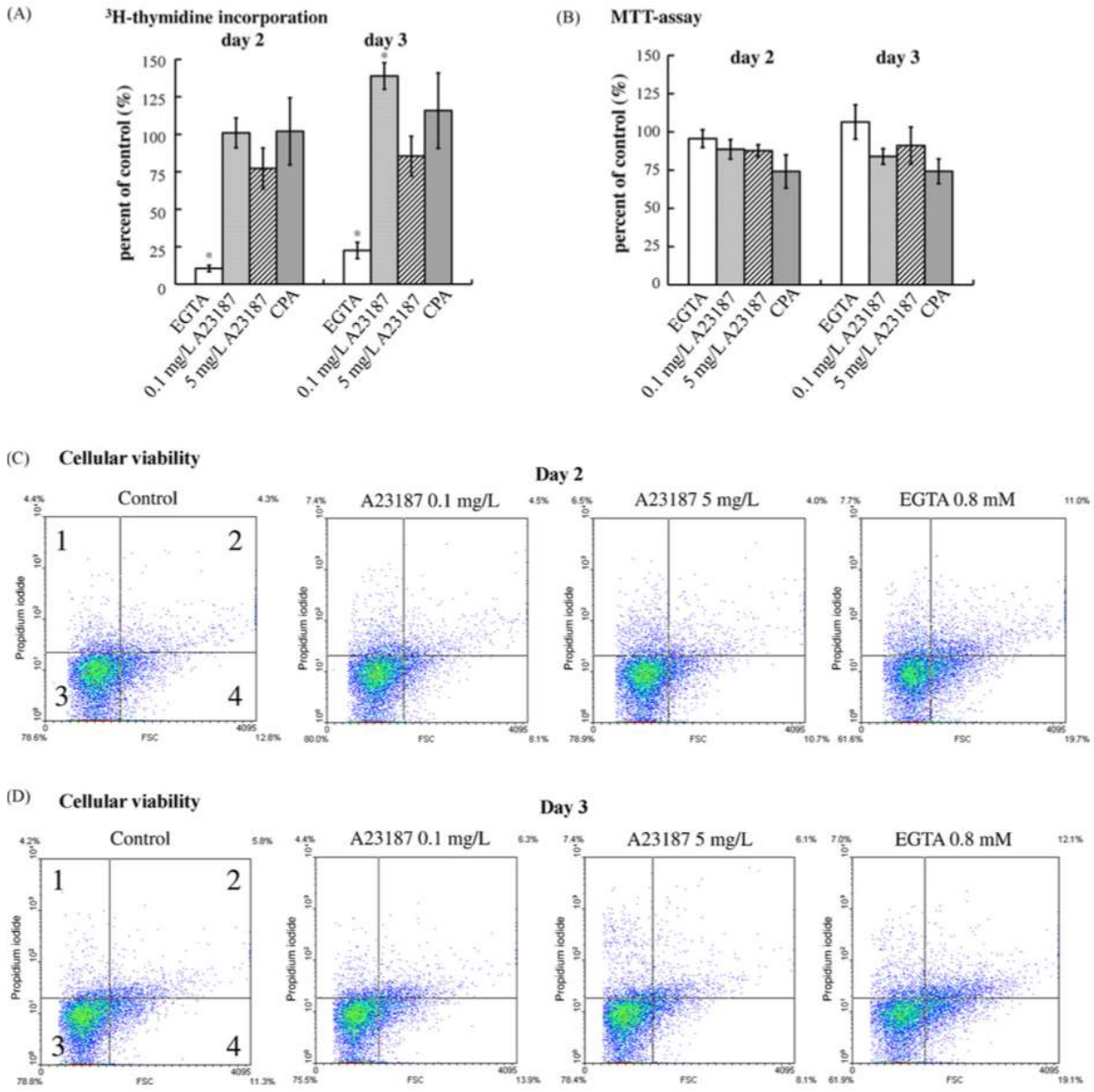

FIGURE 7. Effect of 0.8 mM EGTA, 0.1 and 5 mg/L A23187 and 10 µM CPA on cell proliferation (**A**), mitochondrial activity (**B**) and cellular viability (**C**, **D**) of cells of chondrifying micromass cultures. Cell proliferation was assessed by ^{3}H-thymidine incorporation, mitochondrial activity was measured by MTT assay, and cellular viability was determined by FACS analysis. Assays were carried out each day immediately after EGTA, A23187 or CPA treatments. Quadrants 1 and 2 on panels **C** and **D** represent cells containing propidium iodide (*i. e*. dead cells), whereas quadrants 3 and 4 represent unstained (*i. e.* living) cells of various sizes. Data represent mean ± standard error of the

mean of 5 independent experiments and given in percent of the respective untreated cultures. Asterisks indicate significant (*$P < 0.01$) decrease or increase in ^{3}H-thymidine incorporation (A) as compared to control.

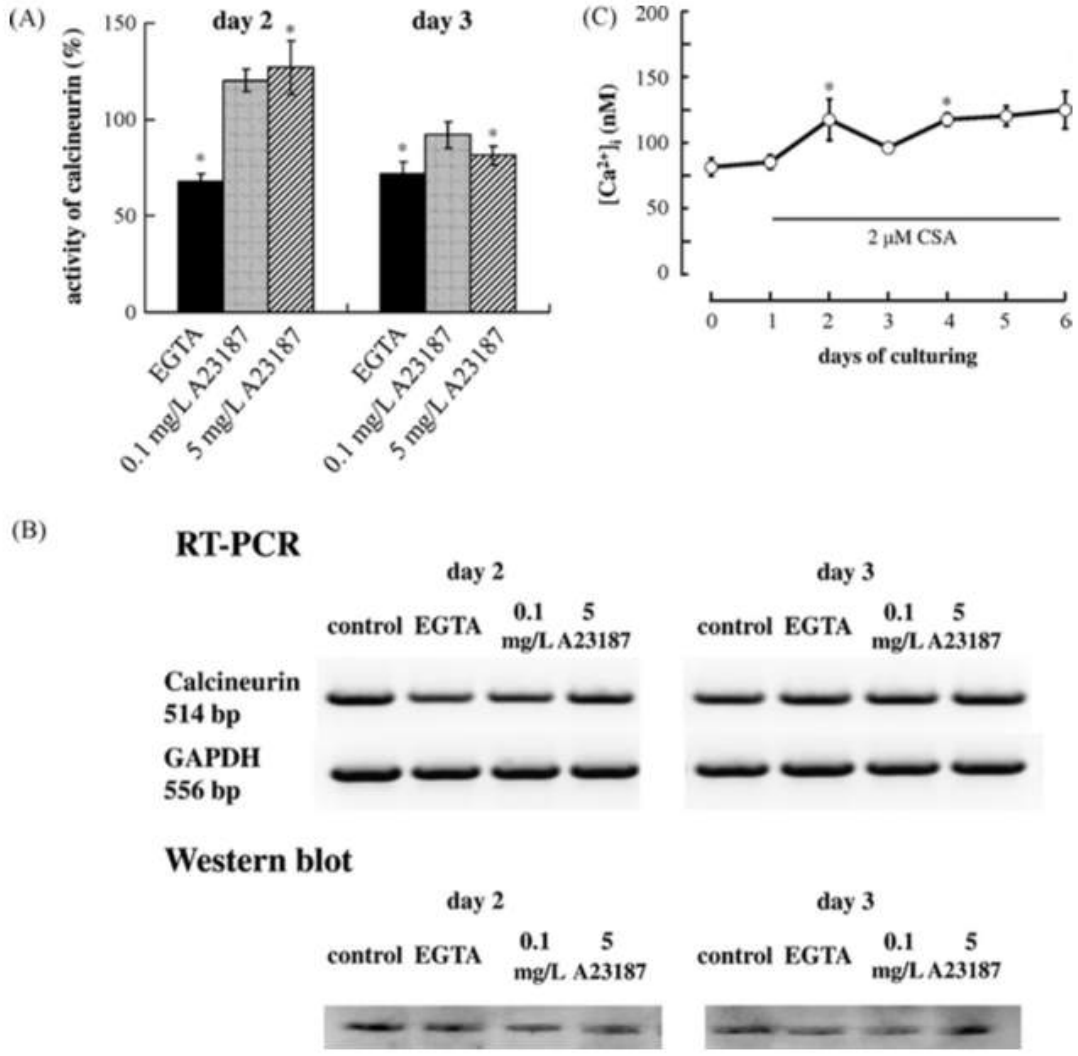

FIGURE 8. Effect of the Ca^{2+} ionophore A23187 and EGTA on the activity of calcineurin (**A**). Data represent mean ± standard error of the mean of three independent experiments and are given in percent of the respective untreated cultures. Effect of A23187 and EGTA on expression levels of calcineurin (**B**). GAPDH was used as a control. Representative data of 3 independent experiments. Role of calcineurin in the regulation of basal cytosolic Ca^{2+} levels of chondrifying mesenchymal cell cultures (**C**). Ca^{2+} levels were determined in Fura-2-loaded cells. Representative data of five independent experiments showing mean values of intracellular Ca^{2+} levels of 30 cells ± standard error of the mean. Statistical analysis by Student's t-test comparing the respective data to the previous culturing day, **P* < 0.01.

ABBREVIATIONS

AAS, atomic absorption spectrometry

AM, acetoxy-methylester

BMP, bone morphogenic protein

CMF-PBS, calcium and magnesium free phosphate buffered saline

CPA, cyclopiazonic acid

CsA, cyclosporine A

DMMB, dimethylmethylene blue

DMSO, dimethyl sulfoxide

dNTP, deoxyribonucleotide triphosphate

DTT, dithiothreitol

ECM, extracellular matrix

EGTA, ethylene glycol tetraacetic acid

ER, endoplasmic reticulum

FACS, fluorescence activated cell sorter

FCS, foetal calf serum

FGF, fibroblast growth factor

GAPDH, glyceraldehide-3-phosphate dehydrogenase

HDC, high density cell culture

IGF, insulin-like growth factor

IP_3, inositol-1,4,5-trisphosphate

MAPK, mitogen-activated protein kinase

MTT, 3-(4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide

N-CAM, neural cell adhesion molecule

OA, okadaic acid

PBS, phosphate buffered saline

PBST, phosphate buffered saline with 0.1 % Tween 20

PKC, protein kinase C

PMCA, plasma membrane Ca^{2+} ATPase

PMSF, phenylmethylsulphonyl

RT-PCR, reverse transcription and polymerase chain reaction

RyR, ryanodine receptor

SDS-PAGE, sodium dodecyl sulphate polyacrilamide gel electrophoresis

Ser, serine

SERCA, sarco(endo)plasmic reticulum Ca^{2+} ATPase

SOCE, store-operated calcium entry

Thr, threonine

TRPV, transient receptor potential ion channel