myo-Inositol 1,4,5-trisphosphate and Ca^{2+} /calmodulin-dependent factors mediate transduction of compression-induced signals in bovine articular chondrocytes

Wilmot B. VALHMU*¹ and Frank J. RAIA†

*Orthopedic Biosciences Laboratory, Department of Orthopedics and Rehabilitation, University of Wisconsin-Madison, Madison, WI 53792, U.S.A., and †Orthopaedic Research Laboratory, Department of Orthopaedic Surgery, Columbia University, New York, NY 10032, U.S.A.

Although the effects of mechanical loading on chondrocyte metabolic activities have been extensively characterized, the sequence of events through which extracellular mechanical signals are transduced into chondrocytes and ultimately modulate cell activities is not well understood. Here, studies were performed to map out the sequential intracellular signalling pathways through which compression-induced signals modulate aggrecan mRNA levels in bovine articular chondrocytes. Bovine articular cartilage explants were subjected to a compressive stress of 0.1 MPa for 1 h in the presence or absence of inhibitors or antagonists of the phosphoinositol and Ca^{2+} /calmodulin signalling pathways in order to determine the roles of second messengers and effector molecules of these pathways in transducing the compression-induced signals. In the absence of the inhibitors, aggrecan mRNA levels were stimulated by compression 2–4-fold relative to levels in tare-loaded (see below)

explants. Treatment of the explants with graded levels of the protein kinase C inhibitor chelerythrine or bisindolylmaleimide I, followed by 1 h compressive loading, did not significantly alter the load-induced elevation of aggrecan mRNA levels. In contrast, thapsigargin, which depletes the $\text{Ins}(1,4,5)\text{P}_3$ -sensitive intracellular Ca^{2+} stores, completely blocked the load response without significantly altering aggrecan mRNA levels in tare-loaded explants. Similarly, antagonists of the Ca^{2+} /calmodulin signalling pathway dose-dependently or completely blocked the load-response. The results obtained demonstrate that transduction of the compression-induced aggrecan mRNA-regulating signals requires $\text{Ins}(1,4,5)\text{P}_3$ - and Ca^{2+} /calmodulin-dependent signalling processes in bovine articular chondrocytes.

Key words: aggrecan, Ca^{2+} /calmodulin-dependent protein kinase II, calcineurin, mRNA, thapsigargin.

INTRODUCTION

Mechanical forces are key regulators of the normal maintenance and turnover of cartilaginous tissues. Clinical and experimental studies indicate that prolonged withdrawal of mechanical input to the articular cartilage of diarthrodial joints leads to loss of extracellular matrix (ECM) components of the tissue [1–3]. Traumatic injuries or impact loading of diarthrodial joints can induce degenerative changes in the articular cartilage and subchondral bones of the joints [4–6]. It has also been shown that altered loading patterns in diarthrodial joints contribute to the development of osteoarthritis [7].

The mechanical environment of the chondrocytes in articular cartilage is complex, and various physico-electrochemical signals are generated when articular cartilage is subjected to mechanical loads. Compressive loading of articular cartilage results initially in pressurization of the interstitial fluid, followed by the movement of fluid from regions of high to low fluid pressurization. As the pressure dissipates, greater amounts of the applied loads are borne by the solid matrix and chondrocytes. The movement of fluid and dissolved ions relative to fixed charges in the tissue generates streaming potentials and streaming currents that may also act as regulators of chondrocyte activities [8]. Various *in vitro* mechanical-loading models have shown that mechanical loads modulate chondrocyte biosynthetic activities in a dose-

and frequency-dependent manner. Static loading of articular cartilage explants at stresses that exceed 0.2 MPa is generally inhibitory to chondrocyte biosynthetic activities [9–14]. However, at stresses ≤ 0.2 MPa, gene expression [15] and synthesis of cartilage ECM components [11,16] may be stimulated, depending on the mode of loading. Cyclic or dynamic loading has been observed to stimulate the synthesis of cartilage ECM components [17]. Even chondrocytes grown in hydrogels retain their abilities to respond to mechanical loading in a frequency- and dose-dependent manner [18,19]. Despite these findings, the mechanisms through which chondrocytes perceive and transduce extracellular mechano-electrochemical signals are not well understood.

In recent years, major focus has been given to understanding the sequence of events through which extracellular physical signals are transduced into intracellular biochemical signals in chondrocytes, leading ultimately to changes in gene expression and modulation of cell activities. One of the earliest means of chondrocyte response to extracellular mechanical input is change in intracellular Ca^{2+} levels, occurring within seconds to minutes. Guilak et al. [20] observed transient increases in intracellular Ca^{2+} concentration ($[\text{Ca}^{2+}]_i$) in response to deformation of cultured chondrocytes via micropipette stimulation. Similarly, fluid flow elicits Ca^{2+} transients in chondrocytes [21–23]. In monolayer cultures, chondrocytes have been shown to propagate

Abbreviations used: BAPTA/AM, bis-(*o*-aminophenoxy)ethane-*N,N,N',N'*-tetra-acetic acid tetrakis(acetoxymethyl ester); $[\text{Ca}^{2+}]_i$, intracellular Ca^{2+} concentration; CaM, Ca^{2+} /calmodulin; CaMKII, CaM-dependent protein kinase II; CREB, cAMP response element-binding protein; CBP, CREB-binding protein; DAG, diacylglycerol; DMEM, Dulbecco's modified Eagle's medium; FBS, fetal-bovine serum; ECM, extracellular matrix; KN-93, 2-[*N*-(2-hydroxyethyl)-*N*-(4-methoxybenzenesulphonyl)]amino-*N*-(4-chlorocinnamyl)-*N*-methylbenzylamine; PKA, protein kinase A; PKC, protein kinase C; PLC, phospholipase C; Tg, thapsigargin; W-7, *N*-(6-aminoethyl)-5-chloro-1-naphthalenesulphonamide hydrochloride; SNK, Student–Newman–Keuls (test).

¹ To whom correspondence should be addressed (e-mail valhmu@surgery.wisc.edu).

intercellular Ca^{2+} signals through gap junctions [24–26]. Grandolfo et al. [26] showed further that this process involves activation of phospholipase C (PLC) and postulated that the cell-to-cell Ca^{2+} signalling may be mediated through permeation of gap junctions by the PLC-generated $\text{Ins}(1,4,5)\text{P}_3$, thus stimulating Ca^{2+} transients in recipient cells. We have previously shown that the transient elevation of aggrecan mRNA levels in bovine articular cartilage explants in response to low-level compression is dependent on signalling through the PLC/PtdIns and adenylate cyclase/cAMP signalling pathways [15]. In the present study we performed further characterization of the role of downstream effector molecules and second messengers of the PtdIns pathway in the compression-induced regulation of aggrecan gene expression in chondrocytes.

To map out the intracellular path of the compression-induced signal(s) transduced through the PtdIns pathway in bovine articular chondrocytes, a strategy using sequential inhibition or blockage of key steps in the pathway was employed. The cleavage of PtdInsP_2 by PLC generates the second messengers $\text{Ins}(1,4,5)\text{P}_3$ and diacylglycerol (DAG). Generation of $\text{Ins}(1,4,5)\text{P}_3$ elicits release of Ca^{2+} from intracellular stores, while DAG activates protein kinase C (PKC) in a Ca^{2+} -dependent manner. Since we have previously demonstrated a requirement of PLC activity for the compression-induced elevation of aggrecan mRNA levels in bovine articular cartilage explants [15], we first sought to determine the roles of the $\text{Ins}(1,4,5)\text{P}_3$ and DAG/PKC branches of the PtdIns pathway in this process. On the basis of the results obtained, other downstream targets were then sequentially investigated. The data show that the transduction of the compression-induced, aggrecan-regulating signal in chondrocytes does not require the activities of PKC. However, it is highly dependent on $\text{Ins}(1,4,5)\text{P}_3$ - and Ca^{2+} /calmodulin (CaM)-mediated processes.

MATERIALS AND METHODS

Tissue culture and loading of explants

The culturing and loading of bovine articular-cartilage explants were performed essentially as described previously by Valhmu et al. [15]. Briefly, full-thickness cylindrical disks (5 mm diameter and 1–2 mm thickness) of articular cartilage were harvested under sterile conditions from the carpometacarpal joints of a 4–6-month-old calf. The joints were obtained from a local abattoir within 4 h of the animal being killed. Typically, disks (six to eight disks/joint) were obtained from two to four animals at the time of harvesting, pooled, and cultured. Prior to loading, the articular cartilage disks were rinsed and cultured at 37 °C in a CO_2 /air (19:1) atmosphere for 1–3 days in DMEM (Dulbecco's modified Eagle's medium; Mediatech Inc., Herndon, VA, U.S.A.) supplemented with 10% (v/v) fetal-bovine serum (FBS) (Sigma Chemical Co., St Louis, MO, U.S.A.), extra amino acids (Mediatech), buffering agents (10 mM Hepes, 10 mM sodium bicarbonate, 10 mM Tes and 10 mM Bes), and antibiotics (100 units/ml penicillin and 100 µg/ml streptomycin; Mediatech).

At the time of loading, each explant was pre-incubated in culture medium for 1 h in the presence or absence of graded concentrations of inhibitors or antagonists (indicated below) of the PtdIns and CaM signalling pathways. After pre-incubation, the explants were transferred to 60 mm-diameter tissue-culture dishes containing 10 ml of culture medium with or without inhibitors or antagonists of the signalling pathways and equilibrated for 15 min under a tare load (approx. 0.025 MPa) between two sintered-glass filters, as previously described [15]. The tare load consisted of the weight of the loading platen (50 g)

applied to each 5 mm-diameter explant. After pre-incubation, the disks were then subjected to a compressive stress of 0 or 0.1 MPa above tare load for 1 h. (All subsequent references to 0 or 0.1 MPa load indicate levels of compressive stress above tare load.) This loading regime has been previously observed to cause a transient 2–4-fold elevation of aggrecan mRNA levels [15] and up to 60% stimulation of proteoglycan synthesis [16] in bovine articular cartilage explants. At the end of the loading regime, each explant was immediately transferred to 0.5 ml of ice-cold RNA extraction buffer [27].

Inhibitors of the phosphoinositol and CaM signalling pathways

For determining the role of DAG/PKC-dependent processes in the modulation of aggrecan mRNA levels by compression, the PKC inhibitors chelerythrine chloride and bisindolylmaleimide I hydrochloride were used. These, and all subsequent pathway inhibitors or antagonists, were obtained from Calbiochem–Novabiochem Corporation (La Jolla, CA, U.S.A.). Except for bisindolylmaleimide I, each compound was used at concentrations approximately equivalent to 0, 2 and 20 times its K_i or IC_{50} where applicable. These concentrations were chosen sufficiently low ($2 \times \text{IC}_{50}$) to minimize non-specific effects and sufficiently high ($20 \times \text{IC}_{50}$) to ensure that maximal inhibition was achieved. In either case, the concentrations were within range of those normally reported in the literature. Bisindolylmaleimide I was used at 0, 10 and 100 times its IC_{50} . The specific concentrations used for each compound are indicated in the Figures in the Results and Discussion sections. For an assessment of the role of $\text{Ins}(1,4,5)\text{P}_3$ -dependent processes, the Ca^{2+} -ATPase pump inhibitor thapsigargin (Tg) was used to ablate $\text{Ins}(1,4,5)\text{P}_3$ -mediated Ca^{2+} release from internal stores. The dependence of the compression-induced stimulation of aggrecan mRNA levels on changes in $[\text{Ca}^{2+}]_i$ was investigated further by pre-incubating explants with 0, 5 and 50 µM bis-(*o*-aminophenoxy)ethane-*N,N,N',N'*-tetra-acetic acid tetrakis(acetoxymethyl ester) (BAPTA/AM), followed by loading the explants in the presence or absence of the compound. Cheng and Benton [28] have shown that in chondrocytes 10 µM BAPTA buffers or clamps $[\text{Ca}^{2+}]_i$ at resting levels. Other inhibitors or antagonists used were *N*-(6-aminohexyl)-5-chloro-1-naphthalenesulphonamide (W-7) hydrochloride and trifluoperazine (calmodulin inhibitors), 2-[*N*-(2-hydroxyethyl)-*N*-(4-methoxybenzenesulphonyl)]amino-*N'*-(4-chlorocinnamyl)-*N*-methylbenzylamine (KN-93; an inhibitor of CaM-dependent protein kinase II) and cyclosporin A (a calcineurin inhibitor). Where DMSO was used to solubilize the compounds, the untreated controls also received concentrations of DMSO equivalent to those in the treated cultures. In each case, the concentration of DMSO was $\leq 0.1\%$.

Quantification of aggrecan mRNA levels

The levels of aggrecan mRNA in tare- and 0.1 MPa-loaded explants were measured by quantitative PCR as previously reported ([15], [29], but see [29a]). Briefly, after loading, each explant was homogenized in RNA extraction buffer, and total RNA was extracted from the homogenate using a total RNA isolation kit (RNAgents Total RNA Isolation System; Promega Corporation, Madison, WI, U.S.A.) based on the method of Chomczynski and Sacchi [27]. This was followed by further purification using the silica-based RNeasy Total RNA spin column (Qiagen Inc., Valencia, CA, U.S.A.). A 10 µl aliquot of each total RNA sample was reverse-transcribed using oligo-dT₁₅ primers (Promega) and the Superscript II reverse transcriptase (Life Technologies, Grand Island, NY, U.S.A.) in a total volume of 25 µl at 37 °C. The aggrecan cDNA in 10 µl of 500-, 2500- and

Figure 1 Effect of ablation of $\text{Ins}(1,4,5)\text{P}_3$ -mediated Ca^{2+} mobilization from internal stores

To preclude $\text{Ins}(1,4,5)\text{P}_3$ -dependent Ca^{2+} release from internal stores in bovine articular chondrocytes in response to compression, the explants were treated for 1 h with graded concentrations of TG before compressive loading at 0 or 0.1 MPa ($n = 8$) for 1 h. The indicated load levels represent stresses above tare load (≈ 0.025 MPa). (A) Representative ethidium bromide-stained gel of quantitative-PCR reaction products. Lanes 1–9 represent standards with starting copy numbers ranging from 10^3 to 3.98×10^4 copies/reaction. Lanes 13, 14 and 15 contained, respectively, reaction products of 1:500, 1:2500 and 1:12500 dilutions of a tare-loaded, untreated control. Samples in lanes 16–48 were similarly diluted and amplified. The intensity of each band was quantified and normalized as described in the Materials and methods section. Please note that, because the amount of starting total RNA that was reverse-transcribed for each sample varied, the relative differences between the experimental samples may not be apparent until normalized by the starting quantities. (B) Bar graph of the levels of aggrecan mRNA in the various samples. For each group, the mean $\pm$ S.D. for the combined data from four separate experiments (example in Figure 1A) is presented. Note that, in the absence of TG, aggrecan mRNA levels were stimulated 2.58-fold ($P < 0.05$, $n = 8$) in response to 0.1 MPa compressive stress, while at 0.1 and 1 μM TG, this increase was completely inhibited, indicating a requirement for $\text{Ins}(1,4,5)\text{P}_3$ -dependent Ca^{2+} mobilization.

12 500-fold serial dilutions of each reverse-transcribed total RNA sample was then PCR-amplified in a total reaction volume of 50 μl using intron-spanning primers within 1000 bases of the poly(A)⁺ tail of the aggrecan mRNA. Quantitative PCR standards consisted of parallel reactions containing known copy numbers (10^3 – 10^5) of the aggrecan target sequence cloned in the pCR II cloning vector (Invitrogen Corporation, La Jolla, CA, U.S.A.). After amplification, 20 μl of each reaction product was electrophoresed on a 2% agarose gel (see Figure 1A), and the intensities of the negative image of the ethidium bromide-stained bands were quantified and used to generate standard curves from which the unknown cDNA copy numbers of the samples were determined. The cDNA copy numbers (indicative of the levels of the mRNAs) were normalized per μg of total

RNA in each reaction mixture. The data were statistically analysed by two-way analysis of variance and Student–Newman–Keuls (SNK) multiple-range comparison test ($\alpha = 0.05$). Results are presented as percentages of the values for untreated, tare-loaded, controls.

RESULTS

We previously demonstrated that load-controlled, continuous compression of bovine articular cartilage explants at 0.1 MPa transiently up-regulates aggrecan mRNA levels [15]. This requires the activities of PLC and cAMP-dependent protein kinase A, suggesting involvement of both the PtdIns and cAMP signalling pathways in this process. To further characterize the role of the PtdIns pathway in the compression-induced regulation of aggrecan gene expression, modulators or antagonists of key components of the pathway were used in the present study to determine the contributions of components of the PtdIns pathway to the transduction of the compression-induced signals in bovine articular-cartilage explants.

To determine whether the stimulation of aggrecan mRNA levels in bovine articular-cartilage explants by 0.1 MPa compressive stress requires compression-induced release of Ca^{2+} from $\text{Ins}(1,4,5)\text{P}_3$ -sensitive intracellular Ca^{2+} stores, explants were loaded at 0 or 0.1 MPa above tare load in the presence of 0, 0.01 and 0.1 μM TG. In the absence of TG treatment, aggrecan mRNA levels were increased 2.58-fold relative to the levels observed in the corresponding tare-loaded explants, by compression at 0.1 MPa ($P < 0.05$; SNK) (Figure 1B). A significant interaction ($P = 0.0001$, two-way ANOVA) between the compressive loads and TG was observed. In the presence of 0.01 and 0.1 μM TG, the levels of aggrecan mRNA in explants loaded at 0.1 MPa were respectively only 1.09- and 1.08-fold those of the corresponding tare-loaded explants, indicating no load-induced increase in aggrecan mRNA levels in the presence of TG ($P > 0.05$, SNK).

The inhibitory effect of TG on the compression-induced elevation of aggrecan mRNA levels (Figure 1) indicated a requirement for compression-stimulated release of Ca^{2+} from

Figure 2 Buffering of intracellular Ca^{2+} concentration dose-dependently blocks the load response

Intracellular Ca^{2+} concentration can be buffered or clamped with Ca^{2+} chelators such as BAPTA. To determine whether the response of aggrecan mRNA levels to compression is dependent on change in $[\text{Ca}^{2+}]_i$, bovine articular-cartilage explants were treated for 1 h with 0, 5, and 50 μM BAPTA/AM ($n = 4$) and then loaded at 0 or 0.1 MPa for 1 h. Aggrecan mRNA levels in the explants were then measured. The results in this and subsequent Figures represent the combined data from two separate experiments. Treatment with BAPTA/AM dose-dependently inhibited the load response. Note: $P < 0.05$ for * versus ** and for ** versus ***. Differences between bars marked by *** are not significant ($P > 0.05$).

Figure 3 Inhibition of PKC does not alter the load response of aggrecan gene expression

Bovine articular-cartilage explants were pretreated with chelerythrine for 1 h at the indicated concentrations (representing 2 and 20 times its IC_{50}) and subjected to 0 or 0.1 MPa ($n = 4$). Note that the response of aggrecan gene expression to compression was unaffected by the inhibitor.

Ins(1,4,5) P_3 -sensitive intracellular stores and suggested that the response of aggrecan gene expression to compression is Ca^{2+} -dependent. To investigate the requirement for intracellular Ca^{2+} in modulation of aggrecan mRNA levels by 0.1 MPa compression, explants were treated with graded levels of BAPTA/AM, a cell-permeant Ca^{2+} chelator, in order to buffer the $[Ca^{2+}]_i$, and subsequently subjected to compression at 0 or 0.1 MPa for 1 h. BAPTA dose-dependently inhibited the load-response ($P = 0.0001$, two-way ANOVA) (Figure 2). In the absence of BAPTA, aggrecan mRNA levels were elevated 2.16-fold ($P < 0.05$, SNK) in explants loaded at 0.1 MPa, compared with tare-loaded explants. At 5 μM BAPTA, aggrecan mRNA was stimulated 1.32-fold ($P < 0.05$, SNK) in response to loading at 0.1 MPa (Figure 2). This level of stimulation represents a 72% inhibition of the load response by BAPTA ($P < 0.05$, SNK). At 50 μM , BAPTA completely blocked the load response ($P > 0.05$, SNK).

For an assessment of the role of PKC in the compression-induced regulation of aggrecan mRNA levels, explants were loaded in the absence or presence of graded concentrations of the PKC inhibitor chelerythrine chloride or bisindolylmaleimide I. At chelerythrine concentrations of 0, 1 and 10 μM , loading the explants at 0.1 MPa respectively increased ($P = 0.0001$, two-way ANOVA) aggrecan mRNA levels by 2.80-, 2.67- and 2.65-fold relative to tare-loaded explants (Figure 3). No interaction effects ($P = 0.71$, two-way ANOVA) were observed between the chelerythrine treatments and the compressive loads. The levels of aggrecan mRNA in the tare-loaded explants, irrespective of chelerythrine treatment, were not significantly different ($P > 0.05$, SNK). The PKC inhibitor bisindolylmaleimide I, similar to chelerythrine, had no significant effects on the response of aggrecan mRNA levels to compression ($P = 0.82$, two-way ANOVA; data not shown).

Our finding of a Ca^{2+} -dependence of the compression-induced elevation of aggrecan mRNA levels in articular cartilage explants suggested that transduction of the mechanical signals involves CaM and associated factors. Antagonists or inhibitors of CaM and downstream effectors were therefore used to test this hypothesis. To investigate the role of CaM, explants were treated with graded levels of W-7 or trifluoperazine and loaded for 1 h at 0 or 0.1 MPa. Treatment of the explants with W-7 completely

Figure 4 Inhibition of CaM completely blocks the load response

To determine whether the compression-induced elevation of aggrecan mRNA levels required activities of CaM, explants were pretreated with W-7 at 0, 50, and 500 μM ($n = 4$) and then loaded at 0 or 0.1 MPa for 1 h in the presence of the compound. The compression-dependent increase in aggrecan mRNA levels was completely blocked, indicating that CaM is a crucial transducer of the compression-induced signals.

Figure 5 Modulation of aggrecan gene expression by compression involves CaMKII

Several CaM enzymes and factors, including CaMKII, mediate the downstream effects of changes in $[Ca^{2+}]_i$. To determine whether CaMKII plays a role in transduction of the compression-induced signals in bovine articular-cartilage explants, the explants were treated with the CaMKII inhibitor KN-93 at concentrations of 0, 0.7, and 7 μM and loaded at 0 or 0.1 MPa. Similar to the effects of BAPTA/AM on the load response, treatment with KN-93 dose-dependently inhibited the increases in aggrecan mRNA levels by the compressive load. Note: $P < 0.05$ for * versus ** and for ** versus ***. Differences between bars marked by *** are not significant ($P > 0.05$).

blocked the compression-induced elevation of aggrecan mRNA levels ($P = 0.0001$, two-way ANOVA) (Figure 4), demonstrating a requirement for CaM-dependent signalling. Compared with tare loading, 0.1 MPa compression increased aggrecan mRNA levels by 3.58-fold ($P > 0.05$, SNK). In the presence of either 50 or 500 μM W-7, compression at 0.1 MPa did not change the levels of aggrecan mRNA from baseline levels ($P > 0.05$, SNK). Trifluoperazine, at 25 and 250 μM , similarly blocked the increase in aggrecan mRNA levels in response to 0.1 MPa compressive stress ($P = 0.0001$, two-way ANOVA; results not shown).

The contribution of CaM-dependent protein kinase II (CaMKII) to transduction of the compression-induced signals was investigated using graded concentrations of the CaMKII inhibitor KN-93. KN-93 dose-dependently inhibited the load-induced response ($P = 0.0001$, two-way ANOVA) (Figure 5).

Figure 6 Calcineurin participates in transduction of the compression-induced signals, but is not rate-limiting

Calcineurin, the CaM-dependent protein phosphatase-2B, may modulate the activities of adenylate cyclase. Our earlier finding of a requirement for transduction of the compression-induced signals through the cAMP pathway, along with the current finding of a requirement for CaM, suggested that calcineurin might participate by modulating adenylate cyclase. To determine whether the signalling process requires calcineurin, explants were treated with 0, 0.05 and 0.5 μ M cyclosporin A ($n = 4$) to inhibit calcineurin activities. Loading the explants at 0 or 0.1 MPa for 1 h revealed only partial inhibition of the load response. Note: $P < 0.05$ for * versus ** and for ** versus ***. In-group comparison of differences between members of groups marked by ** or *** are not significant ($P > 0.05$).

In the absence of KN-93, there was a 1.96-fold stimulation of mRNA levels ($P < 0.05$, SNK). At 0.7 μ M KN-93, aggrecan mRNA levels in 0.1 MPa-loaded explants were only 1.21-fold those in tare-loaded explants (Figure 5), representing a 78% inhibition ($P < 0.05$, SNK) of the compression-induced increase in aggrecan mRNA levels. In the presence of 7 μ M KN-93, the load response was completely blocked ($P > 0.05$, SNK).

The CaM-dependent protein phosphatase 2B (calcineurin) has been shown to potentiate the activities of adenylate cyclase in gastric chief cells [30]. Signalling through the adenylate cyclase/cAMP signalling pathway has previously been shown to be required for compression-induced stimulation of activation of aggrecan mRNA levels in bovine articular-cartilage explants [15]. To determine whether calcineurin plays a role in this process, bovine articular cartilage explants were treated with the calcineurin inhibitor cyclosporin A at concentrations of 0, 0.05 and 0.5 μ M and subjected to compressive loading at 0 or 0.1 MPa for 1 h. In the absence of cyclosporin A, aggrecan mRNA levels were stimulated 3.62-fold ($P < 0.05$, SNK), relative to tare-loaded controls, by 0.1 MPa compressive loading. At 0.05 and 0.5 μ M cyclosporin A, loading of the explants at 0.1 MPa resulted in only 2.16- and 2.34-fold stimulation of aggrecan mRNA levels respectively (Figure 6). These values represent 56 and 49% inhibition ($P < 0.05$, SNK) of the load response, indicating involvement of calcineurin in the compression-mediated regulation of aggrecan mRNA levels. There was, however, no significant difference between the load-response data obtained in the presence of 0.05 and 0.5 μ M cyclosporin A ($P > 0.05$, SNK).

DISCUSSION

Chondrocytes in articular cartilage respond to mechanical modulation by altering their biosynthetic activities, consistent with the type and duration of the mechanical input. This response may be an attempt by the cells to remodel their ECM so that it can meet the mechanical demands exerted upon it. In recent years, greater attention has been given to characterization of the mechanisms

whereby the cells sense and transduce signals from biophysical forces applied to the tissue. Theoretical modelling predicts spatial and time-varying changes in the mechanoelectrochemical environment of the chondrocyte under mechanical loads [16,31]. Such changes in the cell's environment serve as direct modulators of chondrocyte biosynthetic activities. Despite recent progress, the sequence of events through which the cells sense and respond to the various mechanical signals is not fully understood. Valhmu et al. [15] have previously reported a requirement for activities of the PLC and protein kinase A (PKA) in the transient elevation of aggrecan mRNA levels in bovine articular cartilage explants by low-level compressive loading, indicating involvement of the PtdIns and cAMP signalling pathways in this process. Here we report further characterization of the roles of components of the PtdIns pathway, as well as downstream CaM-dependent signalling, in compression-induced modulation of aggrecan mRNA levels in bovine articular-cartilage explants.

Ablation of $\text{Ins}(1,4,5)\text{P}_3$ -dependent Ca^{2+} release from internal stores, via TG treatment, revealed a strong dependence of the compression-induced stimulation of aggrecan mRNA levels on $\text{Ins}(1,4,5)\text{P}_3$ -mediated intracellular Ca^{2+} mobilization (Figure 1). In contrast, inhibition of PKC activity had no significant effects on response of the aggrecan gene to 0.1 MPa compressive stress (Figure 3), indicating that PKC activity is not required for transduction of the compression-induced signals that modulate aggrecan mRNA levels. This is consistent with the reported lack of a role for PKC activity in mediating the mechanical response in carbachol- or histamine-induced sustained contraction of canine bronchi, despite dependence of this process on intracellular Ca^{2+} mobilization [32]. The requirement for intracellular Ca^{2+} mobilization in compression-induced modulation of aggrecan mRNA levels was further demonstrated by the dose-dependent inhibition of the load response (Figure 2) by treatment of the explants with graded levels of BAPTA/AM to buffer the $[\text{Ca}^{2+}]_i$.

Treatment of chondrocytes with TG at concentrations ranging from 0.05 to 1.7 μ M has previously been shown to induce both a sustained [33,34] and a transient [35] rise in $[\text{Ca}^{2+}]_i$, with amplitudes reaching as high as 914 nM in the presence of extracellular Ca^{2+} [34]. In the present study the inability of TG to significantly alter baseline aggrecan mRNA levels, despite slight dose-dependent increases of up to 4%, may be due to the fact that TG treatment abolishes Ca^{2+} oscillations in chondrocytes [35]. Activation of the PtdIns pathway often leads to cytosolic Ca^{2+} oscillation. The frequency of such cytosolic Ca^{2+} oscillation could serve as a signal for gene-specific regulation. Dolmetsch et al. [36] have demonstrated that certain transcription factors are activated in a Ca^{2+} -oscillation-frequency-dependent manner at the same amplitude of $[\text{Ca}^{2+}]_i$. The factors that regulate the aggrecan gene in response to compressive stress might similarly be controlled by Ca^{2+} oscillation frequency. TG treatment, which increases the amplitude and inhibits oscillation of $[\text{Ca}^{2+}]_i$, had no effect on aggrecan levels in the absence of applied load. Thus we postulate that the generation of $\text{Ins}(1,4,5)\text{P}_3$ in response to the compressive load induced a series of spikes in $[\text{Ca}^{2+}]_i$, which in turn activated downstream effectors such as CaM (Scheme 1), whose activity was demonstrated in these studies to be required for stimulation of aggrecan mRNA levels by compressive loading (Figure 4).

The strong inhibitory effects of TG or BAPTA/AM on stimulation of aggrecan mRNA by compression, coupled with the lack of significant effects on the baseline levels of aggrecan mRNA, suggests that modulation of $[\text{Ca}^{2+}]_i$ alone is not sufficient to elicit the observed stimulation of aggrecan mRNA by compressive stress. Our previous observation of a requirement for

Scheme 1 Model of mechanotransduction mechanism of compression-induced stimulation of aggrecan gene expression in chondrocytes

Putative mechanoreceptor(s), when stimulated by compressive stress, activate(s) both the PtdIns and cAMP signalling pathways, which lead(s) to regulation of gene expression. PKC activity is not required for this signalling process. The various antagonists or inhibitors of key signalling molecules used in the present studies and their points of action are indicated. Abbreviations used: ER, endoplasmic reticulum; IP₃, Ins(1,4,5)P₃; PIP₂, PtdIns(4,5)P₂.

both PLC and PKA activities in transducing the compression-induced signals [15] supports this interpretation. The data suggest, therefore, that compression of articular-cartilage explants at 0.1 MPa activates, in parallel, PLC [generating Ins(1,4,5)P₃] and adenylate cyclase (generating cAMP and activating PKA) to transduce signals which converge upon either a common transcription complex [e.g., the cAMP response element-binding protein (CREB)–CREB-binding protein (CBP) complex] (see Scheme 1) or a group of transcription complexes that control expression of the aggrecan gene. Cross-talk between these pathways in transducing the compression-induced signals may exist at the level of calcineurin, a CaM-dependent protein phosphatase that has been demonstrated to modulate the activities of adenylate cyclase in various cell types [30,37–40]. This enzyme has also been shown to modulate glucagon gene transcription through the cAMP response element ('CRE') [41]. As indicated in Figure 6, inhibition of calcineurin blocks the compression-induced increase in aggrecan mRNA levels by approx. 50% at both the low and high concentrations tested. The partial inhibition of the load response by calcineurin suggests that its activity is not rate-limiting. Thus the role of the enzyme in this process is likely to be potentiation of the compression-induced activity of adenylate cyclase.

Mechanotransduction in chondrocytes in response to various mechanical stimuli has been studied by a number of laboratories (see, for example, [15,20,22–26,42–48]). Like various other cell types, chondrocytes utilize the classical as well as other signalling pathways to transduce signals derived from applied mechanical

forces. Increases in cAMP levels in response to tensile stress [46] and cyclical pressure-induced strain [47] have been reported. Consistent with these observations, Valhmu et al. [15] reported a requirement for PKA activity in modulation of aggrecan gene expression by compressive stress. Involvement of PLC, PKC, CaM and the cytoskeleton in the hyperpolarization response of isolated human chondrocytes to cyclic pressure-induced strain has also been reported [48]. The present study demonstrated requirements for Ins(1,4,5)P₃- and CaM-dependent signalling but, unlike those of Wright et al. [48], found no requirement for PKC activity modulation of aggrecan mRNA levels in bovine articular-cartilage explants in response to low-level constant compression. The reason for this difference is not known, but it could lie in the experimental model (i.e., isolated chondrocytes in the study by Wright et al. [48] versus cartilage explants in the present study) or the mode of loading (cyclic pressure versus compression).

In interpreting the data presented here, it should be borne in mind that certain limitations of the studies exist with regard to non-specific effects of the various compounds used. For instance, treatment of chondrocytes with TG has been reported to inhibit general protein synthesis and induce the synthesis of stress proteins within 24–48 h [33,34]. Additionally, the CaMKII inhibitor KN-93 is known to non-specifically block voltage-dependent K⁺ channels in myocytes independent of CaMKII activities [49], and cyclosporin A affects activity of mitochondrial permeability transition pores [50]. The contributions of these non-specific effects to the current data are not known. Thus

future studies that utilize transfection of chondrocytes with dominant-negative mutants of the effector molecules under investigation are warranted in order to verify the observations presented here.

In conclusion, we propose the following signalling model (Scheme 1) for transduction of the aggrecan gene-modulating, compression-induced signals: (1) compression of bovine articular cartilage at 0.1 MPa activates both the PtdIns and cAMP signalling pathways; (2) the generation of $\text{Ins}(1,4,5)\text{P}_3$ via PLC activity releases Ca^{2+} from intracellular stores and activates downstream CaM-dependent enzymes such as CaMKII and calcineurin; (3) calcineurin potentiates the activity of adenylate cyclase that is already induced by the compressive stress; (4) adenylate cyclase activates PKA via production of cAMP; and (5) both PKA and CaMKII translocate to the nucleus, where they activate transcriptional complexes that then bind the aggrecan gene and mediate its transcription.

The data presented here provide insights into key steps in the sequence of events leading to stimulation of aggrecan mRNA levels by low-level compressive stress. Detailed characterization of this process at the level of transcriptional activation is needed in order to provide a complete picture of the mechanotransduction mechanisms through which chondrocytes regulate ECM gene expression in response to applied compressive stress.

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