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**Osteoarthritis and Cartilage**

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# Signal transduction in electrically stimulated articular chondrocytes involves translocation of extracellular calcium through voltage-gated channels[1]

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## Summary

*Objective*: To ascertain, using specific inhibitors, the potential role of calcium-related signal transduction pathways in the mechanism of cartilage matrix protein gene induction and metalloproteinase gene suppression by capacitively coupled electric fields.

*Methods*: Articular chondrocytes were isolated from adult bovine patellae and cultured in high density for 7 days. To study matrix protein expression, cells cultured in the absence or presence of specific calcium pathway inhibitors were exposed to a capacitively coupled electrical field (60 kHz, 20 mV/cm): for aggrecan 1 h at 50% duty cycle and for type II collagen 6 h at 8.3% duty cycle. To study metalloproteinase expression in the presence of interleukin 1 beta (IL-1β), cells were cultured as above but exposed for only 30 min to a 100% duty cycle signal. At harvest, total mRNA was isolated and aggrecan, type II collagen, matrix metalloproteinase (MMP-1, -3 and -13) and aggrecanase [a disintegrin and metalloproteinase with thrombospondin repeats (ADAMTS-4 and -5)] mRNA expression were measured by quantitative real-time polymerase chain reaction (qPCR).

*Results*: (1) In the absence of inhibitors, appropriate electrical stimulation induces a 3–4-fold up-regulation of both aggrecan and type II collagen mRNA and a 3.7–9.6-fold down-regulation of IL-1β-induced metalloproteinases; (2) the presence of inhibitors alone does not affect any target mRNA levels; (3) inhibitors of intracellular calcium regulation and inositol 1,4,5-triphosphate ($IP_3$) formation [8-(diethylamino)octyl-3,4,5,-trimethoxybenzoate hydrochloride (TMB-8) and neomycin, respectively] have no effect on regulation of target mRNA levels by electrical stimulation; and (4) inhibitors of voltage-gated calcium channels (verapamil), calmodulin activation (*N*-(6-aminohexyl)-5-chloro-1-naphthalenesulfonamide hydrochloride, W-7), calcineurin activity (cyclosporin A), phospholipase C activity (bromophenacyl bromide, BPB) and prostaglandin $E_2$ ($PGE_2$) synthesis (indomethacin) completely inhibit the effects of electrical stimulation.

*Conclusions*: The results are consistent with the effects of electrical stimulation involving a pathway of extracellular $Ca^{2+}$ influx *via* voltage-gated calcium channels rather than from intracellular $Ca^{2+}$ repositories; and with downstream roles for calmodulin, calcineurin and *n*uclear *f*actor of *a*ctivated T-cells (NF-AT) rather than for phospholipase C and $IP_3$.
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*Key words*: Articular chondrocytes, Electrical stimulation, Signal transduction, Collagen, Aggrecan, Metalloproteinase, Calcium.

## Introduction

Osteoarthritis (OA), a debilitating disease affecting approximately 21 million Americans, is chiefly characterized by a progressive loss of the articular surface of many joints including those of the hips, knees, lower back, neck, thumbs and the distal ends of the fingers[1]. Although the precise etiology of OA is still unknown, it is clear that the defining cartilage matrix destruction involves the active participation of the resident chondrocytes[2] and members of the MMP (matrix metallo*p*roteinase) and ADAMTS (a disintegrin *a*nd metalloproteinase with thrombo*s*pondin repeats) families of proteases[3].

Chondrocytes in articular cartilage exist in a relatively isolated environment since this tissue lacks blood vessels, lymphatic vessels and nerves. As a result, it is the role of mechanical forces during weight bearing and joint movement to provide the synovial fluid flow necessary for chondrocyte nutrition and maintenance. The fact that the cartilage matrix is rich in fixed ionized macromolecules, this fluid flow results in strain- and diffusion-generated electric potentials that are important in cartilage homeostasis[4–7]. Changes to the cartilage matrix that occur in OA result in a loss of the fixed ionic environment and presumably lead to a disruption of the physiologic electrical fields required for normal matrix maintenance. One modality to restore this important signaling system is the provision of a capacitively coupled electric field. The term ''capacitive'' is used since the arrangement of electrodes and targeted cells/tissue resembles a capacitor (an electrical element used to temporarily store a charge) with the tissue/cells in their dielectric medium placed between a pair of electrodes. Stimulation occurs by a transfer of electrical energy from the capacitor to the targeted tissue (coupling) *via* the induced electric field[8]. A schematic diagram

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of the *in vitro* stimulation apparatus is shown in Brighton *et al.*[9].

Recent results from our laboratory have shown that articular cartilage chondrocytes or explants (from normal adult bovine or osteoarthritic human cartilage) exposed *in vitro* to capacitively coupled electric fields respond by increasing aggrecan and type II collagen gene and protein expression[9–11], and by suppressing metalloproteinase gene expression[9]. This response pattern would be appropriate for the treatment of OA in which loss of matrix (especially aggrecan) and increase of metalloproteinase activity are pathognomonic[12,13]. Although it appears that this modality might be suitable, the mechanism(s) by which capacitive coupling leads to gene regulation in chondrocytes remains to be determined.

In a previous study using known signal transduction pathway inhibitors *in vitro*, it was concluded that the transduction of a capacitively coupled electrical signal regulating bone cell proliferation is *via* $Ca^{2+}$ translocation through voltage-gated calcium channels (inhibited by verapamil) resulting in elevated intracellular $Ca^{2+}$ levels and a concomitant increase in phospholipase $A_2$ ($PLA_2$) activity (blocked by bromophenacyl bromide, BPB). The latter increase leads to cyclooxygenase (COX)-dependent prostaglandin $E_2$ ($PGE_2$) synthesis (blocked by indomethacin), and the former increase results in activation of calmodulin (blocked by *N*-(6-aminohexyl)-5-chloro-1-naphthalenesulfonamide hydrochloride, W-7)[14,15].

Articular chondrocytes are also known to possess voltage-gated calcium channels[16]. Moreover, using a specific voltage-gated calcium channel inhibitor (PD-0200347), Boileau *et al.* have shown that chondrocyte metalloproteinase expression is inhibited, and that development of OA is consequently retarded in a canine disease model[17,18]. Recent data have also demonstrated that elevated intracellular $Ca^{2+}$ in chondrocytes can induce chondrogenesis through a calcineurin/NF-AT (*n*uclear *f*actor of *a*ctivated *T*-cells) signaling pathway[19].

The goal of the experiments described in this *in vitro* study was to ascertain, using specific inhibitors, the potential role of calcium-related signal transduction pathways in the mechanism of cartilage matrix protein induction and metalloproteinase suppression by capacitively coupled electric fields in adult bovine articular chondrocytes. The data show that the effects of capacitively coupled electrical stimulation are blocked by verapamil, *N*-(6-aminohexyl)-5-chloro-1-naphthalenesulfonamide hydrochloride (W-7), cyclosporin A, BPB and indomethacin; but not by neomycin or 8-(diethylamino)octyl-3,4,5,-trimethoxybenzoate hydrochloride (TMB-8). This is consistent with a pathway involving an extracellular $Ca^{2+}$ influx through voltage-gated calcium channels rather than from intracellular $Ca^{2+}$ repositories; and with roles for calmodulin, calcineurin and NF-AT rather than for phospholipase C and inositol 1,4,5-triphosphate ($IP_3$).

## Materials and methods

### CHONDROCYTE ISOLATION AND CULTURE

Chondrocytes were isolated by proteolytic digestion of cartilage slices from the articular surface of freshly collected adult (18–30 months) bovine patellas as described previously[10]. Briefly, under aseptic conditions cartilage slices were minced and incubated sequentially at 37°C with 0.04% (w/v) bovine testicular hyaluronidase (Sigma Chemical Corp., St Louis, MO) in 150 ml of calcium- and magnesium-free (CMF) Hanks' balanced salt solution (Life Technologies, Inc.) for 90 min followed by 0.2% trypsin (w/v) (Life Technologies, Inc., Rockville, MD) in the same medium for 30 min. The undigested pieces were allowed to settle before being resuspended in 0.2% bacterial collagenase (Type I-S, Sigma) in 150 ml of α-MEM (Life Technologies, Inc.) containing 5% fetal bovine serum (FBS) and digested overnight at 37°C. The resulting suspension was centrifuged at 500–1000 × *g*, the cell pellet washed three times in serum-free medium and resuspended in serum-containing α-MEM. Aliquots were stained with Trypan Blue for hemocytometer cell counting and viability assessment. We routinely obtained ~$50 \times 10^6$ cells/g wet weight cartilage with a viability >95%. Chondrocytes were plated at a concentration of 500,000 viable cells/$cm^2$ onto the glass coverslip portion of specially modified Cooper dishes, covered with a normal Petri dish top and incubated for 7 days in medium supplemented with 1% FBS and 50 μg sodium ascorbate/ml. Under these conditions, histologic examination showed individual chondrocytes or pairs of chondrocytes embedded in a toluidine blue-stained matrix layer 2–3 cells thick (unpublished results cited in Ref. 10). All experiments were performed using primary cultures[10,20].

### CAPACITIVELY COUPLED ELECTRICAL STIMULATION

Figure 1 shows a schematic representation of the experimental design. On the day of stimulation, the medium was changed and appropriate additions (cytokine and/or inhibitor) were made to the cultures 2 h prior to initiation of electrical stimulation. All cultures that were used to assess metalloproteinase expression were grown in either the absence or presence of 10 ng/ml interleukin 1 beta (IL-1β, R&D Systems, Minneapolis, MN), and all cultures used to assess the signal transduction pathways were incubated in the absence or presence of various specific inhibitors including verapamil (Sigma, St. Louis, MO) to block voltage-gated calcium channels[21,22], neomycin (Pharma-Tek, Huntington, NY) to block phospholipase C-mediated hydrolysis of phosphatidylinositol 4,5-bisphosphate ($PIP_2$)[23], 4′-BPB (Sigma) to block $PLA_2$ activity[24], TMB-8 (Sigma) to block calcium release from intracellular stores[25], indomethacin (Sigma) to block prostaglandin (PG) synthesis from arachidonic acid (AA) *via* COXs[26,27], *N*-(6-aminohexyl)-5-chloro-1-naphthalenesulfonamide hydrochloride (W-7, Sigma) to antagonize calmodulin interactions[28] and cyclosporin A (Sigma) to block calmodulin-dependent calcineurin-mediated dephosphorylation of the $IP_3$ receptor as well as signaling by NF-AT[29,30]. Figure 2 shows a schematic diagram of the pathway steps known to be inhibited by these compounds. The concentrations of inhibitors used in this study were 40 μM verapamil, 30 μM neomycin, 25 μM BPB, 150 μM TMB-8, 20 μg/ml indomethacin, 4 μM W-7 and 40 μg/ml cyclosporin A. In separate experiments (not shown), these concentrations were determined to be the highest that were not cytotoxic to adult bovine articular chondrocytes. Although we cannot rule out other non-specific effects of these inhibitors, it is important to note that in the experiments performed, the mRNA levels of the cartilage matrix molecules measured were virtually unchanged in the presence of the various inhibitors at the concentrations used (compare *white bars* in Figs. 3–5).

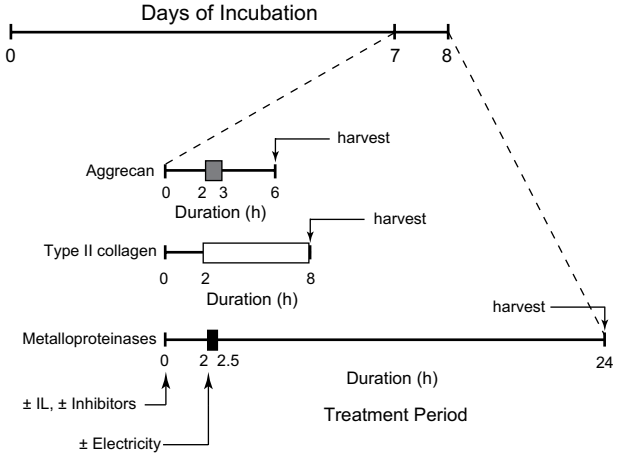



Fig. 1. Experimental design showing the protocol followed for all experiments. All cultures were grown for 7 days and were treated on the 8th day. For aggrecan and collagen experiments, cells were incubated in the absence or presence of signal transduction pathway inhibitors for 2 h prior to initiation of electrical stimulation at 60 kHz and 20 mV/cm (1 h at 50% duty cycle for aggrecan, *gray box*, and 6 h at 8.3% duty cycle for type II collagen, *white box*); samples were harvested 4 h and 6 h after initiation of electrical stimulation, respectively. For metalloproteinase experiments, cells were incubated in the absence or presence of signal transduction pathway inhibitors and/or IL-1β for 2 h prior to initiation of electrical stimulation (30 min at 100% duty cycle, *black box*); samples were harvested 22 h after initiation of electrical stimulation.

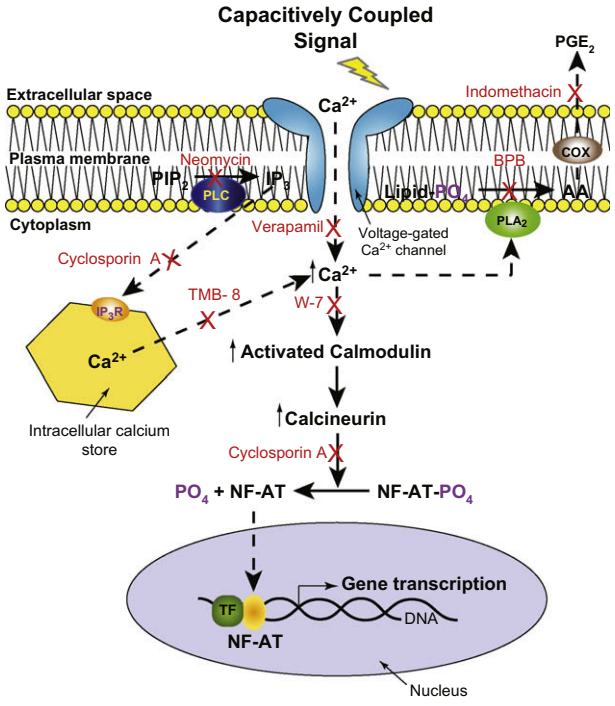



Fig. 2. Schematic diagram of relevant calcium signal transduction pathways showing the steps inhibited (indicated by a red ×) by the specific compounds tested. The main pathway of interest involves the opening of voltage-gated calcium channels (blocked by verapamil) by electrical stimulation, allowing the entry of extracellular $Ca^{2+}$ resulting in elevated cytoplasmic $Ca^{2+}$ levels. This results in activation of calmodulin (blocked by W-7) and the subsequent activation of calcineurin which dephosphorylates NF-AT (blocked by cyclosporin A). NF-AT then translocates to the nucleus where it acts in conjunction with other associated proteins as transcription factors. Another calcium-related pathway involves the conversion of $PIP_2$ to $IP_3$ by the action of phospholipase C (blocked by neomycin) and the $IP_3$-induced release of intracellular $Ca^{2+}$ stores to the cytoplasm (blocked by cyclosporin A and TMB-8). An increase in intracellular $Ca^{2+}$ can also increase in $PLA_2$ activity (blocked by BPB) leading to $PGE_2$ synthesis (blocked by indomethacin). $IP_3R$ = inositol 1,4,5-triphosphate receptor, TF = any one or more generic transcription factors associated with NF-AT (Diagram adapted from Ref. 15).

The unique *in vitro* capacitively coupled electrical stimulation used in this study has been described in detail elsewhere[9,11,15,20]. Electrodes are placed in contact with quartz coverslips on the top and bottom of the culture dish and a uniform field is generated in the medium and cells between the electrodes. To study aggrecan expression, a capacitively coupled 20 mV/cm electric field at a frequency of 60 kHz was applied for 1 h at a 50% duty cycle (1 min on, 1 min off) and samples were harvested 4 h after initiation of stimulation; to study type II collagen expression, a capacitively coupled 20 mV/cm electric field at a frequency of 60 kHz was applied for 6 h at an 8.3% duty cycle (1 min on, 11 min off) and samples were harvested 6 h after initiation of stimulation; to study metalloproteinase expression, a capacitively coupled 20 mV/cm electric field at a frequency of 60 kHz was applied continuously (100% duty cycle) for 30 min and samples were harvested 22 h after initiation of stimulation; these experimental conditions were determined in previous studies[9,10]. Electrical control cultures were treated identically except that electrodes were not connected. At the end of the experiment, each culture was harvested in 2 ml Trizol Reagent (Life Technologies, Inc., Rockville, MD) and frozen at −80°C prior to RNA isolation and analysis as described below.

### RNA ISOLATION AND QUANTITATIVE REAL-TIME POLYMERASE CHAIN REACTION (qPCR)

Total RNA was isolated and characterized as described previously[31]. Briefly, RNA was purified using elements of a one-step method combined with a column purification step (RNeasy Total RNA Kit, Qiagen, Inc., Valencia, CA) and subsequently digested with DNase I (DNA-free kit, Ambion, Austin, TX)[32].

Reverse transcription (RT) followed by qPCR (two-step) was performed as described previously[10,31]. Oligonucleotide primers for bovine aggrecan, type II collagen, MMP-1, -3 and -13, aggrecanase-1 and -2 (ADAMTS-4 and -5) and glyceraldehyde phosphate dehydrogenase (GAPDH, reference gene) were based on database sequences and were designed using Primer-Express 2.0 (Applied Biosystems, Foster City, CA) software. To further ensure that the qPCR signal was generated from cDNA (as opposed to genomic DNA), primer pairs were placed in different exons or across an intron/exon boundary wherever possible. All primer pairs had melting temperatures of 58–60°C and yielded products 73–127 bp (Table I); primers used for bovine aggrecan, type II collagen and GAPDH have been described previously[10]. To verify that the expected products with respect to size and homogeneity were obtained, melting profiles of qPCR products were acquired after completion of the amplification process (Applied Biosystems), and aliquots were analyzed by electrophoresis on 2% agarose gels. Relative quantification of mRNA expression for each of the target genes was accomplished by normalizing to a housekeeping gene as described previously; GAPDH was chosen because its expression did not vary appreciably with electrical stimulation and/or inhibitor addition in these cells[10]. For each experiment, corrected $\Delta C_T$ values were obtained for control (untreated and unstimulated) cultures and experimental (treated and/or stimulated) cultures whose RNA was isolated at the same time, reverse transcribed and analyzed by qPCR; for comparison within and between experiments, these values were expressed as the ratio of Treated to Control.

### STATISTICAL ANALYSIS

The data obtained from treated samples were compared to corresponding untreated groups from the same experiment as described above. In all RT-qPCR experiments, aliquots of samples were analyzed in technical duplicates or triplicates ($n = 1$); each reported value (unless stated otherwise) represents separate RNA isolations and RT reactions from at least two samples ($n \geq 2$) and analyzed as described above. For gene expression, the ratios of Treated/Control samples were reported as the mean ± standard deviation. The means among groups were compared by a one-way analysis of variance and the Tukey–Kramer multiple comparison test for considerable differences between groups using a graphing software package (KaleidaGraph 4.0, Synergy Software, Reading, PA); statistical significance was considered to be $P \leq 0.05$.

## Results

### RESPONSE OF ELECTRICALLY STIMULATED AGGRECAN AND TYPE II COLLAGEN EXPRESSION TO SIGNAL TRANSDUCTION INHIBITORS

Adult bovine articular chondrocytes were cultured in modified Cooper dishes and subjected to capacitive coupled stimulation in the absence or presence of specific signal transduction pathway inhibitors using the optimal signals for aggrecan and type II collagen up-regulation as previously described[10]. The results (Fig. 3) show that: (1) in the absence of inhibitors, electrical stimulation induces a significant 3–4-fold up-regulation of both aggrecan and type II collagen mRNA; (2) the presence of inhibitors alone does not affect aggrecan or type II collagen mRNA levels; (3) inhibitors of intracellular calcium regulation and $IP_3$ formation (TMB-8 and neomycin, respectively) have no effect on the up-regulation of mRNA levels by electrical stimulation; and (4) inhibitors of voltage-gated calcium channels (verapamil), calmodulin activation (W-7), calcineurin activity (cyclosporin A), phospholipase C activity (BPB) and $PGE_2$ synthesis (indomethacin) completely inhibit the up-regulating effects of electrical stimulation.

### RESPONSE OF METALLOPROTEINASES EXPRESSION TO INTERLEUKIN

In the absence of IL-1β treatment, metalloproteinase mRNA levels in adult bovine articular chondrocytes were near the lower limits of accurate detection by RT-qPCR (see Figs. 4 and 5, *None*). However, in the presence of 10 ng/ml IL-1β, relative expression levels increased

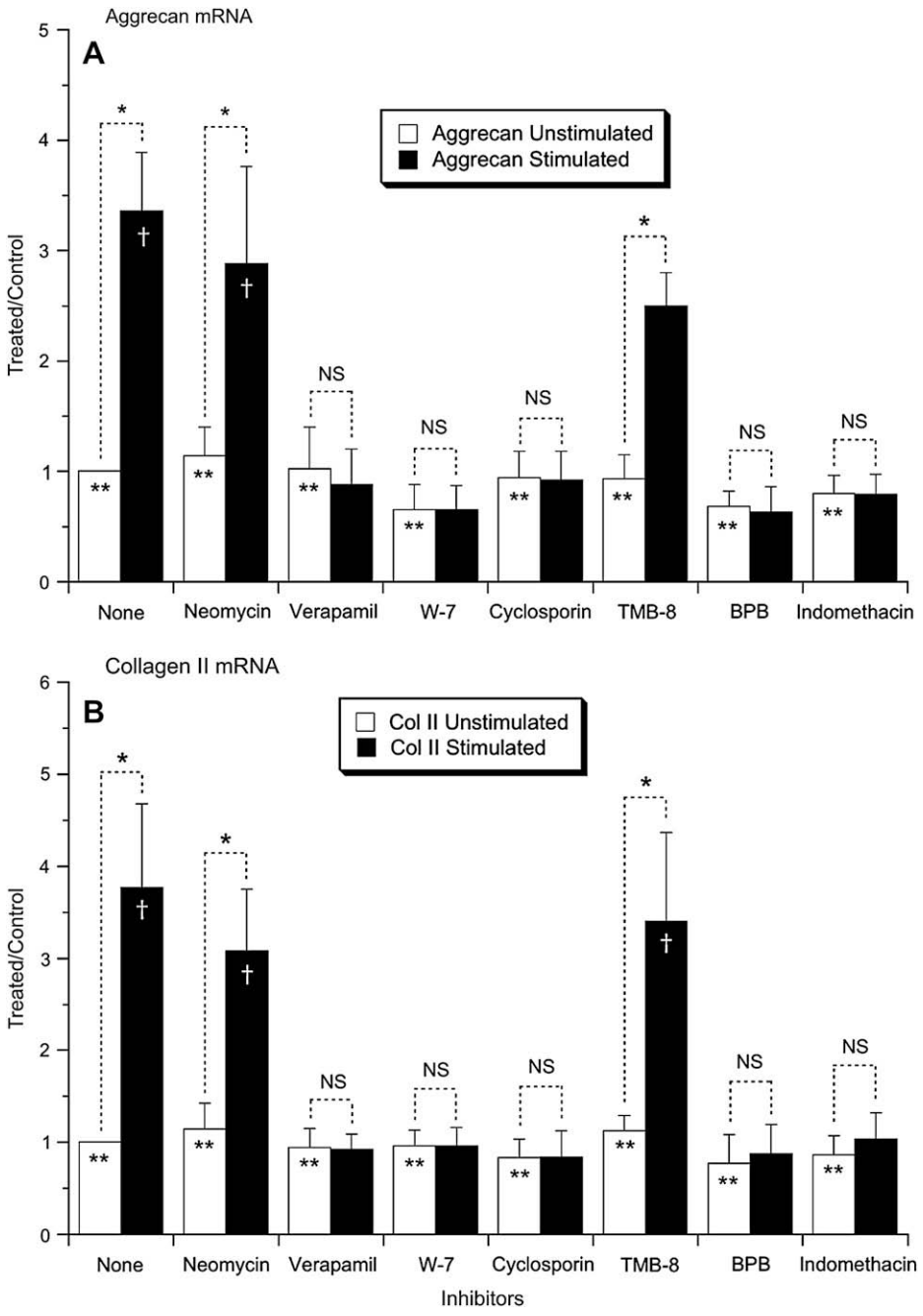
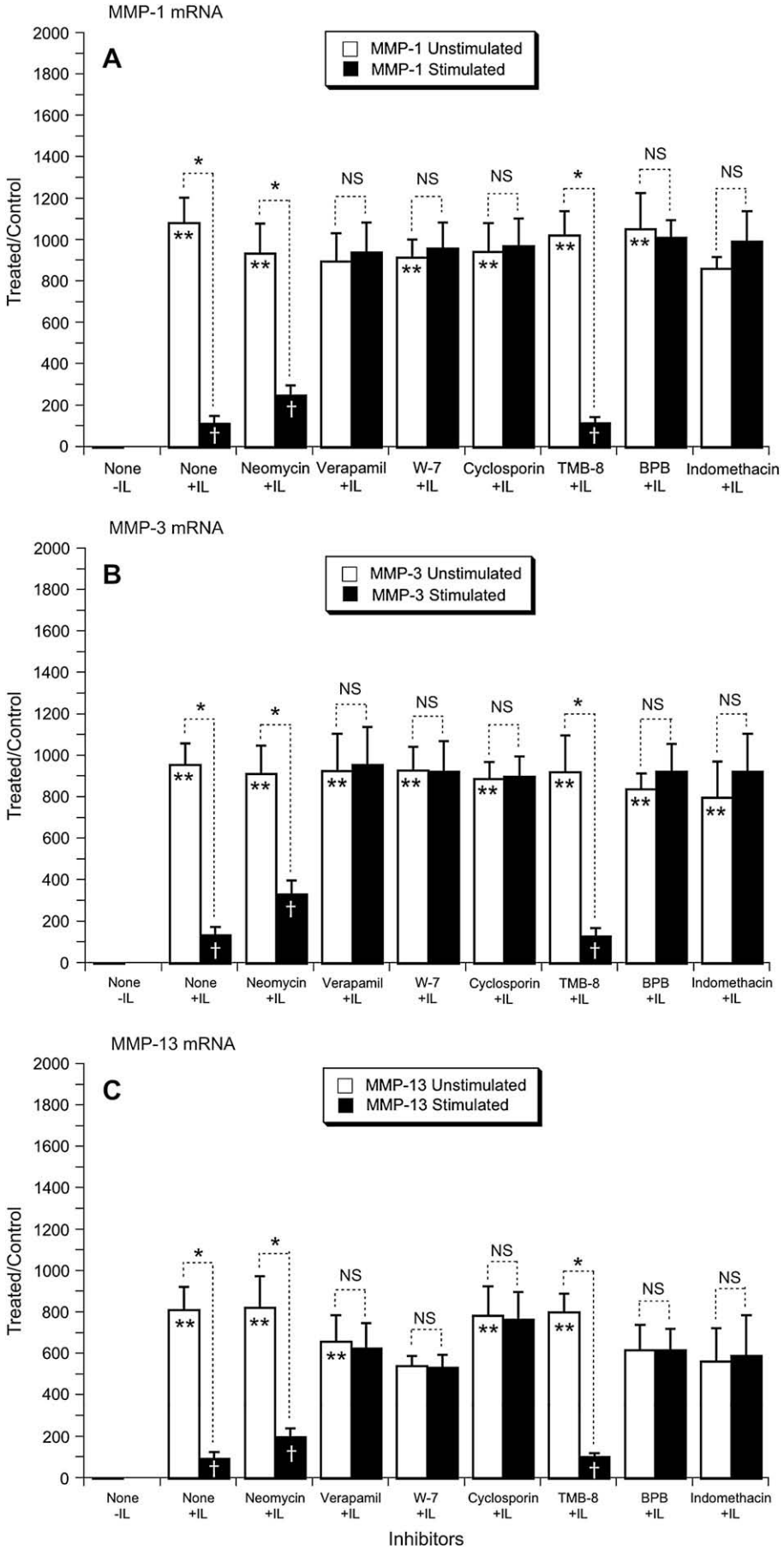



Fig. 3. Effect of signal transduction inhibitors on the regulation of aggrecan and type II collagen mRNA levels by electrical stimulation. For the groups labeled None, Neomycin, Verapamil and Cyclosporin, $n = 12$; for all other groups, $n = 6$. (A) Aggrecan expression. * denotes $P < 0.0001$, NS = not significant ($P > 0.99$); ** denotes unstimulated groups not significantly different from each other ($P > 0.69$); † denotes stimulated groups not significantly different from each other ($P = 0.24$). (B) Type II collagen expression. * denotes $P < 0.0001$, NS = not significant ($P = 1$); ** denotes unstimulated groups not significantly different from each other ($P > 0.99$); † denotes stimulated groups not significantly different from each other ($P > 0.10$).

Fig. 4. Effect of IL-1β and/or signal transduction inhibitors on the regulation of MMP mRNA levels by electrical stimulation. For the groups labeled None, Neomycin, Verapamil and Cyclosporin, $n = 12$; for all other groups, $n = 6$. (A) MMP-1 expression. * denotes $P < 0.0001$, NS = not significant ($P > 0.99$); ** denotes unstimulated groups not significantly different from each other ($P > 0.22$); † denotes stimulated groups not significantly different from each other ($P > 0.55$). (B) MMP-3 expression. * denotes $P < 0.0001$, NS = not significant ($P > 0.99$); ** denotes unstimulated groups not significantly different from each other ($P > 0.47$); † denotes stimulated groups not significantly different from each other ($P > 0.12$). (C) MMP-13 expression. * denotes $P < 0.0001$, NS = not significant ($P > 0.99$); ** denotes unstimulated groups not significantly different from each other ($P > 0.07$); † denotes stimulated groups not significantly different from each other ($P > 0.89$).


MMP-1 mRNA
A
MMP-1 Unstimulated
MMP-1 Stimulated
Treated/Control
2000
1800
1600
1400
1200
1000
800
600
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200
0
None -IL
None +IL
Neomycin +IL
Verapamil +IL
W-7 +IL
Cyclosporin +IL
TMB-8 +IL
BPB +IL
Indomethacin +IL
MMP-3 mRNA
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MMP-3 Unstimulated
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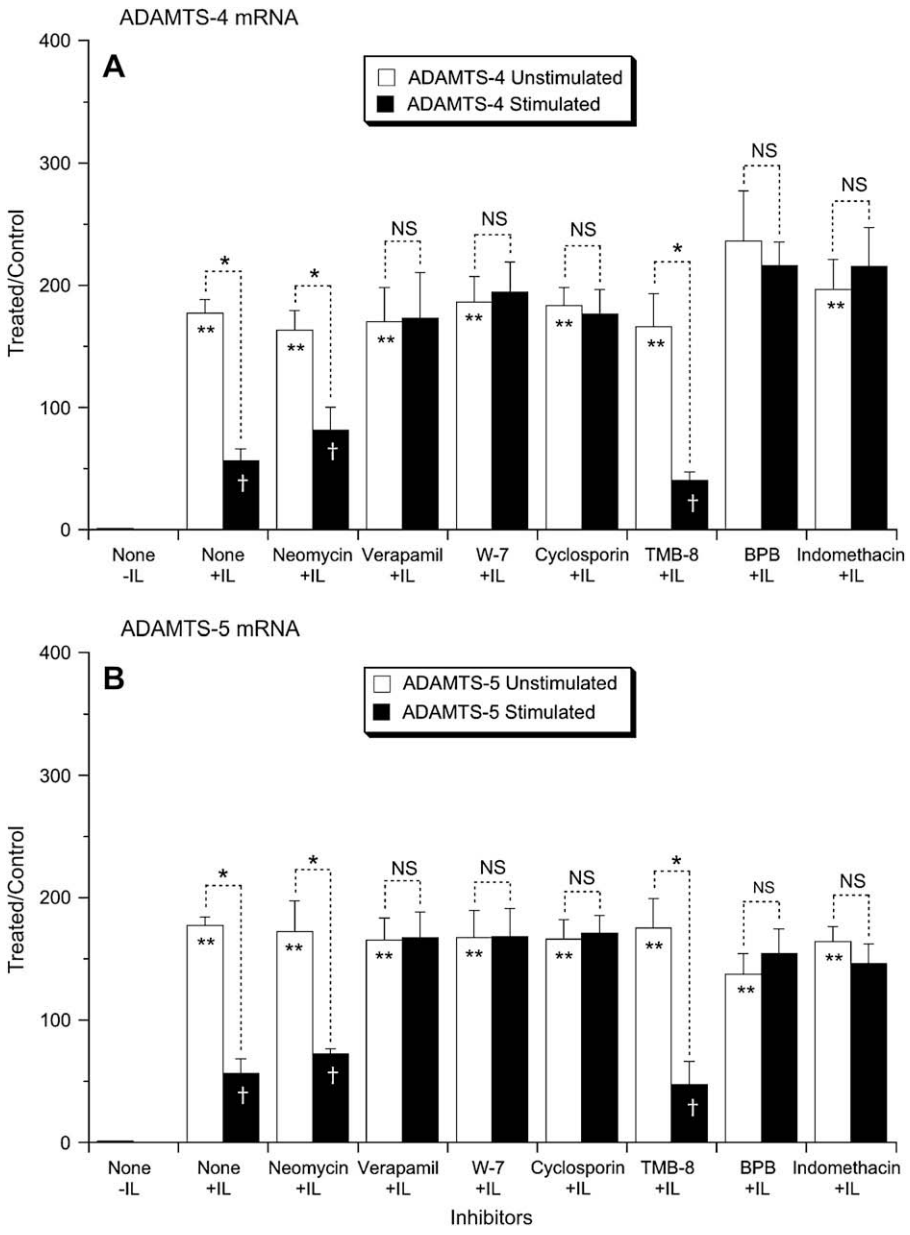


Fig. 5. Effect of IL-1β and/or signal transduction inhibitors on the regulation of ADAMTS mRNA levels by electrical stimulation. For the groups labeled None, Neomycin, Verapamil and Cyclosporin, $n = 12$; for all other groups, $n = 6$. (A) ADAMTS-4 expression. * denotes $P < 0.0001$, NS = not significant ($P > 0.98$); ** denotes unstimulated groups not significantly different from each other ($P > 0.94$); † denotes stimulated groups not significantly different from each other ($P > 0.69$). (B) ADAMTS-5 expression. * denotes $P < 0.0001$, NS = not significant ($P > 0.91$); ** denotes unstimulated groups not significantly different from each other ($P > 0.95$); † denotes stimulated groups not significantly different from each other ($P = 0.82$).

68–676-fold (Table II). For this reason, the experiments involving measurement of metalloproteinase mRNA levels were performed in the presence of the cytokine.

### RESPONSE OF ELECTRICALLY STIMULATED MMP EXPRESSION TO SIGNAL TRANSDUCTION INHIBITORS

The experimental protocol used was identical to that described above for aggrecan and collagen except for the addition of IL-1β to the cultures. In the presence of interleukin, the expression of MMP-1, -3 and -13 was then assessed in the absence or presence of specific signal transduction pathway inhibitors using the optimal signal for MMP down-regulation as previously described[9]. The results (Fig. 4) clearly show that: (1) in the absence of inhibitors, electrical stimulation induces a significant 9.6, 7.1 or 8.6-fold down-regulation of MMP-1, -3 and -13, respectively; (2) the presence of inhibitors alone does not affect MMP mRNA levels; (3) inhibitors of intracellular calcium regulation and $IP_3$ formation (TMB-8 and neomycin, respectively) have no or little effect on the down-regulation of MMP mRNA levels by electrical stimulation; and (4) inhibitors of voltage-gated calcium channels (verapamil), calmodulin activation (W-7), calcineurin activity (cyclosporin A), phospholipase C activity (BPB) and $PGE_2$ synthesis (indomethacin) completely inhibit the down-regulating effects of electrical stimulation.

Table I
*Oligonucleotide primers used for qPCR of bovine metalloproteinases*

| Gene | Genbank number | Nucleotide sequence (5′ → 3′)* | Amplicon size (bp) | Efficiency† (%) |
|---|---|---|---|---|
| MMP-1 | X58256 | 5′-CAAGATGTGGAGACGGTGAAGA-3′<br>5′-CGTTTCTCTGCCTTTCAACTTTC-3′ | 85 | 99 |
| MMP-3 | AB043995 | 5′-GGAAAACTCACCTCACGTACAGAA-3′<br>5′-CAGAGCTTTTTCAATGGCAGAAT-3′ | 86 | 100 |
| MMP-13 | AF072685 | 5′-GGATTCAGGGAAGACGCTCTT-3′<br>5′-TCTCGGGTAGTCTTTATCCATCACA-3′ | 88 | 98 |
| ADAMTS-4 | AF192770 | 5′-TGCGCCATTGTGGAGGAT-3′<br>5′-CGAGTTGTCATGGAGCATGCT-3′ | 90 | 93 |
| ADAMTS-5 | AF192771 | 5′-TCAGCCACCATCACAGAATTTC-3′<br>5′-CGGGAATCTGTTTTCGTGGTA-3′ | 76 | 100 |

*Forward (sense) primer is listed first.
†Efficiency of amplification, calculated as described by Ginzinger[67].

RESPONSE OF ELECTRICALLY STIMULATED AGGRECANASE EXPRESSION TO SIGNAL TRANSDUCTION INHIBITORS

The experimental protocol used was identical to that described above for MMPs. The results (Fig. 5) clearly show that: (1) in the absence of inhibitors, electrical stimulation induces a significant 3.7-fold down-regulation of ADAMTS-4 and -2 mRNA; (2) the presence of inhibitors does not affect ADAMTS-5 mRNA levels; (3) inhibitors of intracellular calcium regulation and $IP_3$ formation (TMB-8 and neomycin, respectively) have no or little effect on the down-regulation of ADAMTS mRNA levels by electrical stimulation; and (4) inhibitors of voltage-gated calcium channels (verapamil), calmodulin activation (W-7), calcineurin activity (cyclosporin A), phospholipase C activity (BPB) and $PGE_2$ synthesis (indomethacin) completely inhibit the down-regulating effects of electrical stimulation.

## Discussion

Articular cartilage chondrocytes *in vitro* respond to a variety of chemical and physical signals including electricity[4,33–40]. Recent studies from our laboratory have investigated the effects of capacitively coupled electrical stimulation on adult bovine articular chondrocytes in mass culture and in cartilage explants[10,11], and on human osteoarthritic cartilage explants[9]. We chose to use the mass culture format rather than cartilage explants primarily because of our concern about the ability of the various metabolic inhibitors to quickly and effectively penetrate intact cartilage. Our experiments showed that articular cartilage chondrocytes respond by increasing aggrecan and type II collagen expression at both the transcriptional and translational levels (in the absence or presence of IL-1β), and by decreasing IL-1β-induced metalloproteinase expression at the transcription level (we have not yet measured metalloproteinase activity and/or protein levels). Notwithstanding the above observations, however, the signal transduction pathways by which capacitively coupled electrical stimulation affects chondrocyte gene expression had not been previously elucidated.

A common cellular signaling pathway involves a $Ca^{2+}$/calmodulin-dependent phosphatase (calcineurin) that is responsible for the dephosphorylation of the cytoplasmic form of the transcription factor NF-AT. This results in the translocation of NF-AT to the nucleus where it binds cooperatively with other transcription factors to the regulatory regions of inducible genes (see Fig. 2)[41]. In the experiments described here, it was clearly shown that in adult bovine articular chondrocytes *in vitro*, inhibitors of voltage-gated calcium channels (verapamil), calmodulin activation (W-7), calcineurin activity (cyclosporin A), phospholipase C activity (BPB) and $PGE_2$ synthesis (indomethacin) completely block both the electrically induced up-regulation of aggrecan and type II collagen expression, and the down-regulation of IL-1β-induced metalloproteinase expression. In contrast, inhibitors of intracellular calcium regulation and $IP_3$ formation (TMB-8 and neomycin, respectively) have no or little influence on the above electrical effects. Thus, a flux of calcium *via* voltage-gated channels is implicated in transducing the capacitively coupled electrical signal in these cells (see Fig. 2). Indeed, it has recently been shown that a specific inhibitor of the voltage-gated calcium channel reduced the expression of MMP-1, -3 and -13 in osteoarthritic canine chondrocytes[18]. This effect could be a result of reduced activation of transcription factors (e.g., Elk-1 *via* protein kinase C and the extracellular signal regulated protein kinase) necessary for MMP transcription[17]. Since voltage-gated ion channels are the key membrane proteins that mediate voltage-evoked cell signaling[42], it should not be surprising that they are implicated in electrical stimulation events. Also, the role of $Ca^{2+}$ as an intracellular messenger leading to gene transcription is amply documented[43]. Nevertheless, it still remains to be determined what are the subsequent downstream pathways ultimately leading to a direct regulation of matrix and metalloproteinase expression at the transcriptional level.

Table II
*Relative mRNA abundance of metalloproteinases (MPs) in adult bovine articular chondrocytes in the absence or presence of IL-1β*

| Enzyme | MP/GAPDH (−IL-1β)* | MP/GAPDH (+IL-1β)* | Fold induction |
|---|---|---|---|
| MMP-1 | $0.0044 \pm 0.0021$ | $1.78 \pm 0.92$ | $398 \pm 36$ |
| MMP-3 | $0.0009 \pm 0.0005$ | $0.26 \pm 0.14$ | $305 \pm 35$ |
| MMP-13 | $0.0005 \pm 0.0004$ | $0.34 \pm 0.26$ | $676 \pm 102$ |
| ADAMTS-4 | $0.0005 \pm 0.0003$ | $0.07 \pm 0.04$ | $132 \pm 17$ |
| ADAMTS-5 | $0.0017 \pm 0.0012$ | $0.12 \pm 0.08$ | $68 \pm 2$ |

*Cells were incubated for 24 h in the absence (−) or presence (+) of 10 ng/ml IL-1β and isolated total RNA was subjected to RT-qPCR ($n = 12$). Relative values for MP mRNA levels were normalized to GAPDH mRNA levels.

Although it has been reported that 10 μM cyclosporin A inhibited IL-1β-induced MMP-13 and ADAMTS-4 and -5—but not MMP-1 and -3—expression[44], whereas ≤0.8 μM inhibited MMP-1 and -3 and ADAMTS-4—but not MMP-13 and ADAMTS-5—expression in chondrocytes[45], our data (Figs. 4 and 5) did not show any inhibitory effect of 33 μM cyclosporin A alone on any of these enzymes. The reason for these disparate results cannot yet be explained. Nevertheless, Little *et al.*[44] found that preincubation of bovine articular chondrocytes with 10 mM cyclosporin A did not inhibit NF-AT activation.

The observed effects of electrical stimulation on chondrocytes—up-regulation of matrix genes and down-regulation of metalloproteinase genes—may not operate directly through the calcium-induced transcription pathway discussed above since NF-AT is usually involved in the up-regulation (not down-regulation) of gene transcription[46]. The transcription results are, however, consistent with the actions of TGF-β, a multipotent regulator of extracellular matrix production. Specifically, TGF-β has been shown to impart a protective role in articular cartilage by counteracting the effects of IL-1[47,48]. Thus, it could be postulated that the electrically induced intracellular calcium cascade would result in the NF-AT-associated up-regulation of TGF-β expression that would subsequently act on the chondrocyte in an autocrine/paracrine fashion to counteract the IL-1β-induced down-regulation of aggrecan and type II collagen, and up-regulation of metalloproteinases. It is relevant to note that, at least in some cells, expression of TGF-β1—among other cytokines—is NF-AT-regulated[49]. Although we have not yet tested for such a scenario in our electrically stimulated chondrocyte cultures, it has been shown by others that PEMF can up-regulate TGF-β in chondrogenic models *in vitro*[50] and *in vivo*[51,52], and that ultrasound-induced higher expression of collagen and aggrecan in chondrocytes is also mediated *via* elevated TGF-β1 expression[53]. In addition, our laboratory has shown previously that capacitively coupled electrical stimulation of bone cells induces TGF-β1 expression *in vitro*[54]. This could explain the roles of calmodulin, calcineurin and NF-AT.

At the same time, our results showed that the presence of BPB and indomethacin, compounds that ultimately control PG synthesis, could also inhibit the effects of electrical stimulation. Thus, whatever the transduction pathways may be, it is apparent that the calmodulin–calcineurin–NF-AT pathway and the $PLA_2$–COX–PG pathway must be co-dependent. It is interesting to note that $PGE_2$, for example, can modulate IL-1β-induced matrix protein and MMP expression in chondrocytes[55,56].

It has also been shown that a number of cytokines, including IL-4, IL-10 and IL-13, are considered anti-catabolic/anti-inflammatory by functioning as down-regulators of IL-1β and MMP expression and up-regulators of IL-1 receptor antagonist expression and $PGE_2$ production among other effects[57–59]. Therefore, in our proposed signaling pathway, such molecules could participate in the anti-catabolic events observed in the presence of electrical stimulation, but these cytokines have not yet been shown to possess the associated anabolic properties (e.g., up-regulation of aggrecan and type II collagen) as TGF-β does.

Chondrocytes contain a number of other relevant signaling pathways—in addition to those discussed above—including the non-canonical WNT[60], nuclear factor-kappa B, mitogen-activated protein kinase and Smad pathways[61,62]; and several of these pathways have been implicated in arthritic diseases[63–66]. It remains to be determined which of these pathways, if any, directly participate in the transduction of capacitively coupled electrical signals. Future experiments will be directed toward this end.

## Conflict of interest

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