ION-CHANNEL DEPENDENT REGULATION OF CHONDROCYTE MATRIX SYNTHESIS IN 3D CULTURE UNDER DYNAMIC AND STATIC COMPRESSION

Janna K Mouw, Stacy M Imler, Marc E Levenston

Parker H. Petit Institute for Bioengineering and Bioscience
George W. Woodruff School of Mechanical Engineering
Georgia Institute of Technology
Atlanta, GA

INTRODUCTION

Mechanical stimulation plays an important role in the maintenance of healthy articular cartilage. In vitro studies have demonstrated that dynamic compression stimulates proteoglycan and protein synthesis, while static compression inhibits matrix synthesis [1]. The mechanisms by which chondrocytes sense the changes in their local mechanical environments produced by these macroscopic stimuli are less clear. Studies with articular chondrocytes in monolayer suggest that mechanosensitive ion channels may participate in chondrocyte mechanotransduction. For example, stretch-activated channels have been suggested to participate in the control of chondrocyte proliferation. Also, blocking the Ca^{2+}-senstitive K^{+} have been demonstrated to have an effect on both proliferation and mRNA level of certain proteins [2]. However, important differences exist between chondrocyte behavior in monolayer and three dimensional cultures. Chondrocytes have been found to dedifferentiate in monolayer, while culture in agarose has been shown to preserve both the morphology and phenotype of chondrocytes. Consequently, the extent to which these same ion channels are involved in the transduction of mechanical stimuli in three-dimensional culture is unclear. The goals of this study were a) to investigate the dose-dependent inhibition of protein and proteoglycan synthesis by four ion-channel inhibitors in both cartilage explants and chondrocytes seeded in agarose, b) to investigate the response of both the explants and gels to static compression when cultured with each of these inhibitors and c) to investigate the response of the gels to dynamic compression when cultured with each of these inhibitors.

METHODS

Sample preparation Articular cartilage cores (4mm diameter) were obtained from immature (2-4 week) bovine stifle joints and trimmed to either 2 or 3 mm thickness. Cores were precultured in basal media (DMEM +10% FBS, NEAA, 50?g/ml ascorbate and 0.4mM proline) for 4 days prior to addition of any ion channel inhibitors. Chondrocytes were isolated via collagenase digestion and seeded at 10 x 10^6 cells/ml in 2% agarose gels (4mm diameter, 2 or 3mm thick). Gels were precultured for 4 weeks in basal media prior to the addition of any ion channel inhibitors. **Inhibitors** We examined the effects of four ion channel inhibitors: Tetrodotoxin (TTX), a sodium channel blocker; Gadolinium (Gad), a stretch-activated channel blocker; 4-Aminopyridine (4AP), a potassium channel blocker; and Nifedipine (Nif), a calcium channel blocker. **Radiolabel incorporation** Media were supplemented with 20?Ci/ml L-5-^{3}H-proline and 10?Ci/ml ^{35}S-sodium sulfate for the final 20 hours of each culture period to measure protein and proteoglycan synthesis, respectively. **Explant response** ***Dose-response*** Cartilage explants (3mm thick, n=6-8/condition) or gels (2mm thick, n=6-8/condition) were cultured for two hours in basal media alone or supplemented with various levels of the four ion-channel inhibitors (Table 1), followed by 20 hours in media containing both inhibitors and radiolabeled precursors. ***Static compression*** Cartilage explants or gels (2mm thick, n=6-8/condition) were cultured in basal media (without radiolabel supplementation) and a single level of each ion-channel inhibitor (Table 1) for two hours prior to loading. Samples were then compressed by 0%, 25% and 50% of the original 2mm cut thickness and cultured for 20 hours under static compression in media supplemented with both an inhibitor and radiolabeled precursors. Additional free-swelling (FS) groups were maintained uncompressed in 48-well plates. ***Dynamic compression*** Gels (3mm thick, n=6-8/condition) were cultured in basal media (without radiolabel supplementation) and the a single level of each ion-channel inhibitor (Table 1) for two hours prior to loading. Gels were then cultured in basal media supplemented with both an inhibitor and radiolabeled precursors under the following conditions: static compression (10% of 3mm thickness), 1Hz oscillatory compression (10%±3%) and FS. **Analysis** Samples were lyophilized, digested with Proteinase K and assayed for DNA, ^{3}H and ^{35}S content. Data were analyzed using General Linear Models ($p<0.05$) and Tukey's test for post-hoc analyses.

RESULTS

Graphs are shown only for the gels as trends were generally consistent for the cartilage explants. Data are grouped and discussed by experiment type. All comparisons are made with non-toxin controls unless specifically mentioned. **Dose response** At the highest (10mM) level of 4AP, protein synthesis in gels (but not tissue) was higher than in controls (Fig. 1a). At the highest level of 4AP, proteoglycan synthesis was signficiantly lower than at the other two 4AP for both tissue and gels (Fig. 1b), although the reduction from controls was not significant for tissue. At the highest (100 μM) level of Nif, protein synthesis in tissue was significantly lower than in controls. No significant changes in synthesis were induced by TTX or Gad at any level tested. **Static compression** For all toxin and control conditions, proline and sulfate incorporation were significantly inhibited under 50% compression compared to 0% and 25% compression conditions. At each compression level, 100 μM Nif (but no other toxin) further inhibited proline incorporation in both explants and gels (Fig. 2a) and further inhibited sulfate incorporation in gels (Fig. 2b) but not in tissue. **Dynamic compression** For all groups except tissue and gels cultured with 100 μM Gad, dynamic compression stimulated proline incorporation compared to the 10% static offset control (Fig. 3a). As in the static compression experiment, 100 μM Nif inhibited proline incorporation for all groups (Fig. 3a). Dynamic compression stimulated sulfate incorporation in controls and all toxin groups (Fig. 3b), with no apparent effect on sulfate incorporation by any of the toxins.

DISCUSSION

Mechanical compression alters many aspects of the chondrocyte's local environment, including cellular deformation, hydrostatic fluid pressure, osmotic pressure, fluid flow and streaming potentials. The chondrocytes have been shown to respond to macroscopic compression with changes increase in aggrecan and collagen II gene and protein expression. However, the mechanisms linking alterations in the cellular environment to specific changes in cell behavior are not well understood. Mechanosensitive ion channels have been hypothesized to have a role in the biosynthetic response of chondrocytes to mechanical stress, based in part on the observed effects of ion channel inhibitors on chondrocytes in monolayer. This study examined whether blocking specific ion channels modifes the cellular response to compression in tissue explants and three dimensional culture.

The treatment levels in this study included toxin levels above and below those typically used in previous monolayer studies. Except for the highest levels of 4AP and Nif, no compression-independent changes in matrix synthesis were observed. While Nif had an additional inhibitory effect on protein synthesis, none of the inhibitors appeared to markedly increase or decrease the relative inhibition by static compression. Interestingly, the stretch activated ion channel inhibitor Gad appeared to inhibit the stimulation of protein synthesis by dynamic compression. This is consistent with previous reports of the inhibitory effects of Gad on the chondrocyte response to cyclic stretch [2] and intermittent hydrostatic pressure [3]. Ongoing studies will examine the effects of ion channel inhibitors on gene expression and the effects of even higher levels of these toxins.

Toxin	Dose-response			Static Compression	Dynamic Compression
	Lower	Middle	Upper		
TTX	0.1	1	10	10	10
Gad	1	10	100	100	100
4AP	100	1000	10000	1000	1000
Nif	1	10	100	100	100

Table 1. Ion-channel inhibitor concentrations (?M)

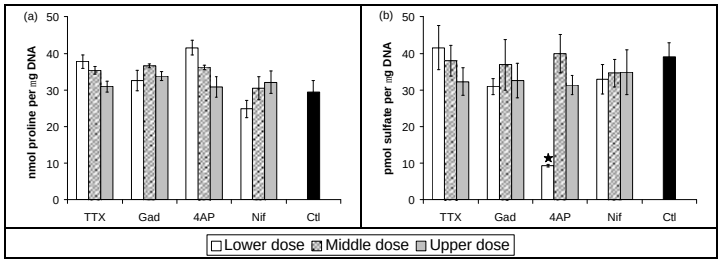

Figure 1. Toxin dose responses

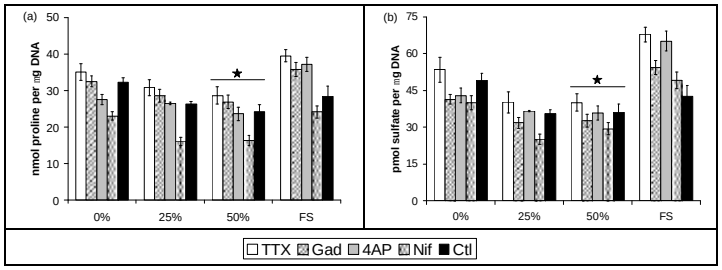

Figure 2. Static compression

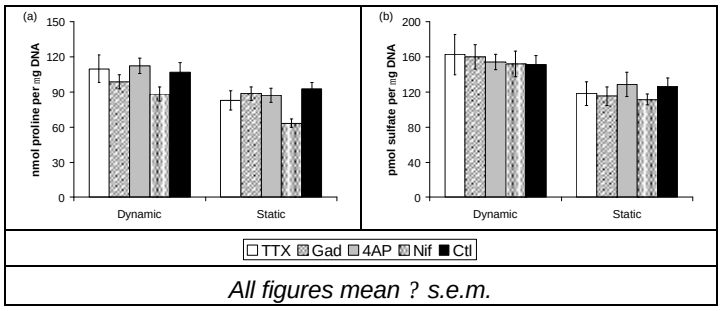

Figure 3. Dynamic compression

ACKNOWLEDGEMENTS

This work was supported by the GTEC ERC (NSF award EEC-9731643) and NSF graduate fellowships for JKM and SMI.

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