

Oscillating fluid flow regulates cytosolic calcium concentration in bovine articular chondrocytes[☆]

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Accepted 3 July 2000

Abstract

Mechanical loading is a well-known regulator of cartilage metabolism. This suggests that a loading-induced physical signal regulates chondrocyte behavior. Previous studies have focused on the effects of steady fluid flow on chondrocytes. In contrast to steady flow, loading induced fluid flow occurs in an oscillatory pattern and includes a reversal of flow direction with each loading event. In this study we examined the hypothesis that oscillating fluid flow increases cytosolic Ca^{2+} concentration ($[\text{Ca}^{2+}]_i$) in bovine articular chondrocytes (BAC) in a frequency-dependent manner and that the presence of serum affects this response. The aims of our study were to examine (1) whether BAC respond to physiologic oscillating fluid flow *in vitro* and compare these results to steady fluid flow, (2) the effect of fetal bovine serum on fluid flow responsiveness of BAC and (3) whether the response of BAC to fluid flow is flow rate and/or frequency dependent. $[\text{Ca}^{2+}]_i$ was quantified using the fluorescent dye fura-2. BAC were exposed to steady, 0.5, 1, or 5 Hz sinusoidal oscillating fluid flow at five different flow rates in a parallel plate flow chamber. Our findings demonstrate that BAC respond to oscillating fluid flow with an increase in $[\text{Ca}^{2+}]_i$ ($p > 0.05$), and furthermore, chondrocyte responsiveness to fluid flow increases with peak flow rate ($p < 0.0001$) and decreases with increasing frequencies ($p < 0.0001$). Finally, the presence of serum in the media potentiated the responsiveness of BAC to fluid flow ($p < 0.0001$). Our results suggest an important role for mechanical load-induced oscillating fluid flow in chondrocyte mechanotransduction. © 2000 Elsevier Science Ltd. All rights reserved.

Keywords: Chondrocytes; In vitro; Mechanotransduction; Fluid flow; Shear stress; Calcium

1. Introduction

Mechanical loading has been shown to regulate cartilage metabolism in the absence of pathology and is necessary to maintain the integrity of the joint *in vivo* (Palmoski et al., 1979). The function of articular cartilage is maintained by chondrocytes via degradation and synthesis of extracellular matrix constituents (Heinegard and Oldberg, 1989; Mow et al., 1994). Therefore, a deeper understanding of how chondrocytes sense and respond to their biophysical environment is critical for prevention and treatment of cartilage diseases.

Previous investigations assessed biosynthetic activity in explanted cartilage tissue in response to controlled mechanical loading. *In vitro* static compression has been shown to decrease proteoglycan synthesis (Chen and Sah, 1998), whereas dynamic intermittent compression increased anabolic activity (Burton-Wurster et al., 1993). Furthermore, studies of oscillatory compression have demonstrated an increased rate of synthesis with increasing loading frequency (Kim et al., 1994; Sah et al., 1989). However, while tissue culture experiments have convincingly characterized the mechanosensitivity of cartilage, the cellular-level physical signal that chondrocytes sense, and to which they respond in their biophysical environment, remains unclear. To isolate the effect of one physical signal from another investigators have employed cell culture methodology.

In vivo compression of cartilage results in electrokinetic effects, hydrostatic pressure, direct cellular deformation and fluid flow (Mow et al., 1984). Fluid flow is an important biophysical stimulus in many different cell types including endothelial (Ballermann et al., 1998;

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[☆]Presented in part at the 44th Annual Meeting of the Orthopedic Research Society in Anaheim, CA 1999 and the 1999 Summer Bioengineering Conference of the American Society of Mechanical Engineers in Big Sky, MT 1999.

Davies, 1995; Nerem et al., 1998) and bone cells (Jacobs et al., 1998; McAllister and Frangos, 1999; Smalt et al., 1997; Weinbaum et al., 1994). Previous studies from our laboratory and others have demonstrated that chondrocytes respond to fluid flow with increases in intracellular calcium concentration ($[Ca^{2+}]_i$), MAP kinase phosphorylation, prostaglandin E2 release, and proteoglycan synthesis (Smith et al., 1995; Valhmu et al., 1998; Yellowley et al., 1997, 1999). However, these studies did not examine a reversing (i.e. oscillating) flow profile to which, due to the cyclic nature of the physiologic loads experienced by cartilage, chondrocytes are more likely exposed in vivo. Articular cartilage in vivo is exposed to periodic mechanical loading events that cause fluid to be repeatedly forced through the extracellular matrix from regions of high relative pressure to regions of low relative pressure. When the loading is removed, fluid returns to compressed regions as the solid-phase reconstitutes. As a result, the flow profile that is experienced by chondrocytes involves a direction reversal and is oscillatory in nature. Therefore, in this study we examined the effect of oscillating fluid flow on $[Ca^{2+}]_i$ in bovine articular chondrocytes (BAC).

We chose to expose cells to fluid flow magnitudes (10–570 $\mu\text{l/s}$), which in our experimental set up produces wall shear stresses ranging from 0.05 to 4.4 Pa, because shear stresses in this range have been demonstrated to alter chondrocyte metabolism in vitro (Smith et al., 1995). Because normal human walking frequency is approximately 1 Hz, we chose oscillating frequencies ranging from 0.5 to 5 Hz. Furthermore, to address the role of chemotransport in fluid flow-induced Ca^{2+}_i signaling in chondrocytes, we investigated the responsiveness of chondrocytes to fluid flow in the presence and absence of fetal bovine serum (FBS). Our hypothesis was that oscillating fluid flow increases $[Ca^{2+}]_i$ in BAC in a frequency dependent manner and that the presence of serum affects this response. The aims of our study were to examine (1) whether BAC respond to physiologic oscillating fluid flow in vitro and compare these results to steady fluid flow (2) the effect of FBS on fluid flow responsiveness of BAC and (3) whether BAC response to fluid flow is flow rate and/or frequency dependent.

2. Materials and methods

BAC were isolated and cultured as described previously (Yellowley et al., 1997). Briefly, articular cartilage from bovine hock joints was chopped into small pieces and digested for 2 h at 37°C in a mixture of 0.15 mg/ml DNase, 2 mg/ml collagenase and 0.1 mg/ml hyaluronidase in a spinner flask. Cells were cultured in media containing RPMI 1640 with HEPES, 20% FBS and 2% Penicillin + Streptomycin. Cells were then subcultured onto microscope quartz glass slides (19 cm²)

(Fridrich & Dimmock, Miville, NJ) and grown to 80% confluency under the same conditions. We have previously demonstrated that cells isolated in this manner display characteristics of the chondrocyte phenotype including expression of type II collagen (Yellowley et al., 1997). Each set of experiments included at least two different isolates. All experiments were done on cells after only one passage.

Cells were exposed to fluid flow as previously described (Jacobs et al., 1998). Briefly, the slides with cells were mounted in a parallel plate flow chamber. The flow chamber was then attached to a custom designed fluid pump via rigid wall tubing, and cells were exposed to steady, 0.5, 1 or 5 Hz oscillating fluid flow at 570, 300, 150, 75 and 10 $\mu\text{l/s}$ peak flow rate. The oscillating flow was provided by a 500 μl syringe mounted in a computer controlled servopneumatic material testing machine (Endura-Tec, Eden Prairie, MN). A sinusoidal oscillating flow profile was achieved via programming an appropriate sinusoidal piston displacement in the computer control software, which produced sinusoidal fluid flow that was verified with an ultrasonic flow meter (T106, Transonic Systems Inc., Ithaca, NY) with respect to waveform and flow rate (Fig. 1). Steady flow was provided by a Harvard syringe pump (Harvard Apparatus, Southnatick, MA) at the same peak flow rates as oscillating flow. The resulting flow rate was monitored with the flow meter. From the given flow channel dimensions, of 38 \times 20 \times 0.28 mm, the resulting shear stress on the cells can be estimated by the wall shear stress equation

$$\tau = \frac{6\mu Q}{bh^2},$$

where Q is the flow rate, τ is the shear stress, μ is the viscosity, b is the chamber width, and h the chamber height (Fox and McDonald, 1985). The corresponding shear stresses for the flow rates used in this study are given in Table 1. The cells were exposed to flow regimes

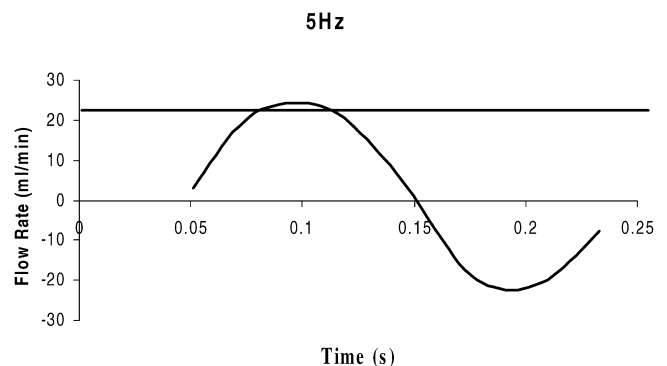


Fig. 1. Wave form utilized in this study. In the oscillating wave form flow reverses resulting in no net flow with an even number of cycles. Steady flow results in a continuous net positive flow.

Table 1
Tested flow rates in $\mu\text{l/s}$ and resulting shear stress

Flow rate ($\mu\text{l/s}$)	Shear stress (Pa)
10	0.073
75	0.55
150	1.1
300	2.2
570	4.2

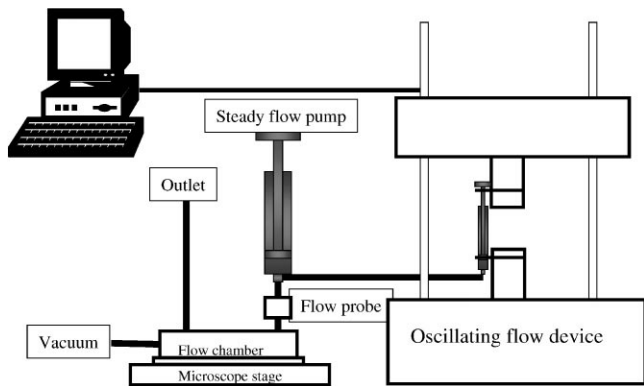


Fig. 2. Experimental setup to apply steady and oscillating flow to cells in vitro. The slide with cells facing the flow channel is held in place via vacuum sealing. The flow chamber is mounted on the stage of an inverted microscope. Flow is applied by either a steady flow pump or a custom made, computer controlled, oscillating flow device. Flow velocity is measured by an ultrasound flow probe. Control experiments were completed with the same setup without applying flow.

utilizing Tyrode's solution with and without 2% FBS. No-flow controls were obtained by following the flow protocol in all aspects without activating the pump. (Fig. 2).

$[\text{Ca}^{2+}]_i$ was quantified as previously described (Yellowley et al., 1997; Jacobs et al., 1998), using the fluorescent Ca^{2+} indicator fura-2. Calibration ratios were determined with calcium standards supplied by the manufacturer (Molecular Probes, Eugene, OR). Cells were loaded with $1\ \mu\text{M}$ fura-2-am for 30 min, and extracellular dye removed. The cells were then mounted in a parallel plate flow chamber and placed on the microscope stage (Nikon Diaphot 300, Nikon, Melville, NY). Cells were alternately illuminated at 340 and 380 nm and the emitted light detected by an ICCD camera at a rate of 1 image every 2.5 s. Data acquisition and analysis software was used to capture and calculate fluorescent signal intensity which was converted to $[\text{Ca}^{2+}]_i$ values (Meta-fluor; Universal Imaging, West Chester, PA). Each 4-min experiment consisted of 1 min of imaging without applied flow (baseline), followed by a 3-min flow period. Each cell in the field (30–60 cells) was manually outlined utilizing analysis software and a time course of $[\text{Ca}^{2+}]_i$ changes

was computed for each cell independently. All intracellular Ca^{2+} data was transferred to a Microsoft Excel spread sheet for further analysis.

Transients in $[\text{Ca}^{2+}]_i$ were examined by utilizing an algorithm which allows one to separate smaller transients superimposed over larger alterations in $[\text{Ca}^{2+}]_i$ (Jacobs et al., 2000). The maximum $[\text{Ca}^{2+}]_i$ transient was determined for both the baseline and the flow periods for each outlined cell. Maximum $[\text{Ca}^{2+}]_i$ transients for the baseline period for the cells in each slide were averaged and twice this value used as a cutoff to eliminate background noise. Cells exhibiting a $[\text{Ca}^{2+}]_i$ transient larger than the cutoff during flow, but not during baseline, were considered responsive. The number of responding cells was expressed as a percentage of total number of cells (89–148) analyzed. Data are shown as percentage of cells responding \pm standard error of proportion.

The study comprised a $5 \times 3 \times 2 \times 2$ design with five different levels of shear stress, three different frequencies, steady and oscillating flow (1 = steady flow, 0 = oscillating flow) and two conditions for FBS (1 = flow profiles with 2% FBS and 0 = a flow profile without 2% FBS). Overall trends in the data were identified on the basis of logistic regression. A logistic regression model was used instead of a linear regression model due to the binary nature of the data (cells were considered responsive or non-responsive). An adequate model was fit for all data points via maximum likelihood estimation and a goodness of fit test was applied. Each estimated parameter of this model was tested via an approximate *t*-test. ANOVA was applied to compare the fraction of cells responding to steady versus oscillating flow at each frequency.

3. Results

To examine how BAC respond to oscillating flow we quantified $[\text{Ca}^{2+}]_i$ response to oscillating flow. Following a 1 min no-flow period, individual cells exposed to 1 Hz oscillating flow at $300\ \mu\text{l/s}$ peak flow rate, displayed a transient increase in $[\text{Ca}^{2+}]_i$ (Fig. 3). Oscillatory fluid flow caused a statistically significant increase in $[\text{Ca}^{2+}]_i$ in all conditions tested ($p < 0.05$) except at 0.5 Hz with 2% FBS at a peak flow rate of $10\ \mu\text{l/s}$ and $75\ \mu\text{l/s}$ and 5 Hz at a peak flow rate of 10 and $75\ \mu\text{l/s}$ without 2% FBS (Fig. 4).

Under all conditions examined there was a flow rate-dependent increase in the percentage of cells responding ($p < 0.0001$). This flow rate-dependent response occurred independent of frequency, in the case of oscillating flow, or the presence of FBS. The flow rate-dependent nature of the response appears greater in the presence of 2% FBS than in its absence. This finding is consistent with the finding that FBS increases responsiveness of BAC (Figs. 4 and 5).

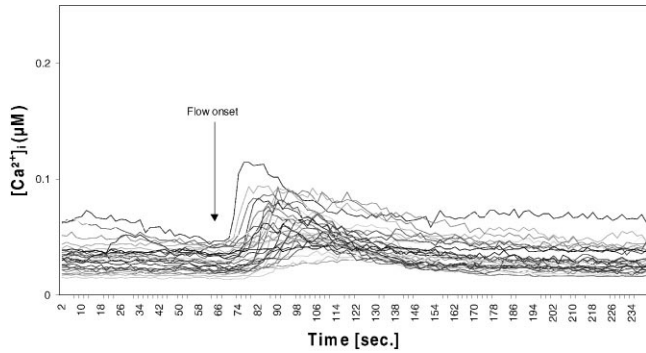


Fig. 3. Timecourse of changes in $[Ca^{2+}]_i$ in BAC. Each line represents 1 individual cell. The arrow indicates the onset of a 1Hz 300 $\mu\text{l/s}$ peak amplitude flow profile with 2% FBS. Note the larger $[Ca^{2+}]_i$ transients, indicating a response, with smaller superimposed transients, after flow onset.

The percentage of cells responding to fluid flow was greater in the presence of 2% FBS for all conditions examined ($p < 0.0001$) (Fig. 5). Interestingly with oscillating flow FBS increased responsiveness to flow to a greater degree at higher flow rates. However, with steady flow FBS increased responsiveness to a lesser degree at higher flow rates.

Logistic regression revealed that steady flow was significantly more stimulatory than oscillating flow ($p < 0.0001$) (Fig. 5). Furthermore, the difference between steady and oscillatory flow showed an interaction with FBS and flow rate such that the difference was more pronounced at higher flow rates in the absence of FBS and lower flow rates in the presence of FBS. ANOVA revealed no significant differences between steady and oscillating flow at 10 and 75 $\mu\text{l/s}$ in the absence of FBS and no significant difference between steady and oscillating flow at 150, 300 and 570 $\mu\text{l/s}$ in the presence of FBS. Finally, the responsiveness of BAC to oscillating fluid flow diminishes with increasing frequency ($p < 0.0001$ by logistic regression for all conditions tested).

4. Discussion

The primary aim of this study was to demonstrate that oscillating fluid flow has the potential to regulate chondrocyte metabolism. Although several previous studies have demonstrated that steady and/or pulsatile flow have an effect on chondrocytes (Smith et al., 1995; Valhmu et al., 1998; Yellowley et al., 1997, 1999), this work is the first investigation of the effect of oscillating flow. This is a critical step since oscillating flow is the predominant flow profile in vivo due to the dynamic nature of the applied loads and, furthermore, other cell types have been shown to respond differently to oscillating flow compared to steady or pulsatile flow (Ajubi et al., 1999; Jacobs et al., 1998). In this study we observed an increased $[Ca^{2+}]_i$ in response to oscillating fluid flow in

primary cultured BAC in vitro. Although oscillating flow tended to be less stimulatory than steady flow, over a broad range of frequencies and flow rates, it was shown to affect Ca^{2+}_i signaling.

Our study design included flow rates from 10 to 570 $\mu\text{l/s}$ and frequencies from 0.5 to 5 Hz as well as steady flow and the presence or absence of FBS. By including all possible combinations of these flow parameters (i.e. a full factorial testing matrix) we were not only able to identify trends in responsiveness for each of the parameters, but also interactions among the parameters. In our study the number of cells responding to oscillating fluid flow was dependent on the peak flow rate in a dose-dependent manner. These findings are consistent with previous observations of a dose-dependent $[Ca^{2+}]_i$ response with flow rate in chondrocytes (Yellowley et al., 1997), bone cells (Hung et al., 1995), and endothelial cells (Geiger et al., 1992). Furthermore, the shear stress levels used in this study bracket those that have been shown by others to stimulate chondrocyte metabolism (Smith et al., 1995; Valhmu et al., 1998; Yellowley et al., 1997, 1999). In articular cartilage this mechanism could be important for maintaining tissue integrity in vivo, where cells in different joints and even different tissue layers experience different magnitudes of mechanical load-induced fluid flow (O'Hara et al., 1990). This concept is supported by a recent study showing proteoglycan synthesis in cartilage is greatest in areas experiencing high rates of fluid flow (Buschmann et al., 1999). Another loading parameter shown to modulate the effect of loading on explanted articular cartilage is frequency (Kim et al., 1994), which was the second experimental parameter in our design.

Chondrocytes exposed to mechanical loading in situ in explanted cartilage plugs exhibit increased biosynthetic activity with increased loading frequency (Kim et al., 1994). In contrast, we found a decreased responsiveness of chondrocytes in vitro to oscillating fluid flow of increasing frequency. These seemingly contradictory findings can be reconciled in two ways. The first possibility is that this apparent contradiction arises due to the limited range of high frequencies addressed in the explant studies, due to a limitation of the experimental compression apparatus. As a result, the frequency range of the current study and the explant studies do not overlap. It is possible that if the explant studies were expanded to frequencies associated with the activities of daily living (0.5 Hz and above) they might show a decrease in biosynthesis with increasing frequency. A second alternative is suggested by theoretical modeling of fluid flow velocities in cartilage, which predict that increased loading frequencies result in dramatically increased fluid velocities (Buschmann et al., 1999). Responsiveness of chondrocytes increases with flow rate in a dose-dependent fashion. Thus, it is possible that the increase in flow rate predicted to occur with increasing frequency in vivo overrides the decrease in cellular sensitivity with increasing frequency we have observed.

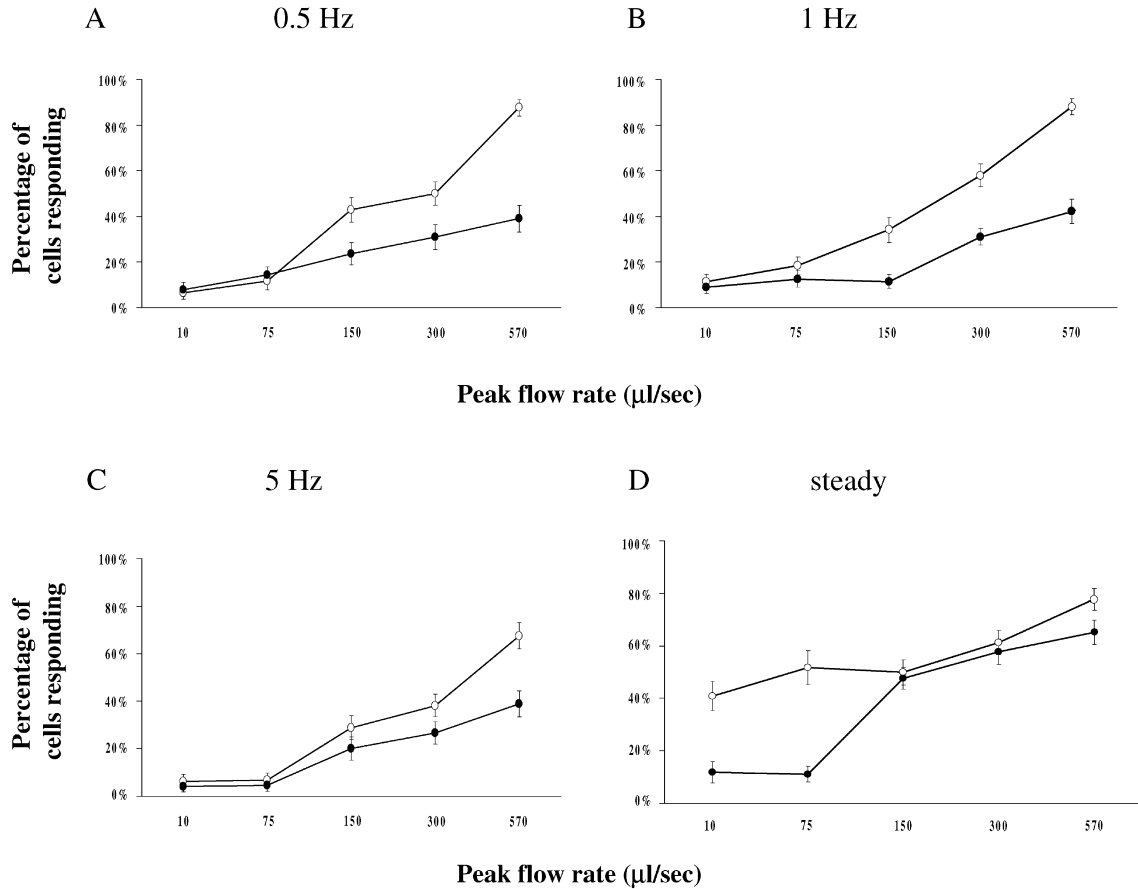


Fig. 4. Flow rate-dependent effect of fluid flow on $[\text{Ca}^{2+}]_i$ in BAC. The y-axis shows the percentage of cells responding \pm standard error of proportion. The x-axis shows all tested flow rates with (empty circles) and without (full circles) 2% FBS. Panel A shows 0.5 Hz oscillating flow, panel B shows 1 Hz oscillating flow panel, C shows 5 Hz oscillating flow and panel D shows steady flow. $N = 89\text{--}148$ individual cells for each condition.

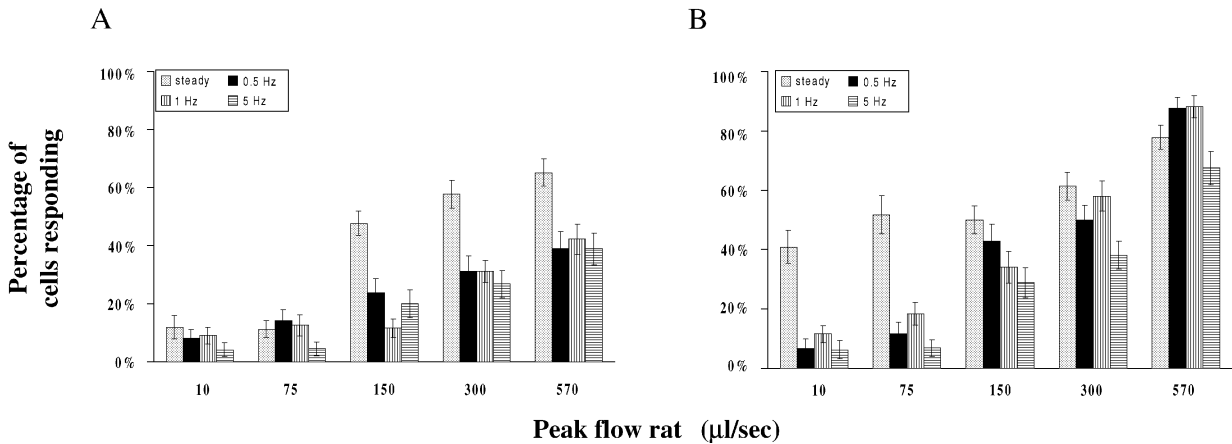


Fig. 5. Effect of flow rate on the number of cells responding. The y-axis shows the percentage of cells responding \pm standard error of proportion. Panel A shows conditions without, and Panel B shows conditions with, 2% FBS. Note the increasing number of cells responding at increasing flow rates ($p < 0.0001$). Steady flow was more stimulatory than oscillating fluid flow over all conditions tested ($p < 0.0001$). However, in the absence of FBS there was no difference between 10 and 75 $\mu\text{l/s}$ peak flow amplitudes between oscillating and steady flow. In contrast, in the presence of 2% FBS there was no significant difference between 300 and 570 $\mu\text{l/s}$ peak flow rate. $N = 89\text{--}148$ individual cells in each condition.

Our finding of decreased sensitivity with increasing frequency may be explained by two cellular-level biophysical mechanisms. First, the mechanical behavior of chondrocytes has been found to be viscoelastic in nature.

Thus, for oscillation frequencies higher than the viscoelastic time constant, cellular deformations due to flow would be minimal. This could result in decreased responsiveness if cellular deformation is an important step in the

transduction mechanism as might be expected if the mechanism involves stretch activated channels or cytoskeletal rearrangement. Alternatively differences in the net convective transport levels associated with the different flow profiles could explain the differences in responsiveness we found. For example, the total fluid volume exchanged in the flow chamber per cycle for an oscillating flow profile decreases with increasing frequency (e.g. for oscillating flow of 300 $\mu\text{l/s}$ at 1 Hz, 95 $\mu\text{l/cycle}$ is exchanged, but only 19 $\mu\text{l/cycle}$ is exchanged at 5 Hz). In contrast, media in steady flow regimes is continuously replenished. Thus, if cellular responsiveness is dependent on a fresh supply of an unknown biochemical agent, one would expect decreasing responsiveness with increasing oscillation frequency and a maximal responsiveness with steady flow, as we observed in these experiments. These results are similar to what has been observed in bone cells (Jacobs et al., 1998). This interpretation suggests that a biochemical agonist is being delivered to the cells via fluid convection. Such an agonist might be a component of serum.

Interestingly, the presence of serum in the media increased the responsiveness of BAC to fluid flow, suggesting that one of the components of FBS might be an important (co)-factor, which sensitizes BAC to fluid flow. Gupta et al. (1998) examined the influence of 2% newborn bovine serum (NBS) on $[\text{Ca}^{2+}]_i$ in human fibroblasts, showing an increase of $[\text{Ca}^{2+}]_i$ in NBS containing media compared to NBS free media at a low flow rate generating a shear stress of 3 dynes/cm², consistent with a role for chemotransport. Smith et al. (1996) showed an increased type II procollagen mRNA in adult human chondrocytes in response to dynamic and static hydrostatic pressure and control to serum containing media. They suggest that serum increases the baseline response. In contrast, our data show no significant increase in the number of cells exhibiting spontaneous (i.e. in the absence of stimulation) $[\text{Ca}^{2+}]_i$ oscillations (data not shown). However, our data is consistent with the hypothesis that one or more components in serum act as potent (co)-factors modulating the $[\text{Ca}^{2+}]_i$ response of BAC to fluid flow.

In the absence of FBS we saw a significantly greater response to steady flow compared to oscillating only at higher flow rates. In contrast, in the presence of 2% FBS the greater response of steady flow over oscillating flow was only observed at lower flow rates. One explanation for this finding may be that steady flow is a more powerful physical signal to the cell, and the presence of 2% FBS potentates this signal. However, the cellular $[\text{Ca}^{2+}]_i$ response can become saturated with virtually all cells responding at high flow rates with FBS, whether the flow is steady or oscillating. Likewise, virtually no cells respond to low flow rates without FBS, regardless of flow type.

While our data clearly demonstrate an effect of oscillating flow on chondrocytes there are some limitations that should be considered. Firstly, the peak fluid velocities utilized in this study are between one and five

orders of magnitude higher than those predicted to occur in vivo or in explant studies (Ateshian et al., 1994; Buschmann et al., 1999). However, the shear stress that these flow rates produce in our chamber are similar to what theoretical models predict chondrocytes might experience in vivo. The large size of the parallel plate flow chamber relative to the microstructural dimensions of cartilage tissue requires that the far-field fluid velocity in the chamber greatly exceeds the velocity expected in cartilage. Unfortunately, no microarchitectural model currently exists describing the biophysical effects and interactions of loading-induced fluid flow and chondrocytes at the cellular level. Until such models become available, it will be difficult to further refine in vitro studies of the effects of biophysical stimuli at the cellular level. Furthermore, physical signals secondary to flow including extracellular matrix compaction, bioelectrical fields, and/or transport effects, may also play important (possibly synergistic) roles in chondrocyte mechanotransduction in vivo (Mow et al., 1999). Secondly, we used an open-loop model for steady flow, delivering fresh media to the cells compared to a closed-loop model for oscillating flow. Although we believe that the total volume of media in any condition is big enough to dilute any possible paracrine factor to a concentration below effectiveness, caution in interpreting steady and oscillating data is necessary. Finally, while $[\text{Ca}^{2+}]_i$ has been shown to influence down stream biological response, (e.g. enzyme activity and gene expression) and has been examined in many different studies of biophysical signal transduction, we have not directly examined flow-induced activity of factors involved in cartilage metabolism. In this regard, further studies must be completed.

In conclusion, in this study we have shown that BAC respond to sinusoidal oscillating fluid flow in a peak flow rate and frequency dependent manner. Furthermore, we have shown that the presence of an additional, as yet unidentified, serum factor has an agonistic effect on the cytosolic Ca^{2+} response to fluid flow. Our results suggest that mechanical load-induced oscillating fluid flow plays an important role in chondrocyte mechanotransduction.

Acknowledgements

This work was supported by National Institutes of Health Grants AG 15107 (HJD) and AR45989 (CRJ) and the Whitaker foundation (CRJ).

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