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Review Article

Hylonome

Early responses to mechanical load in tendon: Role for calcium signaling, gap junctions and intercellular communication

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Abstract

Tendon and other connective tissue cells are subjected to diverse mechanical loads during daily activities. Thus, fluid flow, strain, shear and combinations of these stimuli activate mechanotransduction pathways that modulate tissue maintenance, repair and pathology. Early mechanotransduction events include calcium (Ca^{2+}) signaling and intercellular communication. These responses are mediated through multiple mechanisms involving stretch-activated channels, voltage-activated channels such as Ca_v1, purinoceptors, adrenoceptors, ryanodine receptor-mediated Ca^{2+} release, gap junctions and connexin hemichannels. Calcium, diacylglycerol, inositol (1,4,5)-trisphosphate, nucleotides and nucleosides play intracellular and/or extracellular signaling roles in these pathways. In addition, responses to mechanical loads in tendon cells vary among species, tendon type, anatomic location, loading conditions and other factors. This review includes a synopsis of the immediate responses to mechanical loading in connective tissue cells, particularly tenocytes. These responses involve Ca^{2+} signaling, gap junctions and intercellular communication.

Keywords: Mechanotransduction, Fluid Flow, Strain, Intracellular Calcium, Connective Tissues

Introduction

Tendons, ligaments and other connective tissues are continually subjected to mechanical loads during normal function. Connective tissue cells detect and respond to mechanical stimuli through a variety of pathways involving stretch-activated ion channels, calcium (Ca^{2+}) channels, other ion channels, gap junctions, purinoceptors, integrin interactions with the matrix and cytoskeleton through a mechanosensory complex and second messengers[1-5]. One of the primary second messengers utilized by cells to transduce mechanical to biochemical signals is Ca^{2+} [6,7].

Local and global Ca^{2+} signals co-modulate multiple cell functions including gene transcription, cell growth and proliferation, contraction and apoptosis[6,8-10]. Intracellular Ca^{2+} concentration ($[Ca^{2+}]_{ic}$) can be monitored with ratio imaging systems and fluorescent dyes, such as fura-2, indo-1 and fluo-3[11,12]. Thus, early and real-time responses to mechanical stimulation can be analyzed. Intracellular Ca^{2+} may increase by either the influx of extracellular Ca^{2+} through plasma membrane channels or by release of Ca^{2+} from intracellular stores from the cisternae of the endoplasmic recticulum. Plasma membrane channels permeable to Ca^{2+} include voltage-gated, receptor-gated and mechanogated channels[6,7,9,13-16]. Calcium is released from intracellular stores through an inositol (1,4,5)-trisphosphate (IP_3)-mediated pathway or through ryanodine receptor-mediated Ca^{2+} release[6-8,16-18]. In addition, Ca^{2+} can pass between cells through gap junctions[19].

Gap junctions join the cytosol of two adjacent cells allowing direct cell-to-cell communication. The principal proteins comprising gap junctions are the connexins[19-21]. Six connexins arranged in a floral pattern form a connexon, or hemichannel. Two hemi-channels from separate cells unite to form a gap junction, which cluster in hundreds to thousands in the plasma membrane to form a gap junction plaque. Gap junctions permit both electrical and chemical communication between and among adjacent cells. Electrical communication occurs by passage of ions through the channel, thus

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Tissue	Species/ Cell Line	Flow Type & Magnitude (dyne/cm^2)	Perfusate	Ca^{2+} Response	Increase Ca^{2+} Response	Reduce/ Abolish Ca^{2+} Response	No Effect on Ca^{2+} Response	Refs
Tendon	Human	Laminar, 1-25	EBSS	Y		Ca^{2+} free Celecoxib Indomethacin Pertussis Toxin	Cholera Toxin Gadolinium Verapamil	27,38
	Rabbit	Laminar, 1-25	EBSS or DMEM	N	Serum			46,63
	Avian	Laminar, 1-20	EBSS ± FBS	N			FBS	45
Palmar Fascia	Human	Laminar, 3,25	HBSS	N (3 d/cm^2) Y (25 d/cm^2)	2% NBS (3 d/cm^2)			30
Anterior Cruciate Ligament	Canine	Laminar, 25	HBSS ± 2% NBS	Y	Serum	Ca^{2+} free Neomycin Thapsigargin		32
Medial Collateral Ligament	Canine	Laminar, 25	HBSS ± 2% NBS	Y		Ca^{2+} free (-NBS) Neomycin (-NBS) Thapsigargin	Ca^{2+} free (+NBS) Neomycin (+NBS) Serum	32
Bone	Human	Laminar, Oscillating, & Pulsatile, 20	Tyrode's + 2% FBS	Y				42
	Rat	Laminar, 4, 35	HBSS	N (4 d/cm^2) Y (35 d/cm^2)	ATP NBS	Ca^{2+} free Gadolinium Neomycin U-73122	Verapamil	31,39
		Oscillating 10, 20	DMEM + 2% FBS	Y				41
	ROS 17/2.8 (Rat)	Laminar & Oscillating, 20	Ham's F12 +2% FBS	N				59
	MC3T3-E1 (Mouse)	Oscillating, 20	MEM-α	N	ATP/ UTP 2% FBS	2APB** Apyrase** Nifedipine** Pertussis Toxin** 1 μM Ryanodine** Thapsigargin** U-73122**	Adenosine ATPγs/ ADP/ UDP CTP/ GTP/ TTP PPADS** Gadolinium** 20 μM Ryanodine** U-73343**	49,50
		Laminar, 12		Y		Gadolinium Thapsigargin U-73122	Nifedipine	40
Cartilage	Bovine*	Laminar & Pulsatile, 10, 20, 37		Y		Ca^{2+} free Gadolinium Neomycin Pertussis Toxin Thapsigargin	Caffeine Ryanodine Suramin	36,37
Intervertebral Disc Annulus	Human	Laminar, 1-25	EBSS	Y	IL-1β	Ca^{2+} free		28
Aortic Smooth Muscle	Rat	Laminar, 25	DMEM-F12 +1% P/S or PBS (-Ca^{2+})	N				60
Aortic Endothelial	Bovine	Laminar, 12	HBSS	Y		BAPTA-AM Pertussis Toxin SKF96365 Thapsigargin	Nocodazole Taxol	43
		Laminar, 4	HBSS	N	ATP			54
	Pig	Laminar, 5	1 mM Ca^{2+} 500 nM ATP	Y		ML-9		44

The effects of chemical modulators and other factors on the Ca^{2+} response to fluid flow in connective tissue cells.

Table 1. "Ca^{2+} free" represents a response altered by the removal of extracellular calcium (Ca^{2+}) from the perfusate. "Y" represents an increase in intracellular Ca^{2+} concentration in response to fluid flow. "N" represents a lack of response. Flow perfusates consisted of various solutions including Hank's balanced salt solution (HBSS), Earles' balanced salt solution (EBSS), Dulbecco's modified Eagle's medium (DMEM), minimal essential α medium (MEM-α), fetal bovine serum (FBS), newborn bovine serum (NBS). *Modulator effects were observed on bovine chondrocytes subjected to 37 dynes/cm^2 laminar steady flow. **Modulator effects in the presence of 2% FBS. PPADS (Pyridoxal phosphate 6-azophenyl2', 4'-disulfonic acid).

altering the electric current across the channel. Chemical communication occurs by passage of molecules and ions less than 1000 Da, such as Ca^{2+}, cyclic adenosine monophosphate, and IP_3, thus altering intracellular concentrations[19-21]. Accordingly, gap junctional intercellular communication allows cells to integrate and/or synchronize their activities. Gap junctional intercellular communication modulates synchronous contraction in cardiac myocytes[22,23], the response to injury and healing[24,25] and possibly bone cell differentiation[26]. In tendon cells, gap junctions can regulate load-induced DNA and collagen synthesis[24].

Calcium signaling in response to mechanical load

In connective and other tissues, a mechanical signal can be transmitted to a biochemical signal as a second messenger such as Ca^{2+} or IP_3. Mechanical deformations due to tensile strain, fluid flow, compression and vibration induced an increase in $[Ca^{2+}]_{ic}$ in each of the cell types tested[27-37]. Rat tail tendon tenocytes *in situ* and human tendon surface cells respond to mechanical strains at 1-6% elongation by increasing intracellular Ca^{2+} [29,35] [Knutson and Banes, unpublished observation]. However, there are no other reports of Ca^{2+} responses to tensile strain, compression and vibration in tendon cells that the authors are aware of. Therefore, this section focuses on the Ca^{2+} response to fluid flow in connective tissue cells.

Cells from tendon[27,38], palmar fascia[30], ligament[32], bone[31,39-42], cartilage[36,37], intervertebral disc annulus[28] and endothelium[43,44] increased $[Ca^{2+}]_{ic}$ in response to fluid flow. However, the kinetics of the Ca^{2+} response varied among species, anatomic location, pathway recruitment and flow conditions, which included the type of shear stress applied (steady laminar, oscillating, or pulsatile) and the composition of the perfusate (Table 1).

Human[27,38] but not avian[45] tendon cells responded to fluid flow by increasing $[Ca^{2+}]_{ic}$. Rabbit tenocytes did not increase $[Ca^{2+}]_{ic}$ in response to flow unless serum was present in the perfusate[46]. However, serum did not affect the flow response in avian tendon cells[45]. Thus, the Ca^{2+} response to mechanical stimulation in tendon differs among species.

In human tenocytes, the increase in $[Ca^{2+}]_{ic}$ in response to flow was dependent upon extracellular Ca^{2+} [38]. A similar result was observed in ligament[32], bone[31] and cartilage cells[36]. Analysis of these data raises questions concerning the importance of extracellular Ca^{2+} to mechanotransduction: can altering the extracellular Ca^{2+} concentration ($[Ca^{2+}]_{ec}$) regulate the response to load? Furthermore, is there a threshold $[Ca^{2+}]_{ec}$ at which gating of plasma membrane channels can be controlled? The tissue concentration of free and bound extracellular Ca^{2+} in a digital flexor tendon, for example, should derive from blood plasma and synovial fluid in osmotic balance with the extracellular fluid and the Ca^{2+} pools within the cell. Damage to blood vessels or cells, extravasation, inflammation and swelling likely alter $[Ca^{2+}]_{ec}$. Likewise, the mechanical environment modulates Ca^{2+} influx and efflux from the cell and, hence, changes the $[Ca^{2+}]_{ec}$. Several tissues including bone, cartilage, kidney and parathyroid express calcium-sensing receptors or have some extracellular-Ca^{2+}-sensing mechanism in the plasma membrane that helps regulate systemic $[Ca^{2+}]_{ec}$[47]. However, cells that are less involved in systemic Ca^{2+} regulation such as neurons, kertinocytes, and epithelial cells also express calcium-sensing receptors. Extracellular Ca^{2+}-sensing mechanisms in these cells may be important for modulating $[Ca^{2+}]_{ec}$ in the local microenvironment[47]. Cells can communicate changes in their local microenvironment to neighboring cells. Agonist-induced increases in intracellular Ca^{2+} resulted in an extracellular signal, perhaps the release of Ca^{2+} into the extracellular space, that increased local $[Ca^{2+}]_{ec}$[48]. Calcium-sensing receptors on adjacent cells detected this signal. These cells, in turn, increased their $[Ca^{2+}]_{ic}$. Thus, cells respond to changes in their microenvironment and can communicate these changes to surrounding cells. These Ca^{2+}-sensing mechanisms have yet to be studied in tendon, but they may be important in regulating the detection and the response to load, especially post-injury. Results of recent unpublished work in our lab indicate that the tenocyte response to mechanical load is sensitive to extracellular Ca^{2+}. Rat tail tendon tenocytes do not signal to applied uniaxial strain in the absence of extracellular Ca^{2+} [Knutson and Banes, personal communication]. Tenocytes that were stretched in static strain at 3% elongation signaled by increasing $[Ca^{2+}]_{ic}$ if the $[Ca^{2+}]_{ec}$ was 1.4-1.8 mM Ca^{2+}. These data indicate that a threshold $[Ca^{2+}]_{ec}$ exists where the detection of and/or response to load via a Ca^{2+} signal can be modulated.

Besides the dependence on extracellular Ca^{2+}, the Ca^{2+} response to fluid flow in human tendon cells was sensitive to pertussis toxin[27] (see Table 2 for mechanism of action of all chemical modulators discussed in this review). The fluid flow-induced increase in $[Ca^{2+}]_{ic}$ in osteoblasts[49], chrondrocytes[36] and endothelial cells[43] also involved a pertussis-toxin sensitive, G-protein-dependent pathway. However, further study of additional pathways utilized by other connective tissues is necessary to better understand the mechanisms involved in the Ca^{2+} response of tendon cells to fluid flow. These mechanotransduction signaling pathways involve multiple effectors and mechanisms including IP_3, stretch-activated channels, voltage-gated ion channels and purinoceptors. Calcium release from intracellular stores was involved in the fluid flow response in ligament[32], bone[31,40,50], cartilage[37] and endothelial cells[43]. Inhibition of phosphatidylinositol 4,5-bisphosphate or phospholipase C (PLC) abrogated the increase in $[Ca^{2+}]_{ic}$ in these cells indicating IP_3-mediated release of Ca^{2+} from intracellular stores. Stretch activated (SA) channels are plasma membrane channels that open in response to mechanical stimulation thus allowing various ions to enter the cell[15,51]. Gadolinium, a SA-channel inhibitor, did not affect the load-induced Ca^{2+} increase in tendon cells[27] but was implicated in the laminar fluid flow response in bone[31,40] and cartilage cells[36]. $Ca_v1.2$, an L-type voltage-gated Ca^{2+} channel, is also mechanosensitive[52]. Nifedipine, a Ca_v1 antagonist, blocked

Chemical Modulator	Action/ Location*	References
Amiloride	Inhibits mechanosensitive ion channel	15,102,103
2-aminoethoxydiphyenl borate (2APB)	Inhibits IP_3-induced Ca^{2+} release & store-operated Ca^{2+} influx	104-106
Apyrase (ATP-diphosphohydrolase)	Hydrolyzes nucleotide triphosphates (ATP) and nucleoside diphosphates (ADP)	107,108
1,2-Bis(2-aminophenoxy)ethane-N,N,N',N'-tetraacetic acid (BAPTA)	Ca^{2+} chelator	109
Caffeine	Opens ryanodine receptors	8,110
Celecoxib	Inhibits cyclooxygenase-2	111,112
Cholera Toxin	Catalyzes ADP-ribosylation of Gs_	113-115
Colchicine	Inhibits microtubule formation	116
Cytochalasin D	Inhibits actin polymerization	117
Dantrolene	Inhibits Ca^{2+} release from sarcoplasmic reticulum	118
Gadolinium (Gd^{3+})	Inhibits stretch-activated channels	15,119
Gap 27	Inhibits gap junctions	120,121
18α-glycyrrhetinic acid	Inhibits gap junctions	122
Halothane	Inhibits gap junctions	123
Heparin	Inhibits IP_3-mediated Ca^{2+} release	124
Heptanol	Inhibits gap junctions	125
Herbimycin A	Inhibits tyrosine kinase activity	126,127
Indomethacin	Inhibits cyclooxygenase-1 and -2	111
Manganese (Mn^{2+})	Enters cell through voltage-independent Ca^{2+} influx	128,129
ML-9	Inhibits myosin light chain kinase	130
Lanthanum (La^{3+})	Inhibits calcium channels & capacitative Ca^{2+} entry	131-133
Neomycin	Inhibits IP_3 production	134
Nickel (Ni^{2+})	Inhibits non-specific cation channel and Ca_v3 (T-type voltage-gated Ca^{2+} channels)	128,129,135
Nifedipine	Inhibits Ca_v1 (L-type voltage-gated Ca^{2+} channels)	135-138
Nitrendipine	Inhibits Ca_v1 (L-type voltage-gated Ca^{2+} channels)	139
Nocadozole	Depolymerizes microtubules	140,141
Octanol	Inhibits gap junctions	142
Oxzided ATP	$P2X_7$ receptor antagonist	143
Pertussis Toxin	Catalyzes ADP-ribosylation of G_i	113,115,144
Pyridoxal-5'-phosphate-6-azophenyl-2', 4'-disulphonic acid (PPADS)	P2X purinoceptor antagonist	143,145
Phorbol 12-myristate 13-acetate (PMA)	Activates protein kinase C	146
Rp-cAMP	Inhibits protein kinase A; cAMP antagonist	147,148
Ryanodine	Inhibits ryanodine-sensitive channels at high concentrations; Opens channels at low concentrations	8,17
SKF 96365	Inhibits store-operated Ca^{2+}/cation channels	149,150
Sp-cAMP	Activates protein kinase A; cAMP agonist	147,148
Suramin	Inhibits P2 purinoceptors	151
Taxol	Stabilizes microtubules	152
Thapsigargin	Inhibits endoplasmic recticular Ca^{2+}-ATPase	153
8-Diethylaminooctyl 3,4,5, -trimethoxybenzoate (TMB-8)	Inhibits Ca^{2+} release from intracellular stores and Ca^{2+} influx	154,155
U-73122	Inhibits PLC activation	156
U-73343	Inactive analog of U-73122	156
Verapamil	Inhibits Ca_v1 (L-type voltage-gated Ca^{2+} channels)	136-138

Chemical modulators of mechanical load responses.

Table 2. U-73122 (1-[6-[[17β-3-methoxyestra-1,3,5(10)-trien-17-yl]amino]hexyl]-1H-pyrrole-2,5-dione), U-73343 (1-[6-[[17β-3-methoxyestra-1,3,5(10)-trien-17-yl]amino]hexyl]-1H-pyrrole-2,5-pyrrolidine-dione). *For further details about the actions of these modulators, see references listed and those therein.

the Ca^{2+} response in osteoblasts in response to oscillating flow. However, verapamil, which also inhibits Ca_v1, did not affect the Ca^{2+} response to laminar fluid flow in tendon[27] and bone cells[31]. It is likely that there are redundant pathways in the responses to load that can bypass an initial Ca^{2+} influx and still activate downstream mechanisms that promote secondary events and finally, gene activation.

The activation of mechanotransduction pathways may depend not only on the type of cell subjected to load but also on the properties of the applied loading stimuli, i.e. oscillating versus laminar flow. Ca_v1 channels were activated in response to oscillatory but not laminar flow[27,31,40,50]. Pulsatile flow was a greater stimulator of Ca^{2+} signaling than either steady or oscillatory flow in bone cells[42]. Moreover, osteoblasts could be restimulated with multiple flow regimens, but a refractory period existed during which flow did not induce a Ca^{2+} transient[53]. There are no data illustrating the early signaling responses of tendon cells subjected to various fluid flow regimens, including the pathways activated by either pulsatile or oscillatory flow. The Ca^{2+} response in tendon and other cells from dense connective tissues could also depend on their anatomic location. Medial collateral ligament (MCL) cells were more sensitive to fluid flow than anterior cruciate ligament (ACL) cells. In addition, serum enhanced the ACL cell's response but not the MCL cell's response[32]. Collectively, these data illustrate that the mechanosensitive pathways utilized by connective tissues differ among cell type, species and loading conditions.

In addition to the signaling mechanisms illustrated above that mediate the response to flow, the response to mechanical stress may result from a local increase in a chemical modulator rather than the mechanical stimulation itself. Thus, the Ca^{2+} response may result from a chemotransport, or mass transfer, effect. Rabbit tendon cells increased $[Ca^{2+}]_{ic}$ in response to fluid flow only if serum was present in the perfusate[46]. Serum also enhanced the flow-induced increase in $[Ca^{2+}]_{ic}$ in ligament[32], palmar fascia[30] and bone cells[39,49]. Adenosine triphosphate (ATP) enhanced the Ca^{2+} response in aortic endothelial[54] and rat bone cells[39]. However, pretreatment with either ATP or serum altered the mass transfer Ca^{2+} response in the bone cells[39]. The Ca^{2+} response in MC3T3-E1 cells subjected to oscillatory flow further supports the mass transfer hypothesis since the cells only responded to flow when uridine triphosphate (UTP), ATP, or serum were present in the perfusate[49].

Besides evoking a mass transfer response to flow, ATP may also act in a paracrine or autocrine fashion to increase $[Ca^{2+}]_{ic}$ via activation of $P2Y_2$ purinoceptors. Cells subjected to fluid flow and cyclic compression released ATP or UTP[55-57]. Thus, mechanical stimulation of cells could result in the local increase in ATP, which then activates $P2Y_2$ purinoceptors on neighboring cells. The addition of UTP and ATP, but not other nucleotides, nucleosides or additional purinoceptor agonists (not specific for $P2Y_2$ receptors), to the perfusate increased $[Ca^{2+}]_{ic}$ in MC3T3-E1 cells[49] demonstrating that the mass transfer of ATP activates $P2Y_2$ receptors in response to flow. Besides enhancing the Ca^{2+} response to load, ATP may also act in a negative feedback manner to decrease the downstream response. In human tendon cells, cyclic tensile strain decreased ATP release. Furthermore, ATP blocked the load-induced increase in IL-1β, cyclooxygenase 2 and matrix metalloproteinase-3 expression[58]. *Taken together, local changes in the levels of ATP, UTP, serum, extracellular Ca^{2+}, or other effectors may "sensitize" or "desensitize" cells to mechanical stress, and therefore modulate the load-induced Ca^{2+} response.*

The lack of a Ca^{2+} response to mechanical stimulation in some connective tissue cells including avian tendon[45], rat osteosarcoma cells (ROS 17/2.8)[59] and rat smooth muscle[60] raises further questions about which pathways operate in an initial response to load. Do environmental conditions close ion channels necessary for a flow-induced Ca^{2+} response? Does low $[Ca^{2+}]_{ec}$ or high $[Ca^{2+}]_{ic}$ close these channels? What other factors regulate gating of these channels? A nominally closed channel can be opened through a conformational change in the protein comprising the channel. In addition, phosphorylation may be necessary to open or close a channel or engage a receptor[61,62]. Furthermore, is there another class of mechanosensitive channels that are as yet unidentified? If a receptor is involved, do these non-responding cells require an added mediator, such as ATP, lysophosphatidic acid, or in the presence of injury, serum products, to induce a Ca^{2+} response through a mass transfer effect as is required in rabbit tendon cells? Does fluid flow result in the release of an effector that inhibits Ca^{2+} signaling? Finally, can these cells respond to mechanical load through a Ca^{2+}-independent pathway? Studies in tendon suggest the latter hypothesis. In the absence of serum, rabbit and avian tendon cells upregulated mRNA expression of genes in response to fluid flow but did not increase $[Ca^{2+}]_{ic}$[45,63]. Smooth muscle cells contracted in response to flow but did not induce a Ca^{2+} response[60]. Additionally, ROS 17/2.8 cells released prostaglandin E_2 but did not increase $[Ca^{2+}]_{ic}$ in response to fluid flow[59]. Even though fluid flow did not evoke an increase $[Ca^{2+}]_{ic}$ in these cells, they "sensed" the stimulation via a Ca^{2+}-independent mechanism.

The role of gap junctions and intercellular communication in mechanotransduction

Calcium signals propagate to adjacent cells via gap junctions. Tendon cells have immunohistochemically detectable gap junctions comprised of connexins 43 and 32[64,65] and propagate a Ca^{2+} wave via gap junctions in response to mechanical stimulation by micropipet indentation of the plasma membrane (Figure 1)[66-68]. One mechanism of Ca^{2+} wave propagation through gap junctions is an IP_3-mediated response[18]. Results of inhibitor studies indicated that mechanical excitation stimulated IP_3 synthesis and passage through gap junctions, resulting in the release of Ca^{2+} from intracellular stores[69]. Propagation of a Ca^{2+} wave through tendon cells depended on IP_3-mediated release of intracellu-

Cell	Species/ Cell Line	Reduce/ Abolish Ca^{2+} Response in Stimulated Cell	No Effect on Ca^{2+} Response in Stimulated Cell	Reduce/ Abolish Ca^{2+} Wave Propagation	No Effect on Ca^{2+} Wave Propagation	Refs
Tendon Fibroblast	Human			Gap 27		67,68
	Avian		Halothane Heparin	Halothane Heparin		66
Chondrocyte	Rabbit	Ca^{2+} Free	18a-glycyrrhetinic acid Thapsigargin U-73122 U-73343	18α-glycyrrhetinic acid Thapsigargin U-73343	Ca^{2+} Free U-73343	70
	Bovine	Amiloride Ca^{2+} Free Gadolinium	Colchicine Cytochalasin D Dantrolene Lanthanum Octanol TMB-8	Octanol		157,158
Osteoblast	Human*		ATP/ UTP/ UDP/ ADP Ca^{2+} Free Heptanol Suramin Thapsigargin	ATP/ UTP (fast) UDP/ ADP (fast) Ca^{2+} free (slow) Glycyrrhetinic acid (slow) Heptanol (slow) Nifedipine (slow) Nitrendipine (slow) Suramin (fast) Thapsigargin (fast) Verapamil (slow)	ATP (slow) Ca^{2+} Free (fast) Glycyrrhetinic acid (fast) Heptanol (fast) Nifedipine (fast) Suramin (slow) Thapsigargin (slow)	75,77
	ROS 17/2.8 (Rat)	Ca^{2+} Free Gadolinium Herbimycin A PMA* (30 min) Thapsigargin 50 mM Glucose	Heptanol PMA (72 hr) Rp-cAMP Sp-cAMP Verapamil 5, 25 mM Glucose	Ca^{2+} Free Gadolinium Heptanol PMA (30 min) Sp-cAMP Thapsigargin 50 mM Glucose	Herbimycin A PMA (72 hr) Rp-cAMP Verapamil 5, 25 mM Glucose	76
	ROS 17/2.8 (Rat)		Suramin Thapsigargin	Ca^{2+} Free Glycyrrhetinic acid Heptanol Nifedipine	ATP Suramin Thapsigargin	74,75
	UMR 106-01 (Rat)		Suramin Thapsigargin	ATP Suramin Thapsigargin	Ca^{2+} Free Glycyrrhetinic acid Heptanol Nifedipine	74,75
Osteoclast	Human			Oxidized ATP Suramin	ATP/ ADP Heptanol	81
Synoviocyte	HIG-82	Ca^{2+} Free Thapsigargin U-73122	18α-glycyrrhetinic acid U-73343	18α-glycyrrhetinic acid Ca^{2+} Free Thapsigargin U-73122	U-73343	70
Pulmonary Artery Endothelial	Calf	Ca^{2+} Free Manganese Nickel Ryanodine Thapsigargin U-73122	Gadolinium Lanthanum U-73343 Verapamil	Ryanodine Thapsigargin U-73122	Ca^{2+} Free U-73343	71

The effects of chemical modulators on intercellular Ca^{2+} wave propagation in response to mechanical stimulation by micropipette indentation of the plasma membrane.

Table 3. "Ca^{2+} free" indicates a response altered by the removal of extracellular calcium (Ca^{2+}) from the bathing medium. "Y" indicates that the listed cells propagated a Ca^{2+} wave in response to mechanical deformation. *"Fast" and "slow" denotes modulators that affected the "fast intercellular calcium wave" and the "slow intercellular calcium wave" as described by Jorgensen and colleagues[77]. **Effects of PMA on the Ca^{2+} response in ROS cells depended on the time period of the pre-treatment, 30 minutes versus 72 hours. Thirty minutes of pre-treatment with PMA reduced the Ca^{2+} response, whereas pre-treatment for 72 hours had no effect.

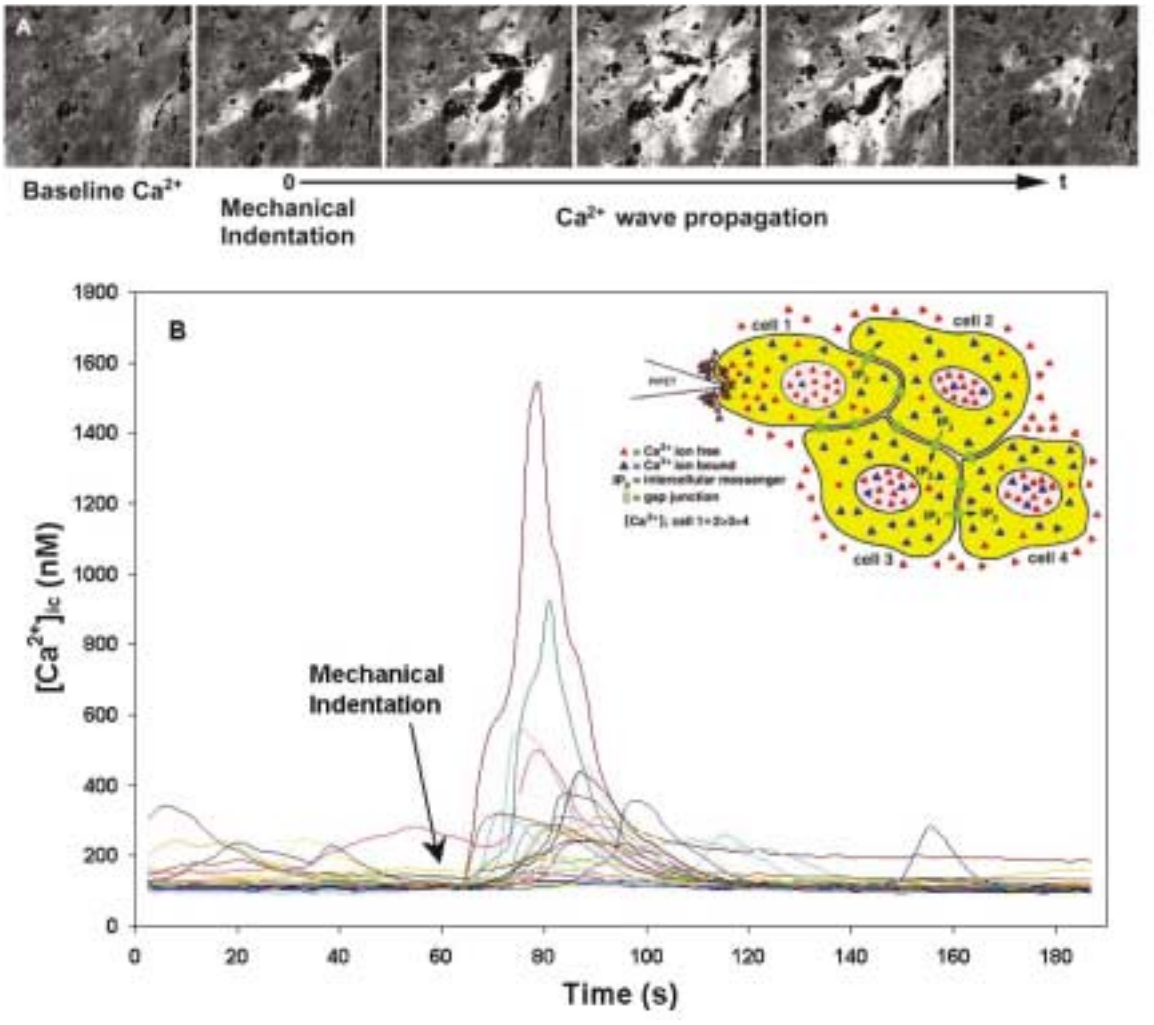

Figure 1. (A) Mechanical stimulation by micropipet indentation induces a calcium (Ca^{2+}) wave in avian tendon cells labeled with fura-2. The first image shows the baseline intracellular calcium concentration ($[Ca^{2+}]_{ic}$) of tendon cells. Once the cell is mechanically stimulated by micropipet indentation (second image), there is a rapid increase in $[Ca^{2+}]_{ic}$ depicted by the change in color of the cells. Dark gray cells represent a basal level of $[Ca^{2+}]_{ic}$. As the $[Ca^{2+}]_{ic}$ increases, the cells become brighter. The Ca^{2+} wave spreads to the cells adjacent to the stimulated cell. After a few minutes, the Ca^{2+} concentration returns to basal levels. (B) The line graph depicts the increase in $[Ca^{2+}]_{ic}$ resulting from mechanical indentation. Each line represents an individual cell's response to the stimulation. The inset cartoon is a schematic of how a Ca^{2+} wave can propagate to adjacent tendon cells via gap junctions.

lar stores as well as cell coupling through gap junctions[66,68]. Further evidence for this mechanism was observed in the inhibition of PLC with U-73122. Inhibiting PLC activation blocked the release of IP_3 into the cytosol, and thus blocked release of Ca^{2+} from intracellular stores. Subsequently, Ca^{2+} wave propagation resulting from mechanical indentation was blocked[70,71]. Furthermore, the absence of extracellular Ca^{2+} did not reduce the intercellular Ca^{2+} wave in chondrocytes indicating that the source of Ca^{2+} in the response was principally from intracellular stores[70]. In airway epithelial and endothelial cells, Ca^{2+} wave propagation to adjacent cells was not blocked when the lack of extracellular Ca^{2+} inhibited the increase in $[Ca^{2+}]_{ic}$ in the stimulated cell[71,72]. Calcium has a diffusion distance in the cytoplasm of 0.1 mm whereas IP_3 can diffuse up to 24 μm[73]. Taken together, these data indicate that mechanical stimulation can produce a Ca^{2+} wave that propagates via the passage of IP_3 through gap junctions. IP_3 then acts upon IP_3-receptors in the ER to release intracellular Ca^{2+} into the cytosol (Figure 2). In addition to IP_3-mediated release of intracellular Ca^{2+} stores, ryanodine-receptors also mediate release of Ca^{2+} from intracellular stores. Endothelial cells propagated a Ca^{2+} wave through both pathways[71].

In contrast, Jorgensen and colleagues[74,75] illustrated that Ca^{2+} wave propagation can also result from Ca^{2+} influx. Mechanical stimulation of ROS 17/2.8 cells opened Ca^{2+} channels in the plasma membrane allowing extracellular Ca^{2+} to enter the cell. Mechanical stimulation of these

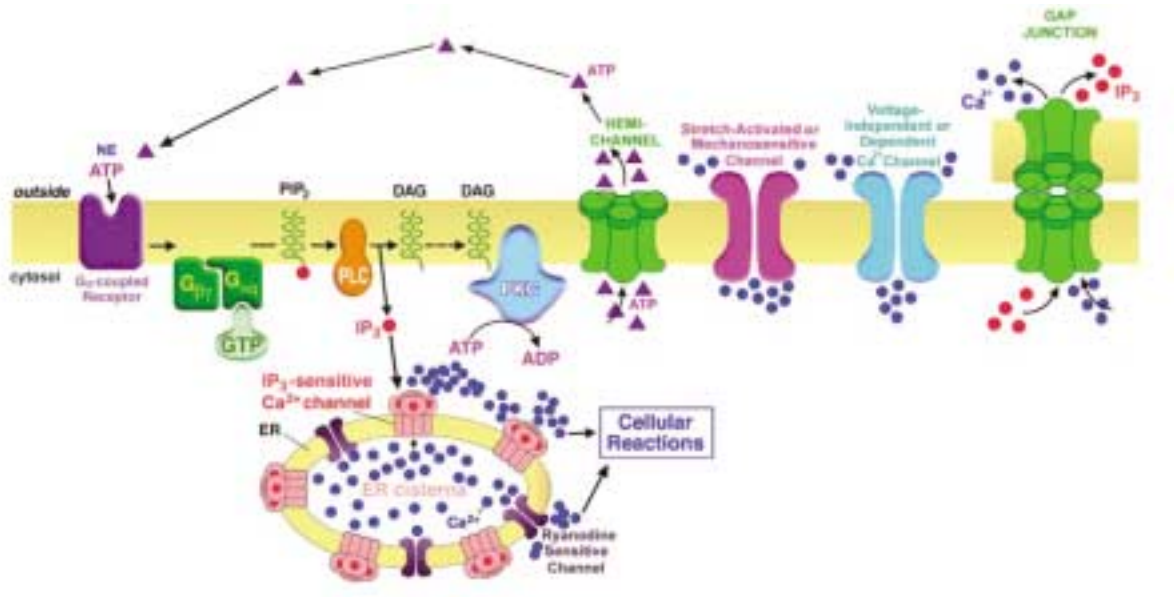

Figure 2. Calcium (Ca^{2+}) wave propagation in response to mechanical deformation by micropipet indentation involves gap junctions and intercellular Ca^{2+} signaling. Cell-to-cell communication can occur by the direct passage of Ca^{2+}, inositol (1,4,5)-trisphosphate (IP_3), or other small signaling molecules between cells through a gap junction. Calcium can induce Ca^{2+} release from ryanodine-sensitive receptors in the endoplasmic reticulum (ER) and thus release Ca^{2+} from intracellular stores. Mechanical deformation also can open stretch-activated and cation channels in the plasma membrane allowing extracellular Ca^{2+} to enter the cell. Furthermore, hemichannels can release adenosine triphosphate (ATP) in response to mechanical load. ATP can activate purinoceptors in an autocrine or paracrine fashion. Ligand activation of a receptor, such as ATP activation of purinoceptors or norepinephrine (NE) activation of adrenoceptors, results in the replacement of bound GDP with GTP on G-proteins. The activated G-protein ($G_{\alpha q}$) interacts with phospholipase C (PLC) resulting in the cleavage of phosphatidylinositol 4,5-bisphosphate (PIP_2) producing IP_3 and 1,2-diacylgylcerol (DAG). IP_3 acts on IP_3-sensitive Ca^{2+} channels in the ER to release Ca^{2+} into the cytosol. DAG activates protein kinase C (PKC), which can phosphorylate several proteins. Altered from Banes et al.[3] with copyright permission from Flexcell International Corp. (Hillsborough, NC).

osteoblasts initiated a "slow [propagating] intercellular calcium wave" that depended on gap junction coupling, extracellular Ca^{2+} and Ca_v1. Another study with ROS 17/2.8 cells had similar results with reduced signaling in the absence of extracellular $Ca^{2+}$and in the presence of gap junction inhibitors[76]. However, verapamil had no effect on the Ca^{2+} wave. Human osteoblasts also propagated a "slow intercellular calcium wave" as well as "fast [propagating] intercellular calcium wave"[75,77]. UMR 106-01 osteoblastic cells propagated a "fast" Ca^{2+} wave in response to mechanical stimulation[74,75]. This Ca^{2+} wave propagation was dependent upon $P2Y_2$ purinoceptor activation and the release of Ca^{2+} from intracellular stores. Similar results were seen in astrocytes and connexin-transfected C6 glioma cells[78]. ATP may be released from the cell when the plasma membrane is compromised and the contents of the cytoplasm diffuse into the extracellular fluid. Wounding of the plasma membrane by mechanical indentation resulted in ATP-mediated intercellular Ca^{2+} signaling in alveolar epithelial cells[79]. When the plasma membrane remained intact during mechanical stimulation, the intercellular Ca^{2+} signal was propagated via gap junctions and was unaffected by apyrase treatment. Furthermore, connexins modulated the release of ATP from cells[78]. ATP can be released from the cell through hemichannels[80] and mediate intercellular Ca^{2+} signaling through a paracrine or autocrine activation of $P2Y_2$ purinoceptors (Figure 2). In addition, other purinoceptors may be involved in intercellular communication. P2X7 receptors are activated in osteoclasts in response to mechanical stimulation[81].

Collectively, the data indicate that multiple chemical modulators affect Ca^{2+} wave propagation resulting from mechanical deformation by micropipet indentation (Table 3). In addition, the initial rise in $[Ca^{2+}]_{ic}$ in the stimulated cell can involve different mechanisms than those used for Ca^{2+} wave propagation (Table 3). For instance, in chondrocytes and endothelial cells, the Ca^{2+} response in the stimulated cell depends on extracellular Ca^{2+} where as the Ca^{2+} wave depends on intracellular Ca^{2+} stores[70,71]. Thus, multiple mechanisms and pathways are involved in the responses to mechanical stimulation and in gap junctional intercellular communication (Figure 2).

Mechanical load also regulates connexin expression and thus gap junctional intercellular communication. Avian tendon cells subjected to 0.05 cyclic strain for 1, 3, 5 and 7 days upregulated connexin-43 (Cx43) mRNA levels[66,82]. Load and norepinephrine together also increased Cx43 mRNA expres-

sion and cell-cell communication in tendon cells[83,84] indicating that perhaps adrenoceptors may also be important in the response to load. Vascular smooth muscle cells subjected to static strain[85] and cardiomyocytes[86] subjected to cyclic strain upregulated Cx43 mRNA expression as early as 30 minutes and Cx43 protein by 4 hours. Rat ventricular myocytes subjected to pulsatile stretch (10% of resting length) for 1, 3, or 6 hours increased Cx43 protein as detected by immunofluorescence for all time periods[87]. Connexin-43 mRNA expression was upregulated after 5 hours in bovine aortic endothelial cells in regions of flow recirculation ("disturbed flow") and flow recovery ("undisturbed flow")[88]. Furthermore, cell-cell communication was decreased after 5 hours of both disturbed and undisturbed flow. Only the cells in the undisturbed flow regions had regained cell coupling by 30 hours of flow. Similarly, vascular endothelial cells also increased Cx43 mRNA expression after 1 hour of applied laminar shear stress (15 dynes/cm^2)[85]. Immunohistochemically detectable Cx43 and Cx45 in the plasma membrane as well as dye-coupling were decreased in both osteoblast-like (MC3T3-E1) cells and osteocyte-like (MLO-Y4) cells after only 1 hour of shear stress[89]. This loss of connexins and dye-coupling increased with both the time and magnitude of applied shear stress. Collectively, the data indicate that load rapidly modulates Cx43 expression and gap junctional intercellular communication.

In addition to load regulating intercellular signaling, gap junctions co-regulate load responses. Gap junctions modulated release of prostaglandin E_2 in MC3T3-E1 and rat osteosarcoma cells in response to oscillatory fluid flow[59,90]. Calcium signaling, Ca_v1, stretch-activated channels, IP_3-mediated release of intracellular Ca^{2+} and G-proteins also regulate load-induced gene expression[40,43,50,91]. Furthermore, gap junctions and intercellular communication may be involved in the response to injury as well as healing[24,25,92]. Thus, further research on the role and function of gap junctions under mechanical load and physiological conditions are necessary to better understand the pathways involved in mechanotransduction as well as the processes involved in connective tissue repair.

Conclusions

Responses to mechanical stimulation involve multiple and redundant pathways[2,3]. Transduction of mechanical load to a biochemical signal involves Ca^{2+} signaling (Ca^{2+} influx, efflux, and release from intracellular stores), IP_3 and perhaps Ca^{2+} diffusion through gap junctions, purinoceptor activation, and mechanosensitive ion channels. These immediate signaling events to load may be useful in regulating the downstream effects of mechanical stimulation on connective tissues. Some positive outcomes of loading are increased matrix gene expression[93,94], mitogenesis[95,96], bone mineralization[97], and blocking cytokine actions[98]. Some negative effects of load are apoptosis[99], loss of membrane integrity[100], and upregulation of cytokines and matrix metalloproteinases[58,63]. Lack of loading altogether can lead to bone resorption and disuse osteoporosis[97,101]. However, the cell responses to load are dose-sensitive, cell-type sensitive, and synergistic or antagonistic with pathway activation/ inactivation. Understanding these responses will further our knowledge of mechanotransduction mechanisms. Furthermore, the importance of mechanical stimulation in normal homeostasis of tissues, response to wounding and in cell-cell communication during passive or active motion during convalescence from injury and repair may be integral in advancing therapeutic approaches and motion therapy regimens, in particular.

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