Full Length Article

T-Type voltage-sensitive calcium channels mediate mechanically-induced intracellular calcium oscillations in osteocytes by regulating endoplasmic reticulum calcium dynamics

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Genevieve N. Brown, Pui L. Leong, X. Edward Guo *

Bone Bioengineering Laboratory, Department of Biomedical Engineering, Columbia University, New York, NY 10027, USA

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ABSTRACT

One of the earliest responses of bone cells to mechanical stimuli is a rise in intracellular calcium (Ca^{2+}), and osteocytes in particular exhibit robust oscillations in Ca^{2+} when subjected to loading. Previous studies implicate roles for both the endoplasmic reticulum (ER) and T-Type voltage-sensitive calcium channels (VSCC) in these responses, but their interactions or relative contributions have not been studied. By observing Ca^{2+} dynamics in the cytosol (Ca^{2+}_{cyt}) and the ER (Ca^{2+}_{ER}), the focus of this study was to explore the role of the ER and T-Type channels in Ca^{2+} signaling in bone cells. We demonstrate that inhibition of T-Type VSCC in osteocytes significantly reduces the number of Ca^{2+}_{cyt} responses and affects Ca^{2+}_{ER} depletion dynamics. Simultaneous observation of Ca^{2+} exchange among these spaces revealed high synchrony between rises in Ca^{2+}_{cyt} and depressions in Ca^{2+}_{ER}, and this synchrony was significantly reduced by challenging T-Type VSCC. We further confirmed that this effect was mediated directly through the ER and not through store-operated Ca^{2+} entry (SOCE) pathways. Taken together, our data suggests that T-Type VSCC facilitate the recovery of Ca^{2+}_{ER} in osteocytes to sustain mechanically-induced Ca^{2+} oscillations, uncovering a new mechanism underlying the behavior of osteocytes as mechanosensors.

1. Introduction

Osteocytes are widely regarded as mechanosensors, capable of detecting changes in the mechanical environment of the bone tissue and modifying cellular responses accordingly [1,2]. Indeed, an intact osteocyte network is required for bone changes in response to unloading [3], and studies have shown that both loading and unloading influence osteocyte expression of proteins that modulate bone turnover [4], such as sclerostin [5,6] and receptor activator of nuclear factor kappa B ligand (RANKL) [7,8]. Still, mechanisms underlying osteocyte mechanotransduction remain unclear. For instance, one of the earliest responses of bone cells to mechanical stimuli is a rise in intracellular, or cytosolic, calcium (Ca^{2+}_{cyt}), but the mechanisms by which osteocytes generate or rely on Ca^{2+} signals to direct bone adaptation are largely unknown.

Osteocytes exhibit robust oscillations in Ca^{2+}_{cyt} in response to mechanical stimulation, a pattern distinct from their osteoblast precursors and attributed to their mechanosensitivity [9,10]. Prior *in vitro* work from our laboratory concluded that Ca^{2+}_{cyt} transients depend on both the extracellular reservoir of Ca^{2+} ions and intracellular storage organelles, in particular the endoplasmic reticulum (ER) [9]. Mechanically-induced ER Ca^{2+} (Ca^{2+}_{ER}) release depends on the purinergic pathway via inositol trisphosphate receptors (IP$_3$R) on the ER membrane. The release of Ca^{2+} from the ER is critical to fluid flow-induced Ca^{2+} oscillations in osteocytes; treatment with the drug thapsigargin to block ER Ca^{2+} reuptake significantly reduced the number of Ca^{2+}_{cyt} transients from an average of five down to a single response. A similar effect was observed in in situ osteocytes, where thapsigargin treatment abolished multiple Ca^{2+} responses induced by dynamic loading of a murine tibia [10].

Ca^{2+}_{cyt} oscillations in osteocytes are also affected by inhibition of a number of membrane channels involved in Ca^{2+} transport, and targeting channels expressed primarily in osteocytes should clarify some mechanisms underlying this unique behavior. For instance, the expression of voltage-sensitive calcium channel (VSCC) subtypes changes as osteoblasts differentiate into osteocytes [11]. Osteoblasts express both low threshold T- and high threshold L-Type VSCC, whereas osteocytes predominantly express T-Type VSCC [12]. Previous studies published from our laboratory explored the effects of VSCC inhibitors when added to the flow medium after shear stimulation [9]. The addition of the T-Type inhibitor NNC 55-0396 interrupted Ca^{2+}_{cyt} responses

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in osteocytes, preventing subsequent Ca^{2+} transients, but had little observable effect on osteoblasts. Treatment of in situ osteocytes with the T-Type inhibitor prior to mechanical stimulation also significantly reduced the number of Ca^{2+} responses [10]. Interestingly, inhibition of these channels in both systems had similar effects as disruption of ER stores, though no link has been demonstrated between them in osteocytes.

VSCC have been shown to interact with the ER and Ca^{2+}_{ER} release pathways in other cells. A direct association of T-Type VSCC with the ER has been previously demonstrated in arterial smooth muscle, where $Ca_V3.2$ channels were found to be localized to ER caveolae by transmission electron microscopy and to bind to ryanodine receptors on the ER by a proximity ligation assay [13]. In addition, a number of proteins participate in Ca^{2+} release from ER stores and Ca^{2+} entry triggered by this release — a phenomenon referred to as store-operated calcium entry (SOCE) — such as Stromal Interaction Molecules (STIMs) and Ca^{2+}-Release Activated Channels (CRACs) [14]. In particular, STIM1 has been shown to interact with L-Type VSCC in rat cortical neurons [15] and vascular smooth muscle cells [16] and $Ca_V3.1$ T-Type VSCC in cardiac myocytes [17]. The potential role of SOCE-related proteins in osteocyte Ca^{2+} signaling have not yet been explored.

Though our previous studies implicate roles for both the ER and T-Type VSCC in osteocyte Ca^{2+}_{cyt} responses, their relative contributions or any interactions between the ER and T-Type VSCC remain unknown. This is largely due to an inability to monitor Ca^{2+} localized to the ER separately from Ca^{2+} entering the cell from the extracellular fluid. However, advances in genetically encoded Ca^{2+} biosensors now enable the targeting of these sensors to subcellular organelles, including the ER [18–20].

Thus, the focus of the current study is to observe Ca^{2+}_{ER} dynamics in bone cells to better understand the role of ER stores in the unique Ca^{2+} oscillations in osteocytes. We hypothesized that an ability to refill ER stores results in Ca^{2+}_{cyt} oscillations in osteocytes. We also hypothesized that the predominant expression of T-Type channels in osteocytes may contribute to their unique Ca^{2+}_{cyt} patterns and further speculated that T-Type VSCC in osteocytes may interact with ER stores.

2. Materials and methods

2.1. Cell culture

Osteocyte-like MLO-Y4 cells (a gift from Dr. Lynda Bonewald, University of Missouri-Kansas City, Kansas City, MO) were cultured on 0.15 mg/ml collagen (rat tail type I, BD Biosciences, San Jose, CA) coated culture dishes in minimum essential alpha medium (α-MEM, Life Technologies, Carlsbad, CA) supplemented with 5% fetal bovine serum (FBS, Hyclone Laboratories Inc., Logan, UT) and 5% calf serum (CS, Life Technologies, Carlsbad, CA). MC3T3-E1 pre-osteoblasts (ATCC, Manassas, VA) were cultured in α-MEM supplemented with 10% FBS. Cells were maintained at 5% CO_2 and 37 °C in a humidified incubator. MLO-Y4 cells were sub-cultured prior to reaching 70–80% confluence in order to maintain an osteocyte-like phenotype.

2.2. Immuno-detection

To detect the presence of L- and T-Type VSCC in osteocytes and osteoblasts, we performed Western blots and immunostaining using antibodies targeted against $Ca_V1.2$ (L-Type) and $Ca_V3.2$ (T-Type) subunits (SDIX, Newark, DE). Protein was extracted from monolayer cell cultures using RIPA lysis buffer with protease and phosphatase inhibitors (Roche Diagnostics, Indianapolis, IN). Western blots were performed on MLO-Y4 and MC3T3-E1 lysates of equal total protein as determined by a BCA assay (Pierce, ThermoFisher Scientific, Waltham, MA). For immunostaining, cells were fixed in 4% paraformaldehyde and permeabilized with 2% Triton™ X-100 (Sigma-Aldrich, St. Louis, MO). Following

antibody incubations, cells were counterstained with DAPI to label nuclei (Molecular Probes, Eugene, OR).

2.3. Inhibitors

All inhibitors used in this study were purchased from Sigma-Aldrich (St. Louis, MO). Thapsigargin is an inhibitor of the Ca^{2+}-ATPase pump on the ER (SERCA) which facilitates the reuptake of Ca^{2+} into the ER, and thapsigargin treatment (1 μM) thereby results in ER depletion. Two VSCC inhibitors were used in this study: the T-Type inhibitor NNC 55-0396 (20 μM) and the L-Type specific inhibitor nifedipine (10 μM). MLO-Y4 cells were also treated with the following inhibitors related to SOCE: YM58483 (3 μM), a CRAC inhibitor [21]; SKF-96365 (50 μM), a SOCE inhibitor that inhibits STIM1 (similar effects as STIM1 silencing) [22] but exhibits off-target effects on VSCC activity [23]; and 2-APB (50 μM), a reliable inhibitor of SOCE and IP_3R [23,24]. Cells were incubated in the inhibitors for 15 min prior to flow exposure, and the inhibitors remained in the flow medium for the duration of the experiment.

2.4. Ca^{2+}_{cyt} indicators and Ca^{2+}_{ER} visualization

To observe Ca^{2+}_{cyt} changes only, MLO-Y4 and MC3T3-E1 cells were stained with Fluo-8 AM (AAT Bioquest, Sunnyvale, CA) dissolved in 20% Pluronic F-127 in DMSO (Invitrogen, Carlsbad, CA). To visualize ER calcium levels in osteocytes and osteoblasts, cells were transiently transfected with the D1ER plasmid (plasmid #36325, Addgene, Cambridge, MA) [25] using standard non-liposomal techniques (Fugene 6, Promega Corporation, Madison, WI). D1ER is a second generation cameleon Ca^{2+} fluorescence resonance energy transfer (FRET) sensor targeted to the ER with a retention sequence. To simultaneously visualize Ca^{2+}_{cyt} and Ca^{2+}_{ER}, cells were transfected with D1ER and then stained with the red-shifted Ca^{2+}_{cyt} indicator Fura Red-AM (20 μM, Life Technologies, Carlsbad, CA) in DMSO and Kolliphor® EL (Sigma-Aldrich, St. Louis, MO) prior to fluid flow stimulation.

To verify the localization of the D1ER plasmid, transfected cells were stained with ER-Tracker Red (Molecular Probes, Eugene, OR). Cells were rinsed and incubated with a warmed 1 μM working solution of ER-Tracker Red for 30 min at 37 °C. Cells were rinsed with fresh medium and post-incubated for 15 min prior to imaging. The FRET biosensor was excited at 430 nm, and fluorescence emission of YFP (530 nm) was collected as the FRET image. The ER Tracker Red dye was imaged using 568 nm excitation/660 nm emission.

2.5. Fluid flow stimulation

Prior to staining with appropriate Ca^{2+} indicators, cells were plated onto 10 μg/mL fibronectin-coated glass slides at ~80% confluency to establish cell–cell contact. Slides were then stained and assembled into a custom parallel-plate flow chamber with a glass bottom that permits live cell imaging under fluid shear stimulation. The chamber was placed on the stage of an inverted microscope (Olympus, Waltham, MA) and attached to a magnetic gear pump (Scilog, Madison, WI) for the application of steady, laminar, unidirectional flow at a shear stress of 35 dyn/cm^2, which has been shown to induce multiple Ca^{2+}_{cyt} responses in osteocytes and fewer, weaker responses in osteoblasts in our previous studies [9]. In addition, the Ca^{2+}_{cyt} patterns observed in vitro under this flow profile are consistent with those observed in ex vivo mouse tibia under physiologic dynamic loads [10], while oscillatory flow induces fewer responses [26]. Baseline fluorescence intensity was captured for 1 min prior to fluid shear stimulation for 9 min.

2.6. Imaging and image analysis

For monitoring ER depletion, time-lapse images were collected 36–48 h post transfection at 40× magnification. The FRET biosensor was