In situ intracellular calcium oscillations in osteocytes in intact mouse long bones under dynamic mechanical loading

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ABSTRACT Osteocytes have been hypothesized to be the major mechanosensors in bone. How in situ osteocytes respond to mechanical stimuli is still unclear because of technical difficulties. In vitro studies have shown that osteocytes exhibited unique calcium (Ca2+) oscillations to fluid shear. However, whether this mechanotransduction phenomenon holds for in situ osteocytes embedded within a mineralized bone matrix under dynamic loading remains unknown. Using a novel synchronized loading/imaging technique, we successfully visualized in real time and quantified Ca2+ responses in osteocytes and bone surface cells in situ under controlled dynamic loading on intact mouse tibia. The resultant fluid-induced shear stress on the osteocyte in the lacunocanalicular system (LCS) was also quantified. Osteocytes, but not surface cells, displayed repetitive Ca2+ spikes in response to dynamic loading, with spike frequency and magnitude dependent on load magnitude, tissue strain, and shear stress in the LCS. The Ca2+ oscillations were significantly reduced by endoplasmic reticulum (ER) depletion and P2 purinergic receptor (P2R)/phospholipase C (PLC) inhibition. This study provides direct evidence that osteocytes respond to in situ mechanical loading by Ca2+ oscillations, which are dependent on the P2R/PLC/inositol trisphosphate/ER pathway. This study develops a novel approach in skeletal mechanobiology and also advances our fundamental knowledge of bone mechanotransduction.—Jing, D., Baik, A. D., Lu, X. L., Zhou, B., Lai, X., Wang, L., Luo, E., Guo, X. E. In situ intracellular calcium oscillations in osteocytes in intact mouse long bones under dynamic mechanical loading. FASEB J. 28, 1582–1592 (2014). www.fasebj.org

Key Words: lacunocanalicular system · mechanotransduction · fluorescence recovery after photobleaching · shear stress · endoplasmic reticulum · purinergic receptor

Osteocytes are mature bone cells that reside in a fluid-filled mineralized bone matrix and are interconnected through numerous intercellular processes to form an extensive network in the lacunocanalicular system (LCS; 1). Osteocytes can extend their dendritic processes to communicate with osteoblasts and possibly osteoclasts on the bone surface and regulate the matrix remodeling activities of these cells through factors such as receptor activator of nuclear factor-κB ligand (RANKL) and sclerostin (2, 3). The osteocyte network has been hypothesized to be the central mechanosensor that orchestrates bone modeling and remodeling by detecting mechanical stimuli applied to the skeleton (4) through interstitial fluid flow (5). Indeed, perturbations in osteocyte networks have been shown to drastically alter bone remodeling and/or modeling in a mechanical disuse model (6). Therefore, understanding osteocyte mechanotransduction has significant clinical implications in diseases involving dysfunctional bone remodeling, such as osteoporosis. To date, however, how in situ osteocytes detect and transduce mechanical stimuli is still poorly understood.

Many in vitro experiments have demonstrated the capability of osteocytes to respond to various forms of mechanical stimuli, such as fluid flow-induced shear stress and hydrostatic pressure (7–10). However, other

Abbreviations: 2D, 2-dimensional; 18α-GA, 18α-glycyrrhetinic acid; α-MMEM, α-minimal essential medium; ANOVA, analysis of variance; CSFBS, charcoal stripped fetal bovine serum; DIC, differential interference contrast; DMSO, dimethyl sulfoxide; ER, endoplasmic reticulum; FBS, fetal bovine serum; FRAP, fluorescence recovery after photobleaching; GJ, gap junction; IP3, inositol trisphosphate; LCS, lacunocanalicular system; PBS, phosphate-buffered saline; PLC, phospholipase C; PPADS, pyridoxalphosphate-6-azophenyl-2′,4′-disulfonic acid; P2R, P2 purinergic receptor; RANKL, receptor activator of nuclear factor-κB ligand; SACC, stretch-activated Ca2+ channel; TG, thapsigargin; VGCC, voltage-gated Ca2+ channel

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types of bone cells, such as osteoblasts and osteoclasts, have also been shown to respond to these physical stimuli (11, 12) and propagate intracellular signaling molecules among neighboring cells (13–15). Thus, a delineated role of osteocytes in bone mechanotransduction remains unclear. Intracellular calcium (Ca\(^{2+}\)) is a pivotal and ubiquitous second messenger regulating many downstream cellular activities, and it is also observed to be one of the earliest mechanotransduction events in bone cells (16, 17). Abundant in vitro studies on Ca\(^{2+}\) signaling in osteoblasts and osteocytes subjected to mechanical stimuli have identified two distinct transduction pathways: fast Ca\(^{2+}\) wave propagation via ATP/P₂ purinergic receptors (P₂Rs), and relatively slow Ca\(^{2+}\) waves via intercellular gap junctions (GJs), such as connexin 43 (13–20). Our group recently discovered that 2-dimensional (2D) in vitro micropatterned osteocyte networks were much more sensitive than osteoblasts to fluid flow stimulation in terms of Ca\(^{2+}\) oscillations (18). Consistent with previous findings (13–15), the ATP-related signaling pathway dominates these uniquely repetitive Ca\(^{2+}\) oscillations. Furthermore, Ca\(^{2+}\) oscillations of micropatterned individual osteocytes were spatiotemporally correlated within a cell network, demonstrating that intercellular communication between neighboring cells is also a fundamental aspect of osteocyte mechanotransduction (4, 19). Thus, osteocyte networks, at least in the 2D in vitro system, demonstrate the capability to detect daily physical mechanical activities and to function as the major mechanical sensors in bone. However, it is unknown whether these intriguing mechanotransduction phenomena hold for in situ osteocyte networks embedded in their native mineralized bone matrix with the natural LCS microenvironment under physiological mechanical loading.

There have been several previous studies using calvarial bone for examining in situ Ca\(^{2+}\) signaling of bone cells (21–23). Ishihara et al. (23) found that both surface osteoblasts and osteocytes in situ exhibited autonomous Ca\(^{2+}\) responses, with the osteoblasts appearing more active. Their study also suggested that inhibition of GJ communication significantly altered autonomous waves in osteocytes but not osteoblasts. In addition, fluid flow on the calvarial surface induced Ca\(^{2+}\) oscillations in both the calvarial and osteocytes, where GJs were once again more critical in osteocytes. Adachi et al. (21) demonstrated that calvarial bone matrix deformation induced by microneedles resulted in a rapid and prolonged increase of Ca\(^{2+}\) concentration in an osteocyte. Although these studies have demonstrated the importance of Ca\(^{2+}\) signaling in bone in situ, the role of mechanical loading in osteocyte mechanotransduction is not clear in a bone not yet adapted for dynamic mechanical events. In this study, we established a novel ex vivo mechanical loading model using mouse tibia for studying real-time Ca\(^{2+}\) signaling in osteocytes. It is worthwhile noting that this axial tibial loading model has been used extensively in in vivo studies of bone mechanobiology (24–28). Our new synchronized loading/imaging model has the capabilities of applying controlled dynamic loading on intact, healthy long bones, measuring and estimating the shear stress on osteocytes in the LCS by a combination of fluorescence recovery after photobleaching (FRAP) imaging and transport modeling, and, most notably, recording the Ca\(^{2+}\) signaling of in situ osteocyte networks in the LCS when the bone is subjected to physiologically relevant dynamic loading. For the first time, we observed Ca\(^{2+}\) oscillations in osteocytes in the form of repetitive Ca\(^{2+}\) spikes in intact long bone under dynamic loading. In addition, we correlated the magnitudes of applied mechanical loads, tissue strains, LCS fluid flow velocities, and shear stresses with the spatiotemporal parameters of Ca\(^{2+}\) responses in osteocyte networks. We further investigated the underlying biochemical mechanisms responsible for osteocyte Ca\(^{2+}\) signaling by examining the roles of 7 Ca\(^{2+}\) signaling-related pathways.

**MATERIALS AND METHODS**

**Bone sample preparation and Ca\(^{2+}\) dye loading**

This study was approved by the Institutional Animal Care and Use Committee at Columbia University. Skeletally mature C57BL/6j female mice were purchased from The Jackson Laboratory (Bar Harbor, ME, USA). After euthanasia by CO₂ inhalation, bilateral intact tibiae with periosteum were immediately dissected from the carcasses, with muscles being gently removed under sterile conditions. Samples were incubated in α-minimal essential medium (α-MEM) containing 5% fetal bovine serum (FBS), 5% calf serum, and 1% penicillin/streptomycin for 2–6 h before imaging (29).

For fluorescent Ca\(^{2+}\) imaging, tibiae were incubated with 15 μM Fluo-8 AM (ABD Bioquest, Sunnyvale, CA, USA) dissolved in dimethyl sulfoxide (DMSO) with pluronic acid F-127 (20% in DMSO; Life Technologies, Carlsbad, CA, USA). After incubation, bones were incubated with 2 μM calcein AM and 4 μM ethidium homodimer-1 dissolved in phosphate-buffered saline (PBS) for 45 min at 37°C. Bone cells inside the tibia were then visualized using a Fluoview FV1000 confocal microscope (Olympus, Tokyo, Japan) with 488/516- and 586/617-nm laser excitation/emission, respectively. Osteocyte viability and function were further confirmed by observing the release of osteocyte Ca\(^{2+}\) spikes in response to the exogenous ATP stimulation. Tibiae (n=9) were incubated with 15 μM Fluo-8 AM for 30 min at 37°C, followed by deesterification for 15 min, and then were imaged using the confocal microscope. ATP solution (Sigma-Aldrich, St. Louis, MO, USA) at 1 mM in α-MEM containing 5% CSFBS was added to the sample. In addition, ionomycin, a potent and selective Ca\(^{2+}\)-ionophore agent (Life Technologies) at 10 or 20 μM dissolved in DMSO and in α-MEM containing 5% CSFBS, was also added to the

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**Cell viability assessment**

The viability of bone cells within the cultured tibiae was assessed by live/dead staining (Live/Dead Viability/Cytotoxicity Kit; Life Technologies) at 2, 12, 24, and 48 h after animal euthanasia (3 tibiae were evaluated at each time point). Tibiae were incubated with 2 μM calcein AM and 4 μM ethidium homodimer-1 dissolved in phosphate-buffered saline (PBS) for 45 min at 37°C. Bone cells inside the tibia were then visualized using a Fluoview FV1000 confocal microscope (Olympus, Tokyo, Japan) with 488/516- and 586/617-nm laser excitation/emission, respectively. Osteocyte viability and function were further confirmed by observing the release of osteocyte Ca\(^{2+}\) spikes in response to the exogenous ATP stimulation. Tibiae (n=9) were incubated with 15 μM Fluo-8 AM for 30 min at 37°C, followed by deesterification for 15 min, and then were imaged using the confocal microscope. ATP solution (Sigma-Aldrich, St. Louis, MO, USA) at 1 mM in α-MEM containing 5% CSFBS was added to the sample. In addition, ionomycin, a potent and selective Ca\(^{2+}\)-ionophore agent (Life Technologies) at 10 or 20 μM dissolved in DMSO and in α-MEM containing 5% CSFBS, was also added to the
samples as positive controls. Only viable cells are expected to show intracellular Ca\textsuperscript{2+} responses (30).

**Mechanical loading system**

A custom-designed system was built for *in situ* osteocyte Ca\textsuperscript{2+} and FRAP imaging of murine tibiae (Fig. 1A). Axial loading of the tibia was applied using a piezoelectric linear actuator (M-227.10; Physik Instrumente, Karlsruhe, Germany). Loading force was measured by a 5-lb load cell (model 31; Honeywell, Columbus, OH, USA). A linear bearing was mounted along the loading axis to constrain the lateral movement of the sample holder during mechanical loading. Actual deformation applied on the tibia was measured using a capacitive displacement transducer (CapaNCDT620; Micro-Epsilon, Raleigh, NC, USA). The force and displacement data were captured using a 16-bit data acquisition (DAQ) card (NI USB-6210) and LabView software (National Instruments, Austin, TX, USA).

**Synchronized tibial mechanical loading and confocal imaging**

Tibiae loaded with a Ca\textsuperscript{2+} indicator were mounted in the loading apparatus, and the anteromedial surface that experi-enced significant tensile strains under compressive axial loading on the whole bone was imaged using the confocal microscope with either ×10 or ×40 objectives and 488-nm laser excitation. Because Fluo-8 AM readily penetrated into live cells, the cells lining the bone surface and osteocytes in the matrix could be easily identified by adjusting the focus depth. Surface cells were typically located at 10 μm below a bright periosteum staining, and osteocytes were identified 30–40 μm below the periosteum. The periosteum on the bone surface was able to act as a barrier against the external fluid movement and thus minimize the potential shear stress generated by the slight medium movement on surface cells. Confocal time-lapse images were synchronized with a rest-inserted mechanical loading protocol to eliminate in-plane and out-of-focus motion artifacts as reported in our previous study (Fig. 1B and ref. 31). A preload of 2 N followed by 175 cycles of 6-, 8-, or 10-N peak compressive load was applied on tibiae (thus resulting in 4-, 6-, and 8-N loading magnitudes). A dwell time of 4 s was applied between each cycle. Confocal images were recorded at 1.1 s/frame during the dwell time after each loading cycle for a total of 180 frames (512×512-pixel images) including 5 frames for baseline recording before loading. The total imaging and loading period was 900 s. Motion artifacts were further removed using the

*Figure 1. Ex vivo mouse long bone mechanical loading and Ca\textsuperscript{2+} and FRAP imaging setups and protocols. A) Schematic representation of the mechanical loading system used for *in situ* Ca\textsuperscript{2+} imaging and FRAP experiments. Axial, cyclic compressive loading was applied on the distal end of the tibia via a piezoelectric linear actuator to generate compression and bending. B) Displacement of the linear actuator produced peak load magnitudes of 4, 6, or 8 N and was synchronized with confocal image acquisition to minimize movement artifacts. Compressive cyclic loads were applied for 175 cycles over 15 min. C) Strain gauges were placed on the anteromedial surface where the Ca\textsuperscript{2+} imaging and FRAP experiments were performed to quantify the load-induced strains. D) Representative fluorescent image showing the selected photobleached and reference lacunae for the FRAP experiment. E) Time course of the fluorescence recovery in the photobleached lacuna. Lacunae were photobleached to ~30–50% of original intensity.*
StackReg image registration algorithm in ImageJ (U.S. National Institutes of Health; Bethesda, MD, USA; ref. 32). Cells were manually traced using MetaMorph 7.0 (Molecular Devices, Downingtown, PA, USA), and the average intensity of the cell body was extracted as a function of time. The average pixel intensity of each individual cell was normalized by its corresponding baseline, and a spike was defined as a transient increase to 1.5 times the baseline intensity of each cell (22, 23). The percentage of responsive cells (number of responsive cells divided by the total cells in the field of view), average number of \( \text{Ca}^{2+} \) spikes (excluding nonresponsive cells), magnitude of \( \text{Ca}^{2+} \) spikes, and time to the first spike were quantified (18, 33).

**Strain measurement**

To quantify load-induced strain on our region of interest (the anteromedial surface), a separate set of nine 3-mo female mouse tibiae were used for mechanical testing. The tensile strain was measured using commercial strain gauges with 0.38-mm grid length and 0.51-mm grid width (EA-06-015DJ-120; Vishay Measurements Inc., Wendell, NC, USA). The strain gauge was glued on the tibia anteromedial surface using cyanoacrylate adhesive (M-Bond 200 adhesive; Vishay Measurements), which was consistent with the \( \text{Ca}^{2+} \) or FRAP imaging region (Fig. 1C). During mechanical testing, the tibiae were immersed in PBS. A 0.5-N preload was applied to secure the sample, and the linear actuator moved at 10 \( \mu \text{m/s} \) to compress the sample until the load reached 11 N. The strain gauge output was connected to a signal conditioning amplifier (Vishay 2100; Vishay Measurements), and strain values were simultaneously recorded by the National Instruments DAQ board.

**FRAP-based measurements of LCS fluid flow and shear stress**

A separate set of tibiae (\( n=14 \)) were incubated in 0.2 mg/ml sodium fluorescein (376 Da) in \( \alpha \)-MEM for 1.5 h at 37°C before imaging. Paired FRAP was performed on randomly selected lacunae \( \approx 40 \mu \text{m} \) below the periosteal surface (Fig. 1D) when bone was mechanically loaded or statically held (31). In brief, the selected lacuna was photobleached using a high-intensity 488-nm laser for \( \approx 3 \) s, reducing its fluorescence intensity to 30–50% of the original level, followed by application of 40 loading cycles with a 4-s imaging period inserted between the adjacent cycles. From the raw image data sets, temporal fluorescence profiles that showed a typical exponential recovery in the photobleached lacuna and a slight autofading because of repeated imaging in far away reference lacuna were obtained (Fig. 1E). After correction of the autofading, the transport rate \( k \) of the tracer (defined as the reciprocal of the characteristic time constant of the exponential recovery) and the transport enhancement \( k/k_0 \) (defined as the ratio of tracer transport rate under loading condition \( k \) over that under static condition \( k_0 \)) were quantified. By simulating the diffusion-convection of tracers in an anatomically accurate LCS model, the canalicular fluid velocity that matched the measured transport enhancement for each loading condition was obtained (31). The corresponding shear stress was calculated from the velocity profile inside the annular canalicular fluid channel using the Brinkman equation (34) and scaled to the lacunar space surrounding the cell body (for details, see Supplemental Material).

**Ca\(^{2+}\) signaling pathway studies**

All 7 of the following biochemical pathways (detailed below) have been shown in our previous studies to be critical for \( \text{Ca}^{2+} \) responses in bone cells (18, 20, 33). Five mice (10 tibiae) were used for each specific pathway group. Left and right tibiae of each mouse were paired and separated into control and inhibitor-treated groups. A cyclic load of 6-N magnitude was applied for all groups. Tibiae were preincubated with the pathway inhibitor for 1 h at 37°C before \( \text{Ca}^{2+} \) dye incubation, and the inhibitor was present in the medium throughout the imaging experiment. The inhibitors for the 7 pathways are specified as follows: 150 \( \mu \text{M} \) pyridoxalphosphate-6-azophenyl-2',4'-disulfonic acid (PPADS), a nonselective P2R blocker; 1 \( \mu \text{M} \) neomycin, a phospholipase C (PLC) inhibitor; 50 \( \mu \text{M} \) GdCl\(_3\), an inhibitor of stretch-activated \( \text{Ca}^{2+} \) channels (SACCs); 20 \( \mu \text{M} \) NNC 55–0396, a selective inhibitor of T-type voltage-gated \( \text{Ca}^{2+} \) channels (VGCCs); 75 \( \mu \text{M} \) 18-glycyrhethinic acid (18s-GA; 0.1% DMSO), a reversible intercellular GJ blocker; 1 \( \mu \text{M} \) thapsigargin (TG; 0.1% DMSO), an inhibitor of the \( \text{Ca}^{2+} \)-ATPase pump of the endoplasmic reticulum (ER); and 10 \( \mu \text{M} \) amiodipine (0.1% DMSO), an L-type VGCC antagonist. Because 18s-GA, TG, and amiodipine were dissolved in DMSO, the contralateral tibiae were incubated in 0.1% DMSO as a vehicle control group. All antagonists were obtained from Sigma-Aldrich. The concentrations of all inhibitors were optimized for bone cells and other type of cells by previous investigations (18, 20, 33, 35–40).

**Data analysis**

All data are presented as means ± sd. One-way analysis of variance (ANOVA) with Bonferroni’s post hoc analysis was performed to compare the \( \text{Ca}^{2+} \) spike number, magnitude of spike intensity, time to reach the first spike and time between the first and second spikes between surface cells and osteocytes across the mechanical loading magnitudes. To determine the correlation between a spatiotemporal parameter and the load level, a linear regression analysis was performed. One-way ANOVA with Bonferroni’s post hoc analysis was used to determine statistical differences between the \( \text{Ca}^{2+} \) spike spatiotemporal parameters of 7 pathway groups. PPADS-, neomycin-, GdCl\(_3\)-, and NNC 55–0396-treated groups were compared with the untreated groups (the contralateral tibiae). 18s-GA, TG-, and amiodipine-treated groups were compared with the vehicle control (DMSO-treated contralateral tibiae). The significance level was set at 0.05.

**RESULTS**

**Validation of the ex vivo \( \text{Ca}^{2+} \) imaging model**

Bone surface cells and osteocytes were imaged at a focal plane \( \approx 10 \) and \( \approx 40 \) \( \mu \text{m} \) below the periosteal surface, respectively (Fig. 2A, C), under a confocal microscope. Differential interference contrast (DIC) images showed the mineral-facing bone matrix with many cavity-like lacunae in the osteocyte layer but not in the bone surface cell layer (Fig. 2B, D). To validate the present ex vivo mouse tibia culture technique, live/dead staining was used to evaluate the cell viability at different time points of tissue culture (2, 12, 24, and 48 h) after tibia dissection (Fig. 2E–H). Our results demonstrate that almost all bone cells were labeled with calcein AM (green color) rather than ethidium homodimer-1 (red color), indicating that the bone cells maintained their viability for \( \approx 12 \) h after animal euthanasia. The viability and function of these osteocytes cultured within 12 h...
after animal euthanasia were further confirmed by the release of robust Ca$^{2+}$ spikes in response to exogenous ATP stimulation (Supplemental Movie S1). In addition, ionomycin, at either 10 or 20 μM, triggered robust and prolonged \textit{in situ} osteocyte Ca$^{2+}$ increases in intact mouse tibiae (data not shown).

Quantification of mechanical loading-induced tissue strain, LCS fluid flow, and shear stress in the mouse tibia

Axial compressive loading was applied on the distal end of the tibia to generate tensile strain on the anteromedial bone surface. The resultant strains of 500–2500 με were measured using strain gauges at 1- to 11-N axial loads (n=9 tibiae; \textbf{Fig. 3A, B}). Significantly linear positive correlation existed between the strain and force values over the tested loading range \([\text{strain (με)} = 193.186 \times \text{force (N)}; \text{Fig. 3B}]\). The average peak tensile strain in the 4-, 6-, and 8-N loading groups was 773, 1159, and 1546 με, respectively. These strain values applied on the mouse tibia fell in the range of the anabolic strain in human long bone \textit{in vivo} (41).

FRAP-based quantification of load-induced fluid flow velocity and shear stress in the LCS is shown in \textbf{Fig. 3C–H}. Mechanical loading increased the fluorescence recovery (\textbf{Fig. 3C}) and enhanced the characteristic transport rate \(k\) \textit{i.e.}, the slope of the fitting line (\textbf{Fig. 3D}). The solute transport enhancement (relative to diffusion, \(k/k_0\)) displayed a positive linear correlation with the load magnitude (\textbf{Fig. 3E}). Using a 3-compartment LCS transport model combined with the measured LCS anatomic parameters listed in Supplemental Table S1 (31, 42), we established a power relationship between load-induced transport enhancement and fluid velocity in the bone LCS of the tibiae studied (\textbf{Fig. 3F}). The calculated peak fluid flow velocity in the bone LCS (detailed in the Supplemental Material) was 34.4 μm/s at 4-N, 46.1 μm/s at 6-N, and 57.8 μm/s at 8-N mechanical loading, which corresponded to 4.7-, 6.3-, and 7.9-Pa fluid shear stress on the osteocyte processes in the canaliculi, respectively (\textbf{Fig. 3G}). The peak fluid flow around the cell body was calculated as 2.7 μm/s at 4-N, 3.7 μm/s at 6-N, and 4.6 μm/s at 8-N loading levels, which corresponded to 0.28-, 0.38-, and 0.47-Pa fluid shear stress, respectively (\textbf{Fig. 3H}). These results demonstrate that the fluid flow within the LCS was positively correlated with loading magnitude under the loading conditions tested.

\textbf{In situ} osteocytes displayed fewer autonomous Ca$^{2+}$ responses than surface cells

Time-lapse fluorescent images of surface cells and osteocytes were captured under static (0-N load magnitude) conditions for 15 min to record the autonomous Ca$^{2+}$ responses \textit{in situ} (\textbf{Fig. 4A, B} and Supplemental Movie S2). The total numbers of bone samples and analyzed cells in each experimental group are shown in Table 1. As shown in \textbf{Fig. 4C}, 13.0% of bone surface cells, but only 1.3% of osteocytes, displayed autonomous Ca$^{2+}$ responses. Responsive surface cells and osteocytes had an average of 1.4 ± 0.8 and 1.3 ± 0.5 spikes during the 15-min imaging period, respectively (\textbf{Fig. 4D}). Our find-
in Fig. 4A, B and Supplemental Movie S2. Bone surface cells and osteocytes displayed dramatically different characteristics in Ca\(^{2+}\) signaling under mechanical loading. A small percentage of surface cells (13.1% at 4 N, 20.5% at 6 N, and 18.7% at 8 N) released 1 or 2 Ca\(^{2+}\) spikes, with slightly higher values under 6- and 8-N conditions. In contrast, osteocytes responded to mechanical loading with Ca\(^{2+}\) signaling in the form of multiple Ca\(^{2+}\) spikes. The percentage of responsive cells was significantly higher than that of bone surface cells over the entire range of loading magnitudes (Fig. 4C). The responsive percentage of osteocytes was linearly proportional to the magnitude of the compressive load applied on the tibiae (47.3% at 4 N, 60.4% at 6 N, and 71.3% at 8 N), whereas bone surface cells showed no consistent trend under increased load. Furthermore, a positive correlation was observed between the number of Ca\(^{2+}\) spikes and loading magnitude in osteocytes but not in surface cells (Fig. 4D). Osteocytes displayed 3.4 ± 3.0 spikes at 4 N, 4.0 ± 3.6 spikes at 6 N, and 5.4 ± 4.3 spikes at 8 N, which were significantly higher than those in the surface cells at all loading levels (P<0.05). Osteocytes also had Ca\(^{2+}\) spikes with a significantly higher magnitude (P<0.05) and took less time to initiate the first Ca\(^{2+}\) spike than surface cells, and both parameters for osteocytes were dependent on the loading magnitude (Fig. 4E, F). In summary, these results demonstrate that embedded osteocytes display unique and robust Ca\(^{2+}\) oscillations that are dependent on the mechanical loading magnitude applied to the long bone.

ER Ca\(^{2+}\) store and ATP-related signaling pathways contribute to mechanical loading-induced Ca\(^{2+}\) oscillations in osteocytes

The numbers of tested tibiae and analyzed cells are shown in Table 2. Inhibitions of P2\(\beta\)R and PLC and depletion of the ER Ca\(^{2+}\) store significantly reduced the percentage of responsive osteocytes (Fig. 5A). The responsive percentage was reduced from 67.3% in the control conditions to 6.3% in the P2\(\beta\)R-inhibited group and to 7.0% in the PLC-inhibited group and from 65.5% under vehicle control conditions to 7.4% in the ER Ca\(^{2+}\) store-depleted group. Thus, these 3 pathways played a critical role in the initiation of Ca\(^{2+}\) responses under loading. Disruption of these pathways also reduced the number of Ca\(^{2+}\) spikes observed over the 15-min loading period (P<0.05; Fig. 5B). The average number of Ca\(^{2+}\) spikes was 1.8 ± 1.2 in the P2\(\beta\)R-inhibited group, 1.5 ± 0.8 in the PLC-inhibited group, and 1.4 ± 0.7 in the ER Ca\(^{2+}\) store-depleted group, all showing a greater than 50% decrease compared with those in the corresponding control groups. Significant inhibition of Ca\(^{2+}\) responses was also observed after the SACCs, T-type VGCCs, GJs, and L-type VGCCs were inhibited. All pathway inhibitions, except GJ inhibition, displayed modest yet significant decreases in Ca\(^{2+}\) spike magnitude (P<0.05; Fig. 5C) and resulted in increases in the time interval between the first and second spikes.
These results demonstrate that both extracellular and intracellular Ca\(^{2+}\)/H\(_{1001}\) sources, including Ca\(^{2+}\)/H\(_{1001}\) -induced Ca\(^{2+}\)/H\(_{1001}\) release mechanisms mediated through inositol trisphosphate (IP\(_3\)), are critical for the initiation and oscillations of Ca\(^{2+}\)/H\(_{1001}\) spikes in osteocytes when bone is subjected to cyclic loading.

**DISCUSSION**

Osteocytes have long been conjectured to be the major mechanosensors in bone because of their unique positioning in the bone matrix, enabling them to directly experience mechanical loads and respond biochemically to the changes in the mechanical microenvironment through their expansive LCS network connections (43). However, how osteocytes respond and transduce the mechanical signals in situ in the LCS is still poorly understood. Several previous studies have investigated osteocyte mechanotransduction in calvarial bone fragments using microneedle displacement (21) or fluid flow over the fragment surface (22). However, to date, critical questions regarding the anatomic site and the usage of physiologically relevant mechanical loading for Ca\(^{2+}\)/H\(_{1001}\) signaling of osteocytes in situ remain unanswered. Understanding the characteristics of Ca\(^{2+}\)/H\(_{1001}\) signaling of bone cells in more clinically relevant load-bearing sites, e.g., tibia and femur, might be more helpful for deciphering the mechanisms of skeletal mechanotransduction. Indeed, osteocytes in long bones display numerous morphological and network differences compared with those in calvarial osteocytes, which possibly involve distinct mechanotransduction and mechanoadaptation mechanisms (44, 45). Furthermore, reproducing physiologically relevant mechanical loading on in situ osteocytes, e.g., fluid flow-induced shear stresses within the LCS resulting from mineralized matrix deformations, is impossible using currently available in vitro or calvarial ex vivo models. The mineralized bone matrix and dynamic nature of physiological loading present significant technical challenges for observing and quantifying Ca\(^{2+}\)/H\(_{1001}\) signaling in osteocytes in intact bones under mechanical loading. In this study, we developed a synchronized loading/imaging approach for investigating in situ osteocyte Ca\(^{2+}\)/H\(_{1001}\) mechanotransduction in long bone, which represents an important advance in organ-level cell signaling studies within dynamically deformed tissues.

In the present study, we observed in situ Ca\(^{2+}\)/H\(_{1001}\) signaling with unique multiple Ca\(^{2+}\)/H\(_{1001}\) spikes in osteocytes responding to dynamic mechanical loading in long bones. In contrast, bone surface cells showed negligible

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**TABLE 1.** Numbers of bone samples and cells (responsive cells together with nonresponsive cells) in different experimental groups for comparison studies of Ca\(^{2+}\)/H\(_{1001}\) signaling between osteocytes and surface cells

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**Figure 4.** Autonomous and mechanical loading-induced Ca\(^{2+}\)/H\(_{1001}\) responses in bone surface cells and osteocytes of intact murine tibiae. A, B) Representative imaging fields (left panels) and Ca\(^{2+}\)/H\(_{1001}\) traces (right panels) of bone surface cells (A) or osteocytes (B) under either static or cyclic mechanical loading with an 8-N peak load magnitude. C) Percentage of responsive cells (had ≥1 Ca\(^{2+}\)/H\(_{1001}\) spike) of total counted cells in the field of view. D) Average number of Ca\(^{2+}\)/H\(_{1001}\) spikes (excluding nonresponsive cells) over the 15-min experimental period. E, F) Peak magnitude normalized with the baseline value (E) and the time between initiation of mechanical loading and the first observed Ca\(^{2+}\)/H\(_{1001}\) spike (F). Data are means ± sd. *P < 0.05.
Ca\textsuperscript{2+} responses in response to mechanical loading. Our findings demonstrate, for the first time, that the osteocyte in the LCS, previously regarded as an inert space occupier, has the unique capability to sense and respond to mechanical loading in bone in the form of robust Ca\textsuperscript{2+} oscillations. Furthermore, we discovered a dose-dependent relationship between the applied loading magnitude on mouse long bone and the spatiotemporal parameters of osteocyte Ca\textsuperscript{2+} spikes. Many previous studies have shown that the anabolic and catabolic responses in bone were dependent on the loading profiles, including the magnitude, frequency, and pattern (46–48). Our study shows that osteocytes not only are sensitive to mechanical loading but also can modulate their immediate biochemical responses according to the loading magnitude, which may presumably regulate downstream bone modeling and/or remodeling activities. These findings provide further evidence to support the hypothesis that osteocytes might act as an orchestrator of the bone modeling and remodeling processes.

The exact nature of the physical signals that trigger Ca\textsuperscript{2+} signaling in osteocytes remains to be determined. When bone is subjected to mechanical loading, multiple physical signals are induced around the osteocytes, which include direct substrate and cell deformation, hydrostatic pressure, interstitial fluid flow in the LCS, and dynamic electrical fields (43, 49, 50). The LCS fluid flow-induced shear stress on the cell membrane of the osteocyte has been posited as a major candidate to induce osteocyte mechanotransduction (29, 34). We have previously shown that shear stresses as low as 0.5 Pa on the cell body were able to induce >95% of in vitro osteocytes to respond with Ca\textsuperscript{2+} spikes (18). The present study demonstrates that physiological loading induced significant LCS fluid flow and shear stress, with the fluid shear positively correlated with the load levels. Moreover, we showed a linear positive correlation between osteocyte Ca\textsuperscript{2+} oscillations and LCS fluid flow. Thus, taken together with our previous in vitro investigations, the present study demonstrates that, within the existence of many potential physical signals, the LCS fluid flow alone has sufficient capacity to induce the observed robust osteocyte Ca\textsuperscript{2+} oscillations. Furthermore, our modeling calculations predicted much higher shear stresses on the cell dendrites than on the cell body. It has been shown that osteocyte dendritic processes displayed a strain amplification mechanism and possess higher mechanosensitivity than the cell body (21, 51, 52). Therefore, osteocytes in situ may initiate Ca\textsuperscript{2+} oscillations from cell processes and result in the subsequent Ca\textsuperscript{2+} signaling in the cell body (52, 53). Together, our findings suggest that load-induced fluid flow may be an important and efficient means of signal transduction in the LCS.

In the present study, reduced osteocyte Ca\textsuperscript{2+} responses were observed when P2R, PLC, or the ER Ca\textsuperscript{2+} store was blocked. We conclude that the ATP-related P2R-PLC-IP3-ER pathway plays a major role in load-induced osteocyte Ca\textsuperscript{2+} oscillations (54). Our previous

Table 2. Numbers of bone samples and cells (responsive cells together with nonresponsive cells) in different experimental groups for Ca\textsuperscript{2+} signaling studies

<table>
<thead>
<tr>
<th>Treatment</th>
<th>PPADS</th>
<th>Neomycin</th>
<th>GdCl\textsubscript{3}</th>
<th>NNC 5-0396</th>
<th>18a-GA</th>
<th>TG</th>
<th>Amlodipine</th>
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<td>807</td>
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Figure 5. Investigation of the roles of various Ca\textsuperscript{2+} signaling pathways in 6-N mechanical loading-induced Ca\textsuperscript{2+} oscillations. Extracellular ATP P\textsubscript{2}X/P\textsubscript{2}Y receptors (P\textsubscript{2}R), PLC, SACCs, T-type VGCCs (T-type), L-type VGCCs (L-type), GJs, and ER Ca\textsuperscript{2+} store (ER) have all been implicated in osteocyte Ca\textsuperscript{2+} signaling. These pathways were inhibited using small molecule inhibitors. A DMSO vehicle control was used for statistical comparisons of GJ, ER, and L-type groups. Percentage of responsive cells (A), average number of Ca\textsuperscript{2+} spikes (excluding nonresponsive cells; B), normalized spike magnitude (C), and time between first and second spikes (D) over the 15-min experimental period. *P < 0.05 vs. corresponding control group; †P < 0.05 vs. vehicle control group.

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in vitro studies also demonstrated that extracellular ATP diffusion played a paracrine and/or autocrine role in mediating both the intercellular Ca\(^{2+}\) wave propagation and intracellular Ca\(^{2+}\) oscillations in bone cells (18, 20). Extracellular ATP can be released by osteocytes by mechanically induced Ca\(^{2+}\) influx (55) and readily transported to neighboring osteocytes through loading-induced convection and diffusion through the LCS (29, 31). Numerous in vivo studies have shown the significance of both P\(_2\)X and P\(_2\)Y receptors in maintaining bone quality and bone mechanosensitivity (56, 57).

In the present study, we showed similarly prominent reductive effects on osteocyte Ca\(^{2+}\) spikes when P\(_2\)R, PLC, and the ER Ca\(^{2+}\) store were blocked. Furthermore, our findings revealed moderate roles of GJs, VGCCs, and SACCs in modulating mechanically induced osteocyte Ca\(^{2+}\) signaling in long bone. Findings from Ishihara et al. (23) revealed that osteocytes specifically modulated autonomous or bone surface fluid-induced Ca\(^{2+}\) signaling via GJs in calvarial bones. However, dramatically different developmental origins, osteocyte shapes, and LCS structures between long bones and calvarial bones might result in distinct Ca\(^{2+}\)-responding mechanisms (44, 45). Moreover, osteocytes in the surface fluid shearing model might receive minimal direct mechanical stimulation, whereas osteocytes in our current model were subjected to direct LCS fluid shear stress. Therefore, this finding suggests that intercellular communication via GJs may be a critical pathway between bone surface cells and osteocytes in the bone matrix. It is also of interest that calvariae are not considered to be a significant weight-bearing site, whereas tibiae are primarily load-bearing bones. The observed difference between tibial and calvarial responses under mechanical loading may underlie different mechanosensing mechanisms and/or various degrees of mechanosensitivity. Genetic knockout studies have demonstrated a complex role of GJs in bone mechanotransduction, whereas enhanced bone formation in response to mechanical loading was observed in connexin 43-deficient mice (58, 59). The current ex vivo model may not provide complete answers to these complex phenomena, but it may offer an interesting real-time quantification technique in those knockout mice for future studies. Notably, Thi et al. (60) suggested that pannexin might be more important in ATP release than connexin 43 hemichannels. This is consistent with our current finding that GJ inhibition is not as dramatic as direct inhibition of the ATP pathway including either P\(_2\)R or PLC. Taken together, the results of our study reveal that extracellular ATP is critical for initiating the Ca\(^{2+}\) activities of in situ osteocytes in long bone, and Ca\(^{2+}\) oscillations in osteocytes rely highly on the IP\(_3\)-induced Ca\(^{2+}\) release from the ER. It should be noted that the exact mechanism underlying the distinct Ca\(^{2+}\) responses between surface bone cells and osteocytes is not known, although it is consistent with our previous in vitro findings (18, 19). We speculate that the keys lie in the ATP-related P\(_2\)R-PLC-IP\(_3\)-ER signaling pathway and ER refilling dynamics between surface cells and osteocytes.

Osteocytes have been implicated in physiological processes as disparate as bone homeostasis and kidney phosphate metabolism (1). Our discovery of mechanically induced osteocyte Ca\(^{2+}\) responses with unique multiple oscillations in long bone provide direct evidence that the osteocyte acts as a major mechanosensor in bone. The positive correlation between Ca\(^{2+}\) oscillations and LCS fluid shear supports, for the first time, the essential role of load-induced LCS fluid flow in osteocyte mechanotransduction and also suggests that the osteocyte network is capable of detecting and differentiating various loads. We also demonstrated that the ATP-related P\(_2\)R-PLC-IP\(_3\)-ER signaling pathway is a critical mechanism in osteocytic Ca\(^{2+}\) oscillations. Understanding the role of Ca\(^{2+}\) oscillations and associated intercellular communications within osteocyte networks may provide a clearer mechanism of how bone tissue can integrate habitual mechanical loads into varying bone modeling and remodeling activities seen in physiological conditions and pathological states such as osteoporosis and disuse bone loss.

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