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Review

Oscillatory intracellular Ca^{2+} responses in living bone ☆

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ABSTRACT

Intracellular calcium (Ca^{2+}) is an important secondary messenger that modulates many cellular processes. Its oscillatory signaling is considered to participate in the regulation of many different cell functions, including bone metabolism. However, it is not entirely clear whether Ca^{2+} oscillations occur between osteoblasts and osteocytes in integrated bone tissues because of the complex mineralized matrices surrounding bone cells. To address this issue, we have recently developed a novel *ex vivo* real-time imaging system, which made it possible to observe repetitive and autonomous oscillations in the intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) in intact chick calvarial explants. This system revealed that Ca^{2+} release from intracellular stores plays a key role in Ca^{2+} oscillations in bone cells. Additionally, gap junctions are important for the maintenance of these oscillations in osteocytes but not in osteoblasts. In this review, we describe the dynamic oscillatory elevations of Ca^{2+} levels that occur in osteoblasts and osteocytes in living bone.

Contents

1. Introduction

Intracellular calcium (Ca^{2+}) is an essential secondary messenger in various physiological cellular processes, and it transduces various signals to the cell interior and between cells [1]. Increases in intracellular calcium concentration ($[Ca^{2+}]_i$) are characterized by their variety of spatio-temporal patterns and oscillatory behavior, which provide the versatility necessary for regulation of many different cell functions [2–6]. In mammalian cells, $[Ca^{2+}]_i$ oscillation is a complex dynamic process; the frequency, amplitude, and spatial localization of $[Ca^{2+}]_i$ oscillations control the efficiency and specificity of many cellular responses [3–5], and such oscillatory behavior is typically observed in cardiac cells and other excitable cells. [6]

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In bone cells, previous studies have suggested that osteoclasts and osteoblasts, known as non-excitable cells, also show $[Ca^{2+}]_i$ oscillations [7,8]. Although the role of oscillatory Ca^{2+} signals is not fully understood in bone cells, autonomous $[Ca^{2+}]_i$ oscillations have been attributed to osteoclastogenic signaling in osteoclasts and are known to affect the differentiation of mesenchymal progenitor cells into osteoblasts in vitro [8].

The homeostasis of the skeletal system is maintained by a delicate balance between osteoclasts and osteoblasts on the bone surface. Osteocytes, the most numerous cells in bone, are terminally differentiated from the osteoblast lineage and reside in lacunae surrounded by the mineralized bone matrix. It is widely believed that osteocytes can sense external mechanical loads and control mechano-induced bone adaptation [9]. They also directly control bone formation [10,11] and resorption [12,13]. Recently, additional evidence has suggested that osteocytes act as critical regulators of the endosteal hematopoietic stem/progenitor cell niche [14], lymphopoiesis, and fat metabolism [15]. These results suggest that osteocytes also act as a central regulator of distant tissue and organs.

A complex three-dimensional (3D) network consisting of osteocytes coordinates the actions of osteoblasts, osteoclasts, and even myeloid cells in vivo [16]. Osteocytes form an extensive intracellular and extracellular communication system via their slender cell processes and canaliculi throughout bone. This 3D structural network is contained within the mineralized bone matrix of bone and is therefore suited to both sensing the need for bone remodeling and then orchestrating it. Because they are embedded within the mineralized matrix, osteocytes are not easily accessible, and their mechanisms and functionality have thus only been extensively studied in two-dimensional (2D) systems, in which the complexity of the 3D microenvironment is often lost; therefore, our understanding of their role in bone remodeling remains incomplete.

Modulation of the $[Ca^{2+}]_i$ responses of osteoblasts and osteocytes by the local environment has only been demonstrated using traditional 2D cell culture models [17–20]. It is not entirely clear whether the same $[Ca^{2+}]_i$ responses are produced when the cells are present in integrated bone tissues. Therefore, the development of a new 3D *ex-vivo* experimental system is required to determine the physiological $[Ca^{2+}]_i$ oscillation profiles of bone cells, especially osteocytes.

In this review, we introduce our novel *ex-vivo* live Ca^{2+} imaging system, which can be used to capture the dynamic oscillatory elevations in Ca^{2+} levels occurring in osteoblasts and osteocytes in live, intact bone explants.

2. Fluorescent dye loading and time-lapse Ca^{2+} imaging in chick calvarial bone

To monitor the dynamics of the multi-cellular $[Ca^{2+}]_i$ oscillation in intact bone explants, we used embryonic chick calvariae, which are useful for observation of living bone tissue because they are very thin and flat [21]. These anatomical features provide advantages for structural observation using confocal laser scanning (CLS) microscopy from the surface to deeper layers [16,22]. For imaging, calvarial explants were placed on a glass slide (Matsunami, Osaka, Japan) with α-MEM (Invitrogen, Carlsbad, CA, USA) and incubated at 37 °C with 5% CO_2. Coverslips were affixed to each glass slide by adhesive grease, and the glass slide was then mounted onto the CLS system at the air-medium interface. The experiments were conducted at 37 °C and within 30 min of mounting to minimize cellular damage during observation. Live imaging of fluorescent labels also made it possible to visualize the calvariae clearly without decalcification or sectioning, and dye transfer mediated by gap junctions has been previously observed in the living cells in calvariae [23]. The level of $[Ca^{2+}]_i$ was measured using a calcium indicator probe as described previously [24]. After loading of 10 μM fluo-8 acetoxymethyl ester (Fluo-8 AM; ABD Bioquest, Inc., Sunnyvale, CA, USA) and 10% Pluronic F-127 (ABD Bioquest, Inc.) onto the calvarial bone explants, images were obtained using a 488 nm krypton/argon laser for excitation, and the emission was detected at a wavelength of 495 nm at 3 s intervals in each experiment. This experiment allowed us to study physiological drug responses in the setting of 3D morphology and intercellular networks by mimicking features of the native environment. This study was the first investigation of $[Ca^{2+}]_i$ oscillation profiles using a 3D calvarial explant model to examine the $[Ca^{2+}]_i$ signaling interactions that occur among bone cells at high temporal and spatial resolution [24].

3. Autonomous $[Ca^{2+}]_i$ responses of osteoblasts and osteocytes in chick calvarial bone

We obtained 2D confocal fluorescent images of the osteoblasts at the bone surface and osteocytes located 15 μm below the osteoblast layer (Figs. 1A and 2A). This live-cell imaging enabled us to visualize the temporal changes of $[Ca^{2+}]_i$ within living bone specimens and revealed novel aspects of the physiological autonomous $[Ca^{2+}]_i$ oscillations in osteoblasts (Fig. 1B–D) and osteocytes (Fig. 2B–D). The time series data were used to the percentage of responsive osteoblasts and osteocytes, which was $15.6 \pm 4.6\%$ and $7.0 \pm 1.6\%$, respectively, during the 300 s experimental period, indicating a significantly higher response in osteoblasts than in osteocytes [24]. Past in vitro studies reported that osteoblasts display autonomous $[Ca^{2+}]_i$ oscillation [8,25,26]; however, these studies did not determine whether such oscillation actually occurs within living bone explants, and not only in cell culture. Our study was also the first to demonstrate autonomous $[Ca^{2+}]_i$ responses in osteocytes and even in osteocytic cells using a 2D cell culture model.

These findings seem to conflict with the previous in vitro results concerning the percentage of responsive cells and the $[Ca^{2+}]_i$ oscillation frequency. Our data imply that these differences may result from differences in cell culture systems, in the species examined, or in experimental conditions. In a recent study, we provided an approach to investigate mechanical stress-induced $[Ca^{2+}]_i$ oscillation where induction of fluid flow-induced mechanical stress was performed as described previously [27]. Briefly, capillary diffusion-aided fluid flow was applied only to the bone explant surface. The experiments were conducted under identical conditions. The results show that the autonomous $[Ca^{2+}]_i$ response was promoted by fluid flow-induced mechanical stress applied to the bone surface via gap junction-mediated cell–cell communication [27]. The results of this study suggest that the intercellular signaling induced by augmented gap junction-mediated $[Ca^{2+}]_i$ oscillation may serve as an early signaling event in mechanotransduction.

4. The role of the intracellular Ca^{2+} store in the endoplasmic reticulum in the autonomous $[Ca^{2+}]_i$ responses of osteoblasts and osteocytes in bone

Increases in $[Ca^{2+}]_i$ take place following Ca^{2+} release from intracellular calcium stores and with influx of extracellular calcium across the plasma membrane or through gap junctions. Among these mechanisms, endoplasmic reticulum (ER) Ca^{2+} stores are known to play a major role in the Ca^{2+} signaling pathways underlying sustained $[Ca^{2+}]_i$ oscillation [28,29]. To examine the involvement of intracellular calcium stores in the