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Microdamage induced calcium efflux from bone matrix activates intracellular calcium signaling in osteoblasts via L-type and T-type voltage-gated calcium channels

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ABSTRACT

Mechanisms by which bone microdamage triggers repair response are not completely understood. It has been shown that calcium efflux ($[Ca^{2+}]_E$) occurs from regions of bone undergoing microdamage. Such efflux has also been shown to trigger intracellular calcium signaling ($[Ca^{2+}]_I$) in MC3T3-E1 cells local to damaged regions. Voltage-gated calcium channels (VGCCs) are implicated in the entry of $[Ca^{2+}]_E$ to the cytoplasm. We investigated the involvement of VGCC in the extracellular calcium induced intracellular calcium response (ECIICR). MC3T3-E1 cells were subjected to one dimensional calcium efflux from their basal aspect which results in an increase in $[Ca^{2+}]_I$. This increase was concomitant with membrane depolarization and it was significantly reduced in the presence of Bepridil, a non-selective VGCC inhibitor. To identify specific type(s) of VGCC in ECIICR, the cells were treated with selective inhibitors for different types of VGCC. Significant changes in the peak intensity and the number of $[Ca^{2+}]_I$ oscillations were observed when L-type and T-type specific VGCC inhibitors (Verapamil and NNC55-0396, respectively) were used. So as to confirm the involvement of L- and T-type VGCC in the context of microdamage, cells were seeded on devitalized notched bone specimen, which were loaded to induce microdamage in the presence and absence of Verapamil and NNC55-0396. The results showed significant decrease in $[Ca^{2+}]_I$ activity of cells in the microdamaged regions of bone when L- and T-type blockers were applied. This study demonstrated that extracellular calcium increase in association with damage depolarizes the cell membrane and the calcium ions enter the cell cytoplasm by L- and T-type VGCCs.

Introduction

Bone cells work in concert to repair the damaged regions of bone matrix [1–3]. Alterations in matrix strain, local fluid flow patterns or osteocyte apoptosis have been postulated to trigger the repair function of bone cells [4–6]. While there is strong evidence for linear microcrack to induce osteocyte apoptosis and trigger bone remodeling, diffuse microdamage does not have an apoptotic effect [7,8]. It is not fully understood how the damaged regions of bone are targeted for repair, especially for diffuse microdamage.

We have proposed mechanically induced calcium efflux ($[Ca^{2+}]_E$) from damaged regions of bone matrix as a potential stimulus which activates local bone cells [9]. Abundant amount of diffuse damage can be created in a controlled fashion in notched regions of devitalized cortical bone [10,11]. Ion-selective electrode measurements demonstrated increased calcium concentration concomitant with the inception of diffuse damage [12], providing evidence on the possibility of a mechanically induced $[Ca^{2+}]_E$ from bone matrix at post-yield strain levels.

When devitalized notched bone samples were seeded with MC3T3-E1 preosteoblasts and diffuse damage was induced, increased intracellular calcium ($[Ca^{2+}]_I$) was observed among the cells on the damage process zone [9]. This cellular reaction was associated with calcium because the response vanished when the tests were repeated with cells-seeded on notched demineralized bone matrix. These results suggest that mechanically induced $[Ca^{2+}]_E$ triggers $[Ca^{2+}]_I$ signaling on MC3T3-E1 cells. However, it is largely unknown as to how $[Ca^{2+}]_I$ signaling occurs as a result of microdamage induction. Neomycin-sensitive voltage-gated calcium channels (VGCCs) were implied in this extracellular calcium induced intracellular calcium response (ECIICR) [13]; however, the study did not identify the specific type(s) of VGCC involved in the response.

The aim of this study is to affirm the involvement of VGCC on $[Ca^{2+}]_E$ induced $[Ca^{2+}]_I$ activation and to identify the type(s) of VGCC involved in the process. Non-selective VGCC inhibitor was used to affirm the general involvement of VGCC in $[Ca^{2+}]_I$ activation, and specific blockers for known types of VGCC were applied to attain our aim.

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Materials and method

Chemicals

Minimum essential alpha medium (α-MEM), penicillin/streptomycin, trypsin/EDTA, HBSS, HEPES, and Fura-2AM were obtained from Invitrogen (Frederick, MD). Voltage-gated calcium channel blockers, Bepridil, (non-selective voltage-gated calcium channel inhibitor) Verapamil, (L-type calcium channel inhibitor) NNC55-0396 (T-type calcium channel inhibitor) and fetal bovine serum (FBS) were obtained from Sigma (St. Louis, MO). ω-Conotoxin MVIIC(N, P/Q type calcium channel inhibitor) and ω-Conotoxin GVIA(N type calcium channel inhibitor) were obtained from Tocris Bioscience (Ellisville, MO).

Well insert model for 1D calcium efflux from basal aspect of cells

Well insert model is a practical way to emulate the calcium efflux from the bone matrix on which cells are seeded (Fig. 2-a) [13]. Cells were seeded on cell culture inserts (0.4 μm pore size culture plate insert, Corning) and the insert was placed in a culture well, creating a dual compartment. Mechanically induced calcium efflux from basal side of the cells was mimicked by applying higher concentration of calcium solution in the well than the concentration of calcium solution in the insert.

Cell culture

The murine pre-osteoblast MC3T3-E1 (passage 21, subclone 4, ATCC) were cultured in alpha-minimum essential medium (α-MEM; Gibco, Grand Island, NY) containing 10% fetal bovine serum (FBS; Sigma-Aldrich), 1% penicillin–streptomycin (Gibco) under 37 °C, and 5% CO_2. Cells were transferred to 6-well insert (0.4 μm culture plate insert, Corning), at a density of 15,000 cells/cm^2, and cultured for 48 h, in the same culture medium, at 37 °C, and 5% CO_2.

Intracellular calcium concentration was measured by 10 μM Fura-2AM solution prepared in HBSS with 20 mM HEPES. Cells were stained for 60 min at room temperature. After washing three times with HBSS solution, the cells were incubated for additional 30 min either in HBSS solution with the pharmaceutical inhibitor, or only HBSS solution (see Table 1 for concentrations of applied pharmaceutical inhibitors). 2.5 ml of 9.6 mM calcium solution in HBSS was loaded in the well while the HBSS in the insert had 1.5 ml of 0 mM calcium. At this concentration gradient, the concentration of the insert converges to 6 mM of Ca^{2+} efflux toward the cells at the steady state. The media in the insert and in the well contained same concentration of inhibitors. $[Ca^{2+}]_I$ was measured under UV epifluorescent illumination using a ×20 water-immersion objective lens with a UV microscope (BX51, Olympus America). The system was equipped with a calcium imaging module for excitation and collection of fluorescence data (*Incyt™* Basic *Im* Fluorescence Imaging system, Intracellular Imaging Inc.). Six inserts were measured in each of the control and inhibitor treated groups. Intracellular calcium fluorescence measurements were performed in a dark room at room temperature.

Membrane potential changes in MC3T3-E1 cells by $[Ca^{2+}]_E$

Membrane potential change in MC3T3-E1 cells under the influence of $[Ca^{2+}]_E$ was measured by staining cells with 5 μM DIBAC4(3) prepared in HBSS with 20 mM HEPES. Cells were prepared in cell culture insert, incubated for 90 min at 37 °C, 5% CO_2, and transferred to the imaging system without rinsing. Cells were subjected to calcium efflux as elucidated. The media in the insert and in the well contained the same concentration of DIBAC4(3) to prevent the dye molecules from being washed-off from cell membrane during efflux exposure. Membrane potential change was measured under UV epifluorescent illumination using a ×20 water-immersion objective lens with a UV microscope (BX51, Olympus America). The fluorescence changes were measured with fluorescence measuring module (*Incyt™* Basic *Im* Fluorescence Imaging system, Intracellular Imaging Inc.). Membrane potential change measurements were performed in a dark room at room temperature.

Application of pharmaceutical inhibitors of VGCCs

Pharmaceutical blockers specific to different VGCCs were used according to the flowchart shown in Fig. 1. Bepridil, a non-specific blocker of all VGCCs, was applied at the first stage to evaluate and affirm whether VGCCs are involved in the ECIICR of MC3T3-E1. Subsequently, ω-conotoxin-MVIIC was used to verify the involvement N and P/Q-type VGCC. If ω-conotoxin-MVIIC showed inhibitory effect on $[Ca^{2+}]_I$, ω-conotoxin-GVIA was tested to conclude whether the effect of ω-conotoxin-MVIIC was caused by N-type VGCC. If ω-conotoxin-MVIIC showed no effect on $[Ca^{2+}]_I$, NNC55-0396 was used to test role of T-type VGCC, followed by Verapamil to find out effect of L-type VGCC. The concentration of each pharmaceutical inhibitor used in this paper is listed in Table 1 along with the references of studies, which utilized these concentrations effectively in bone cells.

Osteoblast-seeded bone loading test with calcium channel inhibitors

Following the screening of various blockers using the insert model, the blockers which demonstrated a significant reduction in $[Ca^{2+}]_I$ signaling were assessed in an in vitro bone damage model developed by our group (Fig. 2-b) [12,13]. Devitalized cortical bone wafers were sectioned from bovine femur (40 mm × 4 mm, 200 μm thickness). Blunt notches (1.5 mm in depth) were machined using methods described earlier [10]. Bone samples were photobleached under UV light for 30 min to decrease autofluorescent interference from bone substrate during intracellular calcium fluorescence measurements. The samples were sterilized by soaking the sample into 70% ethanol for overnight, washed with ×1 PBS and dried in the cell culture laminar flow hood for three times. MC3T3-E1 preosteoblasts were seeded on bone wafers at a density of 20,000 cells/cm^2, and cultured overnight at 37 °C, 5% CO_2.

Bone slices seeded with cells were stained in 10 μM Fluo-8AM solution in HBSS with 20 mM HEPES for 90 min in the cell culture incubator (37 °C, 5% CO_2). For treatment of cells with pharmaceutical blocker and dye de-esterification, the samples were incubated for additional 30 min either in HBSS solution with pharmaceutical inhibitor, or only HBSS solution. Cell-seeded bone samples were treated with Bepridil or a combination of Verapamil and NNC55-0396 to assess the involvement of L- and T-type calcium channels on the cell response. These blockers were selected for bone loading experiments because the prior insert experiments indicated these blockers to be effective.

Mechanical loading was applied on V-notched bone samples for testing the effect of mechanically induced calcium efflux on ECIICR in

Table 1
Pharmaceutical inhibitors and their concentrations used in the study.

Type of VOCC	Inhibitor	Concentration	References
Non-selective calcium channel blacker	Bepridil hydrochloride	30 μM	[37–39]
L-type calcium channel blocker	Verapamil hydrochloride	100 μM	[40–42]
T-type calcium channel blocker	NNC55-0396 dihydrochloride	5 μM	[43–45]
N, P/Q-type calcium channel blocker	ω-Conotoxin MVIIC	1 μM	[46–49]
N-type calcium channel blocker	ω-Conotoxin GVIA	2 μM	[49–51]