Voltage Sensitive Calcium Channel Structure in Osteocytes: Implications in Bone Remodeling

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INTRODUCTION:
Regulation of skeletal remodeling is the focus of great attention in translational osteoporosis research. A recent association study1 which included 200 single-nucleotide polymorphisms within the human chromosome 3p21 revealed that CACNA2D2, which encodes for one of four αδ subunits within the voltage sensitive calcium channel (VSCC) complex, is a novel susceptibility marker for bone mineral density (BMD) variation and may be involved in osteoporosis pathogenesis. VSCCs span the plasma membrane and open in response to external stimuli to alter Ca2+ permeability. Upon entering the cell, Ca2+ acts as a second messenger eliciting responses such as secretion and changes in gene expression. VSCCs are a complex of polypeptide units consisting of a pore forming α1 subunit, an intracellular β subunit, a dimer of disulfide linked α2 and δ subunits, which form an extracellular auxiliary complex and a γ subunit in some tissues, but not osteoblasts. The structure and function of VSCCs has been established in mechanosensitive tissues including skeletal and cardiac muscle and osteoblasts. Osteoblasts predominantly express L-type Ca1.2 (α1C) VSCCs; however, osteocytes express the T-type Ca3.2 (α1βδ) subunit more abundantly. T-type VSCCs have lower conductances than L-type channels, a property that may be significant for osteocyte physiology. Given the recent evidence associating αδ subunit polymorphisms with variation in BMD, the goal of this study was to examine the expression of both L- and T-type VSCC subunits in an osteocyte cell line, MLO-Y4, and in mature cortical bone. The overall aim of this study was to develop further understanding of the structure and functional properties of the VSCC complex in the osteocyte that could account for its role in maintaining Ca2+ homeostasis during normal bone remodeling and its impact on pathological states such as osteoporosis.

METHODS:
MLO-Y4 cells were cultured on collagen I coated plates and brought to approximately 80% confluence. RT-PCR was performed using total RNA extracts followed by gene amplification with self-designed primers. Western blots were performed after transfer of proteins from total cell lysates and electrophoresed on 4-12% Bis-Tris acrylamide gels. MLO-Y4 cells were fixed with parafomaldehyde (PFA) and non-specific epitopes were blocked prior to incubation with VSCC subunit specific antibodies or WGA-FITC (directly conjugated) and viewed using a Zeiss LSM 510 confocal microscope.

Long bones used for immunohistochemistry were harvested from eighteen week old male C57B46J mice, cut into 0.5mm sections, fixed in PFA and decalcified with EDTA. All assays involving animal use were approved by the IACUC at the University of Delaware.

RESULTS:
We characterized the subunit structure of the VSCC complex in osteocytes. Using RT-PCR, Western blot, and immunostaining assays we demonstrate that the T-type Ca3.2 (α1βδ) subunit is the predominant pore forming α1 subunit expressed in osteocytic cells grown in vitro and residing in cortical bone. This finding represents a shift in the expression of VSCC type during the maturation of osteocytes from osteoblasts from long lasting (L-type) to transient (T-type) channels. We did not detect the presence of P/Q, R, or N-type VSCCs within osteocytes, further reinforcing the idea of a key physiologic role of T-type VSCCs in these highly specialized cells. The auxiliary subunits of the VSCC complex modulate expression and function of the pore forming subunit and play a role in the ability of VSCCs to interact with the extracellular environment. Here we demonstrated expression of αδ, β1, β2, and β3 but not γ1 and γ2 transcripts within MLO-Y4 cells. The presence of the newly identified gamma subunits (3-8) has not yet been assessed in this cell line. An association of these auxiliary subunits with the pore forming subunit may stabilize the functional channel complex in the osteocyte.

Immunocytochemistry assays confirmed expression of Ca3.2 throughout the osteocyte cell body as well as along the cytoplasmic processes of MLO-Y4 cells. In contrast, there was little to no staining of Ca1.2 in these cells. Additionally, we demonstrated co-localization of Ca3.2 with wheat germ agglutinin (WGA), a lectin that recognizes sialic acid residues expressed on the highly glycosylated αδ subunit. The gamma subunit also immunostained with sialic acid residues; however, our findings indicate an absence of a gamma subunit in osteocytes. The pattern of immunostaining we obtained supports the hypothesis that the αδ subunit is in close proximity or part of the T-type VSCC complex in osteocytes.

DISCUSSION:
Osteoporosis is a debilitating disease affecting an estimated 75 million individuals in the US, Europe, and Japan, including 1 in 3 postmenopausal women. Diseases affecting BMD, such as osteoporosis, are a major worldwide health problem; and therefore, a substantial amount of clinical and basic science research is underway to understand the mechanisms underlying these pathologies. VSCCs play key roles in conferring the mechanosensitive properties of osteoblasts which secrete and mineralize bone matrix. Osteocytes develop from osteoblasts that become embedded deep within the mineralized matrix. They make up nearly 90% of bone cells and are the primary mechanosensitive cell in bone. Despite the growing evidence that VSCCs are important players in physiologic processes contributing to bone remodeling, very little is known about the VSCC complex structure or mechanistic function in osteocytes.

We assessed the expression of the majority of known VSCC subunits in the MLO-Y4 osteocyte cell line. We rigorously showed by RT-PCR as well as Western blotting and immunostaining that osteocytes express the T-type Ca3.2 (α1βδ) subunit more abundantly than the L-type Ca1.2 (α1C) subunit. T-type VSCCs have lower conductances than L-type channels, a property that is expected to influence osteocyte physiology. Additionally, we demonstrated the expression of αδ, as well as β1, β2, and β3 subunit encoding transcripts; however, γ1 and γ2 transcripts were not present. Immunocytochemistry staining revealed co-localization of WGA with Ca3.2 suggesting that these subunits associate in the MLO-Y4 cell line. T-type VSCCs are activated by weak depolarization and open transiently to permit Ca2+ entry in response to external stimuli. The presence of the T-type Ca3.2 subunit in terminally differentiated, post-mitotic osteocytes as opposed to the long lasting currents of L-type channels can maintain cell viability by preventing Ca2+ induced apoptosis in these isolated cells. Intracellular Ca2+-dependent signaling supported by T-type VSCCs can support key aspects of osteocyte function including signaling, secretion, and changes in gene expression in response to external stimuli.

The αδ subunit increases the density of the α1 subunit at the cell membrane and regulates current amplitude of VSCCs. Recent evidence indicates that the αδ subunit interacts with the extracellular matrix, providing an additional mechanism through which the conductance of these channels may be modulated by mechanical stimulation. An association of αδ subunit with T-type channels in osteocytes can stabilize the functional channel, and provide a mechanism for interaction with the extracellular environment. This key protein complex thus can provide a crucial function for mechanosensitive osteocytes in bone, which has broad implications in normal skeletal remodeling, BMD and risk of developing bone related pathologies.

REFERENCES: