HHS Public Access

Author manuscript

Curr Osteoporos Rep. Author manuscript; available in PMC 2022 April 01.

Published in final edited form as:

Curr Osteoporos Rep. 2021 April ; 19(2): 206–221. doi:10.1007/s11914-020-00647-7.

Skeletal Functions of Voltage Sensitive Calcium Channels

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Abstract

Voltage-sensitive calcium channels (VSCCs) are ubiquitous multimeric protein complexes that are necessary for the regulation of numerous physiological processes. VSCCs regulate calcium influx and various intracellular processes including muscle contraction, neurotransmission, hormone secretion, and gene transcription, with function specificity defined by the channel••s subunits and tissue location. The functions of VSCCs in bone are often overlooked since bone is not considered an electrically excitable tissue. However, skeletal homeostasis and adaptation relies heavily on VSCCs. Inhibition or deletion of VSCCs decreases osteogenesis, impairs skeletal structure, and impedes anabolic responses to mechanical loading. While the functions of VSCCs in osteoclasts is less clear, VSCCs have distinct but complementary functions in osteoblasts and osteocytes. This review details the structure, function, and nomenclature of VSCCs, followed by a comprehensive description of the known functions of VSCCs in bone cells and their regulation of bone development, bone formation, and mechanotransduction.

Keywords

Calcium channels; bone; mechanical loading; osteoblast; osteocyte; osteoclast

Components and Physiological Roles of Voltage-Sensitive Calcium Channels

Voltage-sensitive calcium channels (VSCCs) are widely distributed protein complexes serving a variety of functions by transducing electrical potentials into intracellular signals.

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Conflicts of Interest: None

Acting as a secondary messenger, intracellular calcium (Ca^{2+}) influences physiological processes including muscle contraction, hormone secretion, neurotransmission, and transcriptional regulation [1]. VSCCs are members of a transmembrane cation channel complex superfamily, which includes voltage-gated sodium and potassium channels [2]. This review provides an overview of VSCC structure and function, and how VSCCs control skeletal function.

Structure & Function of VSCCs

VSCCs are multimeric structures consisting of a pore-forming subunit and 2–3 auxiliary subunits that influence channel activity. The α_1 subunit forms the channel pore and consists of four homologous voltage-sensing domains (VSD1–4) each with six transmembrane segments (S1-S6) (Fig. 1). While the α_1 subunit enables Ca^{2+} selectivity, the auxiliary subunits ($\alpha_2\delta$, β, and γ) contribute unique structural and regulatory properties by modulating Ca^{2+} influx, altering trafficking of the α_1 pore, and regulating interactions with the extracellular matrix (ECM) [3, 4] (Table 1).

The cytosolic β subunit was discovered in purified skeletal muscle dihydropyridine (DHP) receptors [5] and has four isoforms ($\beta_{1–4}$) [6–8]. Binding of β to the α_1 pore is mediated via the β interacting domain (BID) [9]. This interaction regulates current amplitude [10, 11], voltage dependence [12], activation kinetics [13], and trafficking of α_1 to the plasma membrane [14]. Trafficking is mediated by masking an endoplasmic reticulum (ER) retention signal on the α_1 subunit by the β subunit‘s BID [15]. The four isoforms of the β subunit interact uniquely with α_1, enabling VSCCs to function differently in various tissues [16].

Of the three auxiliary subunits, the function of the γ subunit is least clear. Originally thought to only be present in skeletal muscle, γ has wide tissue distribution. All eight isoforms ($\gamma_{1–8}$) of the γ subunit are glycoproteins with four transmembrane domains (~32 kDa) and an intracellular amino terminus [17–21]. The exact functions of the γ subunit remain unclear but appear to vary based upon the isoform and tissue type. γ_1 modulates the biophysical properties, but not membrane trafficking, in skeletal muscle [22], while $\gamma_{1–4}$ and γ_7 alter the activation and/or inactivation kinetics of Ca^{2+} currents in neural cells [23, 20, 21]. Continued research will elucidate additional function(s) of the γ subunits.

The $\alpha_2\delta$ subunit has four isoforms ($\alpha_2\delta_{1–4}$) with varying tissue distribution and functions (Table 1) [24–27]. The α_2 (~150 kDa) and δ (17–25 kDa) subunits are encoded by a single gene (*Cacna2d*), which is post-translationally cleaved and then relinked together by a disulfide-linkage [28]. The α_2 portion is entirely extracellular while δ secures the subunit within the cell membrane through a glycosyl-phosphatidylinositol (GPI) anchor [28, 29]. In addition to heavily glycosylated cysteine residues, $\alpha_2\delta$ has three functional domains including a Von Willebrand Factor A (VWA) domain [28, 30] and two Cache domains [30–32]. The VWA domain initiates binding of α_2 to extracellular proteins through its metal ion-dependent adhesion site (MIDAS) [33], while the Cache domains further support extracellular interactions [30]. Most importantly, the VWA and Cache domains of $\alpha_2\delta$ stabilize and interact with the α_1 subunit, enabling modulation of α_1 by $\alpha_2\delta$.

The $\alpha_2\delta$ subunit regulates α_1 pore functions including gating kinetics, membrane density, and interactions with extracellular ligands. The mechanism(s) regulating $\alpha_2\delta$ trafficking of α_1 to the membrane is unclear, but interactions with the MIDAS motif appears essential [34, 35]. Binding of extracellular ligands to $\alpha_2\delta$ subunits can also alter channel activity. In particular, $\alpha_2\delta_1$ and $\alpha_2\delta_2$ bind gabapentinoids including gabapentin and pregabalin [36], which bind with greatest affinity to $\alpha_2\delta_1$ [37, 38]. These drugs decrease channel currents [39, 40] and membrane localization [40], decreasing pain receptor activation and overall neural activity.

VSCC Nomenclature

Though structurally similar, there are ten mammalian VSCC variants, each with distinct properties (Table 2) [41–43]. VSCCs are categorized in several ways including date of discovery, activation threshold, channel conductance/latency, cell-specificity, and subunit isoforms. These nomenclature paradigms have been condensed into three families: L-type VSCCs ($Ca_V1.1$–4; α_{1S}, α_{1C}, α_{1D}, α_{1F}), N/P/Q/R-Type VSCCs ($Ca_V2.1$–3; α_{1A}, α_{1B}, α_{1E}), and T-Type Channels ($Ca_V3.1$–3; α_{1G}, α_{1H}, α_{1I}) (Fig. 2) [44].

Though L- & T-type channels are found in a variety of tissues, N, P/Q, and R-type are largely expressed in neurons. L-Type ($Ca_V1.x$) VSCCs exhibit high voltage activation, large single channel conductance, slow voltage-dependent inactivation, and are the most widely distributed. These properties lend L-Type VSCCs to remain open longer, thus being designated as “L” type due to their „long lasting‟ characteristics [45]. L-Type VSCCs are susceptible to Ca^{2+} binding reagents including dihydropyridines, phenylalkylamines, and benzothiazepines, often referred to as Ca^{2+} channel blockers [46].

In contrast, T-Type ($Ca_v3.x$) VSCCs are activated at more negative membrane potentials and have a very fast voltage-dependent inactivation causing small single channel conductance [45, 47–49]. Compared to L-type channels, voltage-dependence for activation of T-type VSCCs is shifted 20–30 mV in the hyperpolarized direction, resulting in faster (τ ~ 15–30 ms vs. 2,000 ms) and more complete inactivation with a more negative membrane potential (~ −50 mV vs. 0 mV) and slower deactivation than L-type VSCCs [50, 51]. As such, T-Type VSCCs are well-suited for rhythmic firing action potentials and are distinguished by their frequent generation of transient (T-type) Ca^{2+} peaks. Unlike other VSCC families, T-type VSCCs are insensitive to Ca^{2+} binding reagents, spider or snake venoms, but are antagonized by inorganic cations, such as Ni^{2+} and Cd^{2+}, the scorpion toxin kurtoxin, the organic inhibitor mibefradil [50], and its analog NNC 55–0396 [52].

N, P/Q, and R-type VSCCs ($Ca_v2.x$) have an intermediate voltage dependence and rate of inactivation, more negative and faster than L-type but more positive and slower than T-type. Though electrophysiologically similar and located predominately in neurons, the N, P/Q, and R-type channels differ based upon voltage dependence and sensitivity to specialized toxins including spider toxin ω-agatoxin IVA, cone snail peptide ω-conotoxin GVIA, and related peptides [53, 54, 45, 55, 56].

Due to their ability to sense membrane voltage changes, VSCCs were once thought to function only in excitable tissues such as muscle and neurons. However, it has become clear

that these channels play vital roles in the skeleton where they influence calcium signaling, bone homeostasis, and mechanical responses.

Skeletal Functions of VSCCs

VSCCs regulate skeletal development, bone turnover, and mechanotransduction. While most studies focused on the function of L-Type VSCCs in osteoblasts, recent work established an important role of T-type VSCCs in osteocytes [57–59] even though the exact mechanism(s) by which T-type channels regulate bone remain unclear. Additionally, little is known regarding the function of VSCCs in osteoclasts, though recent studies suggest both direct and indirect roles of VSCCs in osteoclast activity.

L-type VSCCs regulate osteoblast proliferation and differentiation

The presence of VSCCs in bone was first discovered in primary murine osteoblasts using patch clamp techniques. These studies showed that a rapid, transient, increase in intracellular Ca^{2+} was required for osteoblast function [60]. These channels were identified as the $Ca_V1.2$ (α_{1c}) isoform [61, 62] where both *in vivo* and *in vitro* studies confirmed $Ca_V1.2$ as the primary channel in osteoblasts [63–71]. Additional work showed that the VSCC complex in osteoblasts lacked a γ subunit [72]. By employing L-type Ca^{2+} channel blockers, such as nifedipine, verapamil, and diltiazem, numerous studies revealed that L-type VSCCs regulate osteoblast differentiation, proliferation, and mechanosensitivity [73–75, 62, 57, 71].

Calcium channel blockers decreased Ca^{2+} influx and subsequent downstream signaling pathways in osteoblasts, which helped delineate the function of VSCCs in osteoblasts. Ca^{2+} influx increased osteocalcin release in osteosarcoma cells, which was further enhanced by the L-type agonist BAY K8644 and suppressed by nifedipine [62, 61]. Nifedipine also impaired osteogenesis *in vivo*, resulting in decreased bone formation rates, cancellous bone volume, epiphyseal growth plate thickness, and humeral length [76]. While use of Ca^{2+} channel blockers established the essential function of VSCCs in bone, the cellular mechanisms remained elusive.

When extracellular Ca^{2+} concentrations increase locally, as during bone resorption, osteoblast-like cells (MC3T3-E1) increase Ca^{2+} influx and intracellular Ca^{2+} signaling, triggering a number of responses including increased osteopontin (OPN) expression [77]. OPN is a matrix protein synthesized by osteoblasts during osteoid formation, and is essential for mineralization and remodeling [78]. Inhibition of L-type VSCCs decreased OPN expression and activity [79], suggesting that VSCCs regulate bone formation and remodeling by altering expression of matrix proteins.

In addition to influencing osteoblast matrix production, L-type VSCCs support osteoblast proliferation. Several soluble factors, including epidermal growth factor (EGF) and purinergic signals (i.e. ATP), which activate intracellular signaling by binding P2Y receptors, enhance osteoblast proliferation. Inhibition of L-type VSCCs blocked both EGF [65] and ATP-induced increases in osteoblast proliferation [80]. In the latter case, L-type inhibition impaired c-jun N-terminal kinase-1 (JNK1) phosphorylation [80], demonstrating

that VSCC-mediated Ca^{2+} influx facilitates ATP-stimulated activation of JNK1 to mediate osteoblast proliferation.

While increased osteoblast proliferation provides more matrix-secreting cells, entry into the osteoblast lineage from mesenchymal precursors (MSCs) is essential for osteogenesis. Deletion or inhibition of L-type VSCCs impairs osteogenic allocation of MSCs. The osteoblast markers osteoprotegerin (OPG) [81] and Runx2 [82] are decreased following L-type VSCC inhibition or siRNA knockdown of $Ca_v1.2$ (α_{1C}), leading to decreased alkaline phosphatase and alizarin red staining of MSCs [82]. Additional work demonstrated that $Ca_v1.2$-mediated changes in MSC osteogenesis was induced through the Wnt/β-catenin pathway, as $Ca_v1.2$ knockdown decreased GSK-3β phosphorylation and inactivated β-catenin [83]. Further work showed that expression of $Ca_v1.2$ decreased in transgenic mice exhibiting an advanced-age phenotype. These impairments in osteogenic differentiation of MSCs were rescued by overexpression or activation of $Ca_v1.2$ [83], suggesting loss of VSCCs expression/activity may explain some of the skeletal changes seen in elderly individuals.

More recent *in vivo* work further delineated the function of $Ca_V1.2$ in bone formation. While global deletion of $Ca_V1.2$ impairs cardiac muscle and is embryonic lethal [84], a novel $Ca_V1.2$ haploinsufficient model with ~80% reduction in *Cacna1C* expression (the gene encoding $Ca_V1.2$) showed impairments in bone development and formation, affecting both the shape and size of the mandible in zebrafish and mice [85]. A comparable but modest impairment in bone formation also was found in $Ca_V1.3$ KO mice, decreasing body weight, femur mass, and midshaft cross-sectional area. Deletion of $Ca_V1.3$ simultaneously increased $Ca_V1.2$ expression, possibly explaining the attenuated effect of $Ca_V1.3$ deletion on bone formation [86]. This compensation further suggests an essential role of $Ca_V1.2$ in bone formation.

Additional work using a Timothy syndrome, gain-of-function mutation in *Cacna1C* and the Cre/Lox system allowed conditional overexpression of $Ca_V1.2$ in cells expressing early mesenchymal and osteogenic lineages under the control of Prx1, Col2a1, and Col1a1 promoters. Overexpression of $Ca_V1.2$ in osteogenic cells increased cortical and trabecular bone at multiple sites, which by 12 months of age had nearly closed the marrow cavity. This high bone mass resulted from increased osteoblast differentiation as shown by widespread nodule formation, increased expression of osteogenic markers (*Alpl*, *Runx2*, *Sp7*), excess bone formation, and decreased osteoclast differentiation [71, 87]. Taken together, these studies demonstrate that Ca^{2+}-mediated regulation of osteogenic gene expression as well as osteoblast proliferation and differentiation is controlled by L-type VSCCs.

L-type VSCCs Regulate Mechanotransduction

Bone is sensitive to mechanical signals, relying on physical cues to direct bone modeling and remodeling. As one of the first measurable responses of bone cells to mechanical force is a rapid and transient increase in intracellular Ca^{2+} [88, 89]; tight regulation of Ca^{2+} influx is a key component of anabolic signaling in bone. Early work used pharmacological inhibitors of L-type VSCCs to decrease Ca^{2+} influx and the anabolic response to loading. A single dose of verapamil or nifedipine suppressed load-induced bone formation in rodents by

50–75% [68]. Importantly, global deletion of $Ca_V1.3$ did not alter osteogenic responses following ulnar loading [86], suggesting $Ca_V1.2$ ($\alpha 1_C$) may be the primary driver of osteoblast mechanotransduction. However, L-type inhibitors did not completely eliminate anabolic responses to loading [68, 90], suggesting that other VSCC types influence mechanical bone adaptation.

While *in vivo* studies demonstrated the tissue-level consequences of VSCC inhibition, *in vitro* work uncovered the cell-specific, mechanical functions of VSCCs. Using osteoblasts isolated from murine long bones, confocal microscopy enabled visualization of stretch-induced Ca^{2+} influx, which correlated with changes in OPG and OPN expression [91]. These stretch-induced responses were eliminated in Ca^{2+}-free media or following nifedipine treatment, but further enhanced with VSCC agonists. Using optical tweezers to mechanically stimulate human osteoblasts, it was found that nifedipine blocked mechanically-induced L-type Ca^{2+} influx [92]. This response was not seen in chondrocytes or osteocytic cells, again further suggesting that osteoblasts utilize channels that are distinct from other mesenchymal cells.

As VSCCs are inherently sensitive to changes in membrane electrical potential, the mechanism(s) by which these channels regulate bone responses is debatable. One possibility is that mechanical stimuli directly activate “mechanosensitive channels” such as transient receptor cation channels (i.e., TRPV4) causing a small, local membrane depolarization (Fig. 3A). This local membrane change then is sufficient to open VSCCs generating Ca^{2+} influx and downstream responses that further increase intracellular Ca^{2+} concentrations [89]. It also is possible that the VSCCs are directly responsive to mechanical stimuli, not requiring other channels, a notion supported by the ability of auxiliary VSCC subunits to interact with the ECM [93] and the internal cytoskeleton [94].

Once Ca^{2+} enters the cell, several signaling pathways are activated, including release of adenosine triphosphate (ATP), prostaglandin E_2 (PGE2), and nitric oxide (NO) (Fig. 3B–D). ATP acts in an autocrine manner, further increasing Ca^{2+} influx by binding the purinergic P2X receptor [95], and releasing ER Ca^{2+} stores through cleavage of phosphoinositol-4,5-bisphosphate into diacylglycerol and inositol triphosphate (IP3) via phospholipase C (PLC) [96, 97]. ATP-induced increases in intracellular Ca^{2+} require L-Type VSCCs, as inhibition of L-type channels in osteoblasts suppress ATP release and Ca^{2+} influx [68, 96]. VSCC-mediated ATP release activates several pathways including MAPK, protein kinase C (PKC), and Src signaling in osteoblasts to stimulate anabolic responses including ERK1/2 phosphorylation [98, 99]. Mechanically-induced ERK1/2 phosphorylation not only is crucial for osteogenesis, but also osteoblast proliferation, differentiation [100–104], and anabolic responses to loading [105, 106]. In fact, mechanically-induced activation of ERK1/2 is necessary for TGFβ-mediated osteogenic differentiation of MSCs [107] as well as downregulation of RANKL and upregulation of NO synthase (NOS) [108].

Mechanically-induced release of PGE2 [109–112] is VSCC-dependent [96], and serves an important role in osteoblast mechanotransduction [113, 114]. PGE2 is synthesized by cyclooxygenase-2 (COX-2) [115] and osteoblast-specific prostanoid synthases [116] following load-induce activation of protein kinase A (PKA) [117]. PGE2 elicits anabolic

responses in osteoblasts in response to mechanical stimuli including activation of cAMP [118], PKA, PKC, and MAPK signaling [119–121], ultimately increasing osteoblast proliferation and differentiation [122, 116, 109, 123].

An important regulator of homeostatic processes, NO is synthesized by NOS following increases in intracellular Ca^{2+}. A labile, water-soluble molecule, NO production is increased following mechanical loading in bone explants [124] and osteoblasts [125]. Importantly, VSCC inhibition restricts mechanically-stimulated NO release [124], dramatically reducing anabolic responses to loading [126]. NO is generated by one of three isoforms of NOS, neuronal NOS (nNOS or NOS1), endothelial NOS (eNOS or NOS3), and inducible NOS (iNOS or NOS2) [127–129]. Of these isoforms, eNOS plays the most significant role in load-induced bone formation [130–132, 125, 133–135]. Once synthesized, NO interacts with guanylate cyclase to generate cGMP and increase the activity of cGMP-dependent protein kinases (PKG1 and PKG2) [136, 131]. This increase in PKG1 and PKG2 activity elicits an anti-apoptotic and pro-proliferative effect in osteoblasts, activating MEK/Erk and Akt signaling pathways [137, 138], inactivating the pro-apoptotic protein BAD [139], stimulating the Wnt/ β-catenin signaling pathway [138], and increasing expression of Runx2 and *Fos* genes which collectively promotes osteoblast proliferation, differentiation, and survival [138, 140, 141].

Mechanical loading increases Ca^{2+} influx in osteoblasts predominantly through L-type VSCCs, leading to release of ATP, PGE2, and NO, which activate downstream signals to stimulate osteoblast proliferation, differentiation, and bone formation. While L-type channels predominate responses in osteoblasts, this is not true for all bone cells.

Transition from L-type to T-type VSCCs: Increased evidence for VSCC role in osteocytes

Osteoblasts are characterized by a “plump”, cuboidal morphology attributed largely to an extensive amount of ER and golgi (Fig. 4A) [142]. These secretory organelles enable the high output of ECM necessary for bone formation and remodeling. Influx of Ca^{2+} through the plasma membrane accounts for only a small amount of the Ca^{2+} required to initiate intracellular signaling events. The greatest contributor to the change in cytosolic [Ca^{2+}] is Ca^{2+}-induced Ca^{2+} release from the ER. Thus, it is likely that osteoblasts require the long-lasting activation times of L-type channels to enable full release of Ca^{2+} from ER stores and high output of ECM.

As osteoblasts are buried in matrix and differentiate into osteocytes (Fig. 4B), expression of L-type channels is downregulated leaving T-type VSCCs to perform necessary functions. This transition is observed in immunostaining images where L-type ($Ca_V1.2$) and T-type ($Ca_V3.2$ and $Ca_V3.1$) channels are predominantly expressed at sites of active bone formation in osteoblasts throughout murine skeletal development [143, 144]. However, $Ca_V1.2$ expression decreases as T-type $Ca_V3.2$ VSCC expression increases upon differentiation into osteocytes [143–145, 59]. Subsequently, regulation of VSCC-dependent Ca^{2+} influx and subsequent intracellular responses in osteocytes is mediated by T-type and not L-type VSCCs.

In contrast to the extensive ER/golgi of osteoblasts, osteocyte cell bodies are much smaller with very little secretory machinery (Fig. 4B). As osteocytes are terminally differentiated, preventing the death of cells that control both osteoblast and osteoclast activity is crucial for maintaining tight regulation of bone remodeling. Excessive concentrations of cytosolic Ca^{2+} induces apoptosis [146]. Thus, one possible explanation for the transition from long-lasting (L-type) to transient (T-type) VSCCs in osteocytes is to limit Ca^{2+} influx to prevent cell death. Recent work applied an overload of mechanical stimuli to osteoblasts resulting in highly elevated intracellular Ca^{2+} concentrations and subsequent cell death. Importantly, treatment with the L-type inhibitor verapamil restricted apoptosis due to excessive cytosolic Ca^{2+} [147]. Similar effects were seen in renal and neural cells where blocking L-type VSCCs attenuated apoptosis [148, 149]. These studies support the hypothesis that osteocytes down regulate L-type VSCC expression to prevent excessive cytosolic Ca^{2+} concentrations as a means of self-preservation and ultimately to maintain bone remodeling capacity.

T-type VSCCs and osteocyte mechanotransduction

Shifting the predominant VSCC from L- to T-type in osteocytes facilitates lower Ca^{2+} permeability and current potential, enabling osteocytes to retain sensitivity to external stimuli without generating cytotoxic Ca^{2+} levels. Just as L-type channels enable mechanical responses in osteoblasts, T-type VSCCs serve a similar and distinct function in osteocytes. T-type VSCCs are uniquely sensitive to fluid shear vs. membrane strain. In one study, fluid shear induced repetitive spike-like peaks of Ca^{2+} in nearly all osteocytes (~97%), while a lesser number of osteoblasts responded to the same mechanical stimulation, only showing a single large Ca^{2+} peak followed by a few smaller Ca^{2+} peaks [150]. Furthermore, osteocytes showed a greater number of Ca^{2+} peaks with lower latency periods, which maintained their magnitude throughout the loading cycle compared to osteoblasts [144, 145].

While these *in vitro* studies highlight differences in Ca^{2+} responses of osteoblasts and osteocytes, recent work used intravital imaging to delineate osteocyte responses to loading. Use of a transgenic mouse expressing GCaMP under the direction of Dmp1-Cre enabled real-time visualization of osteocyte Ca^{2+} influx in the 3rd metatarsal of mice while subjected to mechanical loading [151]. The study examined strains ranging from 250–3,000 micro-strain and frequencies of 0.5–2 Hz. The number of responding osteocytes incrementally increased with increasing magnitudes and frequencies, with a more exponential increase at 2 Hz. However, the Ca^{2+} signal intensity within responding osteocytes remained consistent and unaltered with increasing magnitudes and frequencies, suggesting a threshold to mechanically-induced Ca^{2+} influx. This study suggests that the thresholding capacity of osteocytes limits Ca^{2+} influx, possibly to prevent Ca^{2+} cytotoxicity and further supporting the function of T-type VSCCs, but also shows that the response to mechanical loading is regulated by the recruitment of osteocytes and not total Ca^{2+} influx.

In addition to the work demonstrating differences in Ca^{2+} responses of osteoblasts and osteocytes, several studies confirm that inhibition of T-type VSCCs impairs osteocyte responses to mechanical stimuli. In one study, treatment with NNC55–0396, a T-type VSCC inhibitor, decreased fluid shear-induced Ca^{2+} influx in osteocytic cells; decreasing the amplitude and number of Ca^{2+} peaks as well as total number of responding osteocytes. In

contrast, treatment with the L-type inhibitor nifedipine decreased anabolic responses to mechanical loading in osteoblasts, but elicited no change in Ca^{2+} influx [144, 145] nor the release of PGE2 and NO to loading in osteocytes [124]. T-type VSCC inhibition of *in situ* osteocytes prior to mechanical stimulation also showed similar decreases in total number of Ca^{2+} responses [152]. Unpublished work from our group demonstrated that deletion of $Ca_V3.2$ in mice impairs bone formation and decreases responses to mechanical stimuli, further highlighting the skeletal function of T-type VSCCs.

As mentioned earlier, VSCCs are multi-subunit complexes composed of auxiliary subunits which influence properties of the channel pore. In particular, the large extracellular region of the $\alpha_2\delta_1$ subunit provides a means for VSCCs to interact with the ECM or ligands. While there are four genes that encode unique $\alpha_2\delta$ proteins, we demonstrated that $\alpha_2\delta_1$ is the predominantly expressed isoform in osteocytes, and even though $\alpha_2\delta_1$ does not bind T-type channels in all tissues, it associates with $Ca_V3.2$ in osteocytes [93]. Furthermore, we showed that knockdown of $\alpha_2\delta_1$ in osteocytic cells dramatically impaired mechanically induced ATP release. The $\alpha_2\delta_1$ subunit also was necessary for activation of ERK1/2 signaling in response to mechanical signals in osteocytes [93].

Purinergic signaling mediates the response of bone to mechanical stimuli and is initiated by Ca^{2+} signaling. VSCC-mediated Ca^{2+} influx results in ATP release, which subsequently activates purinergic receptors releasing IP3 by activation of PLC. This cascade enables release of Ca^{2+} stores from the ER. Mechanical loading in osteocytes releases ER Ca^{2+} stores; however, following the initial Ca^{2+} spike, subsequent intracellular Ca^{2+} peaks only occur after recovery of ER Ca^{2+} stores, as nearly 85% of all intracellular Ca^{2+} peaks were associated with a reciprocal decrease in ER Ca^{2+} stores in MLO-Y4 cells [145, 152]. As such, direct inhibition of P2R/PLC, or the prevention of ER Ca^{2+} store repletion decreases mechanically-induced Ca^{2+} oscillations in MLO-Y4 cells [145] and osteocytes *in situ* [152, 153]. In contrast, osteoblasts do not require full recovery of ER Ca^{2+} stores for repetitive mechanical responses, possibly due to their larger ER structure [145]. Therefore, mechanical responses of osteocytes depend on purinergic signaling mediated by T-type VSCCs [145, 152, 144].

Calcium channel functions in osteoclasts

In contrast to mesenchymal osteoblasts and osteocytes, the function of VSCCs in hematopoietic osteoclasts is less clear. While several studies suggest that osteoclasts lack VSCCs [71, 154], others show that these channels are produced in osteoclasts [155]. Additionally, osteoclast differentiation [156] and OPG/RANKL/ANK axis [157] require MAPK/ERK signaling, both of which are influenced by Ca^{2+} influx. In fact, RANKL-mediate osteoclastogenesis is accompanied by increases in Ca^{2+} influx [158]. Furthermore, deletion of Ca^{2+}-sensing receptors impairs osteoclast differentiation and activity as well as induces apoptosis [159, 160]. These data support an essential function of Ca^{2+} signaling in osteoclasts.

Recent *in vivo* and *in vitro* data show L-type VSCCs are highly expressed in osteoclasts and influence osteoclastogenesis. Immunostaining and qPCR analyses revealed $Ca_V1.3$ expression in primary murine osteoclasts [155], which increased 4-fold upon ovariectomy,

suggesting that upregulation of $Ca_V1.3$ is associated with increased osteoclast activity. Additionally, over-expression of $Ca_V1.3$ in RAW264.7 cells increased osteoclast number and expression of numerous osteoclast-specific genes which were decreased upon Cav1.3 knockdown [155]. While data delineating the function of VSCCs in osteoclasts is limited, the dependence of osteoclasts on oscillating Ca^{2+} concentrations for proper function [159–161] warrants further investigation to study VSCCs influence on osteoclast-mediate bone resorption.

While evidence for direct functions of VSCCs in osteoclasts remains unclear, several studies suggest that channel activity in other bone cells has downstream effects on osteoclasts. One such example is the increased release of OPG by osteoblasts following activation of VSCCs, which leads to decreased osteoclast activity and survival [91, 96, 81, 71, 87, 162]. Likewise, inhibition or deletion of $Ca_V1.2$ increased RANKL-induced osteoclast activity predominantly due to reduced OPG release [71, 87, 81].

Another example of indirect regulation of VSCCs on osteoclasts is the increased release of NO following VSCC activation. The anti-resorptive effects of skeletal loading are modulated at least in part by NO production from osteoblasts and osteocytes [112, 163], which decreases RANKL [164, 106]. Additional work showed that inhibition of NOS or L-type VSCCs prior to mechanical loading abrogated the anti-osteoclastogenic effects of loading [165, 105, 106, 68]. These data indicate that mechanical loading regulates release of NO from osteoblasts/cytes, which then influences osteoclast activity and subsequent bone remodeling.

Taken together, these studies suggest that the primary function of VSCCs within the skeleton are mediated through osteoblasts and osteocytes, which then influence osteoclast activity. However, VSCCs may regulate osteoclast function directly in ways that have yet to be determined.

Conclusions

All cells are designed with various receptors and channels to enable interaction with the external environment. As skeletal formation and remodeling rely on spatial, hormonal, and mechanical signals, tight regulation of the membrane electrochemical gradient provides a mechanism by which cells use Ca^{2+} influx to trigger various signaling events. VSCCs allow bone cells to rapidly respond to voltage changes at the plasma membrane. Furthermore, bone cells take advantage of the specific properties afforded by different types of VSCCs, allowing matrix producing osteoblasts to respond to stimuli differently than osteocytes. While the coordinated responses of bone cells are regulated in part by VSCCs, many questions remain regarding other classes of molecules or pharmacological agents which influence these channels in musculoskeletal tissues. Understanding these questions provides a rich field for future investigation.

Acknowledgments

Funding: 1R01AR074473–01 to WRT, MCFC, and AGR; R15AR069943–01 to WRT, and 1F32AR074893–01 to CSW

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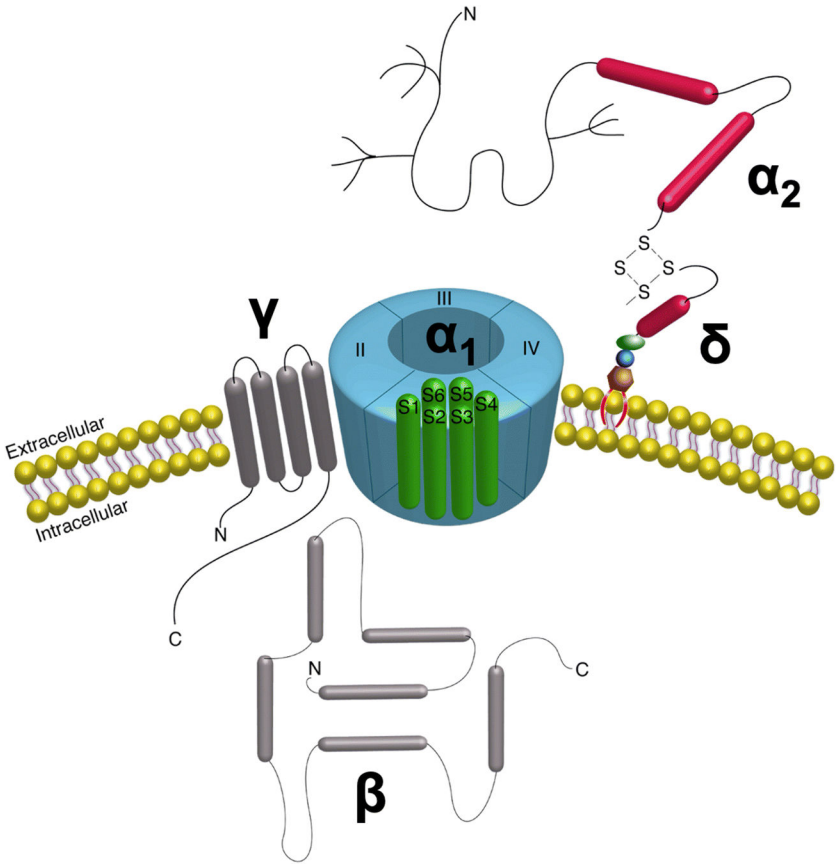

Fig 1. VSCC Structure.

The VSCC complex is composed of the α_1 pore-forming subunit with auxiliary β, γ, and $\alpha_2\delta$ subunits bound to the pore, positioned to alter gating kinetics of the channel. The entirely extracellular $\alpha_2\delta$ subunit is anchored to the membrane via a GPI-anchor.

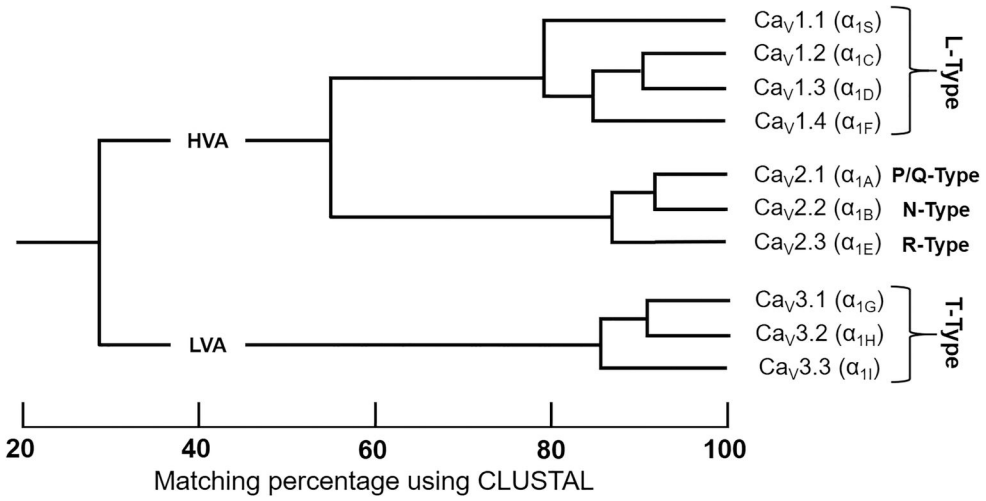

Figure 2. Phylogeny of Voltage-Sensitive Calcium Channel α_1 Subunit.

Only the membrane-spanning segments and the pore loops (~350 amino acids) were compared. By only comparing sequencing pairs, three distinct VSCCs families can be identified with intrafamily sequence identities above 80% ($Ca_V1.x$, $Ca_V2.x$, $Ca_V3.x$). High voltage-activated (HVA); low voltage-activated (LVA). Adapted from Ertel EA et al [44] & Dolphin AC [43].

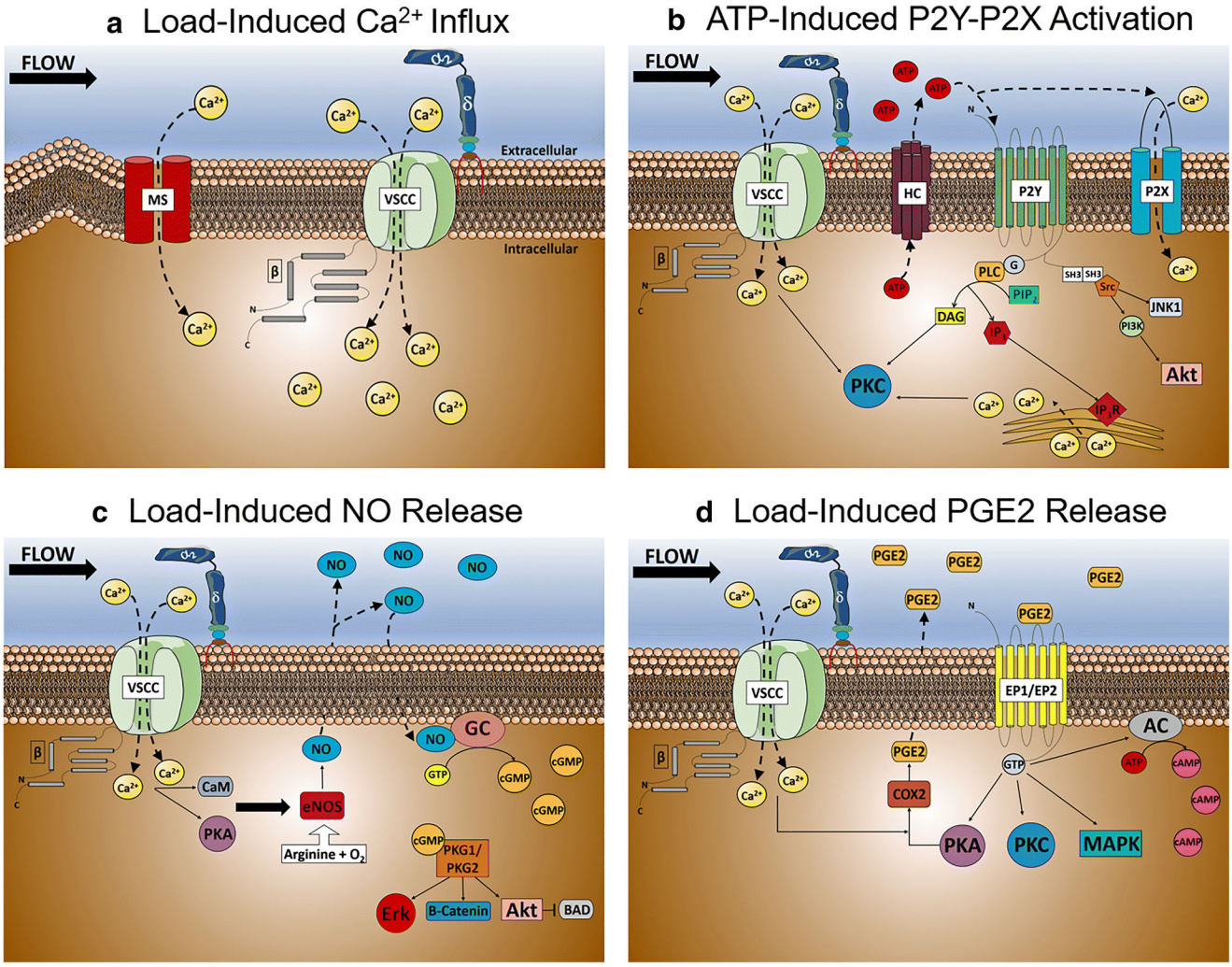

Figure 3. Role of L-type VSCCs in Osteoblast Mechanotransduction.

A) Calcium influx following load-induced shear stress in osteoblasts. Following a relatively small, but rapid influx of calcium via mechanosensitive channels, the plasma membrane is depolarized triggering VSCCs to further increasing intracellular calcium influx. B) Load-induced ATP release following calcium influx by osteoblast VSCCs. Increases in intracellular calcium concentrations by VSCCs increases ATP efflux through hemichannels, activating the ATP-gated purinergic P2X and P2Y receptors to increase P2X-dependent calcium influx and release of endoplasmic reticulum calcium stores. C) Load-induced Nitric Oxide (NO) release by osteoblast VSCCs. Endothelial nitric oxide synthase (eNOS) increases NO expression following VSCC calcium influx and activation by calmodulin (CaM) and protein kinase A (PKA), causing NO-induced guanylate cyclase activation and increases in cyclic guanosine monophosphate (cGMP) intracellular concentrations. D) Load-induced Prostaglandin E_2 (PGE2) release by VSCCs in osteoblasts. Following VSCC calcium influx and PKA activation, PGE2 is synthesized by cyclooxygenase-2 (COX-2) and prostanoid synthases, causing an increase in PGE2 release and the activation of Prostaglandin E1/E2 Receptors (EP1/EP2). ATP, Adenosine triphosphate; AC; Adenylate cyclase; BAD, Bcl-2-associated death promoter; cAMP, Cyclic adenosine monophosphate;

JNK1, c-Jun N-terminal kinase; DAG, diacylglycerol; eNOS, endothelial nitric oxide synthase; Erk, Extracellular-signal-regulated kinase; IP_3, Inositol triphosphate; IP_3R, Inositol triphosphate receptor; MAPK, Mitogen-activated protein kinase; PI3K, Phosphoinositide 3-kinases; PLC, Phospholipase C; PIP_2, Phosphoinositol-4,5-bisphosphate; PKA, Protein kinase A; Akt, Protein kinase B; PKC, Protein kinase C; PKG1/PKG2, Protein kinase G; Src, Src kinase; SH3, Src homology 3 domain

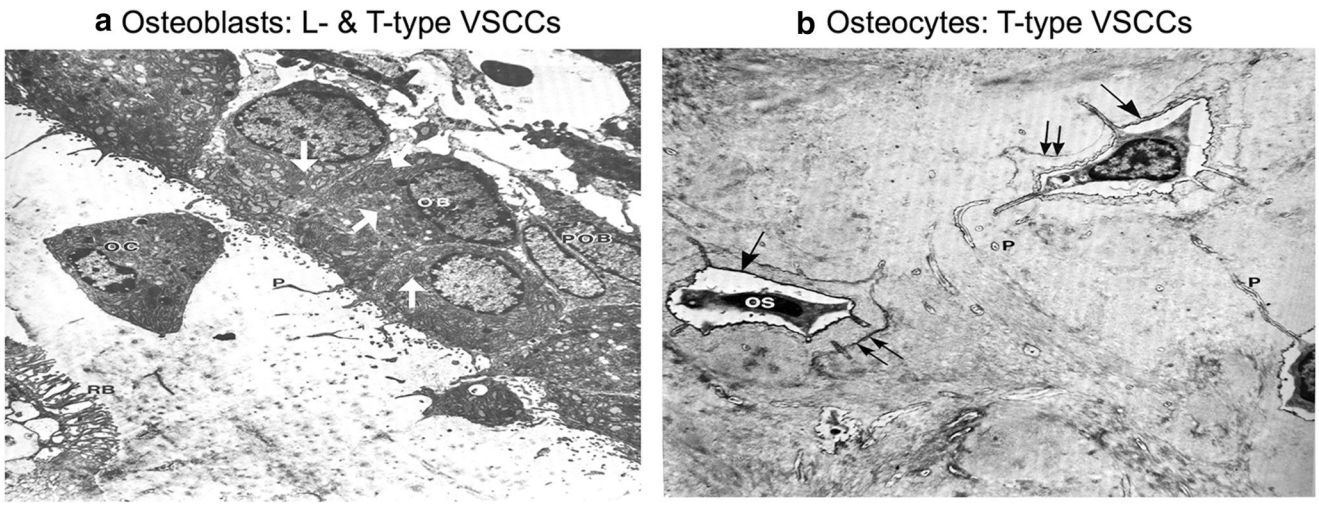

Fig. 4: Predominate VSCC composition coincides with the morphology and function of osteoblasts and osteocytes.

A). The enlarged ER and golgi secretory organelles (white arrows) of osteoblasts (OB) gives its characteristic “plump”, cuboidal morphology and high output of extracellular matrix. The predominate $Ca_V1.2$ L-type VSCCs in osteoblasts have a large single channel conductance and slow voltage-dependent inactivation, allowing for „long-lasting“ calcium influx, the full release of ER calcium stores, and the stimulus necessary for bone formation and remodeling. B) With its flat, stellate shape, mature osteocytes (OS) have much smaller secretory organelles in comparison to osteoblasts. By shifting from expression of L-type to T-type VSCCs during differentiation, the presence of $Ca_V3.2$ VSCCs in osteocytes enables faster and more complete channel inactivation than L-type VSCCs, allowing osteocytes to retain its sensitivity to calcium influx without generating cytotoxic Ca^{2+} levels. Figures adapted from Butler TW [142].

Table 1.

Functional effects and tissue distribution of voltage-sensitive calcium channel auxilliary subunits

Subunit	Functional effects	Tissue Distribution
$\alpha_2\delta_1$	Trafficking α_1 to membrane Increases current amplitude Activating/Inactivating calcium gating-kinetetics Site of extracellular ligand interaction Voltage dependence	Brain, heart, skeletal muscle, bone
$\alpha_2\delta_2$	Increases current amplitude	lung, testis, brain, heart, pancreas, prostate, skeletal muscle, bone, spinal cord
$\alpha_2\delta_3$	Amplifying current density	Brain, heart, skeletal muscle
$\alpha_2\delta_4$	Increases current amplitude	Heart, skeletal muscle, intestine, fetal liver, erythroblasts, adrenal gland, pitutary
β_1	Skeletal excitation-contraction coupling Trafficking α_1 to membrane Activating/Inactivating calcium gating-kinetetics Voltage dependence Targeting of $\alpha_1$1.1 to triads	Skeletal muscle, brain
β_2	Trafficking α_1 to membrane Activating/Inactivating calcium gating-kinetetics Voltage dependence Targeting of $\alpha_1$1.4 to retina	Heart, lung, trachea, aorta, brain
β_3	Trafficking α_1 to membrane Activating/Inactivating calcium gating-kinetetics Voltage dependence	Smooth muscle, trachea, aorta, lung, brain
β_4	Trafficking α_1 to membrane Regulating calcium gating-kinetetics Activating/Inactivating calcium gating-kinetetics	Brain
γ_1	Activating/Inactivating calcium gating-kinetetics Inhibiting channel function	Skeletal muscle
γ_2	Activating/Inactivating calcium gating-kinetetics Inhibiting channel function	Brain
γ_3	Activating/Inactivating calcium gating-kinetetics	Brain
γ_4	Inactivating calcium gating-kinetetics	Heart, lunc, brain, prostate, spinal cord
γ_5	?	Brain
γ_6	Reduces current aplitude	Heart, skeletal muscle, brain
γ_7	Reduces current aplitude	Brain, heart, lung, testis
γ_8	?	Brain, testis, spinal cord

Adapted from Arikkath J & Campbell KP (2003) [3]

Table 2.

Structure, Function, and Pharamacological Properlties of VSCC

Calcium Channel Family	α_1 Subunit	Channel Blocker	Primary Tissues	Primary Function
L-Type	$Ca_v1.1$	DHPs, PHAs, BNZs	Skeletal muscle	Excitation-contraction coupling in smooth muscle, regulation of transcription
	$Ca_v1.2$	DHPs, PHAs, BNZs	Smooth and cardia muscle, Brain, pituitary, adrenal	Excitation-contraction coupling in cardiac and smooth muscle, endocrine secretion, neuronal Ca^{2+} transients in cell bodies and dendrites, regulation of enzyme activity, regulation of transcription
	$Ca_v1.3$	DHPs, PHAs, BNZs	Brain, pancreas, kidney, ovary, adrenal, cochlea	Endorine secretion, cardiac packing, neuronal Ca^{2+} transients in cell bodies and dendrites, auditory transduction
	$Ca_v1.4$	DHPs, PHAs, BNZs	Retina	Visual transduction
N-Type	$Ca_v2.1$	ω-CTx-GVIZ	Brain, nervous system	Neurotransmitter release, Dendritic Ca^{2+} transients
P/Q-Type	$Ca_v2.2$	ω-Agatoxin	Brain, nervous system	Neurotransmitter release, Dendritic Ca^{2+} transients
R-Type	$Ca_v2.3$	SNX-482	Brain, cochlea, pituitary, retina, cardiac muscle	Neurotransmitter release, Dendritic Ca^{2+} transients
T-Type	$Ca_v3.1$	Ni^{2+}, Cd^{2+}, KTx, mibefradil, NNC55–0395	Brain, nervous system	Pacemaking and rhymic firing
	$Ca_v3.2$	Ni^{2+}, Cd^{2+}, KTx, mibefradil, NNC55–0396	Brain, cardiac muscle, kidney, liver	Pacemaking and rhymic firing
	$Ca_v3.3$	Ni^{2+}, Cd^{2+}, KTx. mibefradil, NNC55–0397	Brain	

Abbreviations: BNZ, benzothiazepines; DHP, Dihydropyridines; KTx, Scorpin toxin Kurtoxin; MibefradilPHA, Phenylalkylamines; SNX-482, synthetic toxin from Tarantula *Hysterocrates gigas*; ω-CTx-GVIZ, ω-conotoxin GVIA from cone snail *Conus geographus*

Adapted from Catterall WA (2011) & Dolphin AC (2016) [42, 43]