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# Voltage-Dependent Calcium Channels in Chondrocytes: Roles in Health and Disease

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**Abstract** Chondrocytes, the single cell type in adult articular cartilage, have conventionally been considered to be non-excitable cells. However, recent evidence suggests that their resting membrane potential (RMP) is less negative than that of excitable cells, and they are fully equipped with channels that control ion, water and osmolyte movement across the chondrocyte membrane. Amongst calcium-specific ion channels, members of the voltage-dependent calcium channel (VDCC) family are expressed in chondrocytes where they are functionally active. L-type VDCC inhibitors such as nifedipine and verapamil have contributed to our understanding of the roles of these ion channels in chondrogenesis, chondrocyte signalling and mechanotransduction. In this narrative review, we discuss published data indicating that VDCC function is vital for chondrocyte health, especially in regulating proliferation and maturation. We also highlight the fact that activation of VDCC function appears to accompany various inflammatory aspects of osteoarthritis (OA) and, based on in vitro data, the application of nifedipine and/or verapamil may be a promising approach for ameliorating OA severity. However, very few studies on clinical outcomes are available regarding the influence of calcium antagonists, which are used primarily for treating cardiovascular conditions in OA patients. This review is intended to stimulate further research on the chondrocyte 'channelome', contribute to the development of novel therapeutic strategies and facilitate the retargeting and repositioning of existing pharmacological agents currently used for other comorbidities for the treatment of OA.

**Keywords** Osteoarthritis (OA) · Joint inflammation · Cartilage · Chondrocyte · Voltage-dependent calcium channel · Calcium channel blockers · Nifedipine · Verapamil

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## Introduction

A thin layer of hyaline cartilage covers the articulating bones in most load-bearing synovial joints in the human body, where it enables frictionless movement and provides protection for subchondral bone. Adult cartilage contains a single cell type, the chondrocyte, with the main function of maintaining the proper composition of the extracellular matrix (ECM), in which they are embedded. The ECM of hyaline cartilage primarily consists of a dense and cross-linked meshwork of fibrillar collagens (predominantly type II collagen) and other minor collagens (mainly type VI, IX and XI) that are incorporated in the fibrillar collagen network, regulating fibril assembly, organisation and degradation. This matrix is packed with large aggregating proteoglycans (e.g. aggrecan) and their constituent glycosaminoglycans (GAGs; e.g.

chondroitin sulphate, keratan sulphate, hyaluronan, etc.), which possess a net negative charge attracting counteracting cations and high quantities of osmotically obliged water (approx. 70 % of the net weight of ECM) [1]. Small leucine-rich proteoglycans (SLRPs) such as decorin, biglycan and fibromodulin are specialised ECM molecules that contain multiple repeats of a leucine-rich structural motif, flanked by cysteine residues. SLRPs also modify the deposition and arrangement of fibrillar collagen fibres in the ECM. They also interact with cells and soluble growth factors such as transforming growth factor β (TGF-β) that may affect the proliferation of chondrocytes in addition to modifying the composition of the ECM.

As a unique type of connective tissue, mature articular cartilage is avascular, aneural and alymphatic [2]. Chondrocytes are surrounded by a pericellular matrix and are situated in cavities called lacunae within the ECM, where they exist in a hypoxic microenvironment [3, 4] and control ECM turnover and homeostasis in response to mechanical loading [5] despite having a rather slow (mainly anaerobic) metabolism. Due to the fact that chondrocytes reside within the peculiar milieu of the ECM (which is acidic, hypertonic and hyperosmotic [6–8]), and since their function and differentiation is strongly dependent on intracellular calcium homeostasis [9, 10], they deploy a whole range of ion channels to enable ion transport across the plasma membrane; the collection of ion channels is referred to as the chondrocyte 'channelome' [11]. Albeit generally considered to be non-excitable cells, chondrocytes and chondroprogenitor cells have been reported to express various voltage-dependent potassium channels ($K_V$), ATP dependent potassium channels ($K_{ATP}$), large and small conductance calcium-activated potassium channels (BK and SK), transient receptor potential channels (primarily TRPV1 and TRPV4), purinergic receptors (both P2X and P2Y subfamily members), voltage-gated and epithelial sodium channels, chloride channels and also voltage-dependent calcium channels (VDCCs) [11–13].

## Physiology and Pharmacology of Voltage-Dependent Calcium Channels

Under physiological conditions, $Ca^{2+}$ concentration is several thousand times higher in the extracellular space than in the cytoplasm. Activation of VDCCs results in rapid elevation of intracellular $Ca^{2+}$ levels and initiates various cellular responses. VDCCs are typically closed at resting membrane potentials, and their opening requires depolarization. VDCCs, along with other structurally similar voltage-gated ion channels such as $Na_V$ or $K_V$ channels, are tetrameric cation channels with four transmembrane domains (I–IV) surrounding a selective central pore and typically consist of $\alpha_1$, $\alpha_2\delta$, β and γ subunits. Although the main structural and topological features of VDCCs are largely conserved, their physiological and pharmacological properties can be very different. The $\alpha_1$ subunit, besides forming the pore, also determines the type of the channel; to date, 10 different $\alpha_1$ subunits have been isolated, which can be classified into the structurally and functionally related families of $Ca_V1$, $Ca_V2$ and $Ca_V3$ (see Table 1). The other ancillary subunits (four $\alpha_2\delta$, four β and eight different γ subunits are known) modulate the functional properties of these ion channels. There are five different types of VDCCs; members of the L-type (or 'long lasting') group of $Ca^{2+}$ channels contain one of the $Ca_V1.1$, $Ca_V1.2$, $Ca_V1.3$ or $Ca_V1.4$ $\alpha_1$ subunits; the N-type ('neural') contains $Ca_V2.1$; the P/Q-type ('Purkinje') contains $Ca_V2.2$; the R-type ('residual') contains $Ca_V2.3$; and members of the T-type ('transient') group contain $Ca_V3.1$, $Ca_V3.2$ or $Ca_V3.3$ $\alpha_1$ subunits (Table 1) [14•, 15]. It is of note that the L, N, P/Q and R-type calcium channels are activated at high voltage (HVA), whilst T-type calcium channels are activated at negative resting membrane potential (RMP) values and are considered as members of the low-voltage-activated (LVA) family [16].

Inhibitors such as nifedipine or small peptide toxins from certain spider, snail or scorpion species have contributed to our understanding of the roles of these calcium channels in various models. VDCCs are targets of existing drugs for the treatment of various cardiovascular diseases and central nervous system disorders. Dihydropyridines, phenylalkylamines, benzodiazepines and other small molecules are widely used potent L-type channel blockers. Nifedipine is a member of the dihydropyridine family of calcium channel blocking agents. Its mechanism of action is to inhibit calcium influx, thereby causing, for example, smooth muscle cell relaxation and consequent vasodilation. Therefore, nifedipine, along with other dihydropyridines such as amlodipine or nicardipine, is a potent and widely used antihypertensive drug [17]. The phenyl-alkylamine verapamil, another potent calcium antagonist, binds to a distinct site on the ion channel and is also widely used as an antihypertensive drug [18]. The L-type channels are sensitive to the neurotoxin calciseptine, in addition to the conventional blockers [16]. The main modulators of N-type calcium channels are ω-conotoxins; P/Q-type channels are selectively blocked by ω-agatoxins; and the peptide neurotoxin SNX-482 is a selective R-type calcium channel inhibitor. To date, no specific blockers for T-type $Ca^{2+}$ channels have been identified [19].

## VDCCs and the Effects of Nifedipine/Verapamil on Chondrocytes

Whilst the roles of VDCCs in excitable cells such as muscle, nerve or endocrine cells are well established, they act in an even more complex way in non-excitable cells that involves regulating enzyme activity or gene expression [19]. Although

**Table 1** Classification of VDCCs based on their $\alpha_1$ subunits and inhibitors

| Superfamily | Family | $\alpha_1$ subunit | Gene | Pharmacology |
|---|---|---|---|---|
| High voltage activated | L | $Ca_V1.1$ | CACNA1S | Dihydropyridines (nifedipine); phenyl-alkylamines (verapamil); calciseptine |
| | | $Ca_V1.2$ | CACNA1C | |
| | | $Ca_V1.3$ | CACNA1D | |
| | | $Ca_V1.4$ | CACNA1F | |
| | N | $Ca_V2.1$ | CACNA1A | ω-conotoxins |
| | P/Q | $Ca_V2.2$ | CACNA1B | ω-agatoxins |
| Intermediate voltage activated | R | $Ca_V2.3$ | CACNA1E | SNX-482 |
| Low voltage activated | T | $Ca_V3.1$ | CACNA1G | No selective inhibitor discovered to date |
| | | $Ca_V3.2$ | CACNA1H | |
| | | $Ca_V3.3$ | CACNA1I | |

there is now an accumulating body of data demonstrating the presence and function of VDCCs in chondrocytes, their specific roles in different models are still not entirely clear. We therefore provide a detailed overview of what has been published in the literature, including our own findings, concerning VDCCs and the effects of the most frequently applied inhibitors such as nifedipine and verapamil in differentiating and adult chondrocytes.

The first implication that VDCCs might be involved in early steps of chondrogenic differentiation was published in 1989 [20•]. When nifedipine (40–100 μmol/kg) was administered to rabbits per os on day 16 of gestation, a range of digital defects developed in the foetuses, including reduction, total absence or abnormal structure of the distal phalanx of the fourth digit on the hind paws. These defects were mainly brought about by disturbed chondrogenesis and osteogenesis. It must be added, however, that administration of a drug with a different mechanism of action and different targets (i.e. hydralazine that blocks voltage-gated potassium channels) resulted in the same outcome, indicating that the effect of nifedipine on digit formation in the foetuses was not specific. Indeed, decreased utero-placental blood flow caused by excessive hypotension, rather than a direct blockade of the voltage-dependent ion channel, was implicated as the most probable mechanism underlying the observed defects in a follow-up study [21]. No apparent disturbance of chondrogenesis was observed in embryonic chick limb bud-derived micromass cultures at concentrations of nifedipine below $3 \times 10^5$ μM.

Additional, more specifically targeted experiments were carried out subsequently to explore the role of VDCCs in chondrocytes. In bovine articular chondrocytes, administration of endothelin or platelet-derived growth factor (PDGF) evoked either non-oscillatory $Ca^{2+}$ transients or oscillatory $Ca^{2+}$ events during single cell $Ca^{2+}$ recordings, depending on the subpopulation of cells [22]. $Ca^{2+}$ spiking was not altered in the presence of nifedipine; also, depolarization of chondrocytes by high concentrations of $K^+$ did not induce $Ca^{2+}$ transients, and the study concluded that VDCCs were not functional in these cells. In contrast, nifedipine (administered i.v. at 6 mg/kg/day twice daily) interfered with elongation of long bones in vivo in growing rabbits, affecting the epiphyseal growth plate and bone remodelling [23]. This observation was supported by in vitro data; treatment of mouse embryonic limb bud-derived micromass cultures with nifedipine or verapamil during the early stages of differentiation inhibited chondrogenesis, reflecting on an important role of L-type VDCCs during cartilage formation [24]. Furthermore, in high-density primary cultures of human articular chondrocytes, application of verapamil or nifedipine abrogated the stimulatory effect of prostaglandin $E_2$ ($PGE_2$) on IGFBP-3, a protein that regulates the bioactivity and bioavailability of insulin-like growth factor-1 (IGF-1), an important mediator of chondrocyte metabolism [25]. In a follow-up study, IGF-1, IGF-2 and insulin all increased cytosolic $Ca^{2+}$ levels in cultured rabbit articular chondrocytes but through different mechanisms; $Ca^{2+}$ influx mediated by L-type VDCCs was involved downstream of IGF-1 and insulin, since verapamil diminished these responses [26].

In contrast to earlier observations [23], a different study showed that chicken growth plate chondrocytes did not express functional L-type VDCCs; whilst administration of high concentrations of extracellular $K^+$ evoked large intracellular $Ca^{2+}$ transients, nifedipine (or nitrendipine), was unable to block channel activity, even at 10 times higher concentrations than used normally (at 100 μM) [27]. Furthermore, lack of $Ca^{2+}$ transients evoked by BAY K 8644, an agonist that triggers activation and prolonged opening of L-type channels, corroborated the above findings. $Cd^{2+}$, on the other hand, significantly blocked $K^+$-evoked $Ca^{2+}$ transients, indicating that these cells mainly express N-type VDCCs.

To further elucidate the role of L-type VDCCs in bovine articular chondrocytes, their involvement in mediating cartilage ECM gene induction and matrix metalloproteinase (MMP) gene suppression by capacitatively coupled electric field (60 kHz; 20 mV/cm) was investigated [28]. Whilst electrical stimulation significantly upregulated aggrecan and collagen type II mRNA expression, and at the same time downregulated IL-1β induced MMPs, these were completely

eliminated by the L-type VDCC blocker verapamil. However, this study did not demonstrate downstream pathways that link $Ca^{2+}$ influx through VDCCs to the regulation of matrix genes.

To put in context the role of L-type VDCCs, a hypothesis was elaborated to explain the role of these channels in dihydrotestosterone (DTH) signalling, showing that DHT regulates growth plate chondrocytes in a sex-specific manner [29]. Upon binding to its 7-TM domain-containing plasma membrane receptor, DTH was proposed to initiate the phospholipase C (PLC) signalling pathway leading to $Ca^{2+}$ release from internal $Ca^{2+}$ stores through activation of $IP_3$ signalling; furthermore, $G_{\beta\gamma}$ subunits would induce $Ca^{2+}$ influx through L-type VDCCs in resting zone growth plate chondrocytes derived from male rats. The increase in cytosolic $Ca^{2+}$ levels, sourced from both $Ca^{2+}$ release and influx, would eventually activate classic protein kinase C (PKC) isoenzymes, leading ultimately to chondrocyte differentiation.

## VDCC Inhibitors Influence Proliferation and Resting Membrane Potential in Chondrocytes

Every living cell is characterised by RMP, the voltage across the plasma membrane, which is generated by the activities of different ion channels or transporters with specific ion selectivities and permeabilities. As discussed earlier, even though the chondrocyte is a non-excitable cell type, RMP acts as a key biophysical signal modulating important activities such as volume regulation, proliferation and differentiation [30]. To identify what roles VDCCs may play in the regulation of chondrocyte proliferation and RMP, several studies have been conducted. The first work in this field revealed that, in high-density porcine articular chondrocyte cultures, verapamil stimulated $^{3}$H-thymidine uptake both in control conditions and following treatment with IGF-1; the stimulatory effect on cell proliferation was accompanied by a reduced rate of $^{35}$S-incorporation indicative of inhibited GAG secretory processes [31]. In contrast, nifedipine appears to have the opposite effect on chondrocyte proliferation; application of 10 μM nifedipine completely abolished the enhanced proliferation and differentiation of chicken sternum-derived chondrocytes cultured on 3D collagen scaffoldings triggered by cyclic mechanical load [32•]. To address the apparent contradiction between these two results, further experiments were conducted. When the influence of a set of ion channel inhibitors was studied on the RMP and proliferation of human chondrocytes isolated form OA knee cartilage [33], verapamil reduced the RMP of chondrocytes by 18 %; also suggested was that the ion channel modulators investigated in this study might influence chondrocyte cell proliferation. Subsequent work showed marked suppression of proliferation in human articular chondrocytes following longer incubation times with verapamil; however, it must be noted that verapamil treatment also caused pronounced cytotoxic effects in these cells [34].

To gather further information on the effects of verapamil on the proliferation rate of chondrocytes, the influence of verapamil on endochondral ossification using foetal rat metatarsal rudiments was studied [35]. Verapamil reduced growth of the rudiments and the number of hypertrophic chondrocytes, as well as proliferation, indicating that the function of L-type VDCCs is also required for the proliferation of epiphyseal growth plate chondrocytes. Our own results showing an almost complete lack of proliferating cells in embryonic chicken limb bud-derived micromass cultures treated with nifedipine are in complete agreement with these findings [36], indicating an important positive role for VDCCs in regulating the proliferation of differentiating chondrocytes. The effects of nifedipine and verapamil on chondrocytes are summarised in Table 2.

### Subunit-Specific Expression of VDCC: $\alpha_1$ Subunits

In the first study to describe the subunit-specific expression of VDCCs, $Ca_V1.2$ L-type $\alpha_1$ subunit expression was identified in rat tracheal cartilage using immunohistochemistry [37]. Later on, the presence of the $\alpha_1$ subunit protein of VDCCs was confirmed using a nonspecific pan $\alpha_1$-subunit antibody in mouse limb bud organ cultures, in which they co-localised with Na, K-ATPase and epithelial $Na^+$ channels in putative mechanoreceptor complexes [38]. These initial results were verified by analysing subunit-specific expression of L- and T-type VDCCs during cartilage and bone formation in mice [39]. According to these observations, intense immunoreactive signals for $Ca_V1.2$ (L-type) and $Ca_V3.2$ subunits (T-type) were visible along the perichondrium and in the growth plate at embryonic stage E14.5. At later stages (E18.5), $Ca_V1.2$ and $Ca_V3.2$ immunopositivity could still be observed in chondrocytes in the hypertrophic zone. These findings were corroborated using the murine chondrogenic cell line ATDC5.

Our group examined $\alpha_1$ subunit expression profiles in embryonic chicken limb bud-derived chondrifying micromass cultures and identified members of L-type ($Ca_V1.2$ and $Ca_V1.3$), R-type ($Ca_V2.3$), and T-type ($Ca_V3.1$, $Ca_V3.2$ and $Ca_V3.3$) VDCCs [36], in a good agreement with previously published data. Although the above studies showed the presence of functional $\alpha_1$ subunits in mature or differentiating chondrocytes, $Ca_V3.2$ was the first $\alpha_1$ subunit identified as essential for tracheal chondrogenesis in mice; in mice lacking $Ca_V3.2$ channels, Sox9 expression was attenuated, accompanied by disturbed tracheal cartilage formation [40].

**Table 2** Effects of nifedipine and/or verapamil on chondrocytes at different developmental stages and under different experimental conditions

| | Effect(s) of nifedipine and/or verapamil | Experimental conditions | Reference | Notes |
|---|---|---|---|---|
| 1 | Digital defects in rabbit foetuses | 40–100 μmol/kg nifedipine administered to rabbits per os on day 16 | [20•, 21] | Effects were attributed to decreased utero-placental blood flow |
| 2 | *No effect on $Ca^{2+}$ signalling; high $[K^+]$ failed to evoke $Ca^{2+}$ transients* | *Bovine articular chondrocytes* | [22] | *Nifedipine did not interfere with $Ca^{2+}$ oscillations evoked by ET or PDGF* |
| 3 | Influence epiphyseal growth plate and bone remodelling | 6 mg/kg/day nifedipine administered IV twice daily to growing rabbits | [23] | |
| 4 | Nifedipine/verapamil inhibit chondrogenesis at early stages of differentiation | Mouse embryonic limb bud-derived micromass cultures | [24] | |
| 5 | Nifedipine/verapamil abrogate stimulatory effect of $PGE_2$ on IGFBP-3 | High-density primary cultures of human articular chondrocytes | [25] | |
| 6 | IGF-1 and insulin evoke $Ca^{2+}$ influx through L-type VDCCs | Articular chondrocytes from 21-day-old rabbits; 1 μM verapamil | [26] | |
| 7 | *Unable to block high $K^+$ evoked $Ca^{2+}$ transients* | *Chicken growth plate chondrocytes; nifedipine was used at 100 μM* | [27] | *BAY K 8644 had no effect; results indicate N-type channels* |
| 8 | Eliminate positive effects of capacitatively coupled electric field | Bovine articular chondrocytes; 40 μM verapamil | [28] | |
| 9 | Prevents PKC activation downstream of DTH signalling | Male resting zone growth plate chondrocytes from rats; 10 μM nifedipine | [29] | $Ca^{2+}$ release from internal stores was also involved in the pathway |
| 10 | Verapamil stimulate $^3$H-thymidine uptake and | High-density porcine articular chondrocyte cultures; 40 or 80 μg/mL verapamil | [31] | |
| 11 | Abolish enhanced proliferation rate caused by cyclic mechanical load | Chicken sternum-derived chondrocytes on 3D collagen scaffoldings; 10 μM nifedipine | [32] | |
| 12 | Verapamil reduced RMP by 18 % and suppressed proliferation | Human chondrocytes isolated form OA knee cartilage | [33, 34] | Longer incubation with verapamil was cytotoxic |
| 13 | Influence on endochondral ossification and proliferation in foetal rat metatarsal rudiments | Rat epiphyseal growth plate chondrocytes; 10–100 μM verapamil | [35] | |
| 14 | Disrupts regular pattern of calcium oscillations; abrogates chondrogenesis; almost completely blocks cell proliferation | Chicken embryonic limb bud-derived micromass cultures | [36] | |
| 15 | Completely eliminates the strain-induced upregulation of PTHrP | Cyclic mechanical strain on growth ring-derived chondrocytes from ribs of 4-week-old male rats; 10 μM nifedipine | [41] | |
| 16 | Nifedipine more prominently affects protein synthesis compared to GAG synthesis | Static and dynamic compression in 3D bovine articular cartilage explants | [42] | |
| 17 | Prevents retraction after mechanical stimulation | Mechanical compression of bovine chondrocytes; nifedipine was used at 10 μM | [43] | Nifedipine did not interfere with cell spreading |

Note: Entries in *italics* typesetting (lines 2 and 7) report negative results (i.e. implicate no function of VDCCs and/or their inhibitors)

## Mechanical Load is Partly Mediated by L-Type VDCCs in Chondrocytes

Mechanotransduction pathways in chondrocytes are still incompletely understood, but there is a growing body of evidence that stretch-sensitive ion channels are involved in the process. To elucidate the contribution of $Ca^{2+}$ channels in chondrocyte mechanotransduction pathways, 10 μM nifedipine was applied during mechanical stimulation of a 3D chondrocyte culture system established from 17-day-old chicken sterna [32•]. As mentioned previously, nifedipine completely abolished the enhanced proliferation and maturation of chondrocytes triggered by mechanical load, indicating that L-type $Ca^{2+}$ channels play an important role in mechanosensation and mechanotransduction in chicken sternum-derived chondrocytes.

When cyclic mechanical strain (7 or 12 % elongation by a computer-driven vacuum-operated unit) was applied on growth ring-derived chondrocytes obtained from ribs of 4-week-old male rats [41], pre-incubation with nifedipine (at 10 μM) for 1 h before the strain was applied completely eliminated the strain-induced upregulation of parathyroid hormone-related peptide (PTHrP), an important mediator of endochondral bone formation. These results supported the crucial function of VDCCs in mechanotransduction pathways.

Continuing this line of research, another group analysed how ion channel inhibitors, most importantly nifedipine, influenced the effects of static and dynamic compression in 3D

bovine articular cartilage explants [42]. They observed that inhibition of VDCCs by nifedipine affected total protein synthesis more prominently than GAG synthesis and concluded that a rather complex signalling process, including ion channels, may regulate mechanical loading-induced GAG production. To further scrutinise the potential role of VDCCs during cyclic mechanical compression, the ion channel regulation of cell spreading and retraction was studied in bovine chondrocytes [43]. Although nifedipine did not interfere with cell spreading immediately following mechanical compression, it prevented retraction compared to untreated control cells 6 h after stimulation, suggesting the involvement of VDCCs in regulating transient changes in cell shape. These findings led the authors to hypothesise that L-type VDCCs and α5β1 integrin, together with its associated Src kinase, form mechanoreceptor complexes that are involved in mechanosensation and mechanotransduction. As described earlier, a similar hypothesis has been proposed that β1 integrins, VDCCs and other ion channels form functional mechanoreceptor complexes in embryonic mouse limb bud chondrocytes [38].

## VDCCs are Required for Chondrogenesis

During the complex process of chondrogenesis, $Ca^{2+}$ signalling pathways play determining roles [9, 10]. In earlier studies, we detected rapid $Ca^{2+}$ oscillations, in addition to long-term sustained changes in cytosolic $Ca^{2+}$ levels, in chicken limb bud-derived chondrogenic micromass cultures [44]. As mentioned above, treatment of mouse embryonic limb bud-derived micromass cultures with nifedipine during the early stages of differentiation blocked chondrogenesis, implicating the role of L-type VDCCs during the process [24]. Our research group was the first to describe that differentiating chondrocytes in chicken micromass cultures express the $\alpha_1$ subunits of L-, R- and T-type VDCCs at both mRNA and protein levels; in particular, we detected mRNA transcripts encoding $Ca_V1.2$, $Ca_V1.3$ and $Ca_V2.3$, as well as $Ca_V3.1$, $Ca_V3.2$ and $Ca_V3.3$ VDCCs in complete agreement with previously published data (see [39]). We also reported that the L-type VDCCs play important roles in generating $Ca^{2+}$ influx for oscillations, as nifedipine (10 μM) disrupted the regular pattern of repetitive calcium transients. Furthermore, VDCC blockade abrogated chondrogenesis and almost completely inhibited cell proliferation [36]. These results indicate that by mediating $Ca^{2+}$-dependent signalling pathways in chondroprogenitor cells, L-type VDCCs may play a very important role during the initial stages of chondrogenesis.

As mentioned before, the T-type $Ca_V3.2$ was the first VDCC shown to be indispensable for tracheal chondrogenesis both in vivo and in vitro [40]. These findings were substantiated by the fact that mice lacking $Ca_V3.2$ showed congenital tracheal stenosis; conversely, Cav3.2 overexpression in ATDC5 cells augmented chondrogenesis. Presumably, $Ca^{2+}$ influx through $Ca_V3.2$ would activate the calcineurin/NFAT pathway and regulate Sox9 during tracheal chondrogenesis. However, it is unclear why only tracheal ring cartilage, but not other types of cartilage (e.g. articular cartilage), is affected in mice lacking functional $Ca_V3.2$; the authors suggested that $Ca_V3.2$ is predominantly expressed in tracheal cartilage but hardly detectable in the other cartilages. Since these assumptions would contradict previous observations by other groups (see [39], for example), further studies are required to clarify the specific role of VDCCs (and $Ca_V3.2$ in particular) in chondrogenesis.

## VDCCs in the Context of OA Pathogenesis

OA, a debilitating, multifactorial degenerative joint disease, is a main cause of pain, disability and loss of mobility amongst the elderly. OA is characterised by gradual degeneration of articular cartilage, synovial inflammation (synovitis) and alterations to the periarticular and subchondral bone; therefore, OA can be regarded as a disease of the whole joint. The molecular pathogenesis of OA is still not fully understood, which poses limitations to the development of effective disease-modifying therapeutic approaches [45].

As an attempt to shed more light on the molecular and physiological background of OA pathogenesis, the effects of basic calcium phosphate (BCP) crystals, associated with a rare, destructive form of OA phenotype, were investigated on cytosolic $Ca^{2+}$ levels and $Ca^{2+}$ oscillations in bovine articular chondrocytes and cartilage explants [46]. Inorganic BCP crystals elevated intracellular $Ca^{2+}$ concentrations, induced $Ca^{2+}$ oscillations and at the same time enhanced the rate of matrix degradation (i.e. augmented GAG release and upregulated mRNA expression of MMP-3, ADAMTS-4 and ADAMTS-5). Furthermore, functional L-type VDCCs were required for the calcium phosphate crystal-induced $Ca^{2+}$ oscillations to take place, as treatment with verapamil (1 μM) prevented the crystal-induced rise in cytosolic $Ca^{2+}$ concentration. It must be noted, however, that the crystal-induced GAG release, rather than the upregulated mRNA expression of MMP-3, ADAMTS-4 and ADAMTS-5, was associated with the increased cytosolic $Ca^{2+}$ levels. Therefore, these results imply that by blocking VDCC function, the deleterious effects of inorganic crystal-induced $Ca^{2+}$ signals could be inhibited, and thus the associated OA progression and severity might also be reduced.

The above results corroborated previous in vivo data also indicating the involvement of VDCCs in OA. In these papers, the effects of PD-0200347, an $\alpha_2\delta$ ligand of VDCCs that selectively blocks $Ca^{2+}$ currents, were studied in the anterior cruciate ligament resection dog model of OA. PD-0200347

significantly reduced the progression of structural changes in cartilage by reducing MMP-13 and iNOS gene expression and inhibiting ERK1/2 activation via a Ras-independent but PKCα-dependent mechanism [47•, 48]. Taken together, these results suggest that VDCC-related $Ca^{2+}$ signalling may be an important pathway involved in the pathogenesis of OA and that selective modulation and targeting of VDCC function might be beneficial for treating OA.

In a recent paper, a drug repositioning strategy was deployed to screen for already approved drugs that induce FRZB expression (a soluble antagonist of Wnt signalling) and subsequently downregulate the Wnt/β-catenin pathway in human OA chondrocytes [49]. Verapamil, but not other $Ca^{2+}$ channel blockers, upregulated FRZB gene expression and also stimulated chondrogenic marker gene (ACAN, COL2A1, SOX9) transcript levels. At the same time, verapamil reduced MMP3 expression. Furthermore, intra-articular injection of verapamil halted OA progression and ameliorated cartilage damage in knee joints in a rat OA model [49]. Verapamil also inhibited hyaluronan export from IL-1β treated chondrocytes by blocking the ABC transporter MRP5 and prevented the loss of aggrecan in osteoarthritic rat knee joints [50, 51]. However, since the inhibitory concentrations of verapamil that would be effective on the MRP5 transporter can never be reached in the serum using therapeutic and orally administered drug doses, an alternative hypothesis was suggested; the recent discovery that concurrent $K^+$ efflux through $Ca^{2+}$ activated $K_{ir}$ channels are required for hyaluronan export through MRP5 [52] suggests that blocking VDCC function and subsequent $Ca^{2+}$ influx may inhibit $K^+$ efflux and, simultaneously, excessive hyaluronan export. This hypothesis has yet to be validated.

## Clinical Data on the Effects of VDCC Inhibitors in OA Patients

Various types of VDCCs are located throughout the human body participating in a wide range of functions. L- and T-type VDCCs are integral parts of the cardiovascular system, and their over-activation could manifest in hypertension or arrhythmias; thus, they are the preferred targets of currently marketed calcium channel antagonists. Amongst the less frequent adverse effects of these medications, joint pain or *arthralgia* has been reported, but the frequency ranging from 0.5 to 3 % does not differ significantly from that for placebo [53••]. However, there are few studies available on the clinical outcome of calcium antagonists in joint pain, and, collectively, the evidence currently available is not convincing.

There are some case reports on individual patients with hypertension who developed muscle and joint pain whilst taking antihypertensive medication (i.e. diltiazem hydrochloride or amlodipine); on discontinuation of these drugs, the muscle and joint symptoms were resolved [53••, 54]. In a multicentre, randomised, double-blind clinical trial with a 10-week treatment period that compared the effects of controlled-onset extended release verapamil (314 mg/day) with a conventional, gastrointestinal nifedipine therapy (64 mg/day), arthralgia was more frequent in the nifedipine group (6 %) than that in the verapamil group (2 %); and the difference was statistically significant ($p$=0.048) [55]. However, increased frequencies of joint pain have not been reported in other studies [56]. It must be noted however that (1) some of these cases are probably individual occurrences of joint pain caused by the medications; and (2) arthralgia is a broad term that refers to joint pain and does not necessarily imply pain that is directly evoked by OA. In the clinical papers discussed above, no direct evidence was collected regarding the specific cause and the mechanism for joint pain.

As discussed in the previous section, the severity of experimental OA may be ameliorated using a VDCC inhibitor that selectively blocks $Ca^{2+}$ currents [48]. In a recently conducted survey using the standardised Lequesne questionnaire, the severity of OA in osteoarthritic patients receiving calcium antagonists for the treatment of cardiac arrhythmia was investigated. This study reports that long-term treatment with currently used medications such as Adalat® (active ingredient, nifedipine), Amlodipine® (amlodipine), Carmen® (lercanidipine), Nitrendipine® (nitrendipin) and Norvasc® (amlodipine) led to improvements of OA severity, at least based on subjective patient feedback and Lequesne scores, compared to control patients (who were not taking calcium antagonists) [57•]. However, that study has major limitations that must to be taken into account. Firstly, patients in this study received co-medication. Secondly, patients were not randomised for the study; there were notable differences in the demographics between the groups; and no power analysis was performed. Thirdly, a heterogenous group of calcium antagonists was included in the study. Finally, there is no evidence that antihypertensive drugs actually ameliorated the symptoms of OA in these patients; it might also be the case that the subjective disease improvement experienced by patients could have been caused by the desensitising effects of $Ca^{2+}$ antagonists on synovial nerve endings.

## Conclusions and Future Directions

As it is now evident from data published in vitro and in vivo and summarised above, VDCCs are clearly important modulators of various functional aspects of chondrocyte physiology in health and disease, as well as during primary and terminal chondrocyte differentiation during pre- and postnatal growth plate development (i.e. chondrogenesis and endochondral bone formation). However, it is also evident that there are controversies and unresolved questions regarding their specific roles in chondrocytes from different sources (both in terms

of species and tissues of origin) and developmental stages. Depending on the experimental conditions applied, high concentrations of L-type VDCC blockers exert moderate to severe adverse effects on various signalling pathways, proliferation, growth and differentiation potential of chondrocytes both in vitro and in vivo, according to the literature reviewed here. These effects on chondrocytes from various sources analysed in diverse experimental conditions are summarised in Table 2.

It must be noted, however, that some of the effects of the VDCC inhibitors, nifedipine or verapamil, on bone growth [23], or chondrogenic nodule formation in micromass cultures [24] could have been caused by nonspecific effects of long-term exposure (more than 7 days) to supraphysiological concentrations (up to 100 μM) of the drug. Such concentrations may never be achieved in vivo via oral administration of nifedipine and verapamil. Although the half-maximal concentrations of L-type $Ca^{2+}$ channel antagonists have been described in the range of 20 nM to 50 μM, these values vary considerably in different cell types [58]. Concentrations higher than 50 μM are likely to block other calcium entry pathways or even nonspecifically block $Na^+$ and $K^+$ channels; verapamil can also inhibit T-type channels even at intermediate concentrations [18, 58] or the ABC transporter MRP5 involved in hyaluronan export [51]. Given that the concentration of drug that actually reaches the chondrocyte within the articular cartilage of patients receiving medication for unrelated conditions (e.g. hypertension or arrhythmia) is as yet unknown, it is impossible to extrapolate from the in vitro data that is currently available.

Whilst the effects of VDCC blockage with supraphysiological concentrations of nifedipine and verapamil in healthy chondroblasts and chondrocytes are mostly deleterious as they include inhibition of proliferation, ECM synthesis or chondrogenic differentiation, VDCCs and their inhibitors (mainly verapamil) appear to play contrasting roles in inflammatory OA chondrocytes. This may hold promise for the development of novel therapeutic approaches, possibly even through drug retargeting approaches. However, the effects of verapamil on OA chondrocytes might also be attributable to other, nonspecific targets of the drug, which need to be taken into account.

There are a few possible explanations for the observed differences in healthy and OA chondrocytes in terms of the effects of VDCC inhibitors and VDCC function. Firstly, healthy and OA chondrocytes have an altered complement of plasma membrane ion channels and porins, which can at least partially be attributed to ECM degradation during OA. In particular, a set of ion channels including $Ca^{2+}$ activated potassium channels (BK and IK), $Ca^{2+}$-activated $Cl^-$ channels, aquaporin-1 (AQP1), as well as voltage-gated $K^+$ channels or the piezo-type mechanosensitive ion channel 2 (Piezo2) were differentially expressed in healthy vs. OA cartilage [59–61]. Most of these channels play important roles in setting the RMP. Some of them are also involved in chondrocyte volume regulation [62]. Secondly, as a consequence of altered ion channel expression and function, the RMP values in healthy and inflammatory OA chondrocytes are probably different. The RMP in freshly isolated canine (and also equine, ovine and bovine) chondrocytes was much less negative compared to that of excitable cells [62]; this is of critical importance for the function of voltage-dependent $Ca^{2+}$ channels—most importantly, T-type VDCCs. These channels are small conductance, low-voltage-activated channels [63], which may play a role in sustained $Ca^{2+}$ entry in chondrocytes. It must be noted, however, that chondrocyte RMP values available in the literature may not reflect the actual 'physiological' values because of inappropriately chosen experimental conditions—it is impossible to know if isolated and cultured chondrocytes will have the same RMP as chondrocytes in situ. Thirdly, based on the above, OA chondrocytes may be characterised by altered intracellular calcium homeostasis and linked $Ca^{2+}$-dependent signalling pathways, adding a level of complexity to the picture. Clearly, future studies have to incorporate innovative experimental designs to address these possibilities.

As discussed earlier, our current understanding of VDCCs and the effects of the most widely used inhibitors in chondrocyte biology is limited and controversial, mainly because there are very few papers published in this specific area. It should also be added that studies showing no action of VDCC inhibitors in OA patients may have remained unpublished. To resolve the apparent contradictions, and to better understand the mechanism of VDCC function in different in healthy and OA chondrocytes, new basic and clinical studies should be conducted using physiologically and pharmacologically relevant concentrations of VDCC blockers with clearly defined endpoints, including (a) a systematic and comprehensive analysis of VDCC subunit expression and function in various models, (b) more representative in vitro models to enable a more specific identification of cellular and physiological targets of the inhibitors and (c) sensitive biomarkers that predict responses to the drugs [64•, 65••]. Besides providing a better understanding of chondrocyte biology in health and disease, these prospective studies may also lead to the development of novel approaches for improving articular cartilage bioengineering [66].

Paracelsus, the 'father' of toxicology, wrote: 'All things are poison and nothing (is) without poison; only the dose makes that a thing is no poison'. Clearly, it is the concentration that makes a drug toxic. Therefore, new preclinical and clinical studies to determine the therapeutic vs. deleterious doses of calcium channel blockers such as nifedipine and verapamil are crucially needed.

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**Compliance with Ethics Guidelines**

**Conflict of Interest** Csaba Matta, Róza Zákány and Ali Mobasheri declare no conflicts of interest. The authors wrote this paper within the scope of their academic and affiliated research positions. The authors do not have any commercial relationships that could be construed as biased or inappropriate.

**Human and Animal Rights and Informed Consent** This article does not contain any studies with human or animal subjects performed by any of the authors.

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- •• Of major importance

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