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# Gene regulation by voltage-dependent calcium channels

Maud Barbado , Katell Fablet , Michel Ronjat , Michel De Waard \*

GIN, Grenoble Institut des Neurosciences INSERM : U836, CEA , Université Joseph Fourier - Grenoble I , CHU Grenoble , FR

\* Correspondence should be addressed to: Michel De Waard <michel.dewaard@ujf-grenoble.fr >

## Abstract

**Ca<sup>2+</sup> is the most widely used second messenger system in cell biology and fulfills a plethora of essential cell functions. One of the most exciting findings of the last decades was the involvement of Ca<sup>2+</sup> in the regulation of long-term cell adaptation through its ability to control gene expression. This finding provided a link between cell excitation via cytoplasmic Ca<sup>2+</sup> elevation and gene expression. In this review, we chose to focus on the role of voltage-dependent calcium channels in mediating Ca<sup>2+</sup> entry and regulating gene expression. We evidence the different pathways by which these channels are involved in excitation-transcription coupling, including the most recent Ca<sup>2+</sup> ion-independent strategies that highlight the transcription factor role of calcium channels.**

**MESH Keywords** Animals ; Calcium ; metabolism ; Calcium Channels ; genetics ; metabolism ; Gene Expression Regulation ; Humans ; Models, Biological ; Second Messenger Systems ; physiology ; Transcription Factors ; metabolism

## Introduction

Since calcium has been discovered to act as a second messenger it has been implicated in an ever increasing variety of biological functions (reviewed in [1]). Indeed, calcium regulates common cell processes such as proliferation, protein synthesis, and differentiation, but is also engaged in more specific cell functions like muscle contraction, neurotransmitter release, electrical excitability, and synaptic plasticity [2], thereby revealing its versatility in signaling properties. Moreover, Ca<sup>2+</sup> is not only a trophic factor, but is also involved in programmed cell death [3]. Ca<sup>2+</sup> differs from other second messengers in that, as all the elements of the periodic table, it cannot be metabolized. The second differentiating property is that it is present in relatively high concentration in the extracellular space (1 to 2 mM). Consequently, a strict spatio-temporal control of the intracellular concentration of Ca<sup>2+</sup> is required to allow this second messenger to be involved in a wide variety of cell functions. To achieve this goal, Ca<sup>2+</sup> homeostasis is managed by a wealth of ion channels, localized both at the plasma membrane and intracellular organelles, that permit elevation in cytosolic Ca<sup>2+</sup> concentration, and by Ca<sup>2+</sup>-binding proteins and Ca<sup>2+</sup>-pumps that effectively limit this rise and organize Ca<sup>2+</sup> propagation in the cytosol and in the nucleus [4]. One amazing and unique property of Ca<sup>2+</sup> as a second messenger is its ability to control cellular events that develop on a large time scale, from milliseconds to hours [2]. The millisecond scale gives the full measure of the importance of ion channels in Ca<sup>2+</sup> signaling since channels are the sole pathways that allow a rapid and localized elevation in Ca<sup>2+</sup> concentration. For instance, rapid signaling is at the basis of the control of electrical excitability and neurotransmitter release [5]. Due to the intricate balance between import, sequestration and export pathways, intracellular Ca<sup>2+</sup> elevation is generally transient. Long-term Ca<sup>2+</sup> effects consequently occur by the effective recruitment of various cytosolic and/or nuclear signaling pathways whose life-times are of longer durations than Ca<sup>2+</sup> signals themselves. Such a Ca<sup>2+</sup> signaling integration is required to explain the implication of Ca<sup>2+</sup> in development, cell differentiation and synaptic plasticity. The ability of Ca<sup>2+</sup> to control both short- and long-term processes allows this second messenger to link cell activity to cell differentiation and specialization. As an example, late phase of long-term potentiation in the CA1 region of the hippocampus requires new protein synthesis that is under the control of N-methyl-D-aspartate (NMDA) receptors and voltage-dependent calcium channels [6]. Many long-term effects of Ca<sup>2+</sup> as a second messenger imply a direct or indirect effect of Ca<sup>2+</sup> on gene regulation [7, 8]. Recent development of high-throughput analyses methods has provided a vertiginous list of candidate genes whose expression is under the control of Ca<sup>2+</sup> homeostasis [9, 10]. For instance, in T-lymphocytes from severe-combined immunodeficiency patients, 70% of the 111 gene expression modifications (up- or down-regulation), associated to the pathology, are due to defects in Ca<sup>2+</sup> entry [10]. In neuronal cells, exon expression profiling has revealed the modification of the expression of several thousand transcripts by depolarization-induced Ca<sup>2+</sup> influx [9]. In spite of the impressive number of genes potentially affected by Ca<sup>2+</sup> homeostasis, only a small number of Ca<sup>2+</sup>-dependent pathways and mechanisms of gene regulation have been deciphered in details. This leaves ample room for discovering new signaling pathways and gene targets of Ca<sup>2+</sup> regulation.

## Molecular actors intervening in the control of gene regulation by Ca<sup>2+</sup>

The ability of Ca<sup>2+</sup> to diffuse in the intracellular space is very limited owing to the phenomenal buffering capacity of the cell [11]. While the main Ca<sup>2+</sup> reservoir remains the extracellular space, other essential internal sources for Ca<sup>2+</sup> mobilization have been described and are essential in Ca<sup>2+</sup> signaling and gene regulation [12]. For instance, Ca<sup>2+</sup> released from the sarcoplasmic reticulum was shown to

reduce mRNA levels coding for most nicotinic acetylcholine receptors, thereby controlling muscle fiber differentiation [13]. Although the best described intracellular  $\text{Ca}^{2+}$  sources concerned with gene regulation are the endoplasmic reticulum and the nucleoplasmic reticulum, other sources such as the mitochondria, cytoplasmic secretion granules, and nuclear microvesicles [14] represent putative important factors that will deserve attention in the future. These  $\text{Ca}^{2+}$  stores are spatially distributed in such a way that  $\text{Ca}^{2+}$  can be mobilized in any cell region allowing  $\text{Ca}^{2+}$  signaling pathways to overcome the inherent buffering capacity of the cell. More recently, the existence of  $\text{Ca}^{2+}$  nano- and microdomains has been proposed to represent an important factor of the spatial specialization of  $\text{Ca}^{2+}$  function [5, 15]. These domains, corresponding to the opening of a few  $\text{Ca}^{2+}$  channels, provides high  $\text{Ca}^{2+}$  concentration environments restricted to the proteins associated to these channels. They have been shown to play a role in  $\text{Ca}^{2+}$ -dependent processes, including gene regulation. Therefore, it is obvious that  $\text{Ca}^{2+}$  implication in gene regulation should be as complex as its homeostasis. In particular, regulation of intracellular  $\text{Ca}^{2+}$  elevation implies many key actors that all are potential players in the field of gene regulation (Figure 1). The first type of actors that intervene in  $\text{Ca}^{2+}$  signaling and gene regulation are channels from the plasma membrane that respond to stimuli of various nature (voltage, extracellular ligand, state of intracellular  $\text{Ca}^{2+}$  stores). These  $\text{Ca}^{2+}$  channels comprise voltage-gated  $\text{Ca}^{2+}$  channels (VGCC), receptor-activated  $\text{Ca}^{2+}$  channels, such as the NMDA receptor, and store-operated  $\text{Ca}^{2+}$  channels, such as ORAI. The second type of players is composed of intracellular  $\text{Ca}^{2+}$ -release channels that mobilize  $\text{Ca}^{2+}$  from internal stores, namely ryanodine receptors and  $\text{IP}_3$  receptors. An additional degree of complexity in  $\text{Ca}^{2+}$  signaling resides in the intricate relationship existing between these various types of calcium channels. All these channel types have been involved in gene regulation, each with its own specificities. For instance, over-expression of the transient receptor potential canonical type 6 (TRPC6) calcium channel, localized in excitatory synapses, was shown to promote the formation of these synapses by a calmodulin-dependent kinase IV (CaMKIV)/cAMP response element binding protein (CREB)-dependent pathway [16]. CaMKIV is a  $\text{Ca}^{2+}$ -dependent kinase that phosphorylates and activates the transcription factor CREB. CREB-mediated gene transcription is generally involved in synaptic plasticity and memory ([17] and see [18] for review), and accordingly, transgenic mice over-expressing TRPC6 show improved spatial learning and memory. As another illustration, the activation of nuclear and cytosolic  $\text{Ca}^{2+}$ -signaling via the  $\text{IP}_3$ -receptor in skeletal muscle produces extracellular signal-regulated kinase (ERK) and CREB phosphorylation, thereby regulating gene transcription [19]. This signaling pathway is part of the process of excitation-transcription coupling whereby gene expression is regulated by the electrical activity of motor neurons [20]. In lymphocytes, activation of the  $\text{IP}_3$ -receptor has been shown to induce store-operated  $\text{Ca}^{2+}$  entry through ORAI that in turn activates calcineurin. This phosphatase dephosphorylates nuclear factor of activated T-cells (NFAT), a transcription factor that then translocates in the nucleus to activate gene transcription [21]. Although all these channel types promote  $\text{Ca}^{2+}$  elevation inside cells, it has been shown that specific gene regulation pathways can be associated to the activation of one channel type. More precisely, the spatial distribution [22] and the temporal codification of the resulting  $\text{Ca}^{2+}$  elevation also matters in  $\text{Ca}^{2+}$ -regulated gene transcription [23]. For instance, cytosolic  $\text{Ca}^{2+}$  oscillations have been shown to reduce the effective  $\text{Ca}^{2+}$  threshold for activating pro-inflammatory transcription factors NFAT, Oct/OAP and NF $\kappa$ B [24]. In the same trend, the site of entry of  $\text{Ca}^{2+}$  in hippocampal neurons, NMDA receptors versus voltage-dependent L-type channels, determines the kinetics of CREB phosphorylation and thereby the level of expression of the immediate early gene c-fos [25]. Finally, it was also shown that differences in amplitude and duration of the antigen-stimulated  $\text{Ca}^{2+}$  responses in B lymphocytes translates into distinct patterns of activation of transcription factors such as NFAT, ATF-2 and NF $\kappa$ B [26].

In the remainder of this review, we shall focus on the implication of voltage-gated calcium channels in the control of gene expression. These channels represent a model of choice since they are at the core of the excitation-transcription process by transforming electrical activity in  $\text{Ca}^{2+}$ -signaling information. Since they are present at the plasma membrane, they use several original signaling pathways to vehicle information processing from the periphery of the cell towards the nucleus, the locus of the transcription. Voltage-dependent calcium channels being encoded by many genes, and fulfilling very diverse cell functions, from contraction to transmitter release, the excitation-transcription process in which each calcium channel type is involved may respond to specific functional needs (from contractile proteins to synaptic plasticity).

## **Voltage-dependent calcium channels and gene regulation**

### **Voltage-dependent calcium channels: structure and molecular diversity**

All voltage-dependent calcium channels share one common pore-forming subunit at the structural level that is encoded by 10 genes and give rise to low voltage-activated calcium channels (termed T-type channels, 3 types) and high voltage-activated calcium channels (7 types forming L-type channels (4 types), N-type channel (1 type), P/Q-type channel (1 type) and R-type channel (1 type)) [27]. So far, no auxiliary subunits have been clearly identified for low voltage-activated T-type calcium channels. In contrast, the subunit structure of high voltage-activated calcium channels is much better defined. All these channels possess, in addition to the pore-forming subunit, two associated auxiliary subunits:  $\alpha_2\delta$ , a glycosylated protein with a single transmembrane domain, and  $\beta$ , a regulatory subunit of the MAGUK family. The crystal structure of this latter protein has been resolved and shows that the protein is composed of an SH3 domain associated to a guanylate kinase (GK) domain. In some channel isoforms, a protein called  $\gamma$  subunit has been proposed to form the third auxiliary

subunit of high-voltage activated channels. All these auxiliary subunits play a role in normalizing the biophysical properties of calcium channels and controlling their expression levels at the plasma membrane. Auxiliary subunits are also encoded by several genes (4 for  $\alpha_2\delta$  subunits, 4 for  $\beta$  subunits, and 8 for  $\gamma$  subunits) thereby greatly increasing the phenotype diversity of native calcium channels [28]. Many of these channel types are expressed in neuronal tissues, while other tissues express a poorer subset of calcium channels (one type only in skeletal muscles). In neurons, voltage-dependent calcium channels carry out several functions such as dendritic integration of incoming electrical signals and transmitter release at the synapse. This later function is accomplished mainly by P/Q-, N- and R-type channels. As a result of the importance of voltage-gated calcium channels in many physiological processes, genetic mutations of calcium channel genes induce severe pathologies such as epilepsy, autism, ataxia, migraine, myopathies, deafness and blindness [29]. The link between these genetic mutations and defects in excitation-transcription coupling remains largely unexplored so far.

### **First evidences that voltage-dependent calcium channels are involved in gene regulation**

First evidences of voltage-gated calcium channel implication in gene regulation come from two simultaneous studies published in 1986 [30, 31]. In a first study, chronic depolarization of pheochromocytoma (PC12) cells was shown to induce an increase in c-fos expression levels. This effect was blocked by nisoldipine, an L-type channel inhibitor, as well as by trifluoroperazine or chlorpromazine, two antagonists of calmodulin. It was therefore concluded that cytoplasmic  $\text{Ca}^{2+}$  elevation driven by L-type calcium channels turns on a calmodulin-dependent expression of c-fos [30]. This pathway differed from the earlier-described calmodulin-independent activation of c-fos expression observed after application of nerve growth factor (NGF) in PC12 cells. The second study demonstrated that activation of the nicotinic receptors in PC12 cells induces c-fos expression similarly by the activation of L-type channels; this effect being blocked by verapamil, another L-type channel inhibitor [31]. Since this paradigm also induces the expression of  $\beta$ -actin, but in a depolarization-independent manner, it was concluded that activation of nicotinic receptor produces two  $\text{Ca}^{2+}$ -dependent gene regulation: one indirectly mediated by  $\text{Ca}^{2+}$  entry through L-type channels and another one directly induced by  $\text{Ca}^{2+}$  entry through the nicotinic receptor. This study perfectly illustrates that the entry pathway of  $\text{Ca}^{2+}$  matters with regard to the gene regulation process turned on and consequently the gene being activated. Since these first evidences, different gene activation pathways by voltage-dependent calcium channels have been uncovered that are described hereunder and summarized in Figure 2.

### **Voltage-dependent calcium channels potentially use several gene regulation pathways**

Voltage-dependent calcium channels are increasingly depicted as multi-molecular complexes that regroup not only the channel itself and its auxiliary subunits, but also many key molecular players that translate depolarization-induced  $\text{Ca}^{2+}$  influx and/or conformational changes of the channel into functional events. The excitation-transcription process in which calcium channels are involved does not escape this basic rule. Upon activation of voltage-dependent calcium channels, gene regulation can occur following several conceptually different pathways, that all lead to the activation of transcription factors. These pathways are illustrated hereunder.

- Indirect regulation of transcription factors by channel-distant  $\text{Ca}^{2+}$ -activated proteins. In 1993, it was shown that  $\text{Ca}^{2+}$  entry through L-type calcium channels in hippocampal neurons induce the activation of multifunctional CaMK [32]. This activation was linked to the activation of c-fos promoter. At the same time, it was demonstrated that CaMKI, CaMKII and CaMKIV have all the ability to phosphorylate the transcription factor CREB at Ser133 [33, 34]. While phosphorylation of CREB on Ser133 is generally considered as an activating event, the situation turned out to be more complex by the demonstration that CREB can also be phosphorylated at Ser142 by CaMKII, and that this phosphorylation leads to an inhibitory event [35]. CaMKIV is considered as the most reliable CREB stimulating pathway enzyme since it both allows the recruitment of CREB binding protein (CBP) on CREB. Suppression of nuclear CaMKIV disrupts CREB phosphorylation in hippocampal neurons [36]. These results raised the question on how nuclear CaMK are activated by  $\text{Ca}^{2+}$  entering through L-type channels. All the studies illustrate the importance of nuclear calmodulin in the activation of nuclear CaMK and the ratio between apo-calmodulin and  $\text{Ca}^{2+}$ /calmodulin concentrations. Two modes of nuclear accumulation of  $\text{Ca}^{2+}$ /calmodulin have been demonstrated. On one hand,  $\text{Ca}^{2+}$  from L-type channels activate surrounding calmodulin that translocates and accumulates in the nucleus [37, 38]. Indeed,  $\text{Ca}^{2+}$ /calmodulin possess a diffusion coefficient that is 20-fold superior to the one of apo-calmodulin explaining its preferential nuclear accumulation [37]. However, this nuclear accumulation cannot be explained only by diffusion, but would require a cofactor for its transport and stabilization in its  $\text{Ca}^{2+}$ -bound form. On the other hand, a CREB activation pathway, independent of  $\text{Ca}^{2+}$ /calmodulin translocation to the nucleus, has been highlighted in electrically-stimulated hippocampal neurons [39]. In this study, the authors showed that CREB activation following L-type channel stimulation was still observed in the presence of wheat germ agglutinin, used to block protein transfer through nuclear pores. In this case, CREB activation appears to correlate with increases in nuclear  $\text{Ca}^{2+}$  concentration. Depletion of intracellular  $\text{Ca}^{2+}$  stores with the cyclopiazonic acid or thapsigargin results in a complete block of CREB-mediated gene expression. The mechanism behind this regulation consists in  $\text{Ca}^{2+}$  entry following electrical activation that induces  $\text{Ca}^{2+}$  release from internal stores allowing propagation of the  $\text{Ca}^{2+}$  signal from the periphery of the cell to the nucleus. This study support earlier reports showing that nuclear injection of BAPTA-D70, a  $\text{Ca}^{2+}$  chelator that cannot leave the nucleus, completely inhibits CREB activation following L-type channel activation [33, 40]. While the importance of  $\text{Ca}^{2+}$ -activated kinases in CREB phosphorylation and

CRE-mediated transcription can't be dismissed, evidence is building up for a critical role of phosphatases. The only  $\text{Ca}^{2+}$ -dependent phosphatase is phosphatase 2B or calcineurin.  $\text{Ca}^{2+}$  entry through L-type channels controls inositol 1,4,5-trisphosphate type 1 receptor expression in neurons by the activation of calcineurin [41]. Conceptually, since calcineurin is mostly a cytoplasmic phosphatase, it is assumed that calcineurin may act by dephosphorylating the cytoplasmic form of a transcription factor, allowing then its translocation to the nucleus. In spite of clear evidence for nuclear localization of calcineurin, it was found that it is able to dephosphorylate Ser133 of CREB raising suspicion that it may also act directly in the nucleus [42]. Interestingly, L-type-dependent calcineurin activation has also been involved in the temporal control of CREB phosphorylation through a mechanism that may involve reduced channel activity by L-type dephosphorylation [43]. The dual regulation of nuclear signaling by kinase and phosphatase pathways is a general feature in surface to nucleus pathways [44].

Although L-type channels are the best characterized voltage-dependent calcium channels with regard to gene regulation, some studies also suggest the implication of non-L-type channels in excitation-transcription coupling. By examining NFATc1 nuclear translocation in response to specific patterns of electrical activity in rat sympathetic neurons, it was found that 10 hertz stimulation trains were effective in promoting a  $\text{Ca}^{2+}$ -dependent nuclear translocation of this transcription factor while 1 hertz trains were ineffective [45]. Importantly, this activity-dependent translocation of NFATc1 is strictly dependent on the activation of N-type calcium channels since this effect is blocked by  $\omega$ -conotoxin GVIA, a specific N-type channel inhibitor. In another example, depolarization of superior cervical ganglion neurons induces CREB phosphorylation and an increase in c-fos mRNA and protein levels [46]. This N-type channel-dependence of CREB phosphorylation occurs at low frequency stimulation, while at high stimulation frequency a similar CREB regulation is produced by the exclusive involvement of L-type channels. This selective frequency-dependent implication of various calcium channel types is likely based on difference in inactivation kinetics between N- and L-type channels. Finally, specific activation of N-type calcium channels by electrical stimulation of primary sensory neurons results in tyrosine hydroxylase mRNA expression [47]. This effect is reduced by inhibitors of PKA and PKC, while blockers of MAPK and CaMKII have no effect. For all these studies, it was important to use action potential-like electrical stimulations rather than a KCl-induced depolarization since this latter paradigm tends to produce N-type calcium channel inactivation and masks its implication in excitation-transcription coupling. P/Q-type calcium channels have also been implicated in an important gene regulation pathway. In cerebellar granule cells, blocking depolarization-activated P/Q-type calcium channels with the selective blocker  $\omega$ -agatoxin IVA reduces the expression of syntaxin-1A mRNA to undetectable levels [48]. This effect is specifically linked to P/Q calcium channels since selective blockers of L- and N-type are ineffective at blocking the  $\text{Ca}^{2+}$ -dependent expression of syntaxin-1A. The effect of P/Q calcium channels on syntaxin-1A expression requires intact intracellular  $\text{Ca}^{2+}$  stores and the CaMKII and IV, PKA and MAPK kinase pathways. This regulation is of interest knowing that syntaxin-1A is a critical SNARE protein component associated to P/Q calcium channels and crucial in transmitter release.

- Regulation of transcription factors by  $\text{Ca}^{2+}$ -activated proteins associated to voltage-dependent calcium channels. While this pathway also implies a covalent modification of transcription factors by  $\text{Ca}^{2+}$ -activated phosphatases or kinases, it necessitates the action of regulatory proteins directly interacting with the voltage-dependent calcium channel. One extensively studied example of such a regulation implies the CREB transcription factor family. It was shown that L-type channel activation by depolarization in cortical neurons induces CREB Ser133 phosphorylation by the mitogen-activated protein kinase (MAPK) pathway [49]. This effect is specific to L-type channels since blockers of other calcium channel types (N- and P/Q-types) have no effect on this depolarization-induced activation of CREB.  $\text{Ca}^{2+}$  ions are at the core of CREB activation since  $\text{Ca}^{2+}$  activation of calmodulin bound to the IQ binding site of  $\text{Ca}_v$  1.2 (L-type channel pore-forming subunit) allows the recruitment of the MAP kinase pathway. This result also highlights the importance of  $\text{Ca}^{2+}$  microdomains. Indeed, EGTA that buffers cytoplasmic  $\text{Ca}^{2+}$ , but at a speed insufficient to affect  $\text{Ca}^{2+}$  elevations at the immediate proximity of the mouth of the channel, had no effect on the L-type channel-dependent CREB phosphorylation. In this pathway, both the channel itself and the  $\text{Ca}^{2+}$  permeating through the channel are required for CREB phosphorylation. A mutation of the IQ domain of the channel, that prevents calmodulin binding to  $\text{Ca}_v$  1.2, inhibits depolarization-induced activation of CREB-dependent genes. This implies that free calmodulin, not bound to the channel, is unable to induce CREB phosphorylation. Activation of MAPK is indirect since the activity of the kinase is  $\text{Ca}^{2+}$ -independent and involves activation of Ras, a small G protein, followed by MEK1 phosphorylation by a MEK kinase. In turn, activated MEK1 phosphorylates and activates MAPK [50]. Activated MAPK translocate to the nucleus where they phosphorylate CREB. The prevailing view is that CREB is already in the nucleus, bound to CREs within the promoter of CREB-regulated genes. Phosphorylation of Ser133 favors the recruitment of CREB binding protein (CBP), a transcriptional coactivator. The function of CBP in histone acetylation and further recruitment of the RNA polymerase II transcription machinery permits chromatin remodeling and transcription.

In another example, it was shown by using a green fluorescence protein encoding plasmid, whose promoter is under the control of NFATc4, that depolarization of neurons induces activation of NFATc4 [51]. This process is inhibited by blockers of L-type channels while those of N- and P/Q-type channels have no effect. The authors show that activation of NFATc4 relies on activation of calcineurin, a  $\text{Ca}^{2+}$ -calmodulin-dependent phosphatase that by dephosphorylating NFATc4 allows its nuclear activation [52]. Oliveria and collaborators have

recently shown that anchoring of calcineurin to  $\text{Ca}_v$  1.2 via A-kinase anchoring proteins AKAP79/150 is required for activation of the NFATc4-dependent gene regulation pathway [53]. This group demonstrates that RNAi specific of AKAP150 inhibits NFATc4 nuclear translocation induced by depolarization suggesting that calcineurin-dependent NFATc4 dephosphorylation is inhibited. These two examples demonstrate that voltage-dependent calcium channels can be involved in gene regulation by  $\text{Ca}^{2+}$ -sensing elements that are directly bound to the calcium channel itself. These data indicate that one route of signaling, downstream of  $\text{Ca}^{2+}$  elevation, originates in the close molecular vicinity of the channel, therefore reinforcing the importance of strictly restricted local  $\text{Ca}^{2+}$  increases (nono/microdomains).

- Voltage-gated calcium channels and link with  $\text{Ca}^{2+}$ -regulated transcription factors. In the former studies discussed above, transcription factor activity was under the dependency of  $\text{Ca}^{2+}$ -binding proteins such as calmodulin. Here, we illustrate the case of transcription factors that have the ability to directly bind  $\text{Ca}^{2+}$ , providing a more direct link between the intracellular  $\text{Ca}^{2+}$  elevation and the regulation of transcription. As above, the same questioning is raised with regard to the origin of this  $\text{Ca}^{2+}$  elevation, nuclear versus cytoplasmic. By looking for a protein that has the ability to bind to the downstream regulatory element (DRE) of the prodynorphin gene, a 29 kDa DRE antagonist modulator (DREAM) was identified that has the unexpected feature to possess four EF-hands [54, 55]. DREAM binds to DRE as a tetramer and acts as repressor. Binding of  $\text{Ca}^{2+}$  to DREAM promotes its dissociation from DRE, possibly by favoring a conformational transition from a tetrameric state to a dimeric state [56]. In this scheme,  $\text{Ca}^{2+}$  relieves transcriptional repression of DRE-containing promoters. Other DRE-regulated genes have been identified, including c-fos and gonadotropin-releasing hormone (GnRH) [54, 57]. Using immortalized GT1-7 GnRH neurons, it was shown that nimodipine, a L-type channel blocker, prevents GnRH expression and release. Real-time monitoring of the action of DREAM on a luciferase reporter under the control of the GnRH promoter indicates that nimodipine blocks luciferase expression [57]. These data demonstrate the link between  $\text{Ca}^{2+}$  entry through L-type channels and DREAM activation. Similar observations are made when DREAM is immunologically neutralized with an antibody. DREAM possess sequence identity with calsenilin, a presenilin-2 interacting protein, and KChIP-3 (for potassium channel interacting protein 3), which interacts with the amino-terminus of  $\text{K}_v$  4. Three other members of this family have been identified since then (KChIP-1, -2 and -4) whose DNA-binding activity has not yet been established (for review see [58]). These data indicate that KChIP proteins have pleiotropic functions in cells, both in the cytoplasm and in the nucleus.

- Voltage-dependent calcium channel domains that act as transcription factors. Since the pioneering studies on calcium channel implication in gene regulation, a novel concept has emerged centered around the idea that calcium channel domains themselves may act as transcription factors. The very first evidence that this might be the case came from a study of Hibino and collaborators [59]. In this work, the authors demonstrate that a short particular splice variant of  $\beta_4$ , termed  $\beta_{4c}$ , interacts with the chromobox protein 2/heterochromatin protein 1 $\gamma$  (CHCB2/HP1 $\gamma$ ), a nuclear protein involved in gene silencing and transcriptional regulation [60]. The interaction between  $\beta_{4c}$  and CHCB2/HP1 $\gamma$  is required for nuclear translocation of  $\beta_{4c}$ .  $\beta$  subunits are normally auxiliary subunits of voltage-gated calcium channels but this short isoform lacks most of the guanylate kinase domain of  $\beta$  subunits and should therefore not associate with the pore-forming subunit of voltage-gated calcium channels [61]. Nevertheless, the authors report a mild channel regulation by this subunit suggesting a form of loose connection between  $\beta_{4c}$  and the channel. CHCB2/HP1 $\gamma$  recognizes and binds a lysine residue near the carboxy-terminus of histone H3 that is specifically methylated by SUV39H1. Binding of SUV39H1 and CHCB2/HP1 $\gamma$  onto histone H3 are at the origin of a specialized higher order chromatin state that defines heterochromatin and represses gene activity (for review see [62]). Using a GAL4-CAT reporter system, the authors show that CHCB2/HP1 $\gamma$  inhibits CAT expression, and that  $\beta_{4c}$  decreases the gene-silencing activity of CHCB2/HP1 $\gamma$ . The mechanism of this regulation remains unexplained but highlights for the first time the direct implication of calcium channel sequences in the regulation of gene expression.

Another study, in the same vein of this earlier study, showed that a proteolytic fragment of the C-terminus of the pore-forming subunit of L-type channels ( $\text{Ca}_v$  1.2) encodes a transcription factor [63]. This domain was called calcium channel associated transcriptional regulator (CCAT). Using antibodies directed towards the C-terminus of  $\text{Ca}_v$  1.2, the authors show the presence of CCAT in the nucleus of neurons from developing and adult brains. The export of CCAT out of the nucleus is activated by increase in intracellular  $\text{Ca}^{2+}$  resulting from L-type calcium channel opening. CCAT contains a nuclear retention domain, while nuclear localization signals are absent. CCAT was found associated with p54(nrb)/NonO, a transcriptional regulator of, among others, the retinoic acid and thyroid hormone receptors. Using oligonucleotide microarray, they identified several genes that are down- or up-regulated by CCAT. Among these genes, a gene coding for gap junction protein connexin Cx31.1 is up-regulated as shown by the effect of CCAT on the Cx31.1 promoter in a luciferase reporter. The question remains on the identity of the protease responsible for CCAT production in neurons and how it is regulated and connected to L-type channel activity. Alternatively, CCAT could be produced by alternative splicing of the  $\text{Ca}_v$  1.2 gene, a question that would be worth investigating.

### **Role of voltage-dependent calcium channels in post-transcriptional regulation**

Up to now, we focused on the role of voltage-dependent calcium channels on gene transcription. However,  $\text{Ca}^{2+}$  is also involved at a post-transcriptional stage by influencing mRNA splicing and stability. Depleting  $\text{Ca}^{2+}$  stores with thapsigargin in human IMR32 neuroblastoma cells produces alterations in the relative abundance of no less than 3489 different transcripts, as assessed by high density exon-centric microarray [9]. If depolarization is used instead of thapsigargin treatment, then it is no less than 1505 transcripts that vary in copy number. These data illustrate the crucial role of  $\text{Ca}^{2+}$  in gene expression. However, if one looks at the effect of thapsigargin or KCl treatment on alternative splicing, then 8533 (thapsigargin) or 5139 (KCl) transcripts appear to carry a modified number of copies for one or more exons. Increase in cytoplasmic calcium concentration thus results in two things: one is to alter the abundance of a given transcript (transcriptional regulation and/or mRNA stability), and second is to favor alternative splicing resulting in the appearance of new transcripts (post-transcriptional regulation). Interestingly,  $\text{Ca}^{2+}$ -binding proteins, plasma membrane proteins and ion transporters are part of the transcripts that are up-regulated by elevated intracellular  $\text{Ca}^{2+}$  concentration conditions indicating that  $\text{Ca}^{2+}$  itself controls the expression of proteins involved in  $\text{Ca}^{2+}$  homeostasis. In another more specific study on IMR32 cells, a transcript variant encoding a new form of the plasma membrane  $\text{Ca}^{2+}$  ATPase PMCA appears only following depolarization with KCl [64]. In the same trend, chronic KCl depolarization of cortical neurons modulates the splicing of neurexin 2 $\alpha$  [65]. This effect necessitates calcium channel activity, as witnessed by its blockade by  $\text{CdCl}_2$ , and selectively produces exclusion of exon 11 of neurexin 2 $\alpha$ . Other studies have been conducted on the mechanisms of the regulation of alternative splicing by  $\text{Ca}^{2+}$ . Exon 21 of the NMDA receptor type 1 gene encodes a protein sequence that controls several functions of the receptor, among which its targeting at the plasma membrane, and its splicing varies during development [66]. Depolarization of P19 embryonal carcinoma cells results in exclusion of exon 21 from the transcript. This effect is dependent on the activation of CaMKIV and requires two CaRRE (CaMKIV-Responsive RNA Element) motifs of exon 21 [67].

Regarding mRNA stability issues, it was shown that the stability of the long-form mRNA coding for  $\text{Ca}_v2.2$ , the pore-forming subunit of voltage-dependent calcium channels, is increased by KCl depolarization induced by L-type channel activation [68]. This effect results in increased N-type channel density at the plasma membrane of sympathetic neurons. This feedback process, whereby the activity of one calcium channel type controls the expression level of another calcium channel type at the post-transcriptional level, should be taken into account when working with calcium channel knock-out or transgenic mice.

### **Altered involvement in gene regulation by pathological mutations of voltage-dependent calcium channels**

Genes coding for calcium channel subunits are subject to mutations that result in various pathologies [69, 70]. Researches on channelopathies generally focus on biophysical defects of the calcium channels, misfolding and/or mistargeting issues, and more rarely on possibly associated gene expression deregulation [71]. However, considering the implications of voltage-dependent calcium channels in gene expression processes, one link between calcium channel mutations and pathologies could reside at the gene transcription or post-transcription regulation level. Few preliminary evidence illustrate that the pathological phenotype associated to voltage-dependent calcium channel gene mutations results from alterations of gene expression. The rolling mouse Nagoya, an ataxic mutant mouse with severe ataxic gait, carrying a mutation of the gene coding for  $\text{Ca}_v2.1$  of P/Q calcium channels, show altered mRNA and protein expression levels of ryanodine receptor type 1 and 3 [72]. mRNA levels of ryanodine receptor type 1 also are altered in tottering mice that carry another type of mutation of  $\text{Ca}_v2.1$  [73]. Considering the role of voltage-dependent calcium channels in gene regulation, transcriptomic alterations are likely to be at the basis of an ever growing list of channelopathies.

### **Perspectives**

In this review, we have evidenced the link between voltage-gated calcium channels and gene expression regulation, both at the transcriptional and post-transcriptional levels. Several schemes of regulation have been developed that link without ambiguity voltage-dependent calcium channel activity to gene expression. Other pathways link  $\text{Ca}^{2+}$  ions to gene regulation, but there are still missing connections made to voltage-gated calcium channels. Indeed, several transcription factors are known to be regulated by  $\text{Ca}^{2+}$ , directly or indirectly, or to be part of the calcium channel signaling complexes. In particular, a specific class of transcription factors, termed basic helix-loop-helix (bHLH) proteins have the ability to be regulated by  $\text{Ca}^{2+}$ -calmodulin and S100 proteins, other EF-hand containing  $\text{Ca}^{2+}$ -binding proteins. Seven classes of bHLH transcription factors have been described, some acting as transcription activators (e.g. class I), others playing the role of repressors (e.g. class IV and VI) [74]. bHLH proteins bind to DNA onto a consensus E-box sequence, CANNTG, through their basic DNA-binding domain and following the formation of dimers or higher oligomerization states.  $\text{Ca}^{2+}$ -calmodulin may regulate bHLH transcription factors according to one of the following mechanisms: i) by preventing class I and II bHLH binding to DNA, ii) by preventing the oligomerization of bHLH required for their DNA-binding activity [58]. Since several  $\text{Ca}^{2+}$ -calmodulin-dependent gene regulation pathways rely on  $\text{Ca}^{2+}$  entry through voltage-dependent calcium channels, it would be interesting to investigate the possible link between  $\text{Ca}^{2+}$ -calmodulin regulation of bHLH factors and voltage-gated calcium channel activity.

In another series of observations, CASK, a member of membrane-associated guanylate kinase family, has been shown to interact with Tbr-1, a T-box transcription factor [75], implicated in cerebellar development. When associated to Tbr-1, CASK redistributes to the nucleus and is present in a T element complex that binds to a specific T-box DNA sequence. Interestingly, CASK was, at the same time, found to interact with the C-terminal domain of  $Ca_v 2.2$ , the pore-forming subunit of N-type calcium channels, but not with the homologous domain of L- or R-type calcium channels [76]. CASK association to one particular isoform of  $Ca_v 2.2$  (that differs at the C-terminus) directs the channel towards presynaptic nerve terminals in hippocampal neurons, suggesting that this protein plays a channel targeting role in addition to its independent function as transcription factor [77]. The importance of this CASK/channel complex in excitation-transcription coupling, and specifically from the synapse to the nucleus, is still not established but would merit further investigation.

While regulation of transcription factors by channel activity has been widely illustrated, a completely new signaling pathway linking transcription factors to ion channels has recently been discovered. Caraveo and collaborators showed that TFII-I, a ubiquitously expressed transcription factor present in both the cytoplasm and the nucleus, regulates the agonist-induced  $Ca^{2+}$ -entry through TRPC3 calcium channels [78]. Down-expression of TFII-I by siRNA results in an increase of agonist-induced  $Ca^{2+}$  entry. This effect of TFII-I is maintained when wild-type TFII-I is replaced by a nucleus localization-deficient TFII-I mutant indicating a gene transcription-independent regulation of the TRPC3 activity by TFII-I. The authors identified phospholipase  $C\gamma$  as a specific partner of TFII-I. Phospholipase  $C\gamma$  was shown to play a role in the agonist-induced plasma membrane incorporation of the novel TRPC3 channels [78], a process that would be modulated by its association with TFII-I.

This review illustrates the amazing interplay existing between calcium channels,  $Ca^{2+}$  ions,  $Ca^{2+}$ -binding proteins and transcription factors during the processing of signal informations between the plasma membrane, sometimes as remotely as the synapse, and the nucleus. The presence of some transcription factors directly in the calcium channel molecular complexes indicates the incredible level of local integration in information processing. Conversely, the idea that channel fragments may directly act as transcription factors implies a simplification of the signaling scheme behind excitation-transcription coupling. We bet that novel conceptually interesting pathways linking calcium channels to gene transcription will soon emerge to further enrich our understanding of this fascinating research world.

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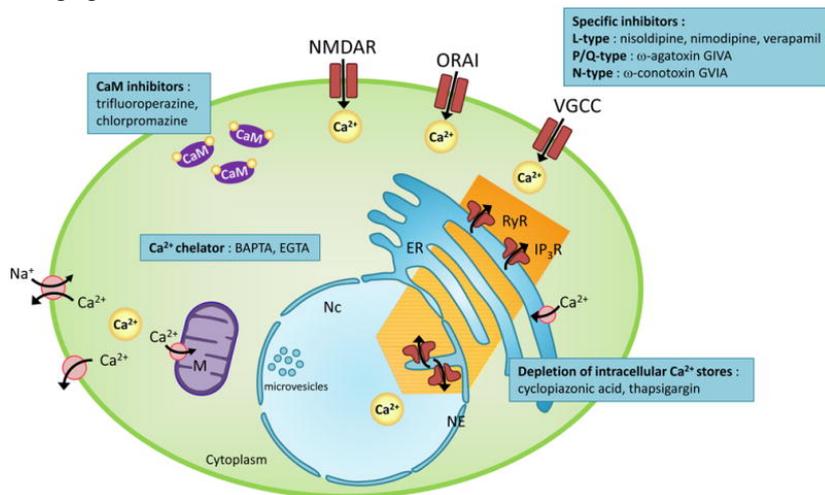
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### Figure 1

Schematic representation of cytoplasmic and nuclear  $\text{Ca}^{2+}$  elevation pathways. Inhibitors of  $\text{Ca}^{2+}$  elevation pathways or  $\text{Ca}^{2+}$ -binding proteins are shown. Abbreviations are: CaM, calmodulin; ER, endoplasmic reticulum;  $\text{IP}_3$  R,  $\text{IP}_3$  receptors; M, mitochondria; Nc, nucleoplasm; NE, nuclear envelope; NMDAR, NMDA receptors; RyR, ryanodine receptors; SOC, store-operated channels; VGCC, voltage-gated calcium channels.



**Figure 2**

Schematic gene transcription pathways regulated by voltage-gated calcium channels. **1.** Activation of transcription factors by  $\text{Ca}^{2+}$ -binding proteins. The example of CREB phosphorylation by CaMKIV following activation of calmodulin by  $\text{Ca}^{2+}$  entry is shown. Calmodulin activation can occur consecutively to  $\text{Ca}^{2+}$  entry through voltage-gated calcium channels, or after an amplification cascade through  $\text{IP}_3$  R- or RyR-sensitive internal stores. **2.** Regulation of transcription factors by  $\text{Ca}^{2+}$ -activated proteins that are part of the calcium channel signaling complex. Two examples are illustrated: MAPK regulation by calmodulin bound on the IQ domain of L-type channels, and calmodulin-activated calcineurin (CaN) regulation of cytoplasmic NFAT. Whether CaN dissociates from the channel to activate NFAT or whether NFAT is in the immediate vicinity of the channel remains unknown. **3.** Regulation of  $\text{Ca}^{2+}$ -binding transcription factors, such as DREAM, by voltage-gated  $\text{Ca}^{2+}$  channels. Whether the  $\text{Ca}^{2+}$  that enters voltage-dependent channels activates directly DREAM by propagating to the nucleus, or whether it is relayed and amplified by  $\text{Ca}^{2+}$  stores as depicted here is not established. **4.** Calcium channel fragments or subunits acting as transcription factors. Two examples are detailed.  $\beta_{4c}$  is a short splice variant of  $\beta_4$  subunit whose association with voltage-dependent calcium channels is challenged by the lack of well-defined guanylate kinase domain required for its association with the pore-forming subunit. It binds onto HP1 $\gamma$  and inhibits its gene silencing function. CCAT is a transcription factor directly derived from the carboxy-terminus of the pore-forming subunit of L-type channels. It translocates to the nucleus where it regulates gene activity. Its export out of the nucleus is controlled by nuclear  $\text{Ca}^{2+}$ . Again, whether internal  $\text{Ca}^{2+}$  stores relay  $\text{Ca}^{2+}$  entry through voltage-dependent channels is not yet established.

