CALCIUM-DEPENDENT INACTIVATION OF NEURONAL CALCIUM CHANNELS

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Calcium ions are ubiquitous intracellular mediators of numerous cellular processes. One of the main mechanisms of Ca^{2+} entry into the cell involves the opening of Ca^{2+} channels in the plasma membrane. To effectively control Ca^{2+} signalling, Ca^{2+} channels inactivate rapidly by a mechanism that depends on an elevation of intracellular Ca^{2+} within tens of nanometres of the channel pore. A structural understanding of this mechanism will provide a framework for understanding the regulation of Ca^{2+} entry and accumulation in neurons. Recent physiological, biochemical and molecular studies have yielded new insights into the regulation of neuronal Ca^{2+} channels.

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doi:10.1038/nrn959

Voltage-gated Ca^{2+} channels (VGCCs) are a large family of integral membrane proteins that control the selective flow of Ca^{2+} ions down their electrochemical gradient in response to changes in membrane potential (BOX 1; TABLE 1). Ca^{2+} ions control processes as diverse as cell proliferation, neuronal development and transmitter release[1,2]. All of these functions have to be accomplished within a narrow range of Ca^{2+} concentrations, as this cation can be toxic if its level is not tightly controlled[3]. So, effective mechanisms, such as pumps and exchangers, remove Ca^{2+} ions from the cytoplasm, restoring the resting concentration once these ions have fulfilled their signalling role. But in addition to these pumps and exchangers, several mechanisms of channel inactivation have appeared during evolution to control Ca^{2+} entry in the face of ongoing electrical activity. So, Ca^{2+} channels can inactivate by three different mechanisms: Ca^{2+}-dependent inactivation (CDI), fast voltage-dependent inactivation (VDI) and slow VDI.

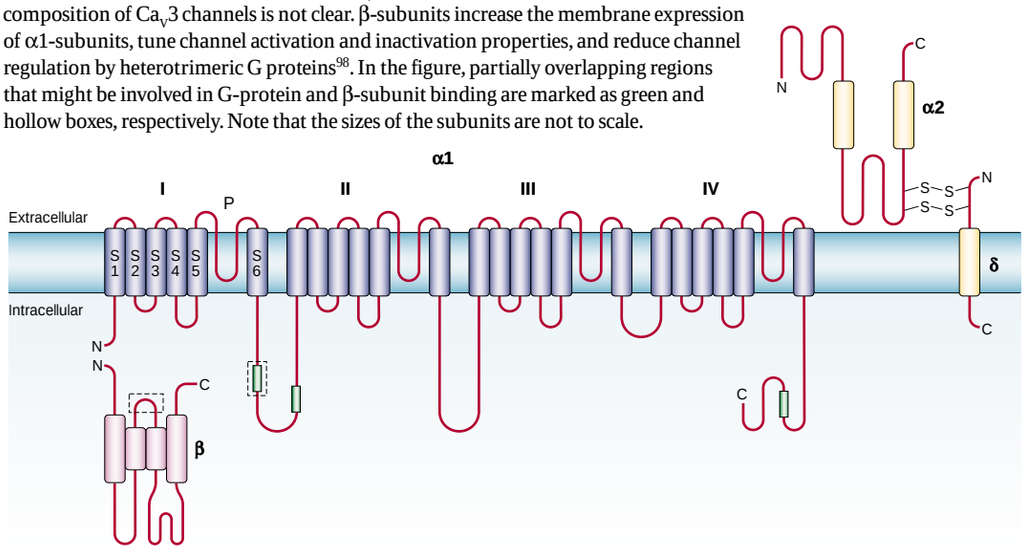
In the case of CDI, Ca^{2+} ions restrict their own entry into the cell by one of the main routes of Ca^{2+} influx, the VGCCs (FIG. 1a). This CDI provides crucial negative feedback in numerous neuronal and non-neuronal settings. The mechanisms that underlie this feedback inhibition have been under investigation for more than 20 years[4,5], but it was only recently that calmodulin (CaM) was identified as an important Ca^{2+} sensor that mediates CDI[6–9]. Here, we review the various mechanisms that have been proposed to mediate CDI, including the involvement of the cytoskeleton, the role of phosphorylation–dephosphorylation processes, and the participation of Ca^{2+}-binding proteins. We then summarize the functional significance of CDI. We conclude by proposing an integrative model of this phenomenon, and suggest some future approaches to the study of CDI. For a detailed discussion of the mechanisms that underlie VDI (BOX 2), we refer the interested reader to REFS 10,11.

Electrophysiological hallmarks of CDI

Brehm and Eckert[4] were the first to study the effect of the local Ca^{2+} chemistry on the function of VGCCs, and they used several criteria to identify CDI. Their experiments in *Paramecium* showed that inactivation was faster in solutions that contained Ca^{2+} than in solutions containing Ba^{2+}. Similarly, they found that Ca^{2+} buffers reduced inactivation, and that the inactivation curve obtained with a double-pulse protocol was U-shaped (see below). On the basis of these findings, they proposed the existence of a Ca^{2+}-dependent mechanism for the inactivation of *Paramecium* Ca^{2+} channels. Similar results were obtained subsequently in molluscan neurons[5,12] and in other ciliates[13].

Box 1 | Molecular composition of VGCCs

Members of the voltage-gated Ca^{2+} channel (VGCC) Ca_V1 and Ca_V2 families consist of a pore-forming α1-subunit, which has four domains (I–IV), each containing six transmembrane segments (S1–S6) and a re-entrant P-loop. The α1-subunit is associated with an intracellular β-subunit and with an α2-subunit, which is entirely extracellular and is linked to the membrane by disulphide bonding to a transmembrane δ-subunit[97]. In some channels, the complex is completed by a γ-subunit (not shown). Members of the Ca_V3 family might contain only a single α1-subunit; however, the exact subunit composition of Ca_V3 channels is not clear. β-subunits increase the membrane expression of α1-subunits, tune channel activation and inactivation properties, and reduce channel regulation by heterotrimeric G proteins[98]. In the figure, partially overlapping regions that might be involved in G-protein and β-subunit binding are marked as green and hollow boxes, respectively. Note that the sizes of the subunits are not to scale.



So, what are the identifying criteria for CDI? To detect its occurrence, several hallmarks of CDI can be probed. First, CDI tends to be fast (FIG. 1b). Channel inactivation is evidenced as a decay of current during prolonged depolarization. The current amplitude at the end of a depolarizing test pulse is divided by the peak current amplitude to define an inactivation ratio, providing a measure of the degree of inactivation. L-type Ca^{2+} channels — the prototypical molecular species that shows CDI (TABLE 1) — are usually subject to a fast CDI and a slower VDI[14].

Second, CDI normally results in a U-shaped inactivation curve (FIG. 1d). The approach of choice to measure this inactivation curve is a double-pulse protocol[4,5] (FIG. 1b), in which a conditioning voltage step to varying potentials is followed by a brief gap and a test pulse to a fixed voltage. The voltage of the test pulse is set to evoke maximal current amplitude, and serves to reveal the number of channels that can be activated. Conditioning steps to very positive or negative voltages evoke minimal Ca^{2+} currents, and yield near maximal Ca^{2+} currents on the test pulse (see FIG. 1 for further details)[15]. By contrast, a conditioning step to the maximum of Ca^{2+} current activation evokes maximal Ca^{2+} entry, and results in a minimal current on the test pulse. This reduction occurs as a result of CDI, and increases in the extracellular Ca^{2+} concentration yield faster inactivation[16]. Consequently,

Table 1 | Classification and inactivation profiles of VGCCs

Native current	α1-Subunit subtypes	Inhibitors	Activation profile	Inactivation profile*	Ca^{2+}-dependent inactivation
P/Q-type‡	α_{1A} ($Ca_V2.1$)	ω-Agatoxin IVA	High voltage	Moderate/slow	Yes/no§
N-type	α_{1B} ($Ca_V2.2$)	ω-Conotoxin GVIA SNX-111	High voltage	Fast	Yes/no‖
L-type	α_{1C} ($Ca_V1.2$) α_{1D} ($Ca_V1.3$) α_{1F} ($Ca_V1.4$) α_{1S} ($Ca_V1.1$)	Dihydropyridines Phenylalkylamines Benzothiazepines	High voltage	Moderate/slow	Yes
R-type	α_{1E} ($Ca_V2.3$)	SNX-482	High voltage	Fast/moderate	No
T-type	α_{1G} ($Ca_V3.1$) α_{1H} ($Ca_V3.2$) α_{1I} ($Ca_V3.3$)	Ethosuximide Mibefradil Kurtoxin	Low voltage	Very fast	No

*The inactivation of specific voltage-gated Ca^{2+} channels (VGCCs) might differ between expression systems and native cells. Furthermore, there might even be differences between neuronal cell types. ‡A single α1-subunit underlies both P- and Q-type Ca^{2+} currents. Q-type Ca^{2+} current can be generated by several independent mechanisms (β-subunits, alternative splicing, post-translational modifications). So, the categorization of P/Q-type currents should be treated with caution. §In thalamocortical relay neurons, P-type currents are not controlled by Ca^{2+}-dependent inactivation. ‖Differing results have been reported.

GH_3 CELLS
A neural cell line that is derived from the rat anterior pituitary gland.

the amplitude of the current evoked by the test pulse typically shows a U-shaped dependency on the conditioning-pulse potential in current–voltage (*I–V*) plots (FIG. 1d), and the maximal rate of inactivation occurs near the peak of the *I–V* relationship. However, it is important to note that a U-shaped inactivation curve might result from voltage-dependent processes[17–20], and so does not necessarily indicate CDI.

Another hallmark of CDI is the effect of Ba^{2+} as charge carrier. As Ba^{2+} is typically less effective than Ca^{2+} at inactivating the channels, the use of Ba^{2+} as the principal charge carrier results in a reduction in the rate of inactivation and a decrease in the inactivation ratio, and to changes in the U-shape of the inactivation curve (FIG. 1c,e). However, it is important to note that the inactivation curve for Ba^{2+} currents can be U-shaped — Ba^{2+} can bind to the inactivation site of L-type Ca^{2+} channels, giving rise to Ba^{2+}-dependent inactivation[21].

CDI can be retarded by increasing the Ca^{2+}-buffering capacity of the cytoplasm through the introduction of exogenous buffers[4]. Importantly, the effect of exogenous Ca^{2+} buffers and the type of buffer that can interfere with CDI provide information about the distance between the source of Ca^{2+} and the Ca^{2+}-binding site, and in turn, about the mechanism of CDI. A weak effect of exogenous chelators has been interpreted as evidence for Ca^{2+} acting very close to the channel protein[22], whereas a clear reduction of CDI by exogenous chelators has been taken to indicate a more distant site of Ca^{2+} action[23–27]. Furthermore, different Ca^{2+} buffers have different capabilities to buffer Ca^{2+} close to the channel. In this respect, BAPTA is believed to buffer Ca^{2+} effectively within a distance as small as 30 nm from the mouth of the inner channel [28]. In the case of EGTA, a substantial buffering effect is observed only 100 nm or more from the channel protein owing to its slower rate of Ca^{2+} binding.

Last, in addition to macroscopic currents, single-channel recordings can also be used to confirm the occurrence of CDI. When compared with Ba^{2+} currents, unitary L-type Ca^{2+} currents are smaller in amplitude and the unitary channel openings less frequent, with only rare openings after several minutes[29,30].

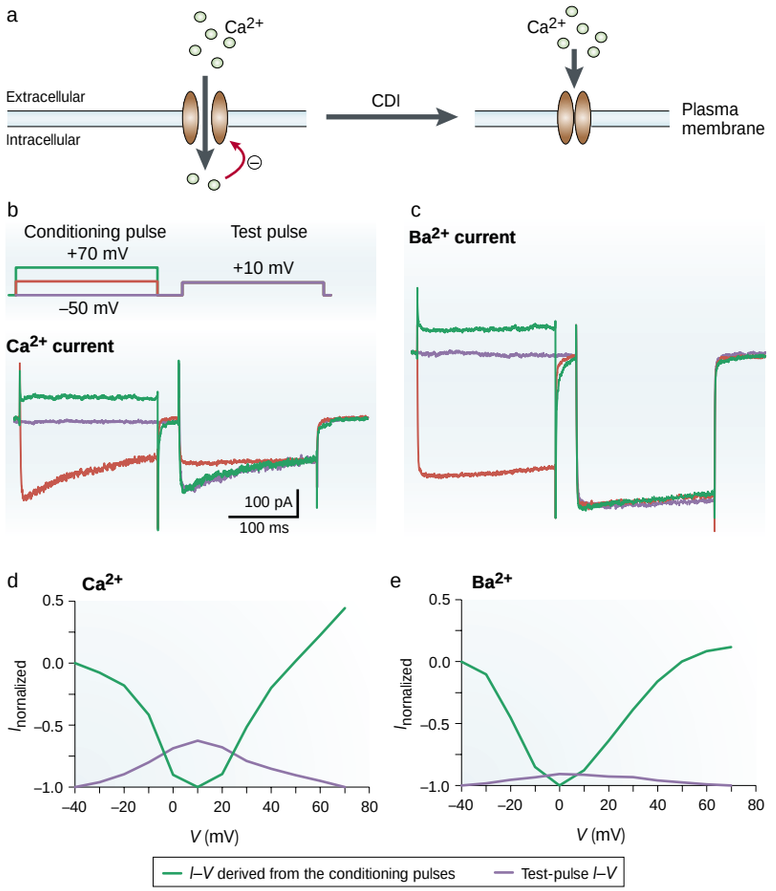


Figure 1 | **Electrophysiological hallmarks of the CDI of VGCCs. a** | Simplified scheme of Ca^{2+}-dependent inactivation (CDI), illustrating the autoinhibitory effect of Ca^{2+} entry through voltage-gated Ca^{2+} channels (VGCCs). **b**–**e** | Whole-cell Ca^{2+} (**b**) and Ba^{2+} (**c**) currents evoked by double-pulse protocols in thalamocortical relay neurons. In this protocol, a conditioning voltage step to varying potentials is followed, after a brief gap, by a test pulse to a fixed voltage. The voltage of the test pulse is set at a value to evoke maximal current amplitude. A conditioning step to a value close to or positive to the Ca^{2+} reversal potential yields a near-maximal Ca^{2+} current amplitude during the test pulse (**b**, green trace). A similar maximal test current amplitude can be obtained from a negative conditioning potential of –50 mV, during which nearly all Ca^{2+} channels are closed (**b**, blue trace). By comparison, a conditioning step that elicits maximal Ca^{2+} current activation results in a decrease in current amplitude on the test pulse (**b**, red trace) owing to CDI. Consequently, the test current amplitude typically shows a U-shaped dependency on the conditioning-pulse potential in current–voltage (*I–V*) plots (**d**, blue line). With Ba^{2+} as the charge carrier, the time-dependent current decay (**b**) and the U-shaped dependency of the test current amplitude on the conditioning-pulse potential (**d**, blue line) are strongly reduced (**c**,**e**). In the experiments shown in **d** and **e**, current was normalized for the maximal inward current (negative sign). Owing to this normalization procedure, the inactivation curve reveals an inverted U-shape with Ca^{2+} as the charge carrier, but not with Ba^{2+}.

Inactivation profiles of VGCC subtypes

The kinetic properties of current inactivation differ significantly between different Ca^{2+} channel subtypes (TABLE 1). The α1-subunit, which forms the channel pore, can be directly inactivated (BOX 1), whereas the β-subunits have an important modulatory role in the inactivation process[31,32]. CDI has been shown to occur, at least partially, in the case of several VGCC α1-subunits (FIG. 2; TABLE 1).

It is generally accepted that L-type Ca^{2+} channels in cardiac and smooth muscle cells inactivate primarily by rapid CDI. The α1-subunits that underlie neuronal L-type Ca^{2+} currents ($Ca_V1.2$ and $Ca_V1.3$) are widely distributed in the nervous system[33,34]. It therefore comes as no surprise that the CDI of L-type Ca^{2+} channels occurs in several neuronal cell types, including dorsal root ganglion (DRG) neurons, thalamic neurons and neural cell cultures (for example, GH_3 CELLS)[35,36]. Furthermore, investigations of L-type Ca^{2+} channels have led to the identification of CaM as the Ca^{2+} sensor of $Ca_V1.2$ Ca^{2+} channels (see below).

Akin to $Ca_V1.2$ channels, the α1-subunit of $Ca_V2.1$ channels, which underlie neuronal P/Q-type Ca^{2+} currents, is subject to feedback regulation by Ca^{2+}/CaM[6]. So, neuronal P/Q-type Ca^{2+} currents also show CDI in native cells and in heterologous expression systems[35,37].

EF HAND
A Ca^{2+}-binding domain, originally identified in parvalbumin, that is also known as the helix–loop–helix domain. The loop can accommodate Ca^{2+} by coordination through several amino acids in a pentagonal pyramid.

IQ MOTIF
A small structural domain that mediates interactions with calmodulin. The motif only loosely defines the amino-acid sequence at 5 of 11 possible residues. Different IQ domains bind calmodulin at varying intracellular Ca^{2+} concentrations or independently of Ca^{2+}.

IFM MOTIF
A cluster of three hydrophobic amino acids located within the inactivation loop between domains III and IV that is required for fast Na^+ channel inactivation.

Box 2 | Voltage-dependent inactivation of VGCCs

As for Na^+ and K^+ channels, the voltage dependency of inactivation of voltage-gated Ca^{2+} channels (VGCCs) is based mainly on the voltage dependency of activation, because inactivation depends primarily on the state of the channel, rather than on voltage directly. This is evident from the fact that, for T-type Ca^{2+} channels or for Ba^{2+} currents through L-type Ca^{2+} channels, inactivation usually increases monotonically with depolarization, saturating at voltages where the channels are fully activated. The fast inactivation of Na^+ and K^+ channels occurs predominantly through the physical occlusion of the pore by cytoplasmic gating particles[99]. K^+ channels have an amino-terminal ball (the inactivation ball) that is connected through a flexible linker of more than 30 amino acids (the chain) to the rest of the channel protein. By binding to an acceptor site on the α-subunit, the ball is supposedly capable of physically plugging the pore during channel inactivation (the 'ball and chain' model of K^+ channel inactivation). For Na^+ channels, a similar model has been proposed that involves the IFM MOTIF as the inactivation particle (the 'hinged lid' model of Na^+ channel inactivation). For VGCCs, the molecular determinants of voltage-dependent inactivation (VDI) are more widely spread over the channel protein and are still not fully understood. Different parts of the channel, including pore-forming transmembrane segments (particularly the S6 regions), intracellular loops, intracellular domain linkers and the carboxyl terminus, seem to be involved in VDI. Structural concepts involving a distortion of the four pore-forming S6 segments, and the intracellular linker between domains I and II as an inactivation particle, have been considered[10,11]. Although these are molecular correlates of fast VDI, VGCCs show a slow inactivation state that they might enter from the open or fast-inactivated state. The molecular determinants of slow VDI are not known. Both fast and slow VDI are modulated by intracellular β-subunits.

CDI has also been described for N-type Ca^{2+} currents in DRG neurons and GH_3 cells[36,38–40]. However, the CDI of N-type Ca^{2+} channels is still a matter of debate. Although the unusual VDI properties of N-type Ca^{2+} channels are sufficient to explain the occurrence of a U-shaped inactivation curve[17,19], a CDI mechanism has been suggested to occur in a BAPTA-inaccessible space in the pore[40].

Other types of VGCC typically show no CDI. In the case of the Ca_V3 family (TABLE 1), this is corroborated by the lack of Ca^{2+}-binding and CaM-binding domains — EF HANDS and IQ MOTIFS, respectively (see below).

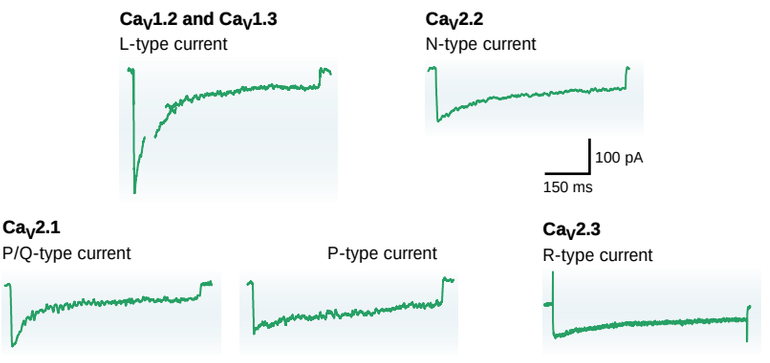


Figure 2 | **Inactivation profile of VGCC subtypes in central neurons.** Native Ca^{2+} currents through voltage-gated Ca^{2+} channels (VGCCs) recorded from thalamocortical relay neurons and isolated through the use of specific blocking agents. Currents were elicited under voltage-clamp conditions by an 800 ms depolarization to +10 mV from a holding potential of –50 mV. Note the unique inactivation profile of each component. Only L-type and Q-type Ca^{2+} currents can be described by a two-exponential function, with the early fast phase of current decay governed by Ca^{2+}-dependent inactivation (CDI)[35]. A single thalamocortical relay neuron can express all high-voltage activated (HVA) Ca^{2+} channel subtypes. Although the exact cellular localization of different α1-subunits is not yet known, thalamocortical relay neurons seem to fit into a general scheme of HVA Ca^{2+} channel expression: most channel subtypes are expressed by a given cell type but are localized in different cell regions to serve different functions[2]. It should be noted here that accessory proteins might markedly affect the kinetic signature of a particular α1-subunit. So, in thalamocortical relay neurons, kinetic properties were used as only one of several criteria to link CDI to different VGCC subtypes[35].

Mechanisms of CDI

The endoplasmic reticulum. In cardiac muscle cells, there is a close functional coupling between L-type Ca^{2+} channels in the plasma membrane and intracellular Ca^{2+}-release channels of the ryanodine receptor (RyR) type in the membrane of the sarcoplasmic reticulum (SR)[41]. This highly localized interaction leads to the release of Ca^{2+} from intracellular Ca^{2+} stores in a process known as Ca^{2+}-induced Ca^{2+} release (CICR)[42], which is followed by cellular contraction. Because of this close coupling, it is not surprising that L-type Ca^{2+} channels are inactivated by Ca^{2+} release from the SR[41] (FIG. 3). Furthermore, it is possible to distinguish two phases in the CDI of cardiac Ca^{2+} channels: an early fast phase that depends on Ca^{2+} released from the SR, and a late slow phase that depends on Ca^{2+} flow through VGCCs[43]. In neurons, there is also a close functional coupling between L-type Ca^{2+} channels and RyRs[44]. Although a contribution of CICR to CDI has not been tested in neurons as yet, CICR has important functional roles in these cells[45]. So, CICR might be at least partially involved in the CDI of neuronal Ca^{2+} channels.

Phosphorylation and dephosphorylation. An early breakthrough in our understanding of the mechanisms of CDI was the finding that promoting the dephosphorylation of VGCCs in molluscan neurons caused a significant increase in the rate of VGCC inactivation. These findings led to the hypothesis of an enzymatic mechanism of CDI[24]. Subsequent studies in mammalian neurons led to the proposal of an antagonistic regulation of VGCC phosphorylation by cyclic-AMP-dependent protein kinase (PKA) and the protein phosphatase calcineurin (PP2B). This antagonistic regulation is now considered to be a general principle of CDI in invertebrates and higher vertebrates[30,46].

Calcineurin is an abundant protein phosphatase that is directly activated by Ca^{2+} and CaM (BOX 3). Protein phosphatase 1 (PP1) is another Ca^{2+}-regulated enzyme, but this regulation occurs only indirectly, through calcineurin (FIG. 3). In the original model of the enzymatic mechanism of CDI in mammalian neurons[46], calcineurin

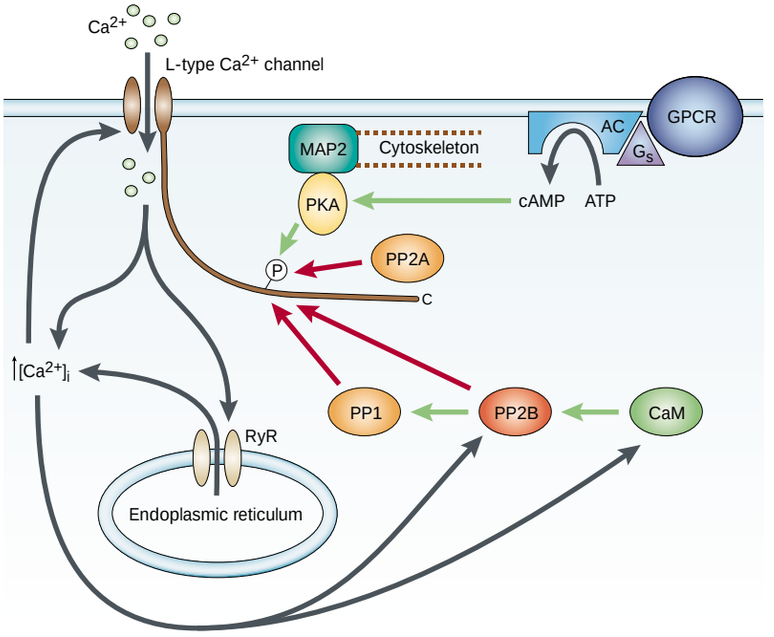


Figure 3 | **Regulation of L-type Ca^{2+} channels by Ca^{2+} and phosphorylation–dephosphorylation reactions.** Ca^{2+} entering the cell through an L-type Ca^{2+} channel might induce Ca^{2+}-dependent inactivation (CDI) by interfering directly with the channel complex. As shown for cardiac L-type channels, the Ca^{2+} responsible for inducing CDI might be released from the endoplasmic reticulum (ER) through ryanodine receptors (RyRs) in a process called Ca^{2+}-induced Ca^{2+} release (CICR)[43]. In addition, CDI might be induced by dephosphorylation of the channel at Ser1928 (REFS 47,48). This reaction is mediated by the protein phosphatase calcineurin (PP2B)[46], an enzyme that is activated by Ca^{2+} and calmodulin (CaM)[100]. The effect of calcineurin might be exerted by protein phosphatase 1 (PP1), which is disinhibited by calcineurin[100]. In mammalian neurons, $Ca_V1.2$ channels are dephosphorylated by PP2A, which is closely associated with the channel[62]. To effectively control channel activity by phosphorylation–dephosphorylation, the counteracting cyclic-AMP-dependent protein kinase (PKA) is also closely targeted to the channel through microtubule-associated protein 2 (MAP2)[50]. As has been shown in thalamocortical relay neurons, the phosphorylation of L-type Ca^{2+} channels by PKA blocks CDI[53]. PKA might be stimulated through different G-protein-coupled receptor (GPCR) cascades. For example, the stimulation of β-adrenoceptors leads to G_S-mediated activation of adenylyl cyclase (AC), increased cAMP production and, finally, stimulation of PKA. In hippocampal pyramidal cells, the β_2-adrenoceptor, G_S, AC, $Ca_V1.2$, PKA and PP2A form a heteromeric signalling complex[89]. Together with further components, such as CaM, cytoskeletal proteins, calcineurin and PP1, this complex might be required for the expression of CDI. Black arrows indicate the movement of Ca^{2+} and cellular metabolites. Green and red arrows indicate activation and inhibition, respectively. It should be noted here that the figure combines results obtained from cardiac cells (CDI induction by CICR) and neuronal cells (the β_2-adrenoceptor pathway in hippocampal pyramidal cells). It is therefore a matter of speculation at present whether all of these events take place in neurons. $[Ca^{2+}]_i$, intracellular concentration of Ca^{2+}.

was proposed to stimulate PP1 activity by the dephosphorylation of an endogenous inhibitor — inhibitor 2 — and the removal of the phosphorylation-dependent inhibition of PP1 activity. As a consequence, PP1 was thought to dephosphorylate L-type Ca^{2+} channels, thereby inducing CDI[46]. In addition, phosphodiesterase and L-type Ca^{2+} channels are substrates of calcineurin and PKA, increasing the complexity of Ca^{2+} channel regulation by Ca^{2+} and cAMP[46].

In recent years, some aspects of this general scheme have been confirmed, whereas others remain controversial. In addition, novel aspects of the regulation of VGCCs by phosphorylation have emerged. So, evidence for the direct phosphorylation of neuronal L-type Ca^{2+} channels by PKA became available only after it was realized that the full-length form of $Ca_V1.2$ could be proteolytically truncated at its carboxyl terminus by the Ca^{2+}-dependent protease calpain[47,48]. Only the long form of $Ca_V1.2$ is effectively and stoichiometrically phosphorylated at Ser1928 by PKA (FIG. 3). Furthermore, in mammalian central neurons, microtubule-associated protein 2B (MAP2B) is associated with $Ca_V1.2$ channels, directly targeting PKA to a VGCC type that is governed by CDI[50] (FIG. 3). MAP2 is a cytoskeletal molecule that is expressed predominantly in neurons, and participates in the stabilization of microtubules and the anchoring of signalling enzymes[49,51]. MAP2 co-purifies and interacts directly with PKA[52]. The biochemical and molecular identification of VGCCs closely complexed with PKA has been corroborated by electrophysiological findings that show a clear reduction of CDI after stimulation of the PKA pathway in different neural cell types[30,53].

Whereas the modulation of CDI by PKA has received much experimental support, the involvement of calcineurin is still a matter of debate. Although there is evidence for the co-localization of calcineurin and VGCCs at the plasma membrane in neurons[54], electrophysiological evidence for and against the calcineurin hypothesis of CDI has been found in different neuronal cell types[36,38,53,55–57]. In this respect, we should mention that assessment of the role of calcineurin in CDI in native cells might be complicated by at least two factors[58]. First, the selectivity of several of the frequently used phosphatase inhibitors is crucial, because a given compound might act on several phosphatase subtypes and, in addition, their effects on other proteins have to be taken into account. Second, calcineurin could exert its physiological effect through PP1.

Although the involvement of calcineurin in CDI remains unclear, other phosphatases, such as PP2A, have come into view. The serine/threonine phosphatases PP1 and PP2A have been shown to regulate cardiac L-type Ca^{2+} channels[59–61]. Furthermore, PP2A directly binds to $Ca_V1.2$ channels and reverses the phosphorylation of Ser1928 in the rat brain[62] (FIG. 3). Indeed, the use of PP1/PP2A blockers in neurons results in a clear decrease in total Ca^{2+} current inactivation[53].

The overall conclusion from these findings is that dephosphorylation seems to be a potent mediator of CDI in neurons of the mammalian nervous system.

The cytoskeleton. The original calcineurin model pointed to a site of Ca^{2+} action that is not part of the channel protein. Indeed, the finding that cytoskeletal stabilizers strongly reduce CDI in neurons indicated that another site of Ca^{2+} binding that is distant from the site of Ca^{2+} entry (that is, an EGTA-sensitive site) might exist. Several studies in neurons from different species have shown that the CDI of VGCCs is sensitive to the state of the cytoskeleton[20,26,27,63]. However, the mechanism of CDI modulation by the cytoskeleton is unclear. Cytoskeletal integrity is one possible mechanism by which Ca^{2+} ions could act on the channel, as both microtubule and microfilament components of the

Box 3 | Ca^{2+}-binding proteins

Calcineurin, or protein phosphatase 2B (PP2B), is a Ca^{2+}-dependent protein phosphatase that is regulated by calmodulin (CaM) and accounts for up to 1% of total mammalian brain protein[100]. The protein is a heterodimer of one catalytic (CnA) and one regulatory (CnB) subunit. Although CnA has high homology with other protein phosphatases, the CnB subunit belongs to the EF-hand-containing Ca^{2+}-binding proteins. The binding of Ca^{2+} to CnB is relatively ineffective at stimulating phosphatase activity. With CaM also bound to the enzyme, phosphatase activity is maximal, indicating high cooperativity.

CaM is a small soluble protein of 17 kDa that is highly conserved throughout evolution. It accounts for about 0.5% of brain protein, and about half of the total amount of CaM is associated with membranes. CaM functions as a monomer with two pairs of EF hands. So, CaM can bind a maximum of four Ca^{2+} ions. The amino-terminal (EF hands 1 and 2) and the carboxy-terminal (EF hands 3 and 4) pairs of EF hands form two separate lobes, and the two lobes are connected by an eight-turn α-helix, giving the whole CaM protein the appearance of a dumb-bell (FIGS 4 and 5). Many unrelated ion channels (ligand-gated channels, Ca^{2+}-activated K^+ channels, Trp (transient receptor potential) channels and intracellular Ca^{2+}-release channels) have been found to be directly associated with CaM, leading to the idea of CaM as a channel subunit [101]. In all cases, the mechanisms of channel modulation by CaM are different.

High-affinity Ca^{2+}-binding proteins (CBPs), such as parvalbumin, calretinin and calbindin-D_{28k}, contain two or more EF-hand-type Ca^{2+}-binding sites, and have been suggested to be important in rapidly buffering Ca^{2+} at the site of Ca^{2+} entry[1]. In addition, CBPs have been considered as Ca^{2+} transporters and/or CaM-like transducers of Ca^{2+} signals to target proteins[102]. Furthermore, the removal of Ca^{2+}-dependent inactivation (CDI) by exogenous Ca^{2+} buffers indicates a possible role of such endogenous binding proteins in this type of feedback control. Indeed, the inclusion of calbindin-D_{28k} in the internal recording solution during WHOLE-CELL PATCH-CLAMP RECORDINGS effectively disrupts CDI in hippocampal granule cells and thalamocortical relay cells[92,103]. Although the exact concentration of exogenous CBPs at their site of action is not known, it seems that the protein reaches sufficiently high levels around the point of Ca^{2+} entry. The available data are also consistent with a close physical association between CBPs and voltage-gated Ca^{2+} channels, as has been shown for other EF-hand proteins, such as CaM and sorcin[104].

cytoskeleton are disrupted by increases in intracellular Ca^{2+} levels[64]. In hippocampal neurons, the microtubule stabilizer taxol and the microfilament stabilizer phalloidin were found to reduce CDI[27,63]. A possible interpretation of these results is that Ca^{2+}-dependent destabilization of cytoskeletal elements that have a structural relationship with the channel leads to CDI. More generally, a model has been proposed in which microtubules stabilize an actin lattice that is contiguous with the plasma membrane[27]. This microfilament lattice is thought to contribute to VGCC localization and could bind directly to the channel or to a closely associated protein. One site of contact of VGCCs with microtubulin in the mammalian brain is the PKA–MAP2 complex[50].

VGCC β-subunits are further elements with which the cytoskeleton can interact. A structural model showed that the $\beta 1_b$-subunit contains three distinct domains that have been shown to participate in protein–protein interactions[65]. These domains included a PDZ DOMAIN, an SH3 DOMAIN and a guanylyl kinase domain. These domains give the $\beta 1_b$-subunit the molecular signature of membrane-associated guanylyl kinase (MAGUK) proteins, which are involved in membrane-protein anchoring[66,67]. So, the targeting of VGCCs to the cytoskeleton through MAP2 and β-subunits might be important for cytoskeleton-dependent CDI and the incorporation of these channels into large CDI-related protein assemblies (see below). It is interesting to note that the downregulation of NMDA (*N*-methyl-D-aspartate) receptor channels by the Ca^{2+}-dependent depolymerization of the channels from actin filaments has also been found in hippocampal neurons, and has been proposed as an important mechanism of ion channel arrest during hypoxia in vertebrate neurons[68,69].

From these observations, it is tempting to conclude that the model of CDI driven by destabilization of the cytoskeleton might be relevant to both invertebrate and mammalian neurons.

The search for a Ca^{2+} sensor. Dephosphorylation and second-messenger production are slow processes that might modulate CDI on a timescale that is not fast enough to constitute the primary inactivation mechanism[70]. So, CDI mechanisms that are associated with the direct binding of Ca^{2+} to the inner surface of the membrane have been explored to explain the fast time course of this process in electrophysiological recordings. As a result, several models that involve the direct binding of Ca^{2+} to the channels have been proposed[70–72]. These include 'domain' models, in which the rapid filling of hemispherical regions (domains) of elevated Ca^{2+} levels only underneath the inner mouth of open channels results in the inactivation of only these channels. By contrast, in 'shell' models, slow filling of a continuous compartment under the membrane (a shell) results in the inactivation of all membrane Ca^{2+} channels. Experimental evidence for both models has been found[22,25,35,73]. In particular, in hippocampal CA1 pyramidal neurons and smooth muscle cells, CDI is preserved in the presence of intracellular BAPTA, indicating a domain model with Ca^{2+} binding at or closely associated with the channel[22,73]. Binding of a single Ca^{2+} ion to an open channel might indeed induce CDI[72]. This suggestion is supported by the fact that the CDI of $Ca_V1.2$ channels is an intrinsic property of the α1-subunit[74], and by the existence of a Ca^{2+}-induced shift of L-type Ca^{2+} channel gating to a mode of low open probability, leaving the direct binding of Ca^{2+} to the channel as a likely chemical initiation event of inactivation[75].

Despite these findings, the nature of the Ca^{2+} sensor of Ca^{2+} channels has only recently been uncovered. In 1995, it was shown that crucial determinants of CDI are located in the proximal third of the carboxyl terminus of the $Ca_V1.2$ channel[76]. Replacing this region of $Ca_V1.2$ with the homologous region of the non-inactivating $Ca_V2.3$ channel abolished CDI, whereas transfer of the $Ca_V1.2$ region to the $Ca_V2.3$ backbone resulted in CDI. So, this region was termed the Ca^{2+}-inactivation (CI) region (see FIG. 5). This short stretch contains a consensus Ca^{2+}-binding motif (an EF hand), and it was proposed that this motif is the Ca^{2+} sensor for CDI. However, it was found that substitution of only the distal two-thirds of the $Ca_V1.2$ carboxyl terminus, which do not contain the EF hand, into $Ca_V2.3$ was sufficient to produce CDI[77]. Experiments in which systematic deletions were made in the $Ca_V1.2$ carboxyl terminus identified two groups of amino acids that were essential

PDZ DOMAIN
A peptide-binding domain that is important for the organization of membrane proteins, particularly at cell–cell junctions, including synapses. It can bind to the carboxyl termini of proteins or can form dimers with other PDZ domains. PDZ domains are named after the proteins in which these sequence motifs were originally identified (PSD95, Discs large, zona occludens 1).

SH3 DOMAIN
Src-homology domains are involved in interactions with phosphorylated tyrosine residues on other proteins (SH2 domains) or with proline-rich sections of other proteins (SH3 domains).

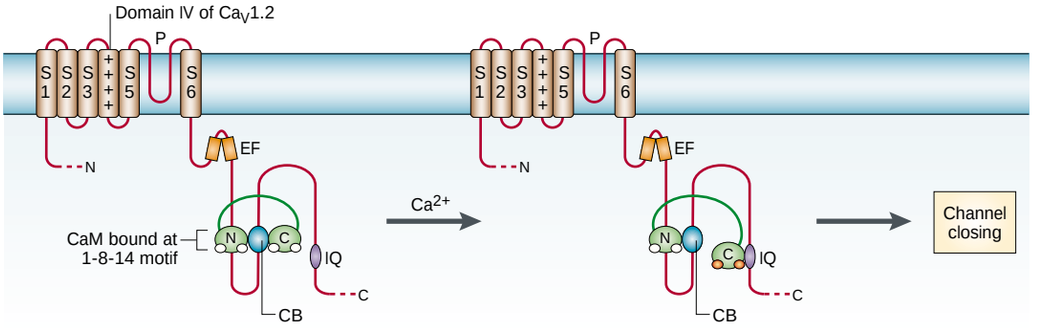


Figure 4 | **Model of CDI induction by CaM in $Ca_V1.2$ Ca^{2+} channels.** Ca^{2+}-dependent inactivation (CDI) depends on the interaction of calmodulin (CaM) with the Ca^{2+} channel. The left panel shows domain IV and the proximal part of the carboxyl terminus of the $Ca_V1.2$ protein. The relative positions of the EF hands (orange boxes) and the IQ motif (purple ellipse) are indicated. The EF hands of CaM are indicated as white circles. Their occupation by Ca^{2+} is indicated by red circles. The amino-terminal lobe of CaM masks the 1-8-14 motif. At resting Ca^{2+} levels (left panel), the amino-terminal lobe of CaM is tethered to the 1-8-14-binding motif, whereas both the amino- and the carboxy-terminal lobes are tethered to the CB region (turquoise). Following the influx of Ca^{2+} (right panel), the Ca^{2+}-binding sites of the carboxy-terminal lobe of CaM become occupied, and the carboxy-terminal lobe binds the IQ motif. Subsequently, processes that lead to channel closure are induced. In this model, the IQ motif is not thought to contribute to CaM tethering at low Ca^{2+} concentrations. P, P-loop.

for CDI. These were located 61 and 103 residues carboxy-terminal of the EF hand, respectively[78]. The second region resembles the consensus pattern for an IQ-type CaM-binding motif[79], raising the possibility that CaM is involved in CDI. Indeed, in 1999, several groups showed that CaM is the dominant Ca^{2+} sensor for CDI in $Ca_V1.2$ and $Ca_V2.1$ channels in heterologous expression systems[6–9]. Furthermore, rather than serving as a Ca^{2+} sensor for CDI, the EF-hand region might be essential for the transduction of Ca^{2+}/CaM binding into inactivation[80].

In conclusion, it is evident that the carboxy-terminal region of VGCCs is of fundamental importance for CDI. Ca^{2+} could bind to the EF-hand motif or to other Ca^{2+}-binding sites of the protein that are, so far, unrecognized. However, it has been suggested that the EF hand contributes to CDI by transducing signals from Ca^{2+}/CaM-binding domains rather than from direct Ca^{2+} binding[81].

Ca^{2+}/CaM-dependent inactivation and facilitation. Ca^{2+} and CaM can both inactivate and facilitate the operation of VGCCs. In contrast to inactivation, facilitation is a subsequent increase in current amplitude after the first channel opening. Experiments in HEK293 (human embryonic kidney) cells using mutant CaM with a selective impairment of Ca^{2+} binding in either the amino- (CaM_{12}) or the carboxy-terminal (CaM_{34}) lobes, each of which harbours two EF hands (BOX 3), revealed lobe-specific effects on CDI[7]. Whereas the expression of mutant CaM_{12} does not affect the CDI of $Ca_V1.2$ channels, CaM_{34} abolishes it. Moreover, the binding of CaM to $Ca_V1.2$ channels and the modulation of this binding by Ca^{2+} seem to be highly complex, and differing results of pre-association between CaM and VGCCs have been reported. Ca^{2+}-insensitive forms of CaM (CaM_{1234}) exert a DOMINANT NEGATIVE effect on endogenous CaM, thereby preventing CDI[7]. On the basis of this observation, it was concluded that Ca^{2+}-free CaM is tethered to the channel protein. Indeed, Ca^{2+}-independent pre-association of CaM with $Ca_V1.2$, $Ca_V2.1$ and, surprisingly, $Ca_V2.3$ channels could be revealed using FLUORESCENCE RESONANCE ENERGY TRANSFER[82]. So, it seems that the pre-association of CaM with VGCCs is coordinated by several segments of the carboxy-terminal region and requires resting concentrations of Ca^{2+}. Short amino-acid segments that are located amino-terminal of the IQ domain have been found to interact with CaM in cardiac myocytes and heterologous expression systems at low levels of Ca^{2+} (less than 100 nM) — levels that approximate to those of resting neurons[83–85]. One of these regions — the 26-amino-acid CB DOMAIN — is located 20 amino acids amino-terminal of the IQ motif[85] (FIG. 4). The CB sequence contains the amino acids that have previously been shown to be necessary for CDI[78,86]. Surprisingly, in protein–protein-interaction studies, a fragment of the carboxyl terminus of the $Ca_V1.2$ channel that includes the IQ domain (and the CB sequence) binds CaM even when the IQ key sequence has been changed completely to alanines[8]. It was later found that CB binds CaM, although with a lower affinity than the IQ motif[85]. However, although IQ peptides from high-voltage activated (HVA) Ca^{2+} channels bind to CaM, only the CB peptide from $Ca_V1.2$ binds to CaM at low Ca^{2+} concentrations. A further region upstream of CB that conforms to a 1-8-14-TYPE A CALMODULIN-BINDING MOTIF[79] can bind CaM and even CaM_{1234} (REF. 83). A model of the CDI of $Ca_V1.2$ channels that is based on CaM switching between binding sites (FIG. 4) could account for several findings in the literature[83]. At rest, with low intracellular Ca^{2+} levels (~50 nM), the 1-8-14 binding region constitutively anchors the amino-terminal lobe of CaM, whereas the CB domain is probably associated with both the amino- and the carboxy-terminal lobes of the same CaM. When the channel opens, Ca^{2+} reaches CaM and binds to the carboxy-terminal lobe. The Ca^{2+}-bound carboxy-terminal lobe then binds to the IQ domain and effects inactivation.

WHOLE-CELL PATCH-CLAMP RECORDING
A high-resolution electrophysiological recording technique in which a very small electrode tip is sealed onto a patch of cell membrane and, with suction, the membrane patch is ruptured to allow low-resistance electrical access to the cell interior. Electrical currents flowing across the cell membrane can then be recorded, although the ion composition of the cell interior is altered to that of the electrode-filling solution.

DOMINANT NEGATIVE
Describes a mutant molecule that can form a heteromeric complex with the normal molecule, knocking out the activity of the entire complex.

FLUORESCENCE RESONANCE ENERGY TRANSFER
(FRET). A spectroscopic technique that is based on the transfer of energy from the excited state of a donor moiety to an acceptor. The transfer efficiency depends on the distance between the donor and the acceptor. FRET is often used to estimate distances between macromolecular sites in the 20–100-Å range, or to study interactions between macromolecules *in vivo*.

CB DOMAIN
A carboxy-terminal 26-amino-acid sequence found in L-type Ca^{2+} channels that binds calmodulin at low concentrations of intracellular Ca^{2+}.

1-8-14 MOTIF
A consensus calmodulin-binding motif that is found, for example, in calcineurin (type A) and fodrin (type B).

The neuronal $Ca_V2.1$ channel also shows CDI, although its magnitude is smaller than that of $Ca_V1.2$ channels (FIG. 2; TABLE 1). In addition, these channels can show Ca^{2+}-dependent facilitation (CDF). Like $Ca_V1.2$ channels, $Ca_V2.1$ channels have more than one CaM-binding site (FIG. 5). One is an IQ-like motif, whereas a second sequence does not fall into the known categories of CaM-binding domains, but strongly resembles a region of the Ca^{2+}/CaM-stimulated adenylyl cyclase type 8 (REF. 79). The latter region (termed CBD; FIG. 5) is necessary for CDI as well as for CDF, because both processes are removed on deletion of this sequence[6,37]. Interestingly, when $Ca_V2.1$ channels are examined using intermediate Ca^{2+}-buffering strength, inactivation is lost whereas facilitation remains. Moreover, CaM_{1234} is dominant negative when co-expressed with $Ca_V2.1$, leading to the loss of CDI and CDF[87]. CaM_{12} and CaM_{34} have completely selective effects: CaM_{12} eliminates CDI, whereas CaM_{34} eliminates CDF. It was recently shown that both CDI and CDF of $Ca_V1.2$ channels can be abolished by mutating five key amino acids in the IQ domain[88].

Although the effect of CaM_{12} on the CDI of $Ca_V2.1$ seems at odds with that on $Ca_V1.2$, these findings indicate that the two lobes can somehow bifurcate the same local Ca^{2+} signal, offering custom-made mechanisms for the initiation of separate functional tasks. Furthermore, it becomes obvious from the bifurcation model that channel-bound CaM has different actions when CaM is differentially loaded with Ca^{2+}.

Most electrophysiological data on VGCC–CaM interactions have been obtained from heterologous expression systems. However, owing to the widespread distribution of VGCCs in the brain and the high structural homology of L-type Ca^{2+} channel isoforms, it is likely that CaM acts as the voltage sensor for CDI in mammalian neurons. In this respect, it is interesting to note that $Ca_V1.2$ channels have recently been found to form large protein complexes that might be relevant to CDI by adding to a CDI-related protein assembly. It has been shown that the β_2-adrenoceptor is directly associated with one of its effectors, the $Ca_V1.2$ channel (FIG. 3). This protein complex also contained the whole signalling cascade, including G proteins, adenylyl cyclase, PKA and the counteracting PP2A[89].

This signalling complex, in close association with other components, such as CaM, calcineurin and the cytoskeleton, might constitute a large functional multi-component protein assembly that is relevant for CDI in neurons.

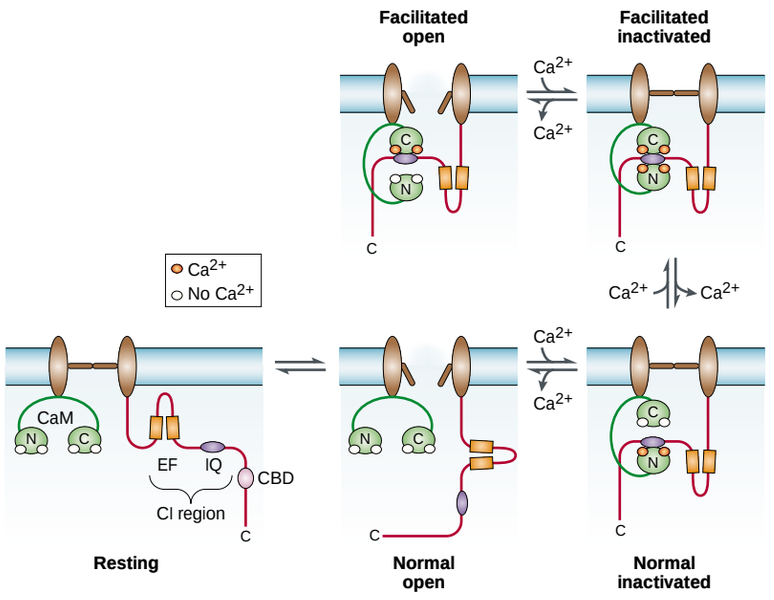


Figure 5 | **Model of CDI and CDF induction by CaM in $Ca_V2.1$ Ca^{2+} channels.** Calmodulin (CaM) contributes to Ca^{2+}-dependent inactivation (CDI) and facilitation (CDF). The $Ca_V2.1$ channel protein and its carboxyl terminus are illustrated schematically. The relative positions of the EF hands (orange boxes) and the IQ motif (purple ellipse) are shown. $Ca_V2.1$ channels contain a second CaM-binding domain (CBD; pink) that resembles a region of Ca^{2+}/CaM-stimulated adenylyl cyclase type 8. As the exact role of the CBD is not understood, it is only shown for the resting state. CaM is pre-associated with the resting channel at a site that provides preferential access to the IQ region. Following the influx of Ca^{2+}, Ca^{2+} binding to the carboxy-terminal lobe of CaM allows its interaction with the IQ site, and induces CDF of the channel. Ca^{2+} binding to the amino-terminal lobe of CaM results in CDI. In this model, the carboxy-terminal lobe might respond preferentially to the fast local changes in Ca^{2+} concentration that are attributable to local Ca^{2+} channel activity. The amino-terminal lobe might detect slower changes in Ca^{2+} concentration that are attributable to cellular Ca^{2+} signalling processes. CI region, Ca^{2+}-inactivation region. Adapted, with permission, from REF. 87 © 2001 Macmillan Magazines Ltd.

The functional significance of CDI

Ca^{2+} entry through presynaptic VGCCs links membrane depolarization to the exocytosis of synaptic vesicles at the nerve terminal. The amount of neurotransmitter or neuropeptide that is released is steeply dependent on presynaptic Ca^{2+} concentrations, such that increases or decreases in Ca^{2+} influx can powerfully alter neurotransmission and hormone release[90]. At many central and peripheral synapses, P/Q-type Ca^{2+} channels mediate transmission[2]. Ca^{2+} channel inactivation in neurohypophysial nerve terminals varies significantly, depending on stimulation frequency and duration[55]. Inactivation increases with the frequency of train pulses (which mimic physiological bursts of action potentials), and intracellular BAPTA abolishes this frequency dependency. As vasopressin and oxytocin have optimal stimulation frequencies for being released, CDI might contribute to the integration of different aspects of complex electrical stimuli — including burst frequency, duration and interburst interval — to determine the amount of hormone release. Furthermore, recent evidence indicates that the CDI and CDF of P/Q-type Ca^{2+} channels in synaptic transmission could contribute to activity-dependent synaptic plasticity[37]. In this way, a combination of the effects of voltage and Ca^{2+} on Ca^{2+} channel inactivation could be associated with the regulation of neurotransmitter and neuropeptide release. However, the particular relevance of CDI to neurotransmission is far from understood. In particular, it is not known to what extent the molecular machinery that underlies CDI is present in presynaptic compartments.

Patients who suffer from chronic temporal lobe epilepsy with Ammon's horn sclerosis, the predominant form of epilepsy in adults, offer a glimpse of the potential

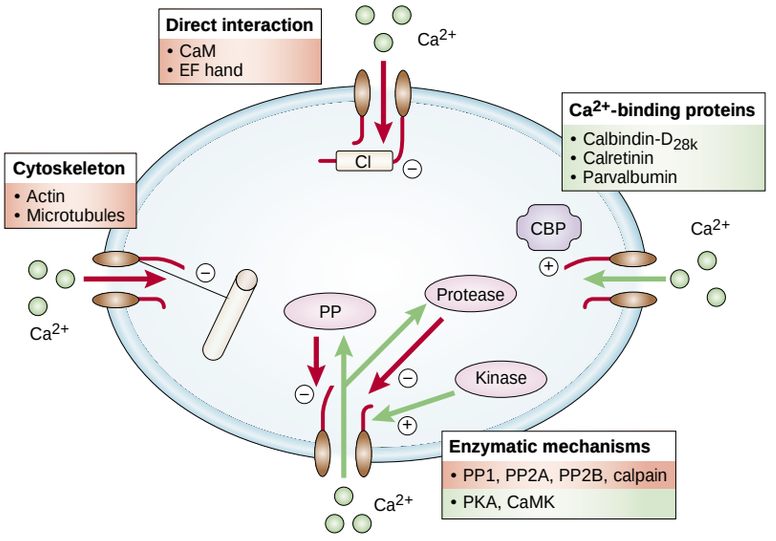


Figure 6 | **Schematic overview of the main mechanisms leading to and influencing CDI in central neurons.** Ca^{2+}-dependent inactivation (CDI) can be caused by direct interactions between Ca^{2+} entering through the pore and the multimeric channel complex — Ca^{2+} binding to the channel's EF hand and/or to calmodulin (CaM). It can also be caused by the activation of Ca^{2+}-dependent protein phosphatases (PPs) such as calcineurin (PP2B), and by a Ca^{2+}-dependent disruption of the interaction between the channel and the cytoskeleton. Ca^{2+} might indirectly activate protein phosphatases such as PP1, whereas other phosphatases, such as PP2A, might modulate CDI in a Ca^{2+}-independent way. Ca^{2+}-dependent activation of proteases such as calpain is also involved in Ca^{2+} channel loss by proteolysis[24]. High-affinity Ca^{2+}-binding proteins (CBP) disrupt CDI by rapidly sequestering Ca^{2+} that is necessary for CDI in the immediate vicinity of the Ca^{2+} channel. A specific cell type might combine the various mechanisms based on the temporal and spatial characteristics of Ca^{2+} transients in relation to the Ca^{2+} sensitivity of the various CDI mechanisms. Green and red arrows indicate activation and inhibition, respectively. CaMK, Ca^{2+}/CaM-dependent protein kinase; CI, calcium-inactivation domain.

clinical relevance of CDI. This condition is associated with a loss of calbindin-D_{28k} (BOX 3) in the affected areas[91]. In agreement with this finding, CDI in surviving hippocampal granule cells that were obtained after neurosurgery revealed markedly increased CDI, thereby diminishing Ca^{2+} influx during repetitive neuronal firing[16,63,92]. These findings point to a neuroprotective role of CDI that is based on a decrease in Ca^{2+} entry.

A unifying model and future work

We have pointed to several mechanisms that underlie the CDI of VGCCs in neurons: direct binding of Ca^{2+} to the VGCC–CaM complex, Ca^{2+}-mediated dephosphorylation of the channel protein, and Ca^{2+}-dependent disruption of the connection between the channel and the cytoskeleton. Although there is evidence in favour of each of these mechanisms, it is difficult to pinpoint the most important one. Furthermore, it is unlikely that these mechanisms are exclusive. Instead, the various mechanisms might operate in a complementary fashion within the same cell (FIG. 6). The speed, amplitude and spatiotemporal patterning of Ca^{2+} signals in combination with the individual Ca^{2+} sensitivity of Ca^{2+} sensors is likely to result in differential and/or combinatorial activation of the various CDI mechanisms, leading to a cell-type-specific CDI profile. This view of custom-made CDI in different cell types is consistent with the observation that even one component — CaM — is used differentially in the CDI of $Ca_V1.2$ and $Ca_V2.1$ channels. Furthermore, the CDI and CDF of $Ca_V2.1$ channels have different Ca^{2+} dependencies, which might reflect differences in Ca^{2+} binding to the amino- and carboxy-terminal lobes of CaM[37]. Ca^{2+} binding to CaM is highly cooperative with Ca^{2+} binding, first to the carboxy-terminal EF hands, which have the highest Ca^{2+} affinity, and subsequently to the lower-affinity sites in the amino-terminal lobes. The degree of Ca^{2+} binding to these sites, and so the activation of CDI and/or CDF, depends on the cell-type-specific speed, amplitude and spatiotemporal patterning of Ca^{2+} signals after Ca^{2+} entry through VGCCs. A similar consideration might apply to the activation of other CDI mechanisms.

The main challenge for future work will be to determine the molecular nature of the transduction mechanism that couples Ca^{2+} binding to channel closure, and to localize protein assemblies that are relevant for CDI in specific functional cellular compartments. The following recent observations could stimulate future work in this direction.

First, the CDI of $Ca_V1.2$ and the VDI of $Ca_V2.1$ channels are influenced similarly by β-subunits. This indicates that the same subunit interactions influence both types of inactivation[93]. It will be interesting to learn whether these channels use a K^+-channel-like 'ball and chain' mechanism (BOX 2), with blocking particles and binding sites encoded by homologous sequences on the $Ca_V1.2$ and $Ca_V2.1$ α1-subunits, making these elements sensitive to the same molecular interactions with the β-subunit.

Second, CaM binds to both intracellular ends of $Ca_V1.2$ channels. This observation has led to the suggestion of a molecular inhibitory scaffold formed by the amino- and carboxy-terminal regions of these channels, which allows its regulation by the βγ-subunits of G proteins and by CaM[94]. This adds to the complexity of VGCC–CaM interactions, and future studies might elucidate the three-dimensional structure of this scaffold.

Third, apart from CaM, Ca^{2+}/CaM-dependent protein kinase II (CaMKII) is involved in the Ca^{2+}-dependent regulation of L-type Ca^{2+} channels[95]. It was recently shown that both CaMKII and an IQ-mimetic peptide facilitate L-type Ca^{2+} currents by favouring prolonged channel openings[96]. Future studies should aim to characterize the common pathway leading to channel facilitation that is used by CaM binding to the IQ domain of Ca^{2+} channels and to CaMKII; more generally, the role of CaMKII in CDI and CDF in neurons should be investigated.

The widespread pre-association of CaM with different VGCC subtypes could motivate a renewed search for Ca^{2+}-dependent modulation of gating of other members of the Ca_V2 family. In addition, as CDI is induced by CICR in cardiac cells, and CICR is a widespread phenomenon in neurons, further research in this area might reveal the involvement of intracellular Ca^{2+} release in CDI in neurons.

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Acknowledgements

This work was supported by Deutsche Forschungsgemeinschaft and Kultusministerium LSA. We thank E. D. Gundelfinger for critical reading of the manuscript before submission, and R. Ziegler for continuous assistance in our work.

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