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Ca^{2+}-dependent modulation of voltage-gated Ca^{2+} channels: analysis in native and heterologous expression systems

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Abstract

Background—Voltage-gated (Ca_v) Ca^{2+} channels are multi-subunit complexes that play diverse roles in a wide variety of tissues. A fundamental mechanism controlling Ca_v channel function involves the Ca^{2+} ions that permeate the channel pore. Ca^{2+} influx through Ca_v channels mediates feedback regulation to the channel that is both negative (Ca^{2+}-dependent inactivation, CDI) and positive (Ca^{2+}-dependent facilitation, CDF).

Scope of Review—This review highlights general mechanisms of CDI and CDF with an emphasis on how these processes have been studied electrophysiologically in native and heterologous expression systems.

Major Conclusions—Electrophysiological analyses have led to detailed insights into the mechanisms and prevalence of CDI and CDF as Ca_v channel regulatory mechanisms. All Ca_v channel family members undergo some form of Ca^{2+}-dependent feedback that relies on CaM or a related Ca^{2+} binding protein. Tremendous progress has been made in characterizing the role of CaM in CDI and CDF. Yet, what contributes to the heterogeneity of CDI/CDF in various cell-types and how Ca^{2+}-dependent regulation of Ca_v channels controls Ca^{2+} signaling remain largely unexplored.

General Significance—Ca^{2+} influx through Ca_v channels regulates diverse physiological events including excitation-contraction coupling in muscle, neurotransmitter and hormone release, and Ca^{2+}-dependent gene transcription. Therefore, the mechanisms that regulate channels, such as CDI and CDF, can have a large impact on the signaling potential of excitable cells in various physiological contexts.

Voltage-gated (Ca_v) Ca^{2+} channels are multi-subunit complexes that play diverse roles in a wide variety of tissues. Multiple Ca_v channels have been characterized ($Ca_v1.x$-$Ca_v3.x$, Table 1), which are comprised mainly of a pore-forming α_1 subunit and for Ca_v1 and Ca_v2 channels, auxiliary $Ca_v\beta$ and $\alpha_2\delta$ subunits [1]. Ca_v channels are generally named according to the identity of the α_1 subunit such that $Ca_v1.2$ channels are those containing the $\alpha_1 1.2$ subunit. Mutations in the genes encoding Ca_v α_1 cause severe human disorders including migraine, deafness, epilepsy, autism, cardiac arrhythmia, malignant hyperthermia and periodic paralysis [2–4]. Ca_v channels mediate an inward Ca^{2+} current that not only depolarizes the cell membrane potential but also provides an intracellular Ca^{2+} signal that

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can activate gene transcription, protein phosphorylation, and neurotransmitter release. Therefore, factors that modulate Ca_v channel properties can significantly affect cellular excitability and signal transduction.

A fundamental mechanism controlling Ca_v channel function involves the Ca^{2+} ions that permeate the channel pore. Ca^{2+} influx through Ca_v channels mediates feedback regulation to the channel that is both negative (Ca^{2+}-dependent inactivation, CDI) and positive (Ca^{2+}-dependent facilitation, CDF). CDI and CDF have been described mainly for "high voltage-activated" Ca_v1 and Ca_v2 Ca^{2+} channels both in native cell-types and heterologous expression systems. CDI and some forms of CDF depend on calmodulin (CaM) binding to the pore-forming Ca_v α_1 subunit. This review will highlight current understanding of the general mechanisms of CDI and CDF with an emphasis on how these processes have been studied electrophysiologically in native and heterologous expression systems.

Ca^{2+}-dependent inactivation (CDI)

Inactivation represents a non-conducting state of many ion channels that is favored by multiple mechanisms. For Ca_v channels, sustained or very positive depolarization promotes voltage-dependent inactivation (VDI). However, Ca_v channel inactivation can also occur via a Ca^{2+}-dependent mechanism, which is evident as a faster decay of the Ca_v current when Ca^{2+} rather Ba^{2+} is used as the charge carrier during sustained depolarizations (Fig. 1a). This was first demonstrated in voltage clamp recordings of *Paramecium* [5, 6], in which Ca^{2+} influx through Ca_v channels regulates reorientation of cilia and swimming in reverse [7]. In addition to faster inactivation of Ca^{2+} currents (I_{Ca}) compared to Ba^{2+} currents (I_{Ba}), inactivation correlated with the amplitude of the peak I_{Ca}. This hallmark of CDI is readily studied in double-pulse voltage-protocols, where the effects of a conditioning (inactivation-inducing) prepulse are reflected in the amplitude of a subsequent test current evoked at a single voltage. A plot of the normalized test I_{Ca} amplitude vs. prepulse voltage is bell-shaped, with greatest inactivation at the prepulse voltage eliciting the maximal inward Ca^{2+} current (Fig. 1b). By contrast, inactivation of I_{Ba} using this protocol should be relatively minor, increasing monotonically with the membrane potential. For such measurements of CDI, the duration of the conditioning prepulse should be short enough to promote greater CDI than VDI, which generally occurs on a longer timescale for most Ca_v1 and Ca_v2 channels.

In their recordings of *Paramecium*, Brehm and Eckert also showed that strong buffering of intracellular Ca^{2+} greatly inhibited inactivation of I_{Ca}, suggesting that Ca^{2+} accumulation in the cell was necessary for CDI ([5, 6]; Fig. 1b,c). However, as will be discussed, the extent to which Ca^{2+} chelators suppress CDI varies between Ca_v channel classes. Nevertheless, these classical studies of *Paramecium* defined approaches for characterizing Ca_v channel CDI, which has proven a fundamental form of negative feedback in various cell types and organisms [8–11]. The mechanisms and properties of CDI vary between Ca_v channel classes and between cell-types, which may have important physiological consequences.

$Ca_v1.2$

Ca_v1 channels mediate L-type Ca^{2+} currents in muscle, nerve, and endocrine cells (Table 1). Of the four classes of Ca_v1 channels, $Ca_v1.2$ is the most ubiquitously expressed [12]. $Ca_v1.2$ regulates excitation-contraction coupling in the heart, which may explain why genetic inactivation of this channel in mice causes embryonic lethality [13]. In ventricular myocytes, $Ca_v1.2$ mediates L-type I_{Ca} that contributes to the plateau depolarization (phase 2) of the action potential. Electrophysiological recordings of these cells show that $Ca_v1.2$ undergoes strong CDI [14–17]. Single-channel analyses of rat myocytes support a model in which Ca^{2+} entry through $Ca_v1.2$ channels shifts their gating to a mode with lower open probability [18,

19]. The fact that $Ca_v1.2$ CDI can be measured in single-channel recordings suggests that CDI requires local Ca^{2+} near the channel pore rather than global Ca^{2+} elevations due to the opening of multiple open channels.

It is now well-established that CDI involes calmodulin (CaM) binding to the cytoplasmic C-terminal domain (CT) the $Ca_v1.2$ α_1 subunit ($\alpha_1 1.2$) [20]. CaM binds to a consensus site, the IQ-domain, in the proximal CT of $\alpha_1 1.2$. Ca^{2+} ions permeating the channel pore bind to the associated CaM, which produces a conformational change supporting CDI. The molecular details underlying CaM interactions with Ca_v channels have been covered in a several excellent reviews [20–22] and will not be a focus here. In whole-cell recordings, CaM inhibitors such as calmidazolium do not prevent CDI of $Ca_v1.2$ in cardiac myocytes [19], but such inhibitors may not antagonize CaM that is tightly associated with some effectors. Overexpression of CaM mutants that cannot bind Ca^{2+} not only inhibit CDI of $Ca_v1.2$ in ventricular myocytes, but also significantly prolongs cardiac action potentials [23]. Since excessively long action potentials can lead to cardiac arrhythmia, the ability of CDI to control action potential duration may protect against aberrant cardiac excitability.

CDI has also been characterized for L-type currents in neuronal and neuroendocrine cells that likely express both $Ca_v1.2$ and $Ca_v1.3$ [24]. However, the functional significance of Ca_v1 CDI in these cell-types is not entirely clear. Ca_v1 channels regulate Ca^{2+}-dependent gene transcription which depends on CaM binding to the IQ-domain, but not on Ca_v1 CDI [25]. Under pathological conditions, such as in epilepsy, Ca_v1 CDI may be neuroprotective by preventing excitotoxic Ca^{2+} overloads in select neurons [26].

$Ca_v1.3$

While $Ca_v1.2$ and $Ca_v1.3$ may be expressed in many of the same cell-types in the brain and heart, $Ca_v1.3$ activates more rapidly and at negative voltages compared to $Ca_v1.2$ [27–30]. These properties allow its contribution to threshold depolarizations that promote spontaneous firing in the sinoatrial node, although $Ca_v1.2$ is also expressed in this tissue [31, 32]. In addition, $Ca_v1.3$ mediates ~90% of the whole-cell I_{Ca} in cochlear inner hair cells [33, 34]. Presynaptic $Ca_v1.3$ channels at the specialized “ribbon synapses” in inner hair cells conduct Ca^{2+} ions that trigger glutamate release at the first synapse in the auditory pathway [33]. The importance of $Ca_v1.3$ for hearing and cardiac pacemaking is underscored by the sinus bradycardia and deafness phenotypes in mice lacking $Ca_v1.3$ and humans with a loss-of-function mutation in the CACNA1D gene encoding $\alpha_1 1.3$ [35].

While $Ca_v1.3$ exhibits intense CDI when expressed heterologously, CDI is more limited in inner hair cells [28, 29, 34]. Whole-cell and perforated patch recordings from auditory hair cells in various species reveal I_{Ca} that inactivates only ~30–40% in 1 second while I_{Ca} in HEK293T cells transfected with $Ca_v1.3$ inactivates 80–90% within the same timeframe (Fig. 2a,b), although the extent of CDI in hair cells can vary with temperature and between species [36–38]. Differences in CDI of $Ca_v1.3$ between hair cells and other cell-types could be accounted for by alternative splicing of $\alpha_1 1.3$ which yields one variant lacking the IQ domain that exhibits little CDI. Since this variant is mainly expressed in outer hair cells, it is unlikely to account for the slow CDI in inner hair cells [39]. An alternate hypothesis is that Ca^{2+}-binding proteins related to CaM oppose CDI of $Ca_v1.3$ in inner hair cells (See below).

$Ca_v1.4$

While the consensus site(s) for binding to CaM are relatively conserved between Ca_v1 channel subclasses, some intriguing differences in CDI have been noted. For example, $Ca_v1.4$, which is the primary Ca_v channel in retinal photoreceptors, undergoes little CDI [40, 41] (Fig. 2c). Similar to the role of $Ca_v1.3$ in inner hair cells, the ability of $Ca_v1.4$ to support

sustained presynaptic L-type I_{Ca} helps maintain tonic glutamate release at ribbon synapses. CaM can bind to the $Ca_v1.4$ α_1 subunit IQ domain, but this interaction may be disrupted by an inhibitory domain (ICDI: inhibitor of CDI) in the distal C-terminus [42, 43]. This domain is weakly conserved in other Ca_v1 channel subclasses, such that its transfer to the $\alpha_1$1.2 or $\alpha_1$1.3 nearly nullifies the normally strong CDI of $Ca_v1.2$ and $Ca_v1.3$ [42, 43]. Multiple mutations in the CACNA1F gene encoding $\alpha_1$1.4 cause congenital stationary night blindness type 2 [2], one of which (K1591X) causes premature truncation of $\alpha_1$1.4 and deletion of the ICDI. As a consequence, $Ca_v1.4$ (K1591X) shows enhanced CDI [42], which could cause vision deficits due to a loss-of-function in photoreceptor transmission.

$Ca_v1.1$

In skeletal muscle, $Ca_v1.1$ channels are the "dihydropyridine receptors (DHPRs)" that mediate excitation/contraction coupling. The voltage-sensing function of $Ca_v1.1$ is directly coupled to activation of Ca^{2+} release by ryanodine receptors in the sarco-endoplasmic reticulum, which initiates muscle contraction. Although Ca^{2+} conductance by $Ca_v1.1$ is not required for excitation/contraction coupling, $Ca_v1.1$ channels do mediate measurable L-type I_{Ca} in skeletal muscle cells [44]. Compared to $Ca_v1.2$, $Ca_v1.1$ undergoes modest CDI [45], which may be due to sequence differences in the IQ-domains that impair CaM binding and/or the affinity of bound CaM for Ca^{2+} [46–48]. In particular, Y1657 and K1662 in the rabbit cardiac $\alpha_1$1.2 are replaced by H1532 and M1537 in the rabbit skeletal muscle $\alpha_1$1.1. Substituting Y1657 and K1662 in $\alpha_1$1.2 with the corresponding residues of $\alpha_1$1.1 weaken CaM binding and eliminate CDI of $Ca_v1.2$ channels in transfected HEK293T cells [46]. However, additional residues that distinguish the IQ domain of $\alpha_1$1.1 and $\alpha_1$1.2, while not directly contacting CaM, alter its Ca^{2+} binding affinity. When bound to the IQ-domain of $\alpha_1$1.2, the C- and N-terminal lobes of CaM bind Ca^{2+} with ~5-fold higher affinity than when bound to the $\alpha_1$1.1 IQ-domain [47]. This latter result could explain why $Ca_v1.1$ CDI is weaker and more strongly inhibited by BAPTA than $Ca_v1.2$ CDI [45]. Sequence differences in the $\alpha_1$1.1 IQ domain are probably not the only determinants of slow CDI in skeletal muscle. The expression of recombinant $Ca_v1.2$ channels in dysgenic myotubes, which lack functional $Ca_v1.1$ channels, results in L-type currents that lack CDI [12]. Thus, as for $Ca_v1.3$ in cochlear hair cells, factors that inhibit CDI of Ca_v1 channels may be endogenously expressed in skeletal muscle. The significance of slow CDI for $Ca_v1.1$ is unknown but may be important for preventing refractoriness of the voltage-sensing capabilities of $Ca_v1.1$ for excitation/contraction coupling [46].

Ca_v2 channels

Previous efforts to characterize CDI of Ca_v2 channels by heterologous expression did not reveal CDI [49] in part because of high concentrations of intracellular EGTA that are typically used for electrophysiological recordings of Ca_v1 channels. However, with a relatively low (0.5 mM) concentration of EGTA in intracellular recording solutions, CDI of $Ca_v2.1$ channels in transfected HEK293T cells is evident as significantly faster inactivation of I_{Ca} compared to I_{Ba} [50] (Fig. 1a). High concentrations (10 mM) of EGTA or BAPTA, which spare CDI of $Ca_v1.2$ and $Ca_v1.3$, completely block $Ca_v2.1$ CDI in transfected cells (Fig. 1c, [51, 52]). These results suggest that $Ca_v2.1$ CDI depends on global elevations in Ca^{2+} while $Ca_v1.2$ CDI depends on local Ca^{2+} elevations near the channel pore. Consistent with this hypothesis, unlike for $Ca_v1.2$ [18], CDI for $Ca_v2.1$ is not evident in single channel recordings [53]. In heterologous expression systems, the local Ca^{2+} sensitivity of $Ca_v1.2$ and $Ca_v1.3$ CDI contrasts with the global Ca^{2+} sensitivity of CDI for all Ca_v2 channels [54].

Similar to $Ca_v1.2$ and $Ca_v1.3$, CDI for Ca_v2 channels involves CaM binding to an IQ-like domain in the Ca_v2 α_1 C-terminal domain. For $Ca_v2.1$, an additional CaM-binding domain (CBD) C-terminal to the IQ-like domain is also involved. Mutations or deletions of both of

these sites impact CDI [50–52, 55]. Besides this difference in CaM binding sites between $Ca_v1.2$ and $Ca_v2.1$, CDI for these channels relies on different Ca^{2+}-binding lobes of CaM. This conclusion is based on electrophysiological experiments where CaM mutants unable to bind Ca^{2+} in the N- or C-lobes were co-transfected with Ca_v channels in HEK293T cells. These studies demonstrate a requirement for the CaM C-lobe for $Ca_v1.2$ CDI whereas the CaM N-lobe is needed for $Ca_v2.1$ CDI [52, 55, 56]. Structural analyses of CaM bound to $\alpha_1 1.2$, $\alpha_1 2.1$, $\alpha_1 2.2$, or $\alpha_1 2.3$ peptides containing the IQ domain indicate that distinctions in how CaM associates with key residues within and/or outside of the IQ domain may underlie the differences in $Ca_v1.2$ and Ca_v2 CDI [57–60].

A second CaM-binding determinant (NSCaTE) in the N-terminal domain of $\alpha_1 1.2$ and $\alpha_1 1.3$, that is not present in Ca_v2 α_1 subunits, contributes to the difference in Ca^{2+} buffer sensitivity of CDI for Ca_v1 and Ca_v2 channels. Transfer of this region to the $Ca_v2.2$ α_1 subunit ($\alpha_1 2.2$) allows for significant CDI in the presence of 10 mM BAPTA. The underlying mechanism may involve distinct interactions of the N- and C- terminal lobes of CaM with the Ca_v1 α_1 NSCaTE and IQ domain, respectively [61].

Although intensely studied in heterologous expression systems, Ca_v2 CDI has been infrequently characterized for Ca_v2 channels in neurons. Ca^{2+} influx through Ca_v2 channels, particularly $Ca_v2.1$ and $Ca_v2.2$, initiates neurotransmitter release at many central synapses. Fluorometric analysis of Ca^{2+} influx in rat brain synaptosomes suggested CDI of P-type currents, which was suppressed by decreasing the extracellular Ca^{2+} concentration [62]. CDI of presynaptic Ca^{2+} channels was also demonstrated in patch clamp recordings of presynaptic nerve terminals in the rat neurohypophysis [63] and for P-type currents at the Calyx of Held synapse in the rat auditory brainstem [64]. Because of the steep dependence of neurotransmitter release on presynaptic Ca^{2+} concentrations, Ca_v2 channel CDI could significantly inhibit neurotransmission. In support of this hypothesis, $Ca_v2.1$ CDI can cause short-term depression at the Calyx of Held and in transfected sympathetic neurons [64, 65].

Ca^{2+}-dependent facilitation (CDF)

Facilitation is an enhanced form of channel opening that can be achieved through multiple mechanisms. CDF is a positive feedback regulation of further Ca^{2+} entry through Ca_v1 and Ca_v2 channels (CDF), which can significantly impact cell physiology in a variety of contexts. Although other forms of facilitation have been described for various Ca_v family members, CDF has mainly been described for native and heterologously expressed $Ca_v1.2$, $Ca_v1.3$, $Ca_v2.1$, and Ca_v3 channels.

$Ca_v1.2$

In cardiac ventricular myocytes, $Ca_v1.2$ CDF helps amplify intracellular Ca^{2+} signals coupled to contraction of the heart [66–68]. During a train of depolarizations, I_{Ca} progressively increases in amplitude, reaching a steady state by the fifth pulse [69]. Facilitation of I_{Ca} is Ca^{2+}-dependent in that it can be induced by photolysis of caged-Ca^{2+} compounds [70] and is abolished by substitution of extracellular Ca^{2+} with Ba^{2+} or Sr^{2+} [69]. CDF can still be observed with 20 mM EGTA or 5 mM BAPTA in intracellular recording solutions, suggesting that $Ca_v1.2$ CDF depends on very local increases in Ca^{2+} near the channel [69].

Like CDI, CDF for $Ca_v1.2$ channels also depends on CaM, which activates CaM-dependent protein kinase II (CaMKII). Two mechanisms have been proposed for the role of CaMKII in $Ca_v1.2$ CDF. In the first, CaMKII binds to the C-terminal domain of $\alpha_1 1.2$ near the IQ-domain, which may position the kinase for phosphorylating two nearby serine residues (S1512/S1570 in the mouse cardiac $\alpha_1 1.2$ [71, 72]. In transgenic mice in which these

residues were mutated to alanine, L-type I_{Ca} shows slower recovery from inactivation and enhanced steady state inactivation, which is consistent with a loss of CDF. CDF was not completely abolished in ventricular myocytes from these mice, suggesting that other processes contribute to CDF (see below). *In vivo* telemetry in these mice revealed electrocardiograms with shorter QT intervals indicative of faster repolarization of cardiac action potentials. Since excessive CaMKII activity such as in heart failure causes QT prolongation and arrhythmia [73], the results suggest that $Ca_v1.2$ CDF, while normally beneficial for improving the force-frequency relationship of excitation/contraction coupling, may contribute to heart disease under pathological conditions.

A second route by which CaMKII enhances CDF is through interactions with the auxiliary $Ca_v\ \beta_{2A}$ subunit. CaMKII binds to and phosphorylates $Ca_v\ \beta_{2A}$ at threonine 498 (rat β_{2A}), which enhances a mode of gating (mode 2) characterized by increased $Ca_v1.2$ channel open probability [74–76]. Overexpression of wild-type $Ca_v\ \beta_{2A}$ in ventricular myocytes causes Ca^{2+} overload and early after-depolarizations [77], which are premature depolarizations between cardiac action potentials that can cause arrhythmia. Moreover, rapid pacing, which would induce CDF, caused premature death of $Ca_v\ \beta_{2A}$-overexpressing ventricular myocytes. By contrast, overexpression of $Ca_v\ \beta_{2A}$ with mutation of threonine 498 to alanine (or leucine 493 mutation that prevents CaMKII binding to $Ca_v\ \beta_{2A}$) reduced $Ca_v1.2$ Ca^{2+} entry and early afterdepolarizations and also prevent $Ca_v1.2$ mode 2 gating [77]. These results confirm that $Ca_v1.2$ CDF can contribute to abnormal excitability that leads to cardiac arrhythmia and a key role for CaMKII/$Ca_v\ \beta_{2A}$ interactions in this process.

An important feature of CDF to consider in whole-cell recordings is the activation of CaMKII by repetitive Ca^{2+} spikes, which stimulates its autophosphorylation at threonine 286 and autonomous activity independent of Ca^{2+}/CaM [78]. If L-type I_{Ca} is the Ca^{2+} source for CaMKII activation, autonomous activity should be more efficiently generated by short repetitive pulses rather than sustained depolarizations. In rat ventricular myocytes, CDF can be evoked with 150-ms pulses from −80 to 0 mV delivered at a frequency of 0.5 Hz. With this protocol, I_{Ca} increases in amplitude ~20% by the 4^{th} pulse [76]. In theory, double-pulse voltage protocols such as those used to measure CDF of $Ca_v2.1$ (see below) could also be applied for analyses of $Ca_v1.2$ CDF. However, the voltage of the conditioning prepulse should not be so depolarized as to evoke voltage-dependent facilitation (VDF). Prepulses up to +160 mV have been used to demonstrate a role for CaMKII in $Ca_v1.2$ facilition [72]. However, little Ca^{2+} influx would be generated during such positive prepulses, due to the small driving force for Ca^{2+} influx near the reversal potential for Ca^{2+} in most electrophysiological recordings. Thus, the measured facilitation with such protocols is likely VDF rather than CDF [72].

While both CDF and CDI characterize $Ca_v1.2$ channels in cardiac myocytes, recombinant $Ca_v1.2$ channels expressed in Xenopus oocytes and HEK 293 cells show primarily CDI and very little CDF, even during repetitive stimuli [79, 80]. CDF for heterologously expressed $Ca_v1.2$ channels is only evident when CDI is inhibited, such as with $\alpha_1 1.2$ subunits containing I-A mutation in the IQ-domain [79]. Coexpression of the CaM-like protein, CaBP1, which also inhibits $Ca_v1.2$ CDI, also allows for $Ca_v1.2$ CDF that is independent of CaMKII [80, 81]. Thus, the presence of overt CDF that is not masked by CDI in cardiac myocytes may depend on cell-type specific differences in the processing of $Ca_v1.2$ channels or on cellular factors which are more abundant in cardiac cells than in heterologous expression systems.

$Ca_v1.3$

CaMKII is also implicated in CDF of $Ca_v1.3$ but through a mechanism distinct from that for $Ca_v1.2$. $Ca_v1.3$ channels do not exhibit CDF when expressed alone or with CaMKII in

HEK293T cells. However, when cotransfected with CaMKII and densin-180, a CaMKII-interacting protein highly enriched at excitatory synapses in the brain [82, 83], $Ca_v1.3$ channels undergo CDF during repetitive depolarizations [84]. Densin-180 binds via its PDZ domain to the distal C-terminus of $\alpha_1 1.3$ and therefore may help scaffold CaMKII to the $Ca_v1.3$ channel complex. All three proteins coimmunoprecipitate from brain lysates, which suggests that densin 180 may permit CaMKII-dependent $Ca_v1.3$ CDF in neurons [84]. CaMKII activation in dendritic spines is mediated by Ca_v1 channels during stimulation protocols that produce long-term potentiation [85]. Thus, densin and CaMKII association with $Ca_v1.3$ may augment Ca^{2+} signals that trigger CaMKII activation and participation in long-term synaptic plasticity.

$Ca_v2.1$

At the Calyx of Held synapse, $Ca_v2.1$ channels initially undergo CDF and then CDI during high-frequency depolarizations, which can influence short-term facilitation and depression of the excitatory postsynaptic responses [64, 86–89]. This facilitation is Ca^{2+}-dependent in that it is only seen for I_{Ca} and not I_{Ba} and reduced by high intracellular EGTA [86]. Similar biphasic CDF and CDI was also reported for $Ca_v2.1$ channels transfected into superior cervical ganglion neurons [65]. Here, CDF and CDI were also associated with short-term facilitation and depression of synaptic transmission [65]. The neurophysiological significance of short-term facilitation due to CDF is not entirely clear, but may ensure reliable transmission by offsetting depression [88].

$Ca_v2.1$ channels in cerebellar Purkinje neurons also undergo CDF as well as CDI during stimulation with action potential waveforms (Fig. 3a,b; [90–94]), but CDF tends to balance the effects of CDI in a way that maintains relatively constant I_{Ca} amplitudes [92]. In Purkinje neurons, P/Q-type I_{Ca} is strongly coupled to Ca^{2+}-activated K^+ channels that regulate Purkinje cell firing rates [95]. Fluctuations in I_{Ca} due to overt CDF or CDI may cause instabilities in firing that could prevent normal correlations of firing rate with synaptic activation [92]. The significance of CDF was also shown for $Ca_v2.1$ channels in presynaptic parallel fiber inputs to Purkinje neurons. Here, $Ca_v2.1$ channels bearing a mutation associated with familial hemiplegic migraine in humans (S218L) do not undergo CDF because they may be in a basally facilitated state. As a consequence, there is no short-term synaptic facilitation at this synapse [94]. Thus, the current data suggest that CDF plays a functionally more significant role for $Ca_v2.1$ in presynaptic terminals than in postsynaptic compartments of neurons.

The mechanisms underlying CDF have been studied extensively for $Ca_v2.1$ channels in transfected HEK293T cells [52, 55]. $Ca_v2.1$ CDF depends on CaM binding to the IQ-domain of $\alpha_1 2.1$, with a reliance primarily on the C-lobe of CaM in contrast to the CaM N-lobe dependence of CDI [52, 55, 57, 59]. In transfected HEK293T cells, $Ca_v2.1$ CDF can be measured using 2–5-ms square pulses or action potential waveforms at a frequency of 100 Hz (Fig. 3b). CDF is plotted as the amplitude of each test current normalized to that for the first in the train. CDF can also be measured with double-pulse protocols with short (~20–50 ms) conditioning prepulses that evoke significant inward I_{Ca} (i.e., −80 mV to +10 mV). With this protocol, facilitation can be measured as the test current amplitude or charge integral normalized to a current in the absence of a prepulse. When plotted against prepulse voltage, facilitation of I_{Ca}, but not I_{Ba}, should be bell-shaped, reflecting its dependence on the amount of inward I_{Ca} during the prepulse (Fig. 3c).

In single-channel recordings, $Ca_v2.1$ CDF is associated with an enhanced open probability of I_{Ca} above that seen for I_{Ba}, rather than an acceleration of activation kinetics [53]. The detection of CDF but not CDI in single-channel recordings [53] suggests a different reliance on local vs. global Ca^{2+} signals for CDF and CDI, respectively. Consistent with this

hypothesis, CDF, unlike CDI, is spared by 10 mM EGTA in whole-cell patch clamp recordings of transfected HEK293T cells [23, 55]. Analyses of CDF of transfected $Ca_v2.1$ channels would therefore benefit by high Ca^{2+} buffering conditions which would limit the competing effects of CDI. However, since the decay of CDF is significantly accelerated by high Ca^{2+} buffering in transfected cells [51], paired-pulse protocols should employ a relatively short interval between the conditioning prepulse and the test pulse for maximal CDF to be achieved.

Ca_v3

Ca_v3 channels are the major Ca_v channels in adrenal glomerulosa cells which secrete aldosterone in response to angiotensin II and physiological increases in K^+ [96]. Angiotensin II stimulates Ca^{2+} release from intracellular stores, activation of CaMKII, and potentiation of Ca_v channel opening by K^+-induced depolarization [97]. The facilitation involves CaMKII binding to and phosphorylation of the cytoplasmic domain linking domains 2 and 3 of $\alpha_1 3.2$ [98]. Phosphorylation of S1198 (human $\alpha_1 3.2$) negatively shifts voltage-dependent activation of $Ca_v3.2$ channels in HEK293 cells. This residue is not present in $\alpha_1 3.1$ and accounts for the absence of CaMKII regulation of $Ca_v3.1$ [98, 99]. Cell-attached single-channel recordings of T-type currents in adrenal glomerulosa cells show that raising the bath Ca^{2+} concentration enhances $Ca_v3.2$ channel open probability, which is accompanied by increased frequency, rather than longer duration, of channel openings [100]. In addition, $Ca_v3.2$ CDF is blocked by CaMKII inhibitors and mimicked by constitutively active CaMKII. Since CDF would cause $Ca_v3.2$ channels to open at more negative membrane potentials, CDF may help set the level of steady state Ca^{2+} influx and angiotensin II-stimulated aldosterone secretion. In heart failure, which is characterized by heightened activation of the renin-angiotensin system, $Ca_v3.2$ CDF may exacerbate the pathological consequences of excessive aldosterone secretion in heart failure, which include structural and functional changes in the heart and vasculature. Since $Ca_v3.2$ channels are highly expressed in the brain [101], CDF may also contribute to the roles of $Ca_v3.2$ in regulating firing patterns in multiple classes of neurons.

Factors that influence CDI and CDF in native cell-types

CDI and CDF, even for a given Ca_v subclass, can be highly variable between cell-types (Fig. 2a,b). Multiple factors can influence the extent to which Ca_v channels undergo CDI and CDF, which can complicate mechanistic inquiries as to their relative significance.

Alternative splicing of Ca_v α_1

Post-translational splicing increases the functional diversity afforded by the 10 genes encoding $Ca_v\alpha_1$ subunits in the human, rat, and mouse genomes. Because of the large size of these genes (~50 exons), the number of splice variants possible for a single Ca_v α_1 subunit may be greater than 1000 [102]. Like the $\alpha_1 1.3$ splice variant lacking the IQ domain in outer hair cells [39], all of the splice variations that have been found to impact CDI or CDF involve alterations in the C-terminal domain. For example, alternative splicing at exon 37 (human $\alpha_1 2.1$) produces two variants (EFa and EFb) with amino acid substitutions in an EF-hand like domain upstream from the CaM binding IQ-like domain and CBD [103]. The EFa but not the EFb variant exhibits CDF [104]. When combined with alternative splicing out of the final exon 47, EFb causes CDF that is blocked by high concentrations of BAPTA only in cells with large current density. Apparently, exclusion of exon 47 permits CDF in EFb containing $Ca_v2.1$ channels that is driven by global, rather than local, increases in Ca^{2+} [104]. How the absence of exon 47 when combined with EFb transforms the Ca^{2+}-sensitivity of $Ca_v2.1$ CDF remains to be elucidated.

The distal C-terminal domain of $\alpha_1$1.3 has also been shown to regulate CDI of Ca_v1.3. Alternative splicing of exons 42 and 42A produces $\alpha_1$1.3 variants that extend >100 amino acids beyond the IQ-domain (exon 42) or are truncated just after the IQ-domain (exon 42A) [28, 105]. When compared in transfected HEK293T cells, the short exon 42A variant exhibits stronger CDI than the long exon 42 variant. FRET experiments suggested that the exon 42 sequence interfered with CaM binding to the proximal $\alpha_1$1.3 C-terminal region including the IQ-domain [106]. Thus, this alternatively spliced exon plays a similar role in $\alpha_1$1.3 as the distal CT in $\alpha_1$1.4 [42, 43], except that the inhibitory effect on CDI is significantly less for Ca_v1.3 than for Ca_v1.4.

Ca_v β subunits

Because inactivation of Ca_v channels can occur by voltage- or Ca^{2+}-dependent mechanisms (VDI or CDI), factors that minimize the former can enhance detection of the latter. For example, CDI is quite minimal in Ca_v2.1 channels that contain the $Ca_v\beta_{1b}$ subunit due to the strong VDI that typifies this subunit composition [107]. Inclusion of the $Ca_v\beta_{2A}$, which produces Ca_v2.1 channels that show significantly weaker VDI than with $Ca_v\beta_{1b}$, results in robust CDI [51]. Considering the heterogeneous expression patterns of different $Ca_v\beta$ subunits [108], the magnitude of Ca_v2.1 CDI may vary between tissues and cell-types.

For Ca_v1.2 and Ca_v1.3, CDI is less dependent on the identity of the $Ca_v\beta$ subunit than for Ca_v2.1 channels. However, Ca_v1.2 CDF due to CaMKII may require $Ca_v\beta_2$ or $Ca_v\beta_1$ subunit. Biochemical studies indicate that autophosphorylated CaMKII stably associates with $Ca_v\beta_2$ and β_1 but not β_3 or β_4 [74]. Although CaMKII can phosphorylate all four Ca_v β subunits to a similar extent, a conserved LXRXXS/T motif present in $Ca_v\beta_2$ and β_1 but not β_3 or β_4 may be required for functionally relevant phosphorylation of $Ca_v\beta$ which ensures stability of the $Ca_v\beta$/CaMKII complex [74]. To what extent $Ca_v\beta$ subunits influence the extent of Ca_v1.2 CDF awaits electrophysiological comparisons of CDF of Ca_v1.2 channels bearing distinct $Ca_v\beta$ subunits.

CaBPs

Due to their similarities with CaM, some neuron-specific CaBPs can substitute for CaM in binding to Ca_v channels, which can significantly alter CDF and CDI [109]. Like CaM, these CaBPs have four EF-hand Ca^{2+} binding domains, at least one of which is nonfunctional [110]. Other structural distinctions including N-terminal myristoylation and a longer central helical domain may confer some CaBPs with the ability to differentially modulate Ca_v channels compared to CaM [111]. For example, CaBP1 is colocalized to some extent with presynaptic Ca_v2.1 channels. Like CaM, CaBP1 binds to the CBD of $\alpha_1$2.1, but does not support CDF and causes intense inactivation independent of Ca^{2+} [112]. Ca_v2.1 also can interact with other CaBPs, such as neuronal Ca^{2+} sensor-1 (NCS-1), which has inhibitory effects on P/Q-type current amplitude in *Xenopus* oocytes and adrenal chromaffin cells [113]. However, injection of purified NCS-1 protein into presynaptic nerve terminals at the Calyx of Held synapse promotes CDF of P/Q-type currents, and activity-dependent facilitation P/Q-type currents at this synapse can be prevented by injection of NCS-1-inhibitor peptides [114]. Therefore, the effects of NCS-1 on Ca_v2.1 may depend on the cellular and subcellular context in which they are coexpressed. A third CaBP that modulates Ca_v2.1 is visinin-like protein-2 (VILIP-2), which is highly expressed in the neocortex and hippocampus and undergoes Ca^{2+}-dependent association with the plasma membrane in neurons and other cell-types [115]. When cotransfected with Ca_v2.1 in mammalian cells, VILIP-2 does not affect CDF, but inhibits CDI [116]. These effects of VILIP-2 may involve displacement of CaM from the CBD, although both CBD and IQ-like domain of $\alpha_1$2.1 are required for the association of VILIP-2 with the channel [116]. How VILIP-2 and CaBP1 can have such opposing effects on Ca_v2.1 function is not entirely clear, but may involve

structural distinctions between the two CaBPs and/or how they interact with $Ca_v2.1$ [117]. The remarkably divergent actions of CaBPs on $Ca_v2.1$ may increase the range of presynaptic P/Q-type Ca^{2+} signals, thus contributing to heterogeneous forms of short-term plasticity at different synapses.

Ca_v1 channels are also differentially regulated by CaBPs. Unlike its inhibitory effects on $Ca_v2.1$, CaBP1 prolongs $Ca_v1.2$ Ca^{2+} currents and completely abolishes CDI [80, 118]. Structural analyses indicate that the opposing actions of CaM and CaBP1 on $Ca_v1.2$ may depend on differences in how the two interact with the IQ-domain [81]. In addition, CaBP1 binds to a site in the N-terminal domain of the $\alpha_1 1.2$, which can affect both CDI and VDI [118, 119]. Interestingly, a CaBP1 variant, caldendrin, causes more modest suppression of $Ca_v1.2$ CDI than CaBP1 through interactions solely with the IQ-domain and not the N-terminal site in $\alpha_1 1.2$ [120]. CaBP1 and caldendrin associate and colocalize with $Ca_v1.2$ in somatodendritic domains of neurons [80, 120], and so may help fine-tune and boost postsynaptic $Ca_v2.1$ Ca^{2+} signals that regulate gene transcription and neuronal excitability.

CaBP1 is highly expressed in inner hair cells where $Ca_v1.3$ channels exhibit anomalously slow CDI at room temperature compared to in transfected cells at room temperature [29, 34, 121, 122]. Consistent with a role for CaBP1 in the mechanism, coexpression of CaBP1 with $Ca_v1.3$ in HEK293T cells strongly inhibits CDI [121, 122]. In addition to CaBP1, CaBP2, 4, and 5 are also expressed in inner hair cells. However, when $Ca_v1.3$ is cotransfected with each of these CaBPs in HEK293T cells, the effects of CaBP1 on slowing CDI and also VDI most closely reproduce the slowly inactivating properties of $Ca_v1.3$ in inner hair cells. In addition, genetic inactivation of CaBP4 in mice has only a minor effect on CDI and no impact on hearing [121]. Confirmation of the role of CaBP1 in CDI suppression awaits similar analyses in inner hair cells from CaBP1 null mice.

In addition to these CaM-like CaBPs, Ca^{2+} binding proteins such as parvalbumin (PV) and calbindin D-28k (CB), which serve more as Ca^{2+} buffers [123], also regulate Ca_v CDI. PV and CB are highly expressed in Purkinje neurons where I_{Ca} is largely mediated by $Ca_v2.1$ [124]. Since $Ca_v2.1$ CDI is sensitive to Ca^{2+} buffers that dampen global Ca^{2+} elevations [51], one would expect that PV and CB might similarly naturally suppress CDI. This hypothesis was partially supported in transfected HEK293T cells [125]. If PV and CB inhibited CDI in Purkinje neurons, then CDI should be increased in neurons from mice with genetic inactivation of the genes encoding PV and CB. However, Purkinje neurons isolated from mice lacking PV and CB showed a decrease in $Ca_v\beta_{2A}$ expression and consistently, increased VDI but not CDI compared to in wild-type neurons [90]. This alteration may have been a compensatory response to prevent excessive $Ca_v2.1$ Ca^{2+} influx in neurons lacking the Ca^{2+} buffering capacity of PV and CB. Thus, whether Ca^{2+} buffering proteins limit CDI of neuronal $Ca_v2.1$ channels remains to be proven.

Despite evidence that Ca_v1 CDI in transfected HEK293T cells is relatively insensitive to Ca^{2+} buffers [54, 61], CDI of Ca_v1 channels in thalamocortical neurons was shown to be inhibited by inclusion of PV and CB in intracellular whole-cell recording solutions in isolated thalamocortical neurons [126]. Further evidence for a role for Ca^{2+} buffering proteins in regulating Ca_v CDI comes from recordings of surviving hippocampal granule cells isolated from the dentate gyrus of patients with mesial temporal lobe epilepsy. These cells have reduced levels of CB, which correlates with increased CDI of Ca_v1 L-type currents [26]. Unlike for Ca_v1 channels in transfected cells, Ca_v1 CDI in these surviving granule cells is inhibited by high intracellular concentrations of BAPTA or EGTA [127]. Why Ca_v1 CDI is sensitive to Ca^{2+} buffers in these neurons but not in HEK293T cells is unclear, but could be related to the presence of Ca_v1 α_1 splice variants that may lack the NSCaTE [61]. Alternatively, CaBPs with distinct Ca^{2+} binding affinities compared to CaM

might compete with CaM for binding to the Ca_v1 IQ domain or NSCaTE in neurons. Regardless of the mechanism, the ability of PV and/or CB to suppress Ca_v1 CDI in neurons may be necessary for supporting Ca^{2+} signals of sufficient duration to activate signaling pathways underlying activity-dependent synaptic plasticity [128]. Moreover, pathological decreases in neuronal Ca^{2+} buffering, such as in temporal lobe epilepsy [26], may be neuroprotective in increasing Ca_v1 CDI and reducing excitotoxic Ca^{2+} overloads.

Other cellular factors

Cells such as myocytes and neurons have complex subcellular features that could also significantly impact the extent to which Ca_v channels undergo CDI and CDF. This seems to be particularly true in skeletal and cardiac muscle cells in which Ca_v1 channels are tightly clustered at junctional membranes in association with intracellular ryanodine receptors (RYRs) in the sarco-endoplasmic reticulum. In cardiac myocytes, Ca^{2+} influx through $Ca_v1.2$ channels enhances Ca^{2+}-induced Ca^{2+} release through activation of RYRs. Due to the tight spatial coupling of RYRs and $Ca_v1.2$ in junctional membranes, $Ca_v1.2$ CDI is enhanced by RYR-mediated Ca^{2+} signals [129]. Such functional coupling between RYRs and $Ca_v1.2$ may also occur in neurons [130], since blockade of RYRs in thalamocortical neurons decreased Ca_v1 CDI [131].

The cytoskeleton has also been implicated as a determinant of CDI in neurons [132, 133]. It is hypothesized that Ca^{2+} influx through Ca_v channels destabilizes cytoskeletal elements, which promotes CDI. In support of this hypothesis, pharmacological stabilization of microfilaments and microtubules reduce Ca_v CDI in neurons [127, 133]. How cytoskeletal dynamics participate in CDI regulation is unclear, but may involve Ca_v α_1 interactions with Ca_v β subunits and/or cytoskeleton-associated proteins [24].

Summary and remaining questions

Electrophysiological analyses have led to detailed insights into the mechanisms and prevalence of CDI and CDF as Ca_v channel regulatory mechanisms. All Ca_v channel family members undergo some form of Ca^{2+}-dependent feedback that relies on CaM or a related Ca^{2+} binding protein. Tremendous progress has been made in characterizing the role of CaM in CDI and CDF of Ca_v1 and Ca_v2 channels at the molecular and atomic scales. Yet, numerous ambiguities remain. First, what determines the variability in CaM-dependent CDI/CDF in native cell types? At one extreme, Ca_v1 channels in photoreceptors and inner hair cells have mechanisms that prevent CDI, to allow for sustained Ca^{2+}-dependent exocytosis required for sensory transmission. By contrast, intense CaM-dependent CDI of Ca_v1 channels in cardiac myocytes is necessary for curtailing cardiac action potentials and maintaining cardiac rhythmicity. Molecular and biochemical analyses of Ca_v splice variation and Ca_v/protein interactions may reveal mechanisms underlying the heterogeneous presentations of Ca_v CDI/CDF in a variety of cell-types.

Second, how does CDI/CDF control cellular excitability and other Ca^{2+} signaling events? Due to the depolarizing influence of Ca_v currents, CDI could directly inhibit neuronal excitability. However, due to the tight coupling of Ca_v channels with Ca^{2+}-dependent K^+ (K_{Ca}) channels, CDF could also inhibit excitability through stronger activation of K_{Ca} channels. Detailed consideration of CDI/CDF within computational models of cellular and network excitability is a necessary first step towards understanding the broader impact of these forms of Ca_v channel modulation in defined physiological contexts.

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Highlights

- Electrophysiological analysis have revealed detailed mechanisms of Ca_v channel modulation.
- Ca_v channels undergo CDI/CDF that relies on Ca^{2+} binding proteins like calmodulin.
- The heterogeneity of CDI/CDF in various cell-types remains largely unexplored.

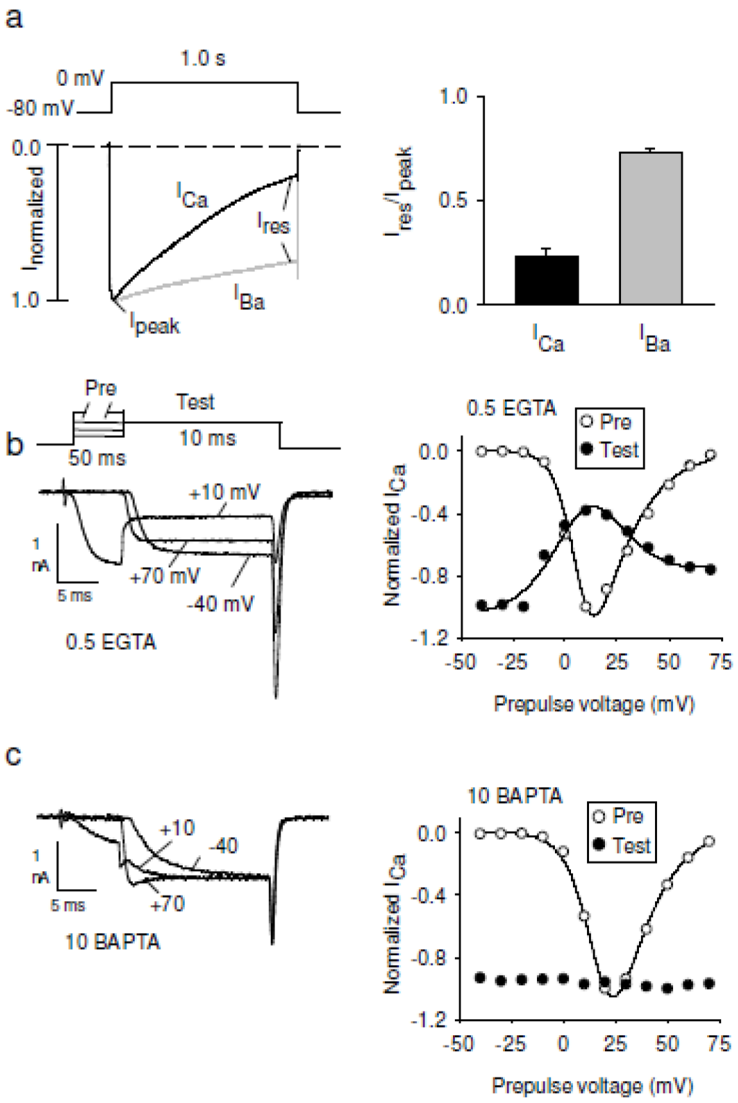

Figure 1.

Characterization of CDI of $Ca_v2.1$ channels in voltage-clamp recordings. a) Comparison of inactivation of I_{Ca} (black trace) and I_{Ba} (grey trace) in HEK293T cells transfected with $Ca_v2.1$ ($\alpha_1 2.1$, β_{2A} and $\alpha 2\delta$). Currents are evoked by 1-s pulses from −80 mV to 0 mV. Extracellular solution contains 10 mM Ca^{2+} or Ba^{2+} and intracellular solution contains 0.5 mM EGTA. *Right,* inactivation is measured by dividing residual current amplitude (I_{res}) by the peak amplitude (I_{peak}). b,c) Double pulse protocol for measuring CDI. *Left*, I_{Ca} is evoked by a 10-ms test pulse after a conditioning prepulse to various voltages as indicated. *Right,* normalized I_{Ca} for the test and prepulse are plotted against prepulse voltage. With physiological Ca^{2+} buffering (0.5 mM EGTA, *b*) but not with higher levels of Ca^{2+} buffering (10 mM BAPTA, *c*), maximal CDI of the test current occurs at prepulse voltages evoking peak inward current.

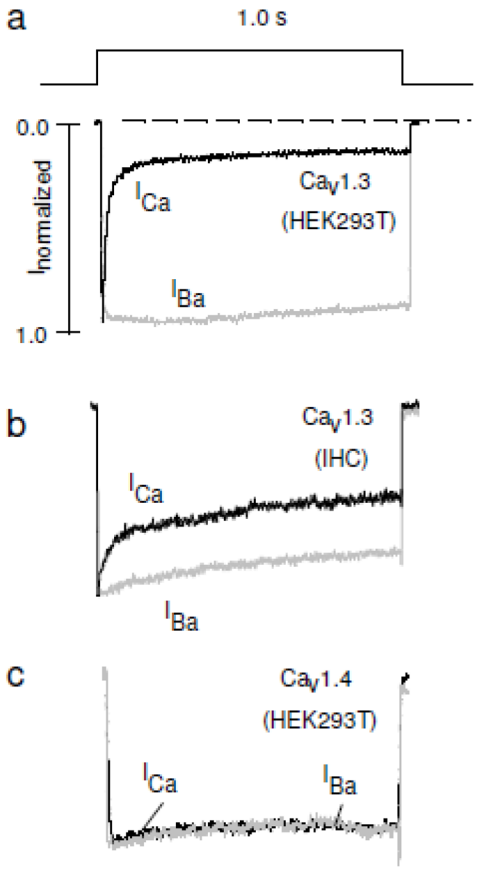

Figure 2.

CDI of Ca_v1 channels. I_{Ca} (black) or I_{Ba} (grey) were evoked by 1-s depolarizing pulses in HEK293T cells transfected with $Ca_v1.3$ (a) or $Ca_v1.4$ (c) or in mouse inner hair cells (IHC), which express predominantly $Ca_v1.3$ (b).

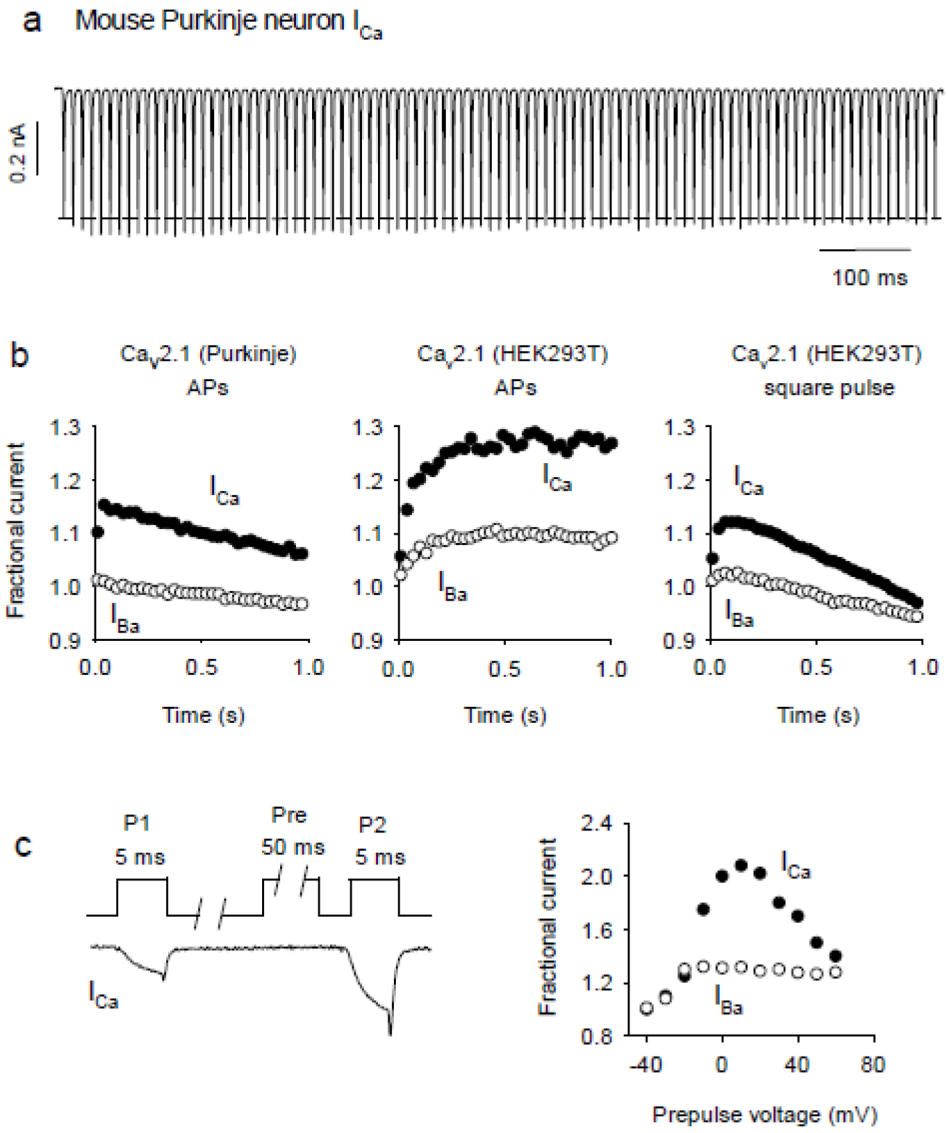

Figure 3.

Characterization of CDF using repetitive and double pulse voltage-clamp protocols. (a) I_{Ca} is evoked in mouse cerebellar Purkinje neurons by a train of action potential (AP) waveforms at 100 Hz. CDF is evident as an initial increase in the amplitude of I_{Ca} above the baseline level (dashed line). (b) CDF is measured by plotting the peak amplitude of test currents normalized to the first in the train for I_{Ca}(filled circles) or I_{Ba} (open circles). Shown are results from AP trains in mouse cerebellar Purkinje neurons and $Ca_V2.1$-transfected HEK293T cells, and square test pulses (−80 mV to +10 mV, 100 Hz) for $Ca_V2.1$-transfected HEK293T cells. (c) Double-pulse protocol measures CDF of $Ca_V2.1$ in transfected HEK293T cells. *Left,* I_{Ca} was evoked by test currents evoked before (P1) or after (P2) a conditioning prepulse (Pre). The prepulse-induced current is not sampled in the representative current trace. The ratio of P2:P1 current amplitude (Fractional current) is plotted against prepulse voltage for I_{Ca} (filled circles) and I_{Ba} (open circles).

Table 1

Classification and expression of voltage-gated Ca^{2+} channels[1]

$Ca_vX.X$	α_1 subunit[2]	Current[3]	Primary tissue distribution
1.1	α_{1S}	L-type	Skeletal muscle
1.2	α_{1C}	L-type	Heart, nervous system, endocrine glands
1.3	α_{1D}	L-type	Heart, nervous system, endocrine glands, inner ear
1.4	α_{1F}	L-type	Retina, T-cells
2.1	α_{1A}	P/Q-type	Nervous system
2.2	α_{1B}	N-type	Nervous system
2.3	α_{1E}	R-type[3]	Nervous system
3.1	α_{1G}	T-type	Nervous system, heart
3.2	α_{1H}	T-type	Nervous system, heart, adrenal gland
3.3	α_{1I}	T-type	Nervous system

[1] See [134] for references;

[2] according to former nomenclature, currently "α_1x.x";

[3] classical nomenclature based on native currents;

[3] $Ca_V2.3$ does not account for all R-type currents in neurons [135, 136]