Chapter 3
Reciprocal Regulation of Neuronal Calcium Channels by Synaptic Proteins

Norbert Weiss and Gerald W. Zamponi

Abstract Voltage-gated Ca^{2+} channels represent one of the main pathways for Ca^{2+} entry into nerve terminals where they play a critical role in the control of synaptic exocytosis. It is traditionally believed that the vesicle-docking/release machinery must be located in the vicinity of the calcium source in order to trigger fast, efficient and spatially delimited neurotransmitter release. This tight coupling is mostly achieved by a physical interaction of the presynaptic calcium channel with several actors of the synaptic vesicle release machinery. Conversely, the binding of synaptic proteins regulates calcium channel activity, providing for fine control of presynaptic Ca^{2+} entry. Here, we review the current state of knowledge of the molecular mechanisms by which synaptic proteins regulates presynaptic Ca^{2+} channel activity.

Keywords SNARE protein • Syntaxin • SNAP-25 • Synaptotagmin • Munc18 • Rim • Cysteine string proteins • Huntingtin

3.1 Introduction

Voltage-gated Ca^{2+} channels (VGCCs) are plasma membrane proteins that convert an electrical signal into intracellular Ca^{2+} elevations. To date, ten genes encoding the pore-forming subunits of mammalian VGCCs have been identified. Seven genes encode the high-voltage activated (HVA) channel subfamily (comprising L-type ($Ca_V1.1$ to $Ca_V1.4$), P/Q-type ($Ca_V2.1$), N-type ($Ca_V2.2$) and R-type ($Ca_V2.3$) channels) and three genes encode the low-voltage-activated (LVA) channel subfamily (composed exclusively of T-type ($Ca_V3.1$ to $Ca_V3.3$) (Ertel et al. 2000). In addition

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to the Ca_V pore-forming subunit, HVA channels are contain auxiliary subunits: β (β$_1$ to β$_4$, a 55 KDa cytosolic protein of the MAGUK (**m**embrane-**a**ssociated **gu**anylate **k**inase) family), α$_2$δ (α$_2$δ$_1$ to α$_2$δ$_4$, a 170 KDa highly glycosylated extracellular protein with a single transmembrane domain), and in some cases γ (γ$_1$ to γ$_8$, a 33 KDa transmembrane protein) (Takahashi et al. 1987). Among this wide diversity of native channels, $Ca_V2.1$ and $Ca_V2.2$ channels have been identified as the predominant Ca^{2+} channels involved in depolarization-evoked neurotransmitter release (Westenbroek et al. 1992, 1995, 1998; Olivera et al. 1994; Wheeler et al. 1994; Dunlap et al. 1995; Day et al. 1996; Timmermann et al. 2002). They support a transient Ca^{2+} microdomain of high concentration (10–50 μM) (Schneggenburger and Neher 2005) within the active zone of the synapse that is essential for the fusion of presynaptic vesicles with the plasma membrane (Llinas et al. 1992; Edwards 2007; Neher and Sakaba 2008; Weber et al. 2010). $Ca_V2.1$ channels support voltage-dependent exocytosis in the central nervous system, whereas $Ca_V2.2$ channels are critically involved at the peripheral synapses. In particular synapses, $Ca_V2.3$ channels are also expressed at sufficiently high density (Day et al. 1996; Hanson and Smith 2002) to support Ca^{2+} entry into presysnaptic terminals (Breustedt et al. 2003; Dietrich et al. 2003) and contribute to some extent to the release of neurotransmitters (Wu et al. 1998; Gasparini et al. 2001; Kamp et al. 2005). Finally, although HVA channels support voltage-dependent exocytosis, release of neurotransmitters at rest (i.e. around the resting membrane potential of neurons) has been demonstrated in some neurons and relies on LVA $Ca_V3.2$ channels (Ivanov and Calabrese 2000; Pan et al. 2001; Egger et al. 2003; Carabelli et al. 2007; Weiss and Zamponi 2012)

In order to efficiently receive Ca^{2+} signals, the vesicle-docking/ release machinery must be located in the vicinity of the source of Ca^{2+}. This is particularly important considering the high Ca^{2+} buffering capability of neurons (Foehring et al. 2009) and the consequent limited diffusion of free Ca^{2+}. In mammalian synapses, this close localization relies on the direct interaction of the Ca^{2+} channels with several members of the vesicle release machinery which is essential for fast (within 200 μs after the arrival of the action potential) and spatially delimited neurotransmitter release (Sabatini and Regehr 1996; Wadel et al. 2007). Conversely, binding of synaptic proteins potently regulates channel activity, providing a reciprocal control of Ca^{2+} entry to fine tune synaptic strength. Interested readers may also refer to the work of Atlas et al., for a discussion of other possible role of biochemical coupling of VGCCs with synaptic proteins (Atlas et al. 2001; Lerner et al. 2006; Marom et al. 2007; Hagalili et al. 2008; Atlas 2010; Cohen-Kutner et al. 2010; Marom et al. 2010; Weiss 2010).

3.2 Basic Principles of Molecular Coupling Between Voltage-Gated Ca^{2+} Channels and SNARE Proteins

SNARE proteins (**s**oluble **N**SF (*N*-ethylmaleimide-sensitive fusion protein) **a**ttachment protein **re**ceptor) comprising the Q-SNAREs syntaxin-1A/1B, SNAP-25 (synaptosomal-associated protein of 25 kDa) and R-SNARE synaptobrevin

(VAMP) (Fasshauer et al. 1998; Sutton et al. 1998) form the SNARE core complex that brings the vesicle and target membranes into close opposition, leading to fusion and exocytosis (Hanson et al. 1997; Otto et al. 1997). Not surprisingly, $Ca_V2.1$ and $Ca_V2.2$ channels are presynaptically colocalized with syntaxin-1A at nerve terminals (Cohen et al. 1991; Westenbroek et al. 1992, 1995) and have been biochemically isolated in complex with SNARE proteins (Bennett et al. 1992; Yoshida et al. 1992; Leveque et al. 1994). Molecular characterization of Ca^{2+} channels/SNARE interaction has identified a *synprint* (*synaptic protein interaction*) locus in $Ca_V2.1$ and $Ca_V2.2$ located within the intracellular loop between domains II and III of the channels (Sheng et al. 1994; Rettig et al. 1996). This motif binds syntaxin-1A and SNAP-25 (but not synaptobrevin). Further biochemical mapping of the *synprint* site has identified two distinct microdomains separated by a flexible linker that independently binds syntaxin-1A and SNAP-25 (Rettig et al. 1996; Yokoyama et al. 2005). The functional relevance of the interaction has been shown by disruption of the Ca^{2+} channel/SNAREs coupling using peptides derived from the *synprint* domain (or by direct deletion of the *synprint* site) that alters synaptic transmission (Mochida et al. 1996; Rettig et al. 1997; Harkins et al. 2004; Keith et al. 2007). However, although the *synprint* site is unambiguously of key importance for fast and efficient neurotransmitter release, there is evidence that some other channel isoforms such as T-type channels, although devoid of the consensus *synprint* site, functionally contribute to presynaptic Ca^{2+} elevations and neurotransmitter release, suggesting the existence of other molecular coupling determinants. Indeed, we recently demonstrated that syntaxin-1A and SNAP-25 biochemically interact with $Ca_V3.2$ T-type channels within the carboxy-terminal domain of the channel (Weiss et al. 2012; Fig. 3.1).

3.3 Functional Interaction of Voltage-Gated Ca^{2+} Channels with SNARE Proteins

SNARE proteins not only bring presynaptic vesicles close to the Ca^{2+} source but also potently modulate channel gating to fine tune presynaptic Ca^{2+} entries and synaptic transmission (Fig. 3.2).

3.3.1 Syntaxin-1A

The notion that SNARE proteins modulate Ca^{2+} influx through VGCC arose from electrophysiological recordings in heterologous expression systems showing that coexpression of syntaxin-1A potently modulates $Ca_V2.1$ and $Ca_V2.2$ gating by shifting the voltage-dependence of inactivation toward more negative membrane potentials (Bezprozvanny et al. 1995; Wiser et al. 1996; Zhong et al. 1999; Degtiar et al. 2000), thus silencing the channels and reducing presynaptic Ca^{2+} entry influx. This regulation was later confirmed in chick ciliary ganglion neurons and

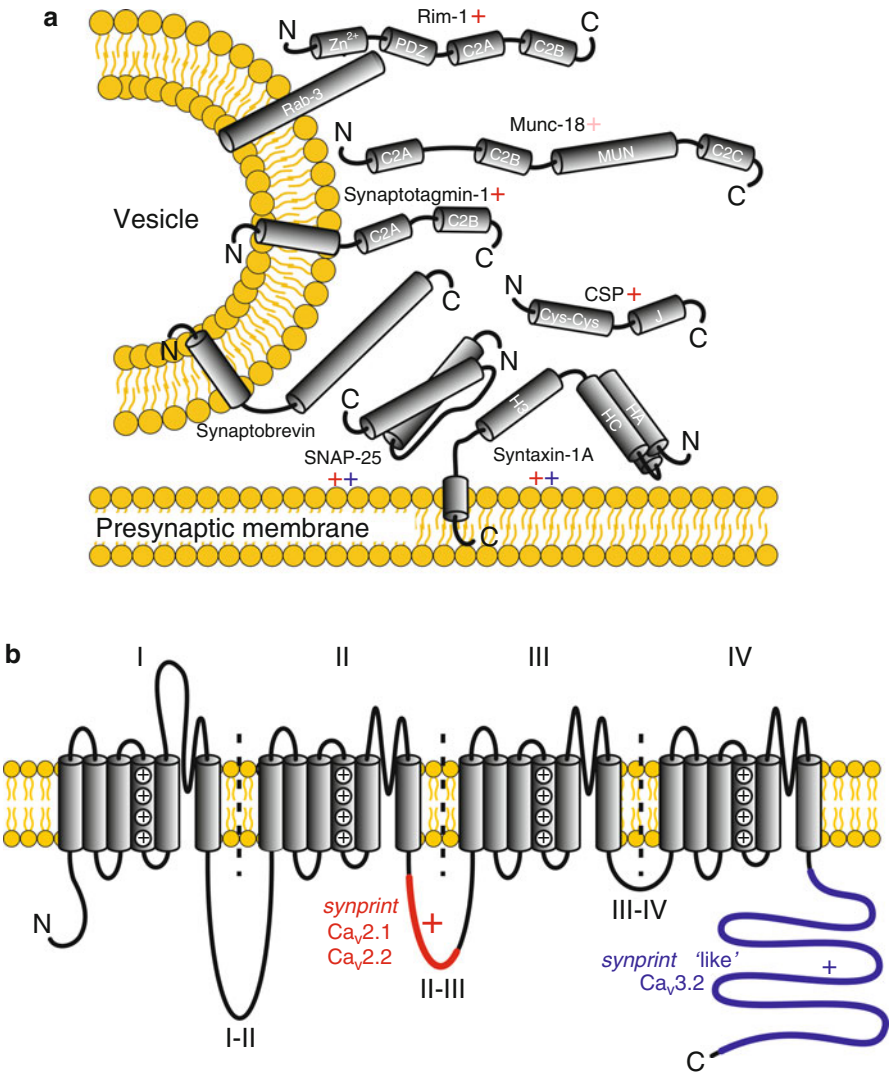

Fig. 3.1 Biochemical interactions between presynaptic Ca^{2+} channels and synaptic proteins. (**a**) Schematic representation of key synaptic proteins involved in the regulation of presynaptic Ca^{2+} channels. (**b**) Putative membrane topology of voltage-gated Ca^{2+} channels. The *synprint* domain found in $Ca_V2.1$ and $Ca_V2.2$ channels (located within the intracellular loop between domains II and III of the channel) is shown in *red*. In contrast, the *synprint* "like" domain found in $Ca_V3.2$ T-type located within the carboxy-terminal of the channel is shown in *blue*. *The red plus* "+" signs indicate proteins that interact directly with the *synprint* site, whereas the *blue plus signs* indicate those interacting with the *synprint* "like" domain of $Ca_V3.2$ T-type channels. Binding of Munc-18 with $Ca_V2.2$ channels has been shown, but the involvement of the *synprint* domain requires further investigations (Adapted from (Davies and Zamponi 2008; Abbreviations: *Rim-1* Rab-3 interacting molecule-1, *CSP* Cysteine String Proteins)

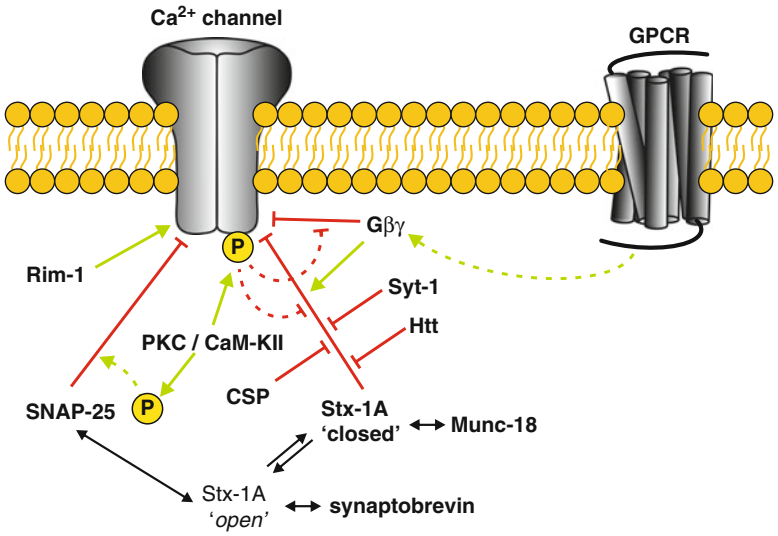

Fig. 3.2 Functional interactions between presynaptic Ca^{2+} channels, synaptic proteins and second messengers. The SNARE syntaxin-1A and SNAP-25, as well as release of free G-protein βγ dimer upon GPCR activation inhibit channel activity. In contrast, synaptotagmin-1, CSP and huntingtin prevent syntaxin-1A-dependent inhibition of the channel. Similarly, channel phosphorylation by PKC or CaM-KII prevents syntaxin-1A and Gβγ-dependent inhibition, while phosphorylation of SNAP-25 promotes its inhibitory effect. Rim-1 directly potentiates Ca^{2+} influx (Abbreviations: *Stx1A* syntaxin-1A, *Syt-1* synaptotagmin-1, *Rim-1* Rab-3 interacting molecule-1, *CSP* Cysteine String Proteins, *Htt* huntingtin, *PKC* protein kinase C, *CaM-KII* Ca^{2+}/calmodulin-dependent protein kinase II, *GPCR* G-protein coupled receptor. *Arrows in red* indicate an inhibitory regulation whereas *arrows in green* indicate a potentiation)

isolated mammalian nerve terminals (synaptosomes) upon application of botulinium neurotoxin C1 (BoNT/C1 which cleaves syntaxin-1A from its membrane anchoring domain). BoNT/C1 treatment shifted the voltage-dependence of inactivation of the channel toward depolarized potentials (Bergsman and Tsien 2000; Stanley 2003). Structure/function studies have identified the transmembrane domain of syntaxin-1A, in particularly the two cysteines (C271 and C272) (Trus et al. 2001), as well as a short stretch within the H3 helical cytoplasmic domain, as fundamental for channel modulation without a direct implication in biochemical interaction with the *synprint* domain (Bezprozvanny et al. 2000; Jarvis et al. 2002). More recently, it was also shown that besides binding the *synprint* site of $Ca_V2.2$ channels, the ten amino-terminal residues of syntaxin-1A might support inhibition of the channel (Davies et al. 2011). Although the exact molecular mechanism of syntaxin-1A mediated regulation of Ca^{2+} channels remains unclear, these results highlight the existence of two kinds of interaction of syntaxin-1A with the channel: (i) a biochemical interaction via the *synprint* domain and (ii) a functional interaction most likely involving additional yet unidentified channel determinants. Consistent with this idea, T-type Ca^{2+} channels that biochemically couple to syntaxin-1A

via different channel binding determinants than the consensus *synprint* domain, are subject to similar syntaxin-1A-dependent modulation as $Ca_V2.1$ and $Ca_V2.2$ channels. Similarly, for $Ca_V2.3$ channels, despite being devoid of the consensus *synprint* domain, various studies indicate that a similar syntaxin-1A-dependent regulation must occur (Bergsman and Tsien 2000; Wiser et al. 2002; Cohen and Atlas 2004). These observations suggest that gating modulation of VGCCs by syntaxin-1A likely involves modulatory channel determinants distinct from the anchoring domains. Dynamic intramolecular interactions between the intracellular loops of $Ca_V2.1$ and $Ca_V2.2$ channels have be reported and appear to be involved in channel gating behavior (Restituito et al. 2000; Raghib et al. 2001; Geib et al. 2002; Page et al. 2004, 2010; Sandoz et al. 2004; Agler et al. 2005; Bucci et al. 2011). Hence, channel remodeling upon syntaxin-1A binding might represent a possible consensus molecular mechanism by which syntaxin-1A modulates $Ca_V2.x$ and $Ca_V3.2$ channels in a similar manner despite distinct coupling molecular determinants. Interestingly, a mutation (A454T) that segregates with familial hemiplegic migraine patients located within the intracellular linker between domains I and II of $Ca_V2.1$ channel alters both syntaxin-1A-dependent channel gating modulation and exocytosis (Serra et al. 2010), suggesting that the I-II channel loop could play an important role in mediating syntaxin-1A modulation. In addition, a conformational switch of syntaxin-1A has been reported, that depends of the molecular partners engaged in the macromolecular complex. Hence, syntaxin-1A presents a *closed* conformation in complex with munc18 (or in isolation) and switches to an *open* conformation when in complex with SNAP-25 or synaptobrevin-2 (Dulubova et al. 1999; Brunger 2001). Interestingly, whereas syntaxin-1A in its *closed* state potently modulates $Ca_V2.2$ and $Ca_V3.2$ channel activity, coexpression of an *open* syntaxin-1A (locked open by two point mutations (Dulubova et al. 1999)) no longer alters channel gating (Jarvis et al. 2002; Weiss et al. 2012). This conformational switch of syntaxin-1A appears to be of key physiological importance since it occurs during the vesicle release cycle (Dulubova et al. 1999; Fiebig et al. 1999; Richmond et al. 2001), suggesting that syntaxin-1A may dynamically and temporally controls presynaptic Ca^{2+} entry during the exocytosis process.

3.3.2 SNAP-25

Like syntaxin-1A, SNAP-25 non-competitively binds onto the *synprint* domain of $Ca_V2.1$ and $Ca_V2.2$ channels, as well as onto the carboxy-terminal domain of $Ca_V3.2$ channels, to produce a similar inhibitory channel gating modulation (Wiser et al. 1996; Zhong et al. 1999; Weiss et al. 2012). The functional modulation produced by SNAP-25 was recently indirectly confirmed in native systems where siRNA silencing of SNAP-25 in glutamatergic neurons produced an increase of Ca^{2+} currents carried by $Ca_V2.1$ channels due to a depolarizing shift of the voltage-dependence of inactivation (Condliffe et al. 2010; Condliffe and Matteoli 2011). Interestingly, this inhibitory regulation is no longer observed when SNAP-25 is

co-expressed with syntaxin-1A (Wiser et al. 1996; Zhong et al. 1999; Jarvis and Zamponi 2001a; Weiss et al. 2012), suggesting that association of SNAP-25 with syntaxin-1A during the vesicle release cycle relieves channel inhibition, allowing timely presynaptic Ca^{2+} elevation required for membrane fusion and exocytosis (Sudhof 2004). Consistent with a key physiological importance of SNAP-25, structural or expression alterations of the protein caused by genetic mutations have been associated with numerous neuropsychiatric and neurological disorders, likely because of mis-regulation of presynaptic Ca^{2+} channels (Corradini et al. 2009). Finally, it was reported that phosphorylation of SNAP-25 by protein kinase C is required for SNAP-25-dependent inhibition of VGCCs (Pozzi et al. 2008), suggesting that like syntaxin-1A, SNAP-25-dependent modulation of channel activity may involve molecular determinants other than the *synprint* site.

3.4 Modulation of Presynaptic Calcium Channels by Non SNARE Proteins

3.4.1 Synaptotagmin-1

Although part of the vesicular release complex, synaptotagmin-1 is not as essential as syntaxin-1A or SNAP-25 in the membrane fusion process per se (Tucker and Chapman 2002), but rather works as a Ca^{2+} sensor, forming the link between presynaptic Ca^{2+} elevation and vesicular fusion, essential for fast and synchronous neurotransmission release (DeBello et al. 1993; Geppert et al. 1994; Augustine 2001; Fernandez-Chacon et al. 2001; Nishiki and Augustine 2001; Tucker and Chapman 2002; Koh and Bellen 2003; Xu et al. 2007). Indeed, synaptotagmin-1 is characterized by an amino-terminal transmembrane region anchored in the vesicle, a variable linker, and two carboxy-terminal rich negatively charged domains (C2A and C2B), each capable of binding Ca^{2+}. Hence, Ca^{2+} binding onto the C2A domain contributes to the insertion of synaptotagmin-1 into the plasma membrane, bringing vesicles docked to the plasma membrane upon Ca^{2+} elevation (Fernandez-Chacon et al. 2001). In contrast, the C2B domain has been reported to biochemically interact with the *synprint* site of $Ca_V2.1$ and $Ca_V2.2$ channels (Sheng et al. 1997). Although binding of synaptotagmin-1 has no major effect on channel gating, it reduces syntaxin-1A-dependent inhibition of $Ca_V2.2$ channels, possibly by Ca^{2+}-dependent binding competition with syntaxin-1A (Sheng et al. 1996). Hence, syntaxin-1A preferentially interacts with the channel at rest (i.e. at low Ca^{2+} level) thus preventing channel activity, whereas presynaptic Ca^{2+} elevation favors its interaction with synaptotagmin-1 and Ca^{2+} entry through VGCCs that is required for the final fusion process of docked vesicles. Moreover, a Ca^{2+}-dependent synaptotagmin-1 interaction with the $Ca_V\beta_{4a}$ auxiliary-subunit of VGCCs has been reported (Vendel et al. 2006), providing another dynamic Ca^{2+} channel/vesicle interaction (Weiss 2006).

3.4.2 Mnc-18

As for synaptotagmin-1, Munc18 belongs to the C2-domain containing protein family, and plays a fundamental role in the assembly/disassembly of the exocytosis machinery (Gulyas-Kovacs et al. 2007; Toonen and Verhage 2007). Genetic ablation of Munc-18 in mice leads to a complete loss of synaptic transmission (Verhage et al. 2000). Although a biochemical interaction of Munc-18 with $Ca_V2.2$ channel within the intracellular linker between domains II and III has been reported (Chan et al. 2007), its coexpression has no effect on channel gating (Gladycheva et al. 2004). Hence, Munc-18 appears to not be a direct modulator of Ca^{2+} channel activity but rather interferes with syntaxin-1A-dependent channel inhibition during the vesicle release cycle as previously mentioned (Dulubova et al. 1999; Brunger 2001). This occurs by stabilizing syntaxin-1A in a *closed* conformation (Jarvis et al. 2002), inhibiting Ca^{2+} channel activity and non-necessary presynaptic Ca^{2+} entry in the absence of docked vesicle.

3.4.3 Rim-1

Rim (Rab-3 interacting molecule) is also part of a family of vesicle-associated proteins whose members share C2 domains. By interacting with numerous components of the presynaptic active zone such as SNAP-25 or synaptotagmin-1 (Coppola et al. 2001), it forms a protein scaffold by participating in the docking and fusion of presynaptic vesicles (Wang et al. 2000; Betz et al. 2001; Coppola et al. 2001; Ohtsuka et al. 2002; Schoch et al. 2002; Kaeser et al. 2011). Essential for short- and long-term synaptic plasticity by affecting the readily releasable pool of vesicles (Castillo et al. 2002, 2002; Blundell et al. 2010; Deng et al. 2011; Han et al. 2011), Rim proteins are also essential for proper targeting of Ca^{2+} channels to presynaptic terminals (Han et al. 2011) and efficient neurotransmitter release (Schoch et al. 2006). Although biochemical studies using native synapstosome membrane preparations failed to demonstrate the existence of a Ca^{2+} channel/Rim complex (Wong and Stanley 2010), various studies report in vitro bindings of Rim with Ca^{2+} channel components. Indeed, direct interaction of Rim-1 with the *synprint* site of $Ca_V2.2$ channels has been shown (Coppola et al. 2001). Moreover, Rim Binding Proteins directly interact with $Ca_V2.2$ channels (and likely with $Ca_V2.1$ channels), possibly providing a molecular link between Ca^{2+} channels and Rim proteins (Hibino et al. 2002). Finally, biochemical interaction of Rim-1 with $Ca_V\beta$ subunits has been reported, slowing $Ca_V2.1$, $Ca_V2.2$ and $Ca_V2.3$ channel inactivation when coexpressed in heterologous systems, thereby increasing Ca^{2+} influx during trains of action potentials (Kiyonaka et al. 2007), and a mutation in Rim-1 (R655H) associated with an autosomal dominant cone-rod dystrophy was found to alter Rim-1-dependent modulation of $Ca_V2.1$ channels gating (Miki et al. 2007) leading to a progressive loss of photoreceptors along with retinal degeneration (Barragan et al. 2005; Michaelides et al. 2005). Altogether, these results highlight the critical role of Rim-1 in the modulation of Ca^{2+} homeostasis at nerve terminals.

3.4.4 Cysteine String Proteins (CSP)

Cysteine String Proteins (CSP) are vesicle-associated protein with a key chaperone role at the synapse (Chamberlain and Burgoyne 2000). It was proposed that CSP may serve as a link between $Ca_V2.2$ channels and presynaptic vesicles (Mastrogiacomo et al. 1994). Indeed, CSP interacts with the *synprint* motif of $Ca_V2.1$ (Leveque et al. 1998; Seagar et al. 1999; Magga et al. 2000) and $Ca_V2.2$ channels (Magga et al. 2000). Moreover CSP promotes presynaptic Ca^{2+} influx by recruiting dormant Ca^{2+} channels (Chen et al. 2002). Although the molecular mechanism by which CSP promotes channel activity remains unknown, considering that CSP interacts with syntaxin-1A (Nie et al. 1999; Wu et al. 1999), it is possible that binding of CSP onto syntaxin-1A prevents syntaxin-1A-dependent channel inhibition. Hence, like synaptotagmin-1, CSP may act as a molecular channel switch activity between undocked and docked vesicles release for timely control or presynaptic Ca^{2+} influx.

3.4.5 Huntingtin

Huntingtin (Htt) is well known for its implication in Huntington's disease (Ross and Tabrizi 2011) but the exact cellular function of the protein remains unclear. However, the observation that genetic ablation of Htt is lethal in mice highlights the fundamental importance of the protein (Nasir et al. 1995). Besides interacting with numerous proteins (to date at least 20 proteins involved in gene transcription, cellular transport or cell signaling has been shown to interact with Htt), Htt directly binds to the *synprint* domain of $Ca_V2.2$ channels (Swayne et al. 2005). However, as synaptotagmin-1, coexpression of Htt with $Ca_V2.2$ channels has no consequence on channel gating, but prevents syntaxin-1A-dependent regulation (Swayne et al. 2005), likely by displacing binding of syntaxin-1A from the channel (Swayne et al. 2006). Hence, Htt is not a direct channel modulator per se, but might represent an important actor of synaptic activity by influencing SNARE modulation. However, it remains unclear if Htt is permanently expressed at the synapse under normal condition, or if it is specifically targeted under particular physiopathological states.

3.5 Modulation of Calcium Channels by Other Signaling Pathways

3.5.1 G-Protein Coupled Receptors

Ca^{2+} entry into presynaptic terminals is also modulated by the activation of numerous G-protein-coupled receptors (GPCRs) and second messengers (Jarvis and Zamponi 2001b). Indeed, activation of specific GPCRs following the liberation of

neurotransmitters initiates a negative feedback regulation on presynaptic VGCCs, inhibiting presynaptic Ca^{2+} entry thus terminating synaptic transmission (Brown and Sihra 2008). This spatially delimited regulation (Forscher et al. 1986) relies on the direct binding of free G-protein βγ dimers release upon GPCR activation (Herlitze et al. 1996; Ikeda 1996) to specific intracellular regions of the channel (De Waard et al. 1997, 2005; Zamponi et al. 1997; Tedford and Zamponi 2006). Interestingly, it was shown that cleavage of syntaxin-1A by the botulinium neurotoxin C1 in chick calyx synapses prevents G-protein-dependent inhibition of Ca^{2+} currents, suggesting the involvement of syntaxin-1A in presynaptic G-protein regulation of Ca^{2+} channels (Stanley and Mirotznik 1997; Silinsky 2005). In vitro studies have later revealed an interaction of syntaxin-1A with G-protein βγ dimers, and although syntaxin-1A is not critical for G-protein regulation it potentiates the inhibition in a receptor-independent manner (Jarvis et al. 2000; Jarvis and Zamponi 2001a; Lu et al. 2001). Moreover, G-protein βγ dimers not only interact with syntaxin-1A, but also with SNAP-25 to mediate presynaptic inhibition (Gerachshenko et al. 2005). Finally, besides to modulate Ca^{2+} channel activity, it was shown that G-protein βγ dimers and synaptotagmin-1 compete for binding to the core SNARE complex in a Ca^{2+}-dependent manner such that at high Ca^{2+} concentration synaptotagmin-1 can displace Gβγ binding (Yoon et al. 2007). Hence, Ca^{2+} elevation in presynaptic terminals may prevent G-protein inhibition, likely by preventing binding of Gβγ with SNARE proteins (Yoon et al. 2007). To add further complexity, it was recently reported that Rim-1 promotes relief of G-protein inhibition of $Ca_V2.2$ channels by modulating channel inactivation (Weiss et al. 2011). Altogether, these results highlight the extreme interplay between GPCR-dependent regulation and the molecular actors of the exocytosis process to fine tune presynaptic Ca^{2+} entry.

3.5.2 Phosphorylation

In vitro studies have shown that the protein kinase C (PKC), as well as the Ca^{2+}-calmodulin-dependent kinase II (CaM-KII) are able to phosphorylate the *synprint* domain of $Ca_V2.2$ channels (Yokoyama et al. 1997, 2005), preventing binding of syntaxin-1A and SNAP-25 (Yokoyama et al. 1997) and thus preventing SNARE-dependent inhibition of the channel (Jarvis and Zamponi 2001a). However, uncoupling of SNARE proteins from the channel upon *synprint* phosphorylation, most likely represent a termination signal for synaptic exocytosis. It was reported that phosphorylation of the *synprint* site of $Ca_V2.1$ channels by the glycogen synthase kinase-3 (GSK-3) prevents SNARE interaction with the channel but also inhibits synaptic exocytosis possibly by interfering with Ca^{2+} channel/SNARE coupling (Zhu et al. 2010). Interestingly, phosphorylation of syntaxin-1A and SNAP-25 by PKC or CaM-KII does not alter interaction with the *synprint* site (Yokoyama et al. 1997), Hence, PKC- and CaM-KII-dependent phosphorylation of

the *synprint* site may serve as a biochemical switch for interaction/modulation of voltage-gated Ca^{2+} channels with SNARE protein complexes.

3.6 Concluding Remarks

Like most key cellular functions, control of neurotransmitter release by presynaptic Ca^{2+} channels is highly regulated. The various components of the exocytosis machinery, besides localizing the vesicles within the vicinity of the source of Ca^{2+}, provide a potent reciprocal control of presynaptic Ca^{2+} influx by modulating channel gating. This dynamic regulation appears to be fundamental to dynamically and temporally fine tune neurotransmitter release. Surprisingly, as highlighted in this chapter, the regulation of presynaptic Ca^{2+} channels appears extremely complex, with intricate interplay between different types of synaptic proteins and second messenger signaling pathways, but also highly redundant. This important redundancy in Ca^{2+} channel regulation by various presynaptic proteins might ensure a security control over a fundamental physiological function. Finally, although $Ca_V2.1$/$Ca_V2.2$ and $Ca_V3.2$ T-type channels use completely distinct channel molecular determinants to interact with the vesicular machinery, they are functionally regulated by syntaxin-1A and SNAP-25 in a strikingly similar manner. This may perhaps underscore the fundamental importance of localizing the exocytosis machinery near the source of Ca^{2+}, and providing tight control over Ca^{2+} entry.

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