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Mini Review

Ubiquitination and proteasome-mediated degradation of voltage-gated Ca^{2+} channels and potential pathophysiological implications

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Abstract. Recent findings have revealed a fundamental role of the ubiquitin-proteasome system (UPS) in the regulation of voltage-gated Ca^{2+} channels (VGCCs). It has been proposed that the ubiquitination-deubiquitination balance dictates the number of channels expressed at the plasma membrane, which in turn influences a number of physiological and pathophysiological processes. This minireview surveys recent studies showing that VGCCs may be ubiquitinated in an unexpectedly complex manner, and highlights the role of the UPS in the regulation of the channels, focusing on the mechanisms that control their cell surface expression. The exciting new findings in this emerging field suggest that the turnover of VGCCs may be determined to a large degree by the activity of the UPS, and that alteration of the UPS molecular machinery may be one of the underlying mechanisms occurring in a number of channelopathies.

Key words: Ca^{2+} channels — Ubiquitination — UPS — VGCC

Voltage-gated Ca^{2+} channels (VGCCs) are multisubunit protein complexes embedded in the plasma membrane of a variety of cell types that couple electrical membrane potential with intracellular Ca^{2+} elevations and downstream Ca^{2+}-dependent signaling processes (Catterall 2011; Felix et al. 2013). Indeed, Ca^{2+} influx through VGCCs serves as a second messenger of electrical signaling, initiating a myriad of physiological events ranging from gene regulation to cell differentiation, hormone secretion to neurotransmission, and heartbeat to movement. To date, 10 genes encoding the pore-forming subunits of VGCCs have been identified and classified into two subfamilies including high voltage-activated (HVA) and low voltage-activated (LVA) channels. While the HVA subfamily consists of 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; the LVA subfamily is exclusively composed of T-type channels ($Ca_V3.1$ to $Ca_V3.3$) (Ertel et al. 2000; Catterall 2011; Felix et al. 2013). The pore-forming subunits share a similar membrane topology that consists of four homologous domains of six putative transmembrane helices (S1–S6), each connected with cytoplasmic loops that provide molecular hubs for interaction and regulation of the channel by regulatory proteins. In addition, these main subunits are surrounded by ancillary subunits including Ca_Vß and $Ca_V\alpha_2\delta$, and in some circumstances $Ca_V\gamma$, which play essential roles in the trafficking and gating of the channels (Catterall 2011; Felix et al. 2013; Geisler et al. 2015; Lazniewska and Weiss 2016).

Posttranslational modification has emerged as an essential mechanism to control the expression and function of ion channels (Lazniewska and Weiss 2014). Over the last few years, it has become evident that the ubiquitin-proteasome system (UPS) plays a major role in regulating the expression of VGCCs. Hence, covalent bound of ubiquitin moieties to the channel protein plays an essential role in regulating channel density at the cell surface. Ubiquitin is a small (~8 kDa) regulatory protein, which binds to lysine residues within the target protein. As for other membrane proteins, ubiquitination of VGCCs requires three successive steps. First, ubiquitin is bound and activated by an ubiquitin-activating enzyme (E1), and then transferred to an ubiquitin-conjugating enzyme (E2). This complex further interacts with an ubiquitin-protein ligase (E3) that ultimately ubiquitinates the substrate. E3 enzymes are responsible for the specificity of the cascade as they bind to a recognition domain within the

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target protein (Hershko and Ciechanover 1998). Similarly to phosphorylation, the ubiquitination process is reversible and requires the activity of deubiquitinating enzymes (DUBs). Likewise, the density of ubiquitinated proteins depends on the pattern of ubiquitination. While monoubiquitinated proteins usually get internalized and are subject to either lysosomal degradation or secondary back recycling to the plasma membrane, polyubiquitinated proteins are generally degraded through the proteasome, a macromolecular complex of proteases (Hershko and Ciechanover 1998).

The role of the UPS is a relatively novel aspect in the control of VGCCs. Initial studies suggested that the Ca_Vß ancillary subunit might enhance surface expression of the channel proteins by antagonizing an endoplasmic reticulum (ER) retention signal in the alpha interaction domain (AID) located in the I-II loop of the pore-forming subunit (Bichet et al. 2000). However, the observation that removing the AID motif in $Ca_V2.1$ channels does not potentiate expression of the channel at the cell surface (Maltez et al. 2005) suggested the existence of other underlying mechanisms controlling Ca_Vß-mediated expression of VGCCs at the cell surface. More recently, it was proposed that the Ca_Vβ ancillary subunit promotes cell surface expression of Ca_V1 and Ca_V2 channels by protecting the channel proteins from proteasomal degradation (Altier et al. 2011; Rougier et al. 2011; Waithe et al. 2011; Fig. 1). Indeed, by preventing the

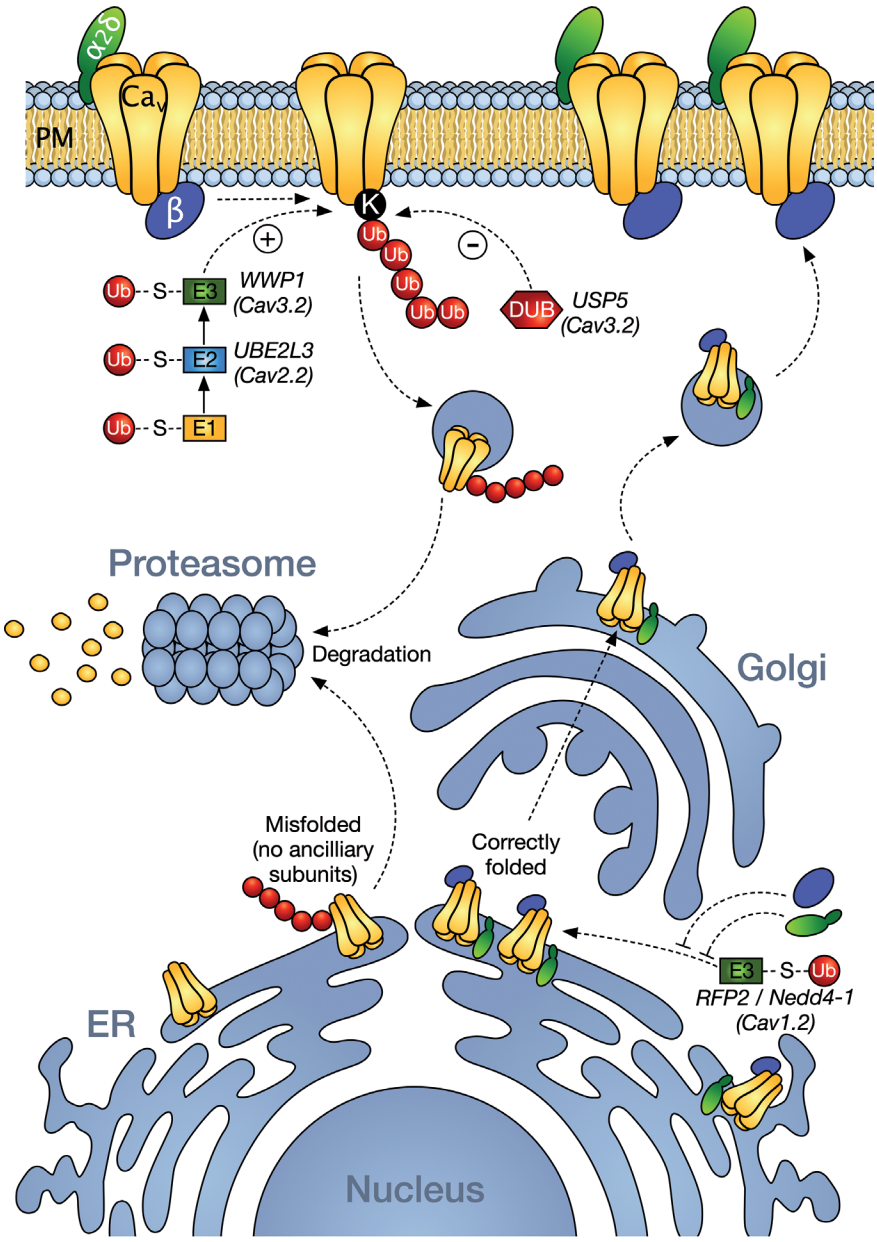

Figure 1. Molecular mechanisms involved in the degradation of voltage-gated Ca^{2+} channels (VGCCs) by the ubiquitin-proteasome system. VGCC pore-forming subunits can be ubiquitinated on lysine residues (K). Ubiquitin (Ub) is sequentially carried by three enzymes: Ub-activating (E1), Ub-conjugating (E2), and Ub-ligase (E3), respectively. Three different E3 enzymes, WWP1, RFP2 and Nedd4-1, has been shown to specifically recognize VGCCs and ubiquitinate them. UBE2L3 is the only E2 found to be involved in the regulation of VGCCs. The auxiliary Ca_Vß and $Ca_V\alpha_2\delta$ subunits may prevent ubiquitination and proteasomal degradation, and consequently promote the functional expression of the channels at the plasma membrane (PM). On the other hand, misfolded channel complexes in the endoplasmic reticulum (ER) are ubiquitinated and targeted for proteasome-mediated degradation. Evidence also suggests that the deubiquitinating enzymes (DUBs) such as the isopeptidase T (USP5) can remove Ub moieties from the VGCC proteins. Though it is acknowledged that the ubiquitination-deubiquitination control the steady state expression of the channel at the cell surface, many aspects of this process are still under investigation.

association of the channel with ubiquitin ligases and/or masking ubiquitination sites, the $Ca_V\beta$ subunit may act as a molecular switch that triggers the exit of the channel from the ER, and diverting $Ca_V1.2$ channels from the ER-associated protein degradation (ERAD) complex (Altier et al. 2011). Interestingly, the E3 ubiquitin ligase RFP2 seems to play a key role in regulating the ubiquitination and expression of $Ca_V1.2$ channels. Originally recognized as a tumor suppressor, RFP2 was shown to be a RING-finger ubiquitin ligase involved in ERAD, and is the typical example of a specific ubiquitin ligase linked to channel regulation at the level of the ER. This notion is supported by the observation that the calcium current density is significantly increased in a neuronal cell line expressing a dominant negative-RFP2 construct (Altier et al. 2011).

In parallel, a $Ca_V\beta$- and Nedd4-1-dependent mechanism controlling the sorting of newly synthesized $Ca_V1.2$ channels was uncovered (Rougier et al. 2011). In this report, the authors proposed that the E3 ubiquitin ligase Nedd4-1 promotes degradation of $Ca_V\beta$-deficient channels by UPS without transiting to the plasma membrane. In agreement with these results, proteasomal degradation of $Ca_V2.2$ channels may also be prevented by the $Ca_V\beta$ subunit (Waithe et al. 2011). This notion is supported by the observation that $Ca_V2.2$ channels containing a mutation (W391A) that prevents binding of the $Ca_V\beta$ subunits with the I-II linker are degraded more rapidly than its wild-type counterpart (Waithe et al. 2011).

The occurrence of alternative channel splice variants may also influence ubiquitination of $Ca_V2.2$ channels. Lipscombe and colleagues demonstrated that the e37a splice variant with restricted tissue expression have reduced ubiquitination levels compared to the more broadly expressed e37b variant, which is closely correlated with a reduced expression of functional $Ca_V2.2$ channels at the cell surface (Marangoudakis et al. 2012; Lipscombe et al. 2013). Indeed, ubiquitination of the $Ca_V2.2$ e37b variant occurs on lysine K1751 and K1762 that are absent in the e37a splice variant. Interestingly, a homologous region in the C-terminal domain of the $Ca_V1.2$ channels has also been reported to be essential in retaining the channel protein in the ER, preventing its trafficking to the plasma membrane (Altier et al. 2011). It is also worth mentioning that the effect of UPS inhibition on the functioning of $Ca_V2.2$ channels was also assessed, and the results showed that the calcium conductance in tsA201 cells after treatment with the proteasome inhibitor MG132 was increased in cells expressing the $Ca_V2.2$-e37a channel variant compared to cells expressing the $Ca_V2.2$-e37b variant (Marangoudakis et al. 2012).

More recently, two interesting reports revealed a novel functional coupling between the light chain (LC) of the microtubule associated protein B (MAP1B) and $Ca_V2.2$ channels, which may contribute to regulate the cell surface density of the channel. In a first study, the authors showed that the $Ca_V2.2$ channel is part of a macromolecular complex including not only LC1 but also the E2 ubiquitin conjugase UBE2L3 (Gandini et al. 2014a). This was the first evidence that a specific E2 ubiquitin conjugase is involved in the regulation of a VGCC. In this context, ubiquitination of the channel protein may be achieved through the action of UBE2L3 with the guidance of an as-yet unidentified ubiquitin ligase, although the selection of the substrate for ubiquitination (i.e. the $Ca_V2.2$ subunit) appears to be mainly under the control of LC1 (Gandini et al. 2014a). In a second report, the authors demonstrated that LC1 may interact with the two main splice variants of the $Ca_V2.2$ channel (e37a and e37b) and that LC1-mediated regulation may involve the internalization of the channel protein *via* a dynamin and clathrin-dependent pathway (Fig. 1). In addition, it was proposed that this regulatory mechanism might be conserved between $Ca_V2.1$ and $Ca_V2.2$ channels (Gandini et al. 2014b).

Likewise, it was established that ubiquitination-deubiquitination balance of $Ca_V3.2$ channels is an important mechanism for regulating T-type channel activity in primary afferent nociceptive neurons. Zamponi and colleagues demonstrated that $Ca_V3.2$ channels are ubiquitinated by the E3 ubiquitin ligase WWP1, whereas the deubiquitinating enzyme USP5 removes ubiquitin moieties from the channel protein, hence controlling the steady state expression of the channel at the cell surface (García-Caballero et al. 2014). In addition, and consistent with the role of T-type channels in peripheral painful neuropathy (Zamponi et al. 2015), *in vivo* inhibition of USP5 activity, either using shRNA to knockdown the expression level of USP5 or by preventing the formation of $Ca_V3.2$/USP5 complex, produced a significant decrease of T-type channel activity, and conferred potent analgesia in both inflammatory and neuropathic mouse models of neuropathic pain (García-Caballero et al. 2014, 2016; Gadotti et al. 2015). In addition to USP5, a role for the deubiquitinase USP2-45 in the control of VGCCs was also documented (Rougier et al. 2015). USP2-45 seems to bind the ancillary $Ca_V\alpha_2\delta$-1 subunit, which may act as an anchor allowing deubiquitination of the $Ca_V1.2$ channels. Hence, USP2-45 may promote the deubiquitination of both $Ca_V1.2$ and $Ca_V\alpha_2\delta$-1 subunits, reducing the steady state expression of $Ca_V1.2$ channels at the cell surface by disrupting the chaperone role of the ancillary subunit towards the $Ca_V1.2$ channel complex (Rougier et al. 2015).

Altogether, these data indicate that the degradation of VGCCs by the UPS may play an important role in channel homeostasis, and open important and novel areas for the study of the dynamics of VGCCs with important physiological and pathophysiological implications. For instance, it has been shown that the inhibition of the UPS may lead to an increased synaptic strength and neurotransmission, possibly by interfering with the expression and activity of

presynaptic VGCCs (Tai and Schuman 2008). Likewise, in some cases, for example in dorsal root ganglion neurons, cell-specific selection of the $Ca_V2.2$ e37a variant over the e37b variant by alternative splicing of the pre-mRNA protects $Ca_V2.2$ channels from ubiquitination and subsequent degradation, suggesting some advantages for pain processing (Marangoudakis et al. 2012). In addition, pharmacological targeting of channel/deubiquitinating enzyme complexes has emerged as a new therapeutic avenue to reverse aberrant upregulation of channel activity associated to neuropathic pain, with possibly limited adverse side effects (García-Caballero et al. 2014). In some other cases (e.g. LC1), degradation of Ca_V2 channels *via* the UPR might play an important role in the regulation of channel surface expression *in vivo* during early neuronal developmental stages. These investigations may also uncover new therapeutic strategies to control channel functional expression by targeting UPS components, as in the case of impaired insulin secretion (Kawaguchi et al. 2006). Likewise, UPS-dependent regulation of cardiac $Ca_V1.2$ channels may help to explain some adaptive mechanisms in the cardiovascular system (Rougier et al. 2011). In addition, because of the role of $Ca_V1.2$ channels in generating the depolarizing plateau of the ventricular cardiac action potential, and to regulate vascular tone, UPS-mediated $Ca_V1.2$ channel regulation might represent a potential pharmacological target for the treatment of cardiovascular diseases.

Our understanding of the detailed underlying mechanisms by which the UPS regulates functional expression of VGCCs has just begun and it is undoubtedly that future studies will reveal the complexity and physiological importance of this regulation. Of significance is the notion that impairment of these regulatory mechanisms may play an essential role in disease states, and pharmacological targeting of the UPS may open new therapeutic avenues.

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