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β subunits of voltage-gated calcium channels in cardiovascular diseases

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Calcium signaling is required in bodily functions essential for survival, such as muscle contractions and neuronal communications. Of note, the voltage-gated calcium channels (VGCCs) expressed on muscle and neuronal cells, as well as some endocrine cells, are transmembrane protein complexes that allow for the selective entry of calcium ions into the cells. The α1 subunit constitutes the main pore-forming subunit that opens in response to membrane depolarization, and its biophysical functions are regulated by various auxiliary subunits–β, α2δ, and γ subunits. Within the cardiovascular system, the γ-subunit is not expressed and is therefore not discussed in this review. Because the α1 subunit is the pore-forming subunit, it is a prominent druggable target and the focus of many studies investigating potential therapeutic interventions for cardiovascular diseases. While this may be true, it should be noted that the direct inhibition of the α1 subunit may result in limited long-term cardiovascular benefits coupled with undesirable side effects, and that its expression and biophysical properties may depend largely on its auxiliary subunits. Indeed, the α2δ subunit has been reported to be essential for the membrane trafficking and expression of the α1 subunit. Furthermore, the β subunit not only prevents proteasomal degradation of the α1 subunit, but also directly modulates the biophysical properties of the α1 subunit, such as its voltage-dependent activities and open probabilities. More importantly, various isoforms of the β subunit have been found to differentially modulate the α1 subunit, and post-translational modifications of the β subunits further add to this complexity. These data suggest the possibility of the β subunit as a therapeutic target in cardiovascular diseases. However, emerging studies have reported the presence of cardiomyocyte membrane α1 subunit trafficking and expression in a β subunit-independent manner, which would undermine the efficacy of β subunit-targeting drugs. Nevertheless, a better understanding of the auxiliary β subunit would provide a more holistic approach when targeting the calcium channel complexes in treating cardiovascular diseases. Therefore, this review focuses on the post-translational modifications of the β subunit, as well as its role as an auxiliary subunit in modulating the calcium channel complexes.

KEYWORDS

voltage-gated calcium channel (VGCC), $Ca_V\beta$ subunits, Ca^{2+}, cardiovascular disease, post-translational modification (PTM)

1. Introduction

Calcium is one of the most important elements in organism, participating various physiological processes, such as heartbeat, muscle contraction, and neuronal communication (1,2). Ca^{2+} enter into the nerve, muscle, and some endocrine cells mainly through voltage-gated Ca^{2+} channels (VGCCs). Based on the membrane voltage required for activation, 10 subtypes of VGCCs were subsequently classified into high-voltage activated (HVA) and low-VA (LVA) calcium channels (3). Further studies classified Ca^{2+} currents into L- (Long-lasting), N-(Neural), P(Purkinje)/ Q-, R-(Residual), and T-(Transient) type currents, which exhibit distinct biophysical and pharmacological properties (4).

The purified channel complex is composed of the pore-forming subunit α_1 (175 kDa) and two auxiliary subunits: $\alpha_2\delta$ (about 160 kDa), β (54 kDa) (**Figure 1**) (5). The α_1 subunit ($Ca_v\alpha_1$) is the principal component of VGCCs and is responsible for their unique biophysical and pharmacological properties. However, proper trafficking and functions of L-, N-, P/ Q-, and R-type channels require the auxiliary subunits. In particular, β subunit is indispensable for HVA Ca_V1 and Ca_V2 Ca^{2+} channels. β subunit acts in many different aspects by enhancing Ca^{2+} channel currents through prevention of proteasomal degradation (6), changing the voltage dependence and kinetics of activation and inactivation, and modulating Ca_v1 and Ca_v2 channels by protein kinases or G protein (7).

β subunit has four subfamilies (β_1–β_4). β_1 subunit is mainly expressed in brain, heart, skeletal muscle, spleen, T-cells (**Table 1**) (4). The β_{1a} isoform, however, is exclusively expressed in skeletal muscle where it partners with skeletal muscle $Ca_v1.1$, and is irreplaceable for excitation-contraction coupling (7). β_2 subunit is found in brain, heart, lung, nerve, nerve endings at the neuro-muscular junction, T-cells, osteoblasts (**Table 1**) (4). It is also the predominant β subunit in the heart, especially β_{2b} (8). β_2 subunit knockout mice die prenatally at embryonic day 10.5 due to lack of cardiac contractions (9, 10), indicating the significant role of β_{2b} subunit in cardiovascular human diseases. More importantly, Antzelevitch et al. (11) pointed out that S481L mutation in the C-terminus of β_{2b} subunit contributed to a type of sudden death syndrome characterized by a short-QT interval and an elevated ST-segment. Cordeiro et al. (12) further found that T11l mutation in the β_{2b} N-terminus led to accelerated inactivation of cardiac L-type channels and was linked to the Brugada syndrome.

β_3 mostly expressed in brain, but also in heart, aorta and smooth muscle, regulating pain, memory, emotion, and even blood pressure (**Table 1**) (4). β_3 subunit knockout mice showed reduced perception of inflammatory pain but not mechanically or thermally induced pain, which is probably due to reduced N-type calcium channel expression in dorsal root ganglia (13). Behaviorally, β_3-null mice exhibit impaired working memory, lowered anxiety and increased aggression and nighttime activity (14,15). Moreover, a high-salt diet induced abnormally elevated blood pressure, reduced plasma catecholamine levels (16) and hypertrophic heart and aortic smooth muscle in β_3 subunit knockout mice (17). These results point to a compromised sympathetic control in β_3 subunit knockout mice, likely due to reduced N- and L-type channel activity (18). A recent study comparing epileptic patients with non-epileptic individuals identified three mutations in β_3 subunit that were present only in patients (19), which further associated β_3 subunit with brain disorders.

β_4 subunit expressed in cerebellum, kidney, and skeletal muscle, but not in the cardiac muscle of adults (**Table 1**) (4). β_4 subunit knockout mice, which was generated by an insertion that causes exon skipping and a premature stop codon in the gene for β_4 subunit, exhibited ataxia, seizures, absence epilepsy, and paroxysmal dyskinesia (20). This could be caused by a 50% up-regulation of T-type Ca^{2+} channels in thalamic neurons (21). Recent studies have, respectively, indicated that lethargic mice also show impairment of parallel fiber volley and Purkinje cell firing in cerebellum (22) and a modified electro-oculogram (23). More importantly, three mutations in the gene encoding of β_4, that is, R468Q, R482X, and C104F, were identified in patients with seizures and epilepsy (24, 25).

Overall, β subunit is crucial not only in the protein expression level of calcium channels, but also in the channel activation and inactivation. Various heterologous expression systems with all four subfamilies of β subunit and all Ca_V1 and Ca_V2 subunits have shown that β subunit can function as a chaperone to dramatically increase the surface expression of Ca_V1 and Ca_V2 channels (26). Moreover, β_1 and β_2 knockout mice have severely reduced Ca^{2+} currents in heart, while overexpression of β subunit using adenoviruses can increase Ca^{2+} channel current density in native cardiac cells (27,28). β subunit can also prevent proteasomal degradation of calcium channels induced by increased ubiquitination *via* K48-linked ubiquitin chain, which was caused by its association with ret finger protein 2 (RFP2), an ER-localized RING finger E3 ligase (6,29). In addition, studies on β subunit knockout mice have shown that β subunit can shift the voltage dependence of activation to more hyperpolarized voltages by 10–15 mV (10). This shift is also visible at the single-channel level, as indicated by Luvisetto et al. (30). Furthermore, β subunit also modulates voltage-dependent inactivation, which reduces the amount of Ca^{2+} entering the cell following depolarization and decreases the number of channels responsible to subsequent depolarizations, causing a shift to more depolarized voltages by 10–20 mV (8, 31).

2. Crystal structure of β subunit

Previous studies have revealed that β subunit is comprised of five distinct regions, namely, guanylate kinase (GK) domain, Src homology 3 (SH3), HOOK region, NH2 terminus, and β-interaction domain (BID), based on amino acid sequence alignment, biochemical and functional studies, and molecular modeling (8,32). The GK domain, SH3 domain, and HOOK region constitute the core of β subunit. GKs can catalyze the reversible phosphoryl transfer from ATP to guanosine monophosphate (GMP) to produce ADP and GDP. SH3 domain contains a well-preserved PxxP motif-binding site and thus has the potential to bind PxxP motif-containing proteins. Therefore, the GK domain and SH3 domain are protein interaction modules in β subunit, engaging in protein-protein interactions (33).

With the development of crystallography, the crystal structure of the β subunit core was reported in 2004, verifying that β core indeed contains an SH3 domain and a GK domain, linked by HOOK region (34–36). Crystal structures of yeast GKs show that these enzymes have a catalytic site harboring the GMP- and ATP-binding pockets (37), which further explains its catalytic ability. The SH3 domain has a similar folding as canonical SH3 domains do. However, its last two β sheets are non-continuous, separated by the HOOK region (34–36). The HOOK region is variable in length and amino acid sequence

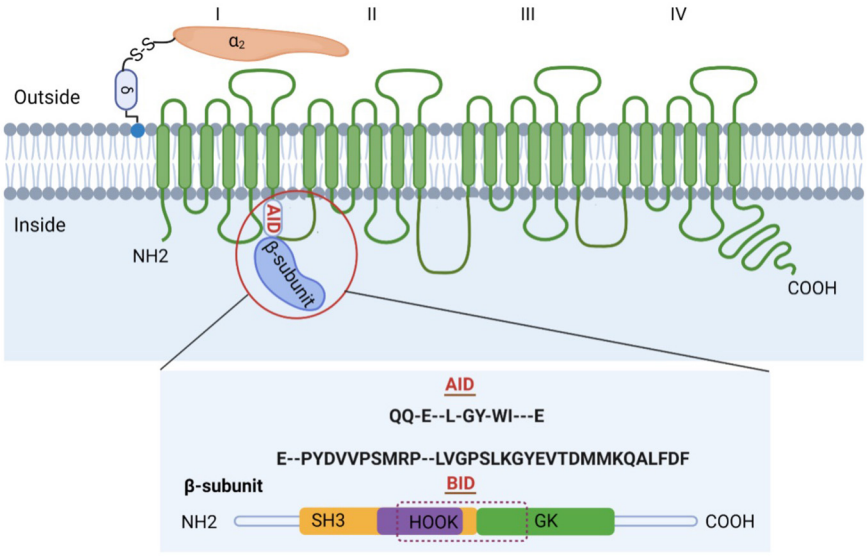

FIGURE 1
Subunit composition of high voltage-gated calcium channels (VGCCs). Schematic representation of the predicted transmembrane topology of VGCCs subunits. The complex is composed of the pore-forming subunit α_1 (175 kDa) and two auxiliary subunits: $\alpha_2\delta$ and β. The AID, marked in red, is located on the I–II loop. β subunit is comprised of GK domain, Src homology 3 (SH3), HOOK region, NH2 terminus, and carboxyl terminus, with BID spanning across SH3-HOOK-GK motif. Both AID and BID have a conserved consensus motif, that is, QQ-E–L-GY-WI—E (AID) and E–PYDVVPSMRP–LVGPSLKGYEVTDMMKQALFDF (BID) (36).

among the β subfamilies. In the crystal structures, a large portion of the HOOK is unresolved due to poor electron density, indicating that it has a high degree of flexibility (34–36). A recent study also revealed the NMR structure of the NH_2 terminus, which consists of two α-helices and two antiparallel β sheets (38). One of the two α-helices in the NMR structure is equivalent to the very first α-helix in the β core structures. Superposition of this helix in the two structures reveals that the NH_2 terminus is oriented away from the core.

Crystal structure of the β core not only verified the component of β core, but also showed that SH3 and GK domains interact intramolecularly (34–36). The interaction is strong enough such that hemi-β fragments containing the NT-$SH3_{\beta strand\ 1-4}$-HOOK module and the $SH3_{\beta strand\ 5}$-GK-CT module can associate biochemically *in vitro* and reconstitute the functionality of full-length β subunits when they are coexpressed in cells (39–41). The strong intramolecular SH3-GK interaction comes from the last β sheet of β-SH3 ($SH3_{\beta strand\ 5}$), which is directly connected to the GK domain, and interacts extensively with both the GK domain and the rest of the SH3 domain, strengthening the otherwise weak interactions at the SH3-GK interface (42). Apart from the interaction between SH3 and GK domains within β subunit, Pragnell et al. (32) also found that β subunit binds to α-interaction domain (AID) domain within α_1 subunit, a high-affinity site located in the cytoplasmic loop connecting the first two homologous repeats of α_1 subunit. AID is comprised of 18 residues, with a conserved consensus motif (QQxExxLxGYxxWIxxxE) in all Ca_V1 and Ca_V2 channels. The AID binds to all four β subunits through the BID, a 31-amino acid segment of β subunit (43). Single mutations in either AID or BID can greatly weaken the α_1–β interaction, as indicated by *in vitro* binding experiments (44).

3. Post-translational modifications of β subunit

The calcium channel β subunits are known to be regulated by various post-translational modifications, namely, phosphorylation and palmitoylation. Pioneering studies from the 1980s revealed the rapid phosphorylation of the RRPTP motif of the β_{1a} subunit by protein kinase A (PKA) and cAMP-dependent kinases

TABLE 1 Tissue distribution and key features of β subunits.

Subunit	Tissue distribution and key features	References
β_1	β_1 mainly expressed in brain, heart, skeletal muscle, spleen, and T-cells. β_{1a} isoform is exclusively expressed in skeletal muscle, partnering with skeletal muscle $Ca_v1.1$, and being irreplaceable for excitation-contraction coupling.	(4, 6)
β_2	β_2 mainly expressed in brain, heart, lung, and nerve. β_{2b} is most abundant β subunit in the heart and is crucial for cardiac contractions.	(4, 7–9)
β_3	β_3 mainly expressed in brain, heart, aorta, and smooth muscle. β_3 is associated with brain disorders, such as, memory loss, emotional disturbance, and epilepsy.	(4, 13, 14)
β_4	β_4 mainly expressed in brain, especially in cerebellum, kidney, and skeletal muscle. β_4 knockout mice exhibit ataxia, seizures, absence epilepsy, and paroxysmal dyskinesia.	(4, 19)

within skeletal muscles (45). Notably, in cardiomyocytes, cAMP-dependent phosphorylation was later identified to be the primary phosphorylation of the β subunits (46). As such, phosphorylation of the β subunits was thought to be a signal transduction pathway for the up-regulation of L-type calcium channels.

3.1. Phosphorylation

β_{2a}, the main β subunit within the cardiac milieu, was empirically reported to be phosphorylated by PKA on three serine residues–Ser459, Ser 478, and Ser479 of the C-terminus (47). Interestingly, these phosphorylation sites were not conserved within the other β subunit isoforms. This therefore raises the question for whether phosphorylation of the β_{2a} subunit is indeed important for its function. However, an overexpression paradigm in cardiomyocytes revealed no significant modulation of the $Ca_V1.2$ channels by the β_{2a} subunit (48). In that study, it was observed that a phosphorylation-deficient β_{2a} subunit mutant equally up-regulated the functions of $Ca_V1.2$ channels in heart cells. This result suggests that the phosphorylation of the β_{2a} subunits in heart cells may be involved in the facilitation of other signaling pathways rather than modulating $Ca_V1.2$ channel functions. Nevertheless, it should be noted that the presence of the β_{2a} subunit is still important for increasing the open probability (P_o) of the α_1 subunit even when compared to the other β subunit isoforms (8).

In a more recent study, the use of liquid chromatography-mass spectrometry (LC-MS/MS) elucidated the *in vivo* phosphorylation of two residues of the β_{1a}–Ser193 and Thr205 (49). Both residues are located within the HOOK domain of the β_{1a} subunit, and, interestingly, are conserved across the various β subunit isoforms. Although *in silico* prediction models suggested the possible phosphorylation of Ser193 of the β_{1a} by casein kinase II, *in vitro* experiments proved otherwise, however, on the other hand, Thr205 of the β_{1a} was observed to be phosphorylated by PKA. Functionally, electrophysiological whole-cell patch clamp techniques revealed the phosphor-modulatory functions of the two residues. Through the site-directed substitutions of the conserved phosphorylated residues, S152E phosphomimetic of the conserved Ser residue within β_{2b} showed a significantly decrease in the peak current density of $Ca_V1.2$ channels. Moreover, the voltage-dependent activation and inactivation of $Ca_V1.2$ channels were right-shifted toward more positive potentials. As expected, S152A phospho inhibitory substitution showed opposing effects. Conversely, it is interesting to note that neither the phosphomimetic nor phospho inhibitory substitution of T164D and T164A of β_{2b}, respectively, revealed no observable differences in either $Ca_V1.2$ peak currents or its voltage-dependent activities. However, it was noted that calcium-dependent inactivation of β_{2b}^{T164D} was significantly increased but not in β_{2a}^{T164D}, thus suggesting a subunit-specific effect. It may be possible that the differential effect arose from other modifications of the β_{2a} subunit, such as palmitoylation. Nevertheless, it is clear that phosphorylation of either the conserved Ser or Thr residues within the HOOK domain of β subunits allowed for the modulation of the α_1 channel properties.

Even more recently, a β subunit-dependent modulation of $Ca_V1.2$ channels by Rad protein was reported (50–52). Rad is a member of the Ras/GTPase superfamily and was characterized for its unique GTPase-activating protein (GAP)-like activity (53). It was reported that substitution of specific residues into alanine in Rad (Arg208 and Leu235) or β_{2a} subunit (Asp244, Asp320, and Asp322) abolished the interaction between these two proteins (54). Furthermore, these mutations also attenuated the hyperpolarizing shift in the current-voltage curve of $Ca_V1.2$ channels, thereby leading the authors to conclude that the interaction between Rad and β_{2a} is necessary for the cAMP-PKA regulation of $Ca_V1.2$ channels. Moreover, using Förster resonance energy transfer (FRET), the authors further showed the robust interaction between Rad and β_{2a}, which complements the reports from earlier studies (50,55). Here, the coexpression of only the PKA catalytic subunit with Rad abolished the interaction between Rad and β_{2a}. It was also reported that similar results were observed between phosphorylated Rad and the other β subunit isoforms. Therefore, taken together, these data strongly suggest that the phosphorylation of Rad functions to inhibit its interaction with the β subunits. Further studies showed that Rad-phosphosite-mutant mice (4SA-Rad) displayed weakened adrenergic activation of calcium channels, which thus produced profound physiological effects: reduced heart rate with increased pauses, reduced basal contractility, attenuated of β-adrenergic contractile response, and diminished exercise capacity (52).

In addition to PKA, protein kinase C (PKC) has also been identified to phosphorylate the β subunit (56). Here, proline and serine residues within the BID were phosphorylated, and are located between the SH3 and GK domains (43). Similarly, phosphomimetic substitutions of phosphorylated residues were made and the function of the resultant phosphomimetic β_{1b} subunit mutants were tested. In their study using *Xenopus* oocyte cells, peak current density of the α_1 subunit was observed to be increased by P221R but decreased by S228R when compared to wild-type β_{1b} subunit. Both P221R and S228R decreased voltage-dependent activation of the α_1 channels. On the other hand, while S237R did not affect α_1 current density, S237R increased voltage-dependent activation of the α_1 channels. Biochemical results revealed that P221R and S228R mutants were able to interact with the α_1 subunit similar to that of the wild-type β_{1b} subunit. However, S237R completely abolished any interactions with the α_1 subunit. Therefore, these results suggest that PKC phosphorylation of residues within the BID serves to regulate specific functions of the β subunit, thereby modulating the α_1 channel properties accordingly.

3.2. Palmitoylation

As aforementioned, the β_{2a} subunit, unlike the other β subunit isoforms, is also regulated by palmitoylation, which describes the process of covalent attachment of fatty acid chains to cysteine residues *via* a thioester linker (57–59). Palmitoylation of the β_{2a} subunit has been reported in various cell lines such as HEK 293 and Sf9 insect cells and was later identified to occur on Cys3 and Cys4 of the N-terminus. Functionally, confocal imaging revealed that palmitoylation of the β_{2a} subunit was important for regulating the localization of the protein toward the plasma membrane (57). In contrast, palmitoylation-deficient β_{2a} subunits remained largely punctate and intracellular. Moreover, through whole-cell patch clamp experiments, it was observed that the $I_{Ca,L}$ of $Ca_V1.2$ channels was much less per amount of charge movement when co-expressed with a palmitoylation-deficient β_{2a} subunit. Palmitoylated wild-type β_{2a} subunits also slowed inactivation of the channel complex. However, it should still be noted that even in the absence of palmitoylation, the β_{2a} mutant was still able to

facilitate $Ca_V1.2$ surface membrane trafficking, presumably through preventing $Ca_V1.2$ turnover *via* ERAD (6).

3.3. S-nitrosylation

β subunits are also modified by S-nitrosylation, a reversible post-translational modification of proteins whereby cysteines are S-nitrosylated at sulfhydryl groups (60). β subunit S-nitrosylation was reported to be required for nitric oxide-mediated $Ca_V2.2$ down-regulation in a subtype-dependent manner as β_1 or β_3 subunits resulted in stronger reduction in Ca^{2+} currents compared with β_2 or β_4 subunits (61). Moreover, Cys346Ala substitution of β_3 subunit significantly ablated the inhibitory effect of S-nitroso-N-acetylpenicillamine (SNAP), a nitric oxide donor, on $Ca_V2.2$ channel function (61). Therefore, while not expressed within the cardiovascular system, N-type calcium channels are important for proper neurological function and S-nitrosylation is currently being investigated for pain treatment, whereby $Ca_V2.2$ channels have been reported to be important for the modulation of pain (62). However, the molecular mechanisms by which β subunit S-nitrosylation affects calcium channel function remain to be investigated. In contrast, a recent study on $Ca_V1.2$ channel reported that β subunit is not involved in the nitric oxide-mediated down-regulation of $Ca_V1.2$ channels (63) as the β subunits-free S-nitrosylated $Ca_V1.2$ channels were still able to degraded at lysosome, a novel mechanism underlying nitric oxide-induced vasodilation. It is noteworthy that β_3 subunit mutant carrying Cys346Ala substitution could be used in the $Ca_V1.2$ study to further confirm the roles of β subunit S-nitrosylation in $Ca_V1.2$ down-regulation by nitric oxide.

4. Roles of β subunit in calcium channel trafficking

More recently, the generally accepted notion that the β subunit is integral for membrane trafficking of α_1 subunits is now brought back into discussion. In cardiomyocytes expressing transgenic α_{1C} mutants that is unable to bind β subunits, α_{1C} subunits were still observed to be trafficked to the sarcolemma *in vivo*, and was also able to sustain normal excitation-contraction coupling (**Figure 2**) (64). However, these α_{1C} mutants were not stimulated by agonists of the β-adrenergic pathway, thereby showing significant impairment of β-adrenergic stimulation of contractility. Taken together, these data suggest that while the β subunit is still integral for modulating channel biophysical properties, it may be dispensable in the context of maintaining proper α_1 subunit membrane trafficking and basal electrophysiological functions in specific cell types. Nevertheless, further work needs to be done to validate this hypothesis as well as identify potential targets that may replace β subunit for the α_1 subunit membrane trafficking in cardiomyocytes.

5. β subunits in cardiovascular diseases

β subunits play important roles in heart and vessels based on their dramatic effects on L-type calcium channels. Cardiac−specific β_{2a} overexpression was reported to induce cardiac hypertrophy with reduced ejection fraction in 6-month-old mice. This could be due to the increased Ca^{2+} influx through $Ca_V1.2$ channels and activated hypertrophic Calcineurin-NFAT3 (nuclear factor of activated T cells) and CaMKII/HADC5 (calcium/calmodulin−dependent protein kinase II/histone deacetylase 5) signaling pathways in β_{2a}-transgenic mice (65). Moreover, another study also reported that the same β_{2a}−transgenic line displayed more severe cardiac hypertrophy under phenylephrine stimulation, while non-phosphorylated mutant β_{2a}-overexpressing mice showed weakened responses to phenylephrine-induced cardiac hypertrophy. This study revealed that CaMKII−mediated phosphorylation of β_{2a} subunits in caveolae is essential for cardiac dysfunction induced by chronic α_1-adrenergic stimulation (66). These results are in line with the study that reduction in Ca^{2+} influx through $Ca_V1.2$ channels by short hairpin-mediated knockdown of β_2 subunits attenuated the cardiac hypertrophy-induced by pressure overload in mice (67).

Although β subunits are involved in the pathogenesis of cardiac hypertrophy, it remains unclear how β subunits regulate $Ca_V1.2$ channel function in cardiomyocytes as $Ca_V1.2$ channels were localized at two different microdomains in ventricular and atrial cardiomyocytes: T−tubule and caveolae (68–71). Moreover, there has been a debate on where is the source of hypertrophic Ca^{2+}. A $Ca_V1.2$-inhibitory domain of REM, a member of the Rad, Rem, Rem2, and Gem/Kir (RGK) GTPase family that is known to potently inhibit $Ca_V1.2$ channel function (72), was fused to a caveolin−binding domain to generate the chimeric protein Caveolin-binding domain (CBD)-REM. CBD-REM was shown to inhibit the NFAT translocation to nucleus in adult feline left ventricular myocytes, although it had no effects on total Ca^{2+} current density (**Figure 3**) (73). This work indicates that $Ca_V1.2$ channels from caveolae could be the pathway for hypertrophic Ca^{2+}, while T-tubule-resident $Ca_V1.2$ channels mainly account for contractile Ca^{2+}. In contrast, with cardiac-specific overexpression of CBD−REM or a caveolae−targeted $Ca_V1.2$ activator (CBD−β_{2a}, a mutated $\beta_{2a}^{C3S/C4S}$ fused C−terminal to CBD) in mice, both

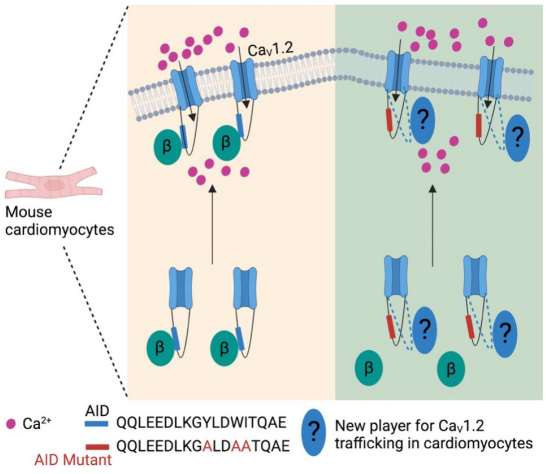

FIGURE 2
β subunit-less $Ca_V1.2$ channels are still trafficked to cell surface in mouse cardiomyocytes. At least in mouse cardiomyocytes β subunit is required for β-adrenergic stimulation on $Ca_V1.2$ channels, but not trafficking to plasma membrane. This indicates new player(s) is involved in the trafficking of cardiac $Ca_V1.2$ channels.

transgenic mice with pressure overload-induced cardiac hypertrophy did not display significant changes in cardiac function compared to wild-type mice (**Figure 3**) although CBD−β_{2a} potentiated the NFAT translocation in feline cardiomyocytes (74).

The mechanisms underlying the differential effects of CBD−β_{2a} in mouse heart and feline ventricular myocyte remain to be investigated. As L-type calcium channels were reported to be redistributed to caveolae from T-tubule in failing human and rat cardiomyocytes, which led to increased open probability of $Ca_V1.2$ channels and early afterdepolarization (75), one could expect that the effect of CBD−β_{2a} on caveolae-resident $Ca_V1.2$ channels may be weakened by increased $Ca_V1.2$ channel numbers in caveolae of failing mouse cardiomyocytes. In addition, it is noteworthy that caveolin-3 was reported to bind to β_{2c} and β_{2a} only, not β_{2b}, β_{2d}, β_3, β_4 in mouse ventricular myocytes, and a Caveolin-3^{P104L} mutant overexpressed in neonatal mouse cardiomyocytes remarkedly reduced the β_{2c} trafficking to cell surface through Caveolin-3 retention in Golgi complex (76). This finding raises a concern that CBD−β_{2a} may not be able to bring sufficient $Ca_V1.2$ channels to caveolae in cardiomyocytes. More importantly, as mentioned above in this review β subunit-less $Ca_V1.2$ channels are still able to be trafficked to cell surface in mouse ventricular myocytes (64), which indicates that overexpression of CBD−β_{2a} may not produce significant effects on $Ca_V1.2$ levels at plasma membrane.

In addition to heart diseases, β subunits also have essential roles in vascular diseases. It has been reported that both total $Ca_V1.2$ channel level in aortas and L-type Ca^{2+} current in isolated aortic smooth muscle cells were reduced in $\beta_3^{-/-}$ mice, although the basal systolic blood pressure remained normal (16, 17, 77). However, compared to wild-type mice, $\beta_3^{-/-}$ mice developed significantly higher systolic and diastolic blood pressure when fed with high salt diet (17) or subcutaneously infused with Angiotensin II for 2 weeks (77). Why global loss of β_3 does not induce the phenotype of hypertension? It could be due to the abundant expression of β_3 subunits in multiple organs as mentioned in section "1. Introduction." $\beta_3^{-/-}$ mice have been found to exhibit enhanced performance in several memory tasks through increased N-Methyl-D-Aspartate receptor (NMDAR) currents and NMDAR-dependent long-term potentiation (14), suggesting that β_3 loss is able to activate NMDAR. Intriguingly, NMDA receptor activation induces renal vasodilation by increasing the epithelial sodium channel-dependent connecting tubule-glomerular feedback responses (78). These results suggest lack of gross phenotype in hypertension in $\beta_3^{-/-}$ mice could be due to the compensatory effects from activation of NMDAR in kidney. A smooth muscle-specific knockout mouse of β_3 subunits may more precisely validate the roles of β_3 subunit in blood pressure regulation.

6. Potential β subunits-targeted therapeutic development

Given that β subunits play key roles in regulating the function of L-type calcium channels, the major calcium channels in cardiovascular systems (29, 79), thus β subunits have been considered as targets for developing new therapeutics of cardiovascular diseases.

Two truncated N-terminus-less β_2 subunit including the BID and C-terminus (β_2-C-BID) or BID only (β_2-BID) have been reported to bind to $Ca_V1.2$ channels intracellularly, while lacking the motifs required to target the $Ca_V1.2$ channel complex to cell surface (80). Both β_2-C-BID and β_2-BID displayed strong dominant-negative effects on L-type Ca^{2+} current density in HL-1 cells as shown in **Figure 4** (80), a cardiac muscle cell line derived from AT-1 mouse atrial cardiomyocyte tumor lineage (81). These β_2 subunit decoys represent potential therapeutics to reduce $Ca_V1.2$ surface expression in cardiovascular pathologies featured by up-regulation of $Ca_V1.2$ channels. However, the ability of β_2-C-BID and β_2-BID to interfere with the protein interactions between L-type calcium channels and β_2 subunits and to reduce total and surface $Ca_V1.2$ channels remains to be tested.

In addition to BID domain, α_1-ID within I–II loop of L-type calcium channels is also considered a target to regulate the protein interactions between β subunits and calcium channels. An AID peptide with *meta-xylyl* (m-xylyl) staple incorporated at N-terminal (AID-CAP) was designed to enhanced helical content that bind to β subunits in a native-like manner (82). AID-CAP was shown to significantly reduce the peak Ca^{2+} current in *Xenopus* oocytes co-expressing β_3 subunits and wild-type $Ca_V1.2$ channels, or β_2 subunits and mutant $Ca_V1.2^{Y437A}$ channels carrying a mutation within AID that weakens the β-binding affinity by about 1,000-fold (**Figure 4**) (83). In contrast, AID-CAP failed to alter the Ca^{2+} current in *Xenopus* oocytes expressing β_2 subunits and wild-type $Ca_V1.2$ channels, suggesting that $Ca_V1.2$-β_{2a} protein complexes stably resist kinetic competition by injected AID-CAP peptides. This could be due to the similar binding affinity of wild-type $Ca_V1.2$ channels or AID-CAP to β_2 subunits (82) or palmitoylation mediated β_{2a} subunit anchoring to the membrane (84). Overall, the stapled AID peptides show strong binding affinity to $Ca_V1.2$ channels with negatively regulatory effects in β isoform-selective manner. However, further studies need to conducted to validate their effects of $Ca_V1.2$ protein levels and the $Ca_V1.2$-β protein interactions by biochemical assays and to test their roles in cardiovascular diseases using multiple rodent models, such as roles in hypertension using spontaneously hypertensive rats.

Regarding the $Ca_V1.2$-β protein interactions, Galectin-1, a member of β-galactoside-binding protein family (85), has been identified as a new $Ca_V1.2$-binding partner that interact with I–II loop in a splice isoform-specific and $Ca_V1.2$ channel-selective manner (86), and has emerged as a novel target for hypertension treatment through disrupting $Ca_V1.2$-β protein interaction and promoting the proteasomal degradation of $Ca_V1.2$ channels (87). Galectin-1 was reported to bind to residues Asp457 and Glu459 within exon 9*-null I–II loop of $Ca_V1.2$ channels, thereby removing β subunits from $Ca_V1.2$ channels and masking the endoplasmic reticulum export signals within exon 9 fragment. β subunit displacement further exposed lysines within I–II loop to poly ubiquitination, which increases the proteasomal degradation of $Ca_V1.2$ channels (**Figure 4**) (87). More importantly, the negative regulation on $Ca_V1.2$ channels function by Galectin-1 was found to effectively and stably control hypertension in spontaneously hypertensive rats when Galectin-1 was overexpressed in smooth muscles using adeno-associated virus as a vector (87). It is noteworthy that the $Ca_V1.2$-binding sites within Galectin-1, which may help to develop anti-hypertensive Galectin-1-based peptides, have not been identified.

Recently an immunized llama nanobody nb.F3 has been isolated and found to interact with β_{2b} with a high binding affinity (K_d = 13.2 ± 7.2 nM) (88). The nanobody itself has no effects on calcium channel function. Intriguingly, when the

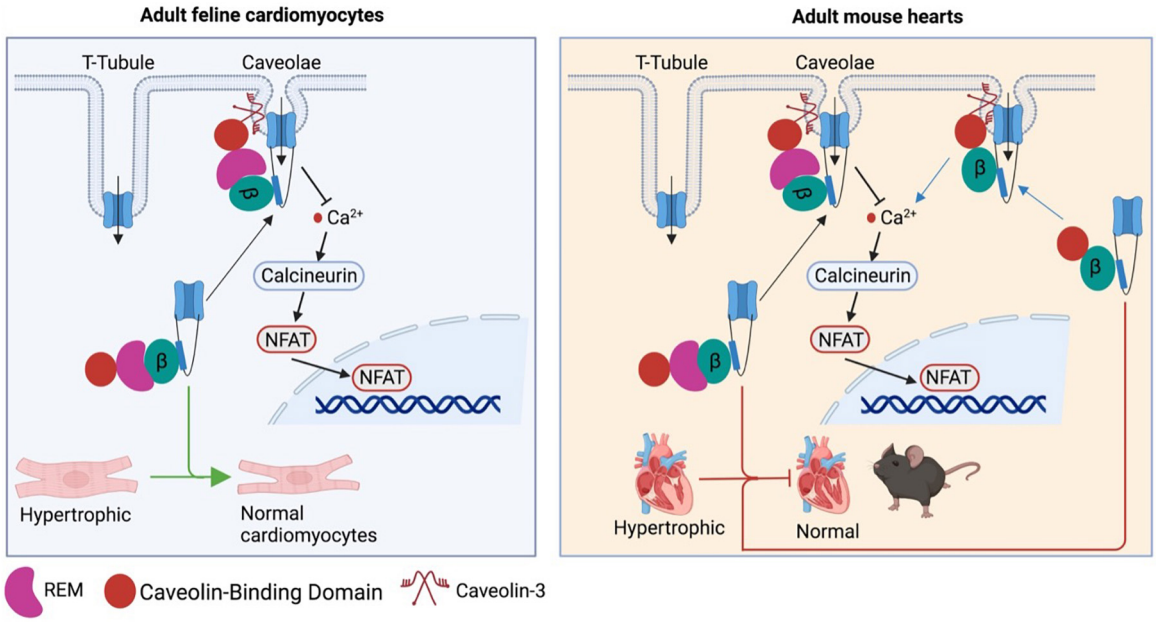

FIGURE 3
Controversy over the caveolae-resident $Ca_V1.2$ calcium channel as the hypertrophic Ca^{2+} source. CBD-REM generated by a $Ca_V1.2$-inhibitory domain of REM fused to a caveolin–binding domain is able to significantly inhibit the hypertrophic signaling by blocking the NFAT translocation to nucleus in adult feline left ventricular cardiomyocytes, while selective overexpression CBD-REM or CBD-CBD–β_{2a} in cardiac muscles fail to alter cardiac function of mice subjected to pressure overload-induced cardiac dysfunction.

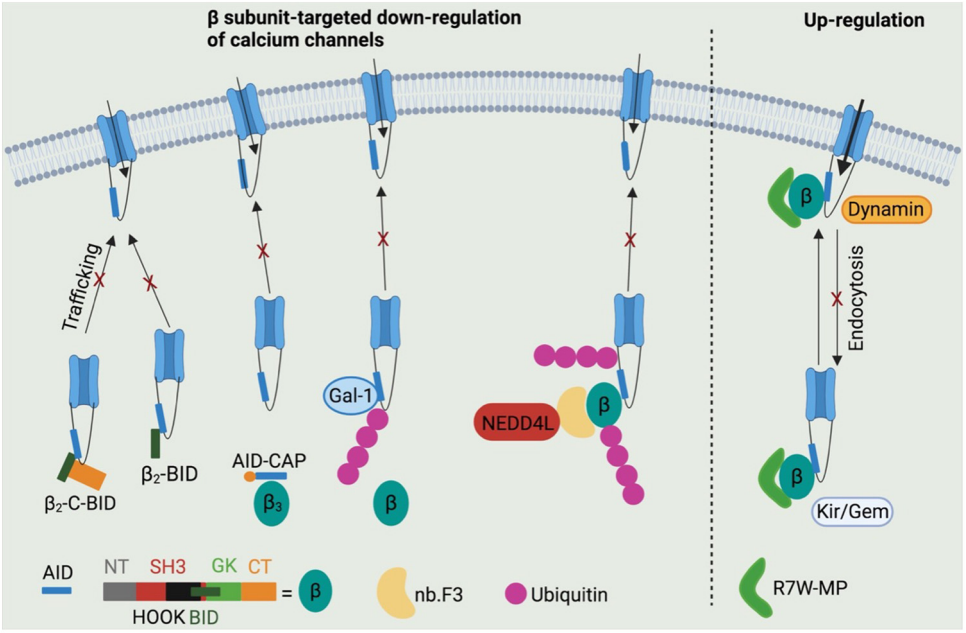

FIGURE 4
Potential β subunits-targeted therapeutic development through modulating calcium channel functions. β_2-C-BID and β_2-BID, lacking for the channel trafficking-required motifs, occupy the β subunits-binding sites within AID domain, thereby leading to reduced trafficking of channels to cell surface. Stapled AID peptides disrupt the interactions between calcium channels and β_3 subunits, but not β_2 subunits, which also results in down-regulation of calcium channel function. Galectin-1 is able to displace β subunits from calcium channels by binding to exon 9 C-terminus and thus to inhibit channel function by exposing lysines within I–II loops to ubiquitination. A β subunit-binding nanobody nb.F3 fused with catalytic domain of NEDD4L E3 ligase significantly blocks the calcium channel function by strongly enhancing the ubiquitination level. R7W-MP peptide, which binds to tail-binding domain (TID) domain within the β_2 subunit Src homology 3 (SH3) domain, is able to up-regulate calcium channel function *via* multiple mechanisms, such as preventing channel degradation *via* ubiquitin-protease system, inhibiting the channel endocytosis by displacing dynamin from β subunits and facilitating the channel trafficking to cell surface by displacing Kir/Gem from β subunits.

C-terminus of nb.F3 was fused to the catalytic Homologous to the E6-AP Carobxyl Terminal (HECT) domain of NEDD4L, an E3 ubiquitin ligase (89), the resulted construct, named Ca_V-aβlator, completely blocked the Ca^{2+} current from various high VGCCs overexpressed in HEK 293 cells, and from endogenous calcium channels in guinea pig ventricular cardiomyocytes, murine dorsal root ganglion neurons and pancreatic β cells (**Figure 4**) (88). This study proposed a potent genetically encoded general inhibitor for β subunit-binding calcium channels, which, however, remains to be validated in disease models.

In addition to β subunits-targeted negative modulation of calcium channel function, a peptide R7W-MP containing an oligoarginine (R7W) cell-penetrating peptide and a fragment of β_2 subunit C-terminal coiled-coil tail was designed to stabilize $Ca_V1.2$ channels. Akt-phosphorylated β_2 subunit C-terminal tail was reported to bind to the tail-binding domain (TID) within β_2 SH3 domain, which induced a structural rearrangement of β_2 subunit and thereby stabilized $Ca_V1.2$ channels by preventing the proteasomal degradation (90,91). R7W-MP peptide, by targeting TID domain within the β_2 subunit SH3 domain, was able to prevent Dynamin from binding to β_2 subunit SH3 domain, thereby protecting $Ca_V1.2$ channels against endocytosis (91,92). Moreover, R7W-MP peptide also facilitated $Ca_V1.2$ chaperoning to the plasma membrane by preventing the interaction between β_2 subunits and Kir/Gem (**Figure 4**) (91), a member of the RGK small GTP-binding protein family reported to decrease the level of L-type calcium channels at cell surface (93, 94). More importantly, R7W-MP restores cardiac function in a diabetic cardiomyopathy mouse model through increasing $Ca_V1.2$ current density.

7. Conclusion

β subunits have been widely studied in various cardiovascular disease conditions and plays important roles in cardiac hypertrophy, diabetic cardiomyopathy, and hypertension, although some controversies remain. More strategies modulating high VGCCs have been developed by selectively targeting β subunit itself or the protein interactions between calcium channels and β subunits. Compared to calcium channel blockers to completely ablate the activity of L-type calcium channels, it may be advantageous to partially reduce Ca^{2+} influx by inhibiting the up-regulation of $Ca_V1.2$ channels in cardiovascular diseases, such as hypertension. These findings provide proof-of-principle for this concept by showing that targeting β subunits could normalize $Ca_V1.2$ channel expression, which may be used as new targets for therapeutics of cardiovascular diseases.

Author contributions

ZH, KL, and CL outlined, drafted, and contributed to the writing of the manuscript. TS critically edited and finalized the manuscript. All authors approved the final version of the manuscript.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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