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A mutation in the β interaction domain of the Ca^{2+} channel α_{1C} subunit reduces the affinity of the (+)-[^{3}H]isradipine binding site

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Abstract The molecular mechanisms of how α_1 and β subunits of voltage-gated Ca^{2+} channels interact with one another are still controversial. Here we show that despite a mutation in the β interaction domain that has previously been shown to disrupt binding, α_{1C}Y467S and β_{1a-myc} still formed immunoprecipitable complexes when coexpressed in tsA201 cells. However, the α_{1C}Y467S–β_{1a-myc} complexes had a decreased affinity to (+)-[^{3}H]isradipine. This indicates that the β interaction domain in the I–II loop of the α_1 subunit is not merely an anchor required for the functional interaction of the two Ca^{2+} channel subunits but is itself part of the effector pathway for β-induced channel modulation. © 2002 Published by Elsevier Science B.V. on behalf of the Federation of European Biochemical Societies.

Key words: Calcium channel; (+)-[^{3}H]Isradipine; Radioligand binding; Immunoprecipitation; Immunofluorescence

1. Introduction

The cytoplasmic β subunits of voltage-dependent Ca^{2+} channels are important determinants of plasma membrane expression and modulators of channel functions of the pore-forming α_1 subunit. Evidence supporting the 'chaperone' function of the β subunit in membrane incorporation of α_1 includes observations of increased current densities [1–4], the increased number of drug binding sites [2,5], and an increased membrane localization observed with immunocytochemistry [6–8]. Evidence for a current-modulating role of the β subunit includes changes in current kinetics, most notably the β isoform-specific effects on inactivation properties [9,10], and changes of single-channel properties [4,11–13].

Increasing evidence suggests the existence of two types of α_1–β interaction sites in the α_1 subunit: a high-affinity binding site in the cytoplasmic loop connecting the homologous repeats I and II [14] and several binding sites at the N-terminus [15,16] and the C-terminus [17,18] of particular α_1 subunits. The amino acid motif QQ-E--L-GY--WI--E constituting the I–II loop β interaction domain is highly conserved between the α_1 subunit isoforms [14] and mutating one or more of these residues prevents subunit association [19]. A possible role of the I–II loop in membrane insertion was suggested, according to which association of β with the I–II loop inhibits the retention of the channel in the endoplasmic reticulum (ER) [20]. An involvement in modulatory functions was suggested by experiments in which a synthetic peptide corresponding to the I–II β interaction domain of α_{1C} reversed the effects of β on the open probability in inside-out patches [21]. However, other studies indicate that multiple sites on the α_1 subunit play specific roles in membrane incorporation and modulation of the Ca^{2+} channel [4,16,22,23].

We have previously shown that the substitution of a critical residue (Y467S) in the β interaction motif in the I–II loop of α_{1C} prevented colocalization of α_{1C} and a β_{1a-GFP} fusion protein as well as the enhanced membrane incorporation seen with immunocytochemistry [4]. Surprisingly, this mutation did not prevent β-induced modulation of current kinetics or the increase in single-channel open probability. Here we demonstrate that α_{1C}Y467S forms immunoprecipitable complexes with β subunits that can explain the previously observed effects of β on α_{1C}Y467S function. Moreover, the observation that the affinity of (+)-[^{3}H]isradipine binding to α_{1C}Y467S–β_{1a-myc} complexes is reduced compared to wild type emphasizes the importance of the β interaction domain in the I–II loop in mediating the effects of β subunits on the properties of the conduction pore.

2. Materials and methods

2.1. Cell culture and transfection

TsA201 cells were grown in F12 medium (Gibco BRL, Vienna, Austria) containing 10% fetal bovine serum to 80% confluence. For transfections 10 μg plasmid DNA and 10 μg unspecific DNA (pUC18) diluted in 250 mM $CaCl_2$ solution were mixed with a solution containing 274 mM NaCl, 40 mM HEPES, 10 mM KCl, 1.4 mM $Na_2HPO_4 \cdot 12H_2O$ and 12 mM dextrose (pH 7.05).

2.2. Expression plasmids

The generation of α_{1C}Y467S has been described elsewhere [4]. The myc-tagged rabbit skeletal muscle β_{1a} subunit (β_{1a-myc}) was generated by fusing the cDNA sequence encoding a single copy of the myc antibody epitope to the 3′ end of β_{1a} in pcDNA3. A PCR fragment containing the 3′ end of β_{1a} fused to the myc cDNA followed by an *Xba*I site was created (antisense primer: 5′-GTC TAG ACT AAT TAA GAT CTT CTT CAG AAA TCA ACT TTT GTT CCA TGG CGT GCT CCT GCT GTT GGG GC-3′). The PCR product

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was digested with *Nar*I (nt 1147 of β_{1a}) and *Xba*I and ligated into a β_{1a}–pBluescript construct. Finally, β_{1a-myc} was inserted into pcDNA3 (Invitrogen, San Diego, CA, USA) via *Kpn*I and *Xba*I. All regions containing PCR-amplified products were verified by sequence analysis.

2.3. Immunofluorescence staining

Cover glasses with cells were removed from the culture dishes before harvesting, fixed and immunostained as previously described [24]. The following antibodies were used: affinity-purified anti-α_{1C} CNC (1:1000) [25] and anti-β com (1:1200) [26]; mouse monoclonal anti-myc 9E10 (Invitrogen, Carlsbad, CA, USA); fluorescein- and Texas red-conjugated goat anti-rabbit and anti-mouse IgGs (Jackson Immuno Research, West Grove, PA, USA).

2.4. Crude membrane preparation

After harvesting transfected tsA201 cells were incubated for 15 min on ice in hypotonic buffer (10 mM Tris–HCl (pH 7.4), 100 mM trypsin, 1 μM pepstatin A, 1 mM leupeptin, 0.5 mM benzamidine, 0.2 mM phenylmethylsulfonyl fluoride (PMSF), 2 mM iodoacetamide) and then homogenized with a tight-fitting glass-glass Dounce homogenizer. The homogenate was centrifuged for 20 min at $400\times g$ at 4°C. Microsomes were collected by centrifuging the supernatant for 10 min at $90\,000\times g$ at 4°C. The pellet was resuspended in the hypotonic buffer and the protein concentration was determined using a Lowry assay.

2.5. Radioligand binding assays

Membrane protein (100 μg/ml) was incubated with increasing concentrations of (+)-[^{3}H]isradipine (0.01–1.3 nM) for 120 min at room temperature in 1 ml of 50 mM Tris–HCl (pH 7.4), 1 mM $CaCl_2$ and 0.1 mM PMSF. Non-specific binding was determined in the presence of 1 μM (±)-isradipine. Bound ligand was determined by filtration through polyethyleneimine-treated GF/C Whatman filters [27]. Filters were washed and the bound radioactivity determined by liquid scintillation counting.

2.6. Immunoprecipitation

Membrane protein (2 mg) was incubated for 2 h at room temperature in 50 mM Tris–HCl (pH 7.4), 0.1 mM PMSF, 1 mM $CaCl_2$ and 0.6 nM (+)-[^{3}H]isradipine. After another 30 min incubation on ice the mixture was centrifuged at $100\,000\times g$ for 15 min to remove free radioligand. The pellet was dissolved in 1 ml solubilization buffer (SB) containing 2% digitonin and TBS (150 mM NaCl, 50 mM Tris–HCl (pH 7.4), 0.1 mM PMSF), incubated for 30 min on ice and insoluble material removed by centrifugation ($100\,000\times g$, 30 min, 4°C). Solubilized protein in the supernatant was subjected to immunoprecipitation or directly analyzed in Western blots. For immunoprecipitation 7 μg protein A-Sepharose (PAS) was incubated with anti-myc antibody 9E10 or equivalent amounts of control IgG, and washed in SB diluted three-fold in TBS. 100 μl of the solubilized membrane protein plus 200 μl TBS were mixed with the PAS-bound antibody for 4 h at 4°C. After washing the antibody-bound radioactivity was determined by liquid scintillation counting. For Western blot analysis the PAS–antibody pellet was resuspended in sodium dodecylsulfate (SDS) sample buffer (10 mM Tris–HCl (pH 7.4), 20% glycerin, 10% SDS, 10 mM dithiothreitol and bromophenol blue), heated to 95°C for 3 min and loaded on 7% polyacrylamide

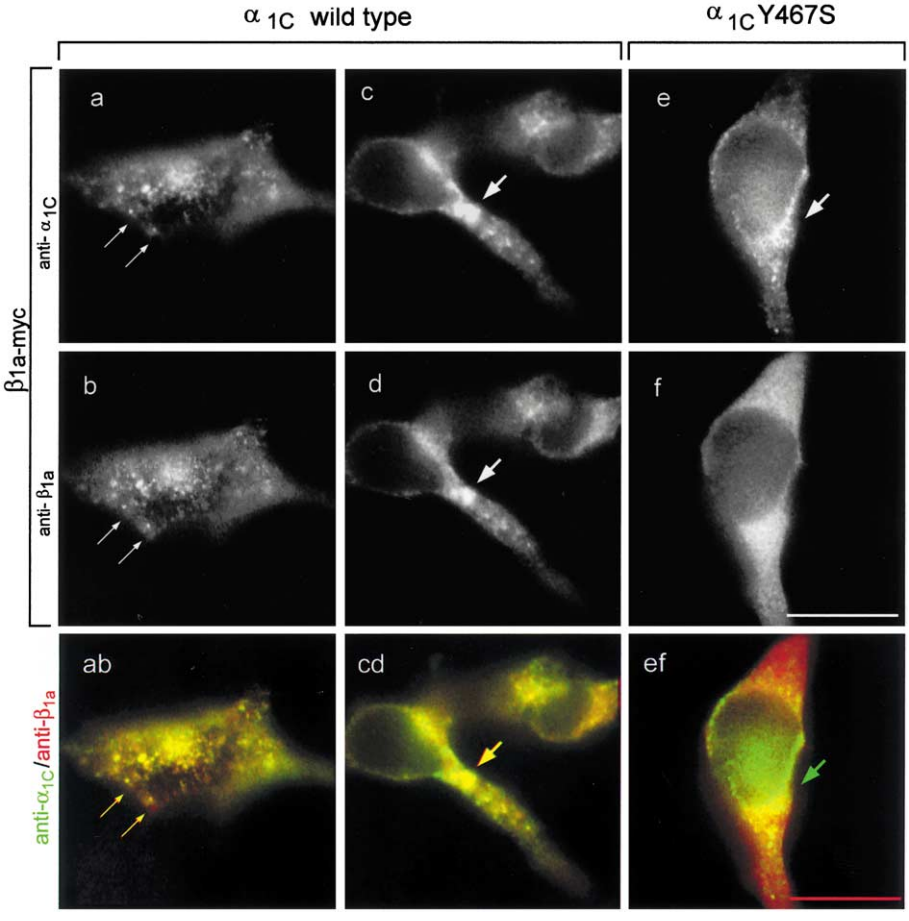

Fig. 1. Double immunofluorescence labeling of wild type α_{1C} and mutant α_{1C}Y467S coexpressed with β_{1a-myc} in tsA201 cells. a,b: Colocalization of α_{1C} and β_{1a-myc} in clusters in the plasma membrane (thin arrows); the focus plane is at the top surface of the cell. c,d: α_{1C} and β_{1a-myc} colocalized in aggregates in the ER (thick arrows). Here and in e,f, the focus plane goes through the center of the cell. e,f: The mutant α_{1C}Y467S is localized in the ER (arrow in e), whereas β_{1a-myc} is diffusely distributed throughout the cytoplasm. The color overlays of the images above show the colocalization of α_{1C} and β_{1a-myc} in a,b and c,d as yellow label, whereas in e,f much of α_{1C}Y467S (green) and β_{1a-myc} (red) is differentially distributed. Bar, 10 μm.

SDS gels. Peroxidase-conjugated IgG (Sigma) was used with a chemoluminescence detection system (ECL; Amersham Pharmacia Biotech, UK).

3. Results

3.1. Differential distribution of wild type α_{1C} and mutant α_{1C}Y467S coexpressed with β_{1a-myc}

Double immunofluorescence labeling of transfected tsA201 cells demonstrated that wild type α_{1C} and β_{1a-myc} are colocalized in surface membrane clusters (Fig. 1a,b) and in internal membrane compartments, presumably the ER (Fig. 1c,d). This colocalization was prevented by the single residue substitution in α_{1C}Y467S (Fig. 1e,f). Whereas α_{1C}Y467S was still expressed in the ER (Fig. 1e) and at a strongly reduced rate in surface membrane clusters, β_{1a-myc} was not colocalized with α_{1C}Y467S in either of these locations. Instead, β_{1a-myc} showed a uniform cytoplasmic staining (Fig. 1f), similar to the labeling pattern observed in tsA201 cells expressing β_{1a-myc} without a α_1 subunit (not shown). This lack of colocalization suggests the loss or a dramatic reduction of α_{1C}–β association due to the Y467S substitution. In addition, detectable surface membrane clusters decreased from 54% in $\alpha_{1C}/\beta_{1a-myc}$ expressing cells to 4% in α_{1C}Y467S/β_{1a-myc} expressing cells (200–300 cells from three separate experiments for each condition).

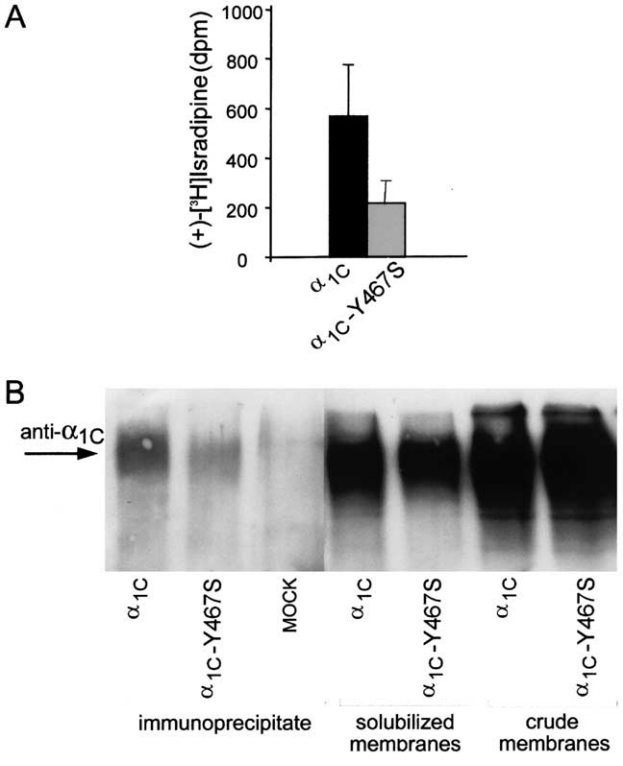

Fig. 2. Co-immunoprecipitation of wild type α_{1C} and mutant α_{1C}Y467S with β_{1a-myc}. A: Anti-myc precipitated (+)-[^{3}H]isradipine binding activity in solubilized membranes of cells transfected with α_{1C}+β_{1a-myc} and with α_{1C}Y467S+β_{1a-myc}; means ± S.D. of three independent experiments. B: Western blot analysis with anti-α_{1C} shows that β_{1a-myc} precipitated α_{1C} and α_{1C}Y467S. Whereas crude and solubilized membranes contained roughly equal amounts of α_{1C} and α_{1C}Y467S, β_{1a-myc} precipitated less than half of α_{1C}Y467S compared to α_{1C}. Mock-transfected cells (MOCK) and equal amounts of control IgGs (not shown) demonstrated that there was no non-specific staining at the position of the α_{1C} band.

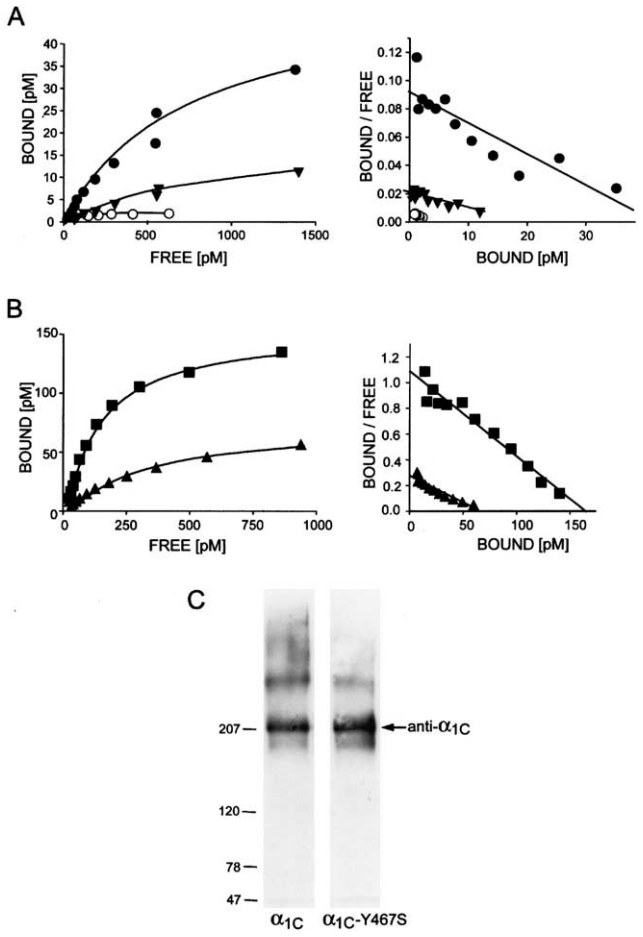

Fig. 3. (+)-[^{3}H]Isradipine binding to wild type α_{1C} and mutant α_{1C}Y467S subunits of L-type Ca^{2+} channels with and without the β_{1a} subunit. A: (+)-[^{3}H]Isradipine saturation curves and corresponding Scatchard plots of membranes of cells expressing α_{1C} alone (○), $\alpha_{1C}/\beta_{1a-myc}$ (●), and α_{1C}Y467S/β_{1a-myc} (▼). Coexpression of the β_{1a} subunit increased B_{max} and the affinity of (+)-[^{3}H]isradipine binding to α_{1C} subunit and, to a lesser extent, of the mutant α_{1C}Y467S (▼). B: Comparison of α_{1C} and α_{1C}Y467S coexpressed with β_{1a-myc} and $\alpha_2\delta$ shows that (+)-[^{3}H]isradipine binds to $\alpha_{1C}/\alpha_2\delta/\beta_{1a-myc}$ (■) with higher affinity and B_{max} than α_{1C}Y467S/$\alpha_2\delta/\beta_{1a-myc}$ (▲). C: Western blot of corresponding samples (20 μg/lane) demonstrates that the concentrations of Ca^{2+} channels were similar in preparations of $\alpha_{1C}/\alpha_2\delta/\beta_{1a-myc}$ and α_{1C}Y467S/$\alpha_2\delta/\beta_{1a-myc}$.

3.2. β_{1a-myc} forms immunoprecipitable complexes with α_{1C} and with α_{1C}Y467S

Whereas the disruption of α_1–β colocalization by the Y467S substitution is consistent with published data on the importance of this residue in β binding [19], it is in apparent conflict with the fully functional β-induced modulation of whole-cell and single-channel currents previously observed with α_{1C}Y467S [4]. To clarify whether α_{1C}Y467S is still capable of forming stable complexes with the β subunit, we performed immunoprecipitation experiments on digitonin-solubilized membranes of tsA201 cells cotransfected with β_{1a-myc} and either α_{1C} or α_{1C}Y467S. An antibody against β_{1a-myc} precipitated (+)-[^{3}H]isradipine binding activity from cells expressing α_{1C} as well as from cells expressing α_{1C}Y467S (Fig. 2A). This

Table 1
Affinities and B_{max} values of α_{1C} and α_{1C}Y467S with and without β_{1a-myc} and $\alpha_2\delta$

	α_{1C} wild type			α_{1C}Y467S			B_{max} reduction[a] (%)
	K_D (pM)	B_{max} (pmol/mg)	(*n*)	K_D (pM)	B_{max} (pmol/mg)	(*n*)	
α_{1C}	528.3 ± 70.4[b]	0.05 ± 0.03	(2)	n.d.	n.d.		
$\alpha_{1C}+\beta_{1a-myc}$	480.0 ± 37.5	1.06 ± 1.19	(3)	825.3 ± 89.0	0.44 ± 0.46	(3)	33, 53 (2)
$\alpha_{1C}+\alpha_2\delta+\beta_{1a-myc}$	154.7 ± 10.0	1.29 ± 0.38	(3)	254.0 ± 113.4	0.56 ± 0.26	(4)	46 ± 13 (3)

[a]Percent reduction of B_{max} of α_{1C}Y467S compared to α_{1C} in parallel experiments.
[b]Values give means ± S.D.

indicates that β_{1a-myc} and α_{1C}Y467S exist in complexes stable enough to withstand solubilization and washing. However, compared to α_{1C}, α_{1C}Y467S forms stable complexes with β at a reduced rate. After normalization to differences in the amounts of α_1 protein measured in the parallel Western blot analysis, the values for immunoprecipitated (+)-[^{3}H]isradipine-labeled α_{1C}Y467S subunits were between 25 and 60% of those for α_{1C} in the same experiments.

This reduced precipitation of (+)-[^{3}H]isradipine could be due to a reduced number of α_{1C}Y467S–β_{1a-myc} complexes, or it could result from an increased dissociation rate of (+)-[^{3}H]isradipine from normal amounts of α_{1C}Y467S–β_{1a-myc} complexes. To discriminate between these two possibilities, the amounts of immunoprecipitated α_{1C} and α_{1C}Y467S were directly compared by Western blot analysis. The results shown in Fig. 2B confirm that anti-β_{1a-myc} still precipitates α_{1C}Y467S, and that the amount of α_{1C}Y467S in the precipitate is about half of that of α_{1C}, even though the concentrations of α_{1C} and α_{1C}Y467S in the starting materials were equal. Thus, the Y467S substitution in the β interaction domain reduced but did not fully abolish the capacity of the mutant to form α_{1C}Y467S–β_{1a-myc} complexes.

3.3. (+)-[^{3}H]Isradipine binding properties of α_{1C} are altered by the Y467S substitution

The effects of decreased formation of α_{1C}Y467S–β_{1a-myc} complexes compared to wild type are also reflected in (+)-[^{3}H]isradipine binding assays. Fig. 3A shows representative saturation curves and Scatchard plots of (+)-[^{3}H]isradipine binding to crude membrane preparations of tsA201 cells transfected with wild type or mutant α_{1C} with and without β_{1a-myc}. (+)-[^{3}H]Isradipine binding to α_{1C} alone was too low (0.05 pmol/mg) to accurately calculate K_D values, but coexpression of α_{1C} with β_{1a-myc} increased the B_{max} of (+)-[^{3}H]isradipine binding 20-fold (Table 1). Similarly, β_{1a-myc} coexpression dramatically increased the B_{max} of (+)-[^{3}H]-isradipine binding to α_{1C}Y467S from below detectability to 0.44 ± 0.46 ($n = 3$ experiments performed in parallel) of wild type levels. Despite the mutation in the β interaction domain of α_{1C}Y467S, this mutant still bound (+)-[^{3}H]isradipine in a β_{1a-myc}-sensitive manner. Direct comparison in two parallel experiments revealed 33% and 53% reduction of (+)-[^{3}H]-isradipine binding sites with α_{1C}Y467S+β_{1a-myc} compared to $\alpha_{1C}+\beta_{1a-myc}$. This is consistent with the reduced amount of α_{1C}Y467S–β_{1a-myc} complexes found in the immunoprecipitation assay (see above). However, not only B_{max} but also the affinity of (+)-[^{3}H]isradipine binding was significantly ($P = 0.0034$) reduced for α_{1C}Y467S (Table 1).

Coexpression of β_{1a-myc} plus $\alpha_2\delta$ with wild type or mutant channels caused a further increase of affinity and B_{max} of (+)-[^{3}H]isradipine binding (Fig. 3B; Table 1). Here again the mutation in the β interaction domain of α_{1C}Y467S reduced B_{max} for (+)-[^{3}H]isradipine (Table 1). Parallel Western blot analysis (Fig. 3C) confirmed that the difference in available (+)-[^{3}H]isradipine binding sites did not result from different expression levels of α_{1C} and α_{1C}Y467S. Taken together this shows that the association of α_{1C} with β_{1a-myc} increases B_{max} and the affinity of (+)-[^{3}H]isradipine binding to α_{1C}, with the major effect on B_{max}, and that the β_{1a-myc}-mediated increase of (+)-[^{3}H]isradipine binding is still observed with α_{1C}Y467S. However, in preparations containing α_{1C}Y467S not only B_{max} but also the K_D values are reduced. Thus, the Y-to-S substitution in the β interaction domain reduced the total number of α_1–β complexes. But it also diminished the ability of β subunits to convert these α_{1C}Y467S–β_{1a-myc} complexes into a high-affinity state for (+)-[^{3}H]isradipine.

4. Discussion

The present results clearly demonstrate that α_{1C}Y467S – an α_{1C} mutant with a single residue substitution in the primary β interaction domain – is still capable of forming stable complexes with the β_{1a-myc} subunit. This is unexpected because the tyrosine in position 467 of α_{1C} is one of three conserved amino acids in the β interaction domain in the I–II loop of voltage-gated Ca^{2+} channels that have been considered essential for β binding. The corresponding substitution in the α_{1A} channel isoform resulted in a loss of binding in an overlay assay and in a strong reduction of current amplitude when coexpressed with β_{1b} [14]. In an in vitro binding assay a 55 amino acid fusion protein containing the mutated β interaction domain showed >90% reduction in β_{1b} binding [19]. A possible explanation of the differences to our present findings could be that this particular residue or even the entire β interaction domain in the I–II loop contributed differently to complex formation in the neuronal and in the muscle isoforms. In previous studies of our own laboratory the mutation in the β interaction domain disrupted the colocalization of the α_{1C} and β subunits as seen with immunocytochemistry, whereas the β-induced increase in current amplitudes and single-channel open probability was still observed [4,23]. The finding of immunoprecipitable complexes of α_{1C}Y467S and β_{1a-myc} in the present study explains the β-induced functional effects on α_{1C}Y467S. However, it is surprising that a two-fold reduction in complex formation detected in (+)-[^{3}H]isradipine binding and immunoprecipitation assays should make such a striking difference in the colocalization and surface membrane clustering detected with immunocytochemistry.

Secondly, the present findings show that the β interaction domain in the I–II loop is involved in the β-mediated stabilization of a high-affinity (+)-[^{3}H]isradipine binding state of the α_1 subunit. It has previously been shown that coexpression of

α_{1C} and β_{1a} increases of affinity of (+)-[^{3}H]isradipine binding to α_{1C} due to a decreased dissociation rate [27,28]. The present findings extend this observation by showing that the conversion of α_{1C} to a high-affinity binding state is affected by the Y-to-S substitution. Not only the concentration of stable α_{1C}Y467S–β_{1a-myc} complexes is decreased, but also the mechanism by which β binding leads to high-affinity (+)-[^{3}H]isradipine binding is directly altered by the mutation. Whereas the decrease of the B_{max} can be explained by a lower occupancy of α_{1C}Y467S by β_{1a-myc} due to a reduced binding affinity of the I–II loop, this does not explain the decreased affinity of (+)-[^{3}H]isradipine to the remaining α_{1C}Y467S–β_{1a-myc} complexes. This finding suggests that the Y-to-S mutation leads to an altered conformation of the dihydropyridine binding pocket, and thus emphasizes the role of the I–II linker in the transmission of conformational changes from the β binding site to the (+)-[^{3}H]isradipine binding site. Since the dihydropyridine binding site in α_{1C} is intimately related to the Ca^{2+} selectivity filter in the channel pore [29,30], such a functional link between the I–II loop and the ion conductance pathway could represent an efficient modulatory switch for channel properties.

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