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Calcium channels: the β-subunit increases the affinity of dihydropyridine and Ca^{2+} binding sites of the α_1-subunit

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Abstract A Ca^{2+} channel α_1-subunit derived from rabbit heart was transiently expressed in COS-7 cells. The dihydropyridine (+)-isradipine had low affinity (K_i = 34.3 nM) for the α_1-subunit in the absence of the β-subunit due to rapid dissociation (k_{-1} = 0.11 min^{-1}). Co-expression of the β-subunit resulted in a >35-fold increase in (+)-isradipine binding affinity (K_i = 0.9 nM) due to decreased dissociation (k_{-1} of 0.007 min^{-1}). Higher DHP binding affinity was associated with an increase of the apparent affinity of Ca^{2+} ions for the channel. Our data suggest that the β-subunit affects the coordination of Ca^{2+} ions with sites that are coupled to the dihydropyridine binding domain and by this mechanism increases the affinity for these ligands.

Key words: Ca^{2+} channel; β subunit; cDNA expression; Ca^{2+} antagonist; Radioligand binding

1. Introduction

Voltage-dependent Ca^{2+} channels in vertebrates are heterooligomeric complexes, which consist of the major α_1-subunit along with up to three accessory subunits $\alpha_2\delta$, β, and (in skeletal muscle) γ [1,2]. Sharing common structural motifs with the principal subunits of voltage-gated Na^+- and K^+-channels, the α_1-subunit contains basic functional elements including the selectivity filter and the voltage sensor [3]. The α_1-subunit of L-type Ca^{2+} channels also bears the domains responsible for Ca^{2+}-dependent binding of organic Ca^{2+} channel blockers, such as dihydropyridines, phenylalkylamines and benzothiazepines [4]. The site of non-covalent interaction with β-subunits has been identified as a 18-amino-acid motif within the intracellular linker between the transmembrane repeats I and II [5].

The functional role of the accessory subunits is not fully established. When co-expressed with α_1 in heterologous expression systems (*Xenopus* oocytes or mammalian cells) $\alpha_2\text{-}\delta$ and γ exert only minor effects on current densities, gating properties and drug binding [1,6]. In contrast, more pronounced effects have been reported for β-subunits. Their expression stabilized physiological Ca^{2+} channel kinetics [7,8] and increased α_1-mediated inward currents [9,10]. The latter effect was explained by a facilitation of channel opening upon membrane depolarization rather than an increased expression of α_1 protein in the plasma membrane [11,12]. In addition, β-subunits enhance the expression of α_1-associated DHP binding activity [9]. Radioligand binding studies in membranes prepared from stably transfected cell lines are consistent with an increase of the number of high affinity DHP binding sites without effects on the equilibrium dissociation constant of drug binding [7,8,12].

Here we report a more detailed analysis of the β-subunit effects on the DHP binding properties of α_1-subunits transiently expressed in COS-7 cells. In contrast to the studies employing stably transfected cell lines we find that β-subunits do not affect DHP binding densities but are required to stabilize the DHP binding domain in a high affinity state. In kinetic experiments we provide evidence that this affinity increase is accompanied by an increased affinity of the α_1-subunit for Ca^{2+} ions. Our data suggest that a β-subunit-induced conformational change results in an improved coordination of Ca^{2+} within one or more of the channel's Ca^{2+} binding domains.

2. Materials and methods

2.1 Construction of expression plasmids and transfection

Plasmid c_{60}CaCH2a was constructed by replacing the first 144 amino terminal residues of $\alpha 1_{Ca}$ (CaCH2a) [13] with the corresponding 60 residues from carp skeletal muscle α_1 [14]. When expressed in yeast this construct yielded higher expression levels than CaCH2a cDNA (unpublished). For reasons of simplicity we refer to this expression-optimized construct as 'α_1' in the text. The 5'-noncoding region of PCR amplified β-subunit cDNA derived from rabbit skeletal muscle (β_1/CaB1) was supplemented with a consensus sequence for initiation of translation [15] as reported to be essential for functional heterologous expression of cardiac β-subunits [10]. The PCR-clone was sequenced on both strands and proven to be identical to the published original clone β_1/CaB1 [16]. 5'-modified cDNAs were cloned into the SV40 late promoter expression vector pSVL (Pharmacia). Large-scale plasmid preparation and purification was performed using QUIAGEN-tips (QUIAGEN Inc.). COS-7 cells (ATCC CRL 1651) were transfected following a modified DEAE-Dextran protocol [17] using 4 μg of c_{60}CaCH2a- and 1 μg of β_1-cDNAs per 100-mm dish.

2.2 Membrane preparation and DHP binding assay

55 hours after transfection cells were washed with PBS (145 mM NaCl, 2.68 mM KCl, 8.1 mM $Na_2HPO_4 \cdot 12H_2O$, 1.47 mM KH_2PO_4), suspended (1×10^6 cells/ml) in ice-cold hypotonic lysis buffer (10 mM Tris-HCl (pH 7.4), 0.1 mM phenylmethylsulfonyl fluoride (PMSF), 30 μM $CoCl_2$, 1 mg/ml bovine serum albumin (fatty acid free, Sigma)), and incubated on ice for 15 min. All further steps were carried out at 2–4°C. The swollen cells were disrupted with a glass–teflon homogenizer (tight pestle, 20 strokes). The homogenate was centrifuged for 15 min at $400 \times g$. The low speed pellet was discarded and membranes in the supernatant collected by centrifugation at $90{,}000 \times g$ for 45 min. The final pellet was resuspended in binding buffer at a protein concentration of 3–6 mg/ml, quickly frozen in liquid nitrogen and stored for up to 4

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Abbreviations: DHP, dihydropyridine; PCR, polymerase chain reaction; SDS, sodium dodecyl sulfate.

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weeks at −80°C until use. Guinea-pig cardiac membranes were prepared as described [18]. Binding assays were carried out in 50 mM Tris-HCl (pH 7.4 (25°C)), 0.1 mM PMSF, 1 mM $CaCl_2$, 1 mg/ml bovine serum albumin (binding buffer) in a final assay volume of 0.505 ml. For inhibition and kinetic studies 0.3–1.4 nM of (+)-[^{3}H]isradipine (83–88 Ci/mmol, New England Nuclear, Vienna, Austria) were incubated with 50–150 μg of membrane protein for 60 min at 25°C (37°C for guinea-pig cardiac membranes) in the absence (control) and presence of 1 μM (±)-isradipine. Specifically bound radioligand at equilibrium was 3–7 pM for α_1 (concentration of total ligand between 0.4 nM and 1.4 nM, 100–150 μg of membrane protein) and 12–20 pM for $\alpha_1\beta$ (concentration of total ligand between 0.3 and 0.8 nM, 80–110 μg of membrane protein) and guinea-pig cardiac membranes (concentration of total ligand 0.3 nM, 40–60 μg of membrane protein). Bound and free ligand were separated by filtration over GF/C glass fibre filters using 10% (w/v) polyethylene glycol 6000, 10 mM Tris-HCl, pH 7.4, 10 mM $MgCl_2$ as the washing buffer. All experiments, unless noted otherwise, were performed at least three times employing membranes from different transfections. K_d and B_{max} values were calculated after Scatchard transformation. K_i values were calculated as $K_i = IC_{50} / (1 + L/K_d)$, where L is the radioligand concentration, K_d the dissociation constant and the IC_{50} value the concentration of unlabeled ligand that inhibits specific binding by 50% [19]. Dissociation rate constants were determined by linear regression analysis of semi-logarithmic plots of the pooled dissociation data as shown in Fig. 2. In case of biphasic dissociation the rate constants were determined by nonlinear curve fitting of the pooled dissociation data to a bi-exponential decay as shown in Fig. 3; the percentage of sites contributing to the respective rate is given in parenthesis.

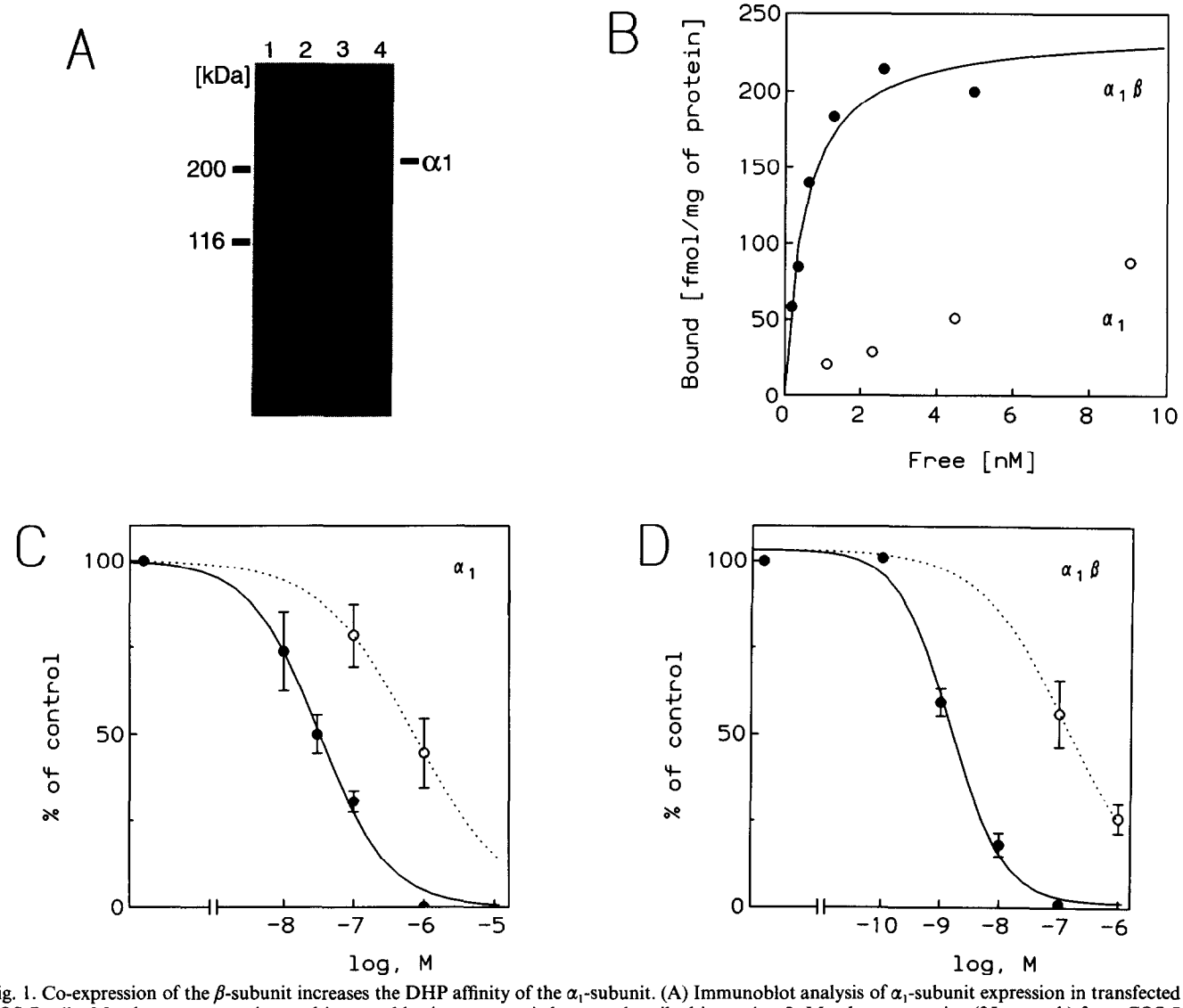

Fig. 1. Co-expression of the β-subunit increases the DHP affinity of the α_1-subunit. (A) Immunoblot analysis of α_1-subunit expression in transfected COS-7 cells. Membrane preparation and immunoblotting was carried out as described in section 2. Membrane proteins (35 μg each) from COS-7 cells transfected with c_{60}CaCH2a (lanes 1,2), c_{60}CaCH2a plus β_1 (lane 3) and β_1 alone (lane 4) were immunostained with the affinity-purified sequence-directed antibody anti-CP(1382–1400). Nonspecific staining (lane 1) was determined by pre-blocking affinity purified anti-CP(1382–1400) with 1 μM of the antigenic peptide. (B) Representative saturation isotherm of (+)-[^{3}H]isradipine binding to membranes from COS-7 cells transfected with c_{60}CaCH2a in combination with β_1 (●). K_d and B_{max} values for the experiment shown were 0.69 nM and 256 fmol/mg of protein, respectively, as calculated after Scatchard transformation ($r = 0.95$). (+)-[^{3}H]Isradipine binding to membranes from COS-7 cells transfected with c_{60}CaCH2a alone (○). Each data point represents the mean of three independent determinations. (C,D) Stereospecific inhibition of (+)-[^{3}H]isradipine binding to membranes from COS-7 cells transfected with c_{60}CaCH2a (C) or c_{60}CaCH2a plus β_1 cDNA (D) by (+)-isradipine (●) and (−)-isradipine (○). Data were normalized with respect to control binding (= 100%). Error bars indicate S.D. from three separate transfections. The K_i values for (+)-isradipine, calculated as described in section 2, are 34.3 ± 6.9 nM ($n_H = 0.8$) for α_1, and 0.9 ± 0.2 nM ($n_H = 1.0$) for $\alpha_1\beta$. No stereospecific binding was detected in nontransfected COS-7 cells ($n = 2$) and COS-7 transfected with or β_1 cDNA or vector DNA ($n = 2$).

2.3 Immunoblotting analysis

Samples were reduced in the presence of 10 mM dithiothreitol, separated by electrophoresis through 8% SDS-polyacrylamide gel and blotted to Immobilon-P membranes (Millipore) by wet electrophoretic transfer [20]. The membrane was blocked for 180 min with 0.05% gelatine, 0.25% non-fat milk in 20 mM Tris-HCl, pH 7.4, 150 mM NaCl, 0.1% Tween-20, 0.5% Triton X-100. Binding of affinity purified anti-CP(1382–1400) [21] was visualized using alkaline-phosphatase-conjugated goat anti-rabbit immunoglobulines and nitroblue tetrazolium salt / 5-bromo-4-chloro-3-indolyl phosphate [20]. Prestained size markers were from Bio-Rad.

3. Results and discussion

COS-7 cells were transiently transfected with α_1-subunit cDNA alone or in combination with skeletal muscle β-subunit cDNA. Immunoblot analysis (Fig. 1A) revealed that the density of α_1 is not altered by co-expression of β-subunit. Equilibrium saturation experiments with the DHP antagonist (+)-[^{3}H]isradipine were performed to assess dissociation constants and receptor densities. Co-expression of the α_1- and β-subunit yielded saturable high affinity DHP binding (K_d = 0.66 ± 0.03 nM; B_{max} = 220 ± 38 fmol/mg of protein). DHP binding to membranes of COS-7 cells transfected with the α_1-subunit alone appeared to be unsaturable up to radioligand concentrations of 10 nM (Fig. 1B). We therefore tested whether the criteria of DHP binding to Ca^{2+} channels (nanomolar affinity, stereoselectivity) were met, by performing binding-inhibition experiments with the enantiomers of unlabeled isradipine. Inhibition of (+)-[^{3}H]isradipine binding was stereoselective for the (+)- and (−)-enantiomer with eudismic ratios of ~20 for α_1 and ~100 for $\alpha_1\beta$. The K_i value was 34.3 ± 6.9 nM for (+)-isradipine when α_1 was expressed alone (Fig. 1C). Scatchard transformation of the binding-inhibition data in Fig. 1C yielded B_{max} estimates ≥411 fmol/mg of protein (n = 3), suggesting that the B_{max} is not reduced in the absence of β. As expected, co-expression of β- subunit decreased the K_i value for (+)-isradipine approximately 38-fold to 0.9 ± 0.2 nM (Fig. 1D). These data from binding-inhibition experiments confirm our results from direct labeling studies indicating that reduced binding of isradipine in the absence of β-subunit is due to a decrease in affinity without major changes in B_{max}. We further confirmed this β-subunit-induced increase in affinity in kinetic experiments (Fig. 2). (+)-[^{3}H]isradipine rapidly dissociated with a half-life of 6.5 min (k_{-1} = 0.11 min^{-1}) when α_1 was expressed alone. Co-expression of β-subunit increased the half-life of the DHP-α_1 complex to 99 min (k_{-1} = 0.007 min^{-1}). As $K_d = k_{-1}/k_{-1}$ calculated association rate constants must remain almost unchanged, suggesting that the β-subunit-induced increase in affinity can be attributed mainly to a decrease of the DHP dissociation rate. Thus our data, obtained from transient expression experiments, demonstrate that co-expression of β-subunit affects the affinity (K_d) and not the density (B_{max}) of DHP receptors.

Since high affinity DHP binding is dependent on the occupation of high affinity Ca^{2+} binding sites of the Ca^{2+} channel [22–26] we tested the possibility that the low DHP affinity observed for α_1 expressed alone was accompanied by a lower affinity for Ca^{2+}. It has previously been shown that ligand-channel complexes are destabilized when free Ca^{2+} is reduced below micromolar concentrations, resulting in a time- and temperature-dependent ligand dissociation, that is determined by the conversion rate of the channel to a very low affinity binding state [24]. This process is reversed by readdition of Ca^{2+} [22,24] and the conversion rates, monitored by ligand dissociation from the low affinity state, have been suggested to reflect Ca^{2+} dissociation from the channel [27]. In order to compare the conversion rate constants of α_1 and $\alpha_1\beta$ we performed kinetic experiments where dissociation of (+)-[^{3}H]isradipine binding was induced by reducing the free Ca^{2+} concentration below 10 nM by the addition of 10 mM EDTA. At 25°C a conversion rate constant of 2.16 min^{-1} for the α_1-subunit was observed (Fig. 3). Co-expression of β-subunits slowed the conversion induced by removal of free Ca^{2+} and resulted in a biphasic decay of (+)-[^{3}H]isradipine binding (Fig. 3). Both rate constants (0.73 min^{-1} for the fast (27%), and 0.014 min^{-1} for the slow (73%) component) were substantially slower (3- and 150-fold, respectively) than the constant for α_1 expressed alone. The biphasic decay of (+)-[^{3}H]isradipine binding prompted us to perform equivalent experiments with guinea-pig cardiac membranes. The conversion reaction was also biphasic (rate constants of 0.43 min^{-1} (35%) and 0.014 min^{-1} (65%)), similar to the co- transfected COS-7 cells (Fig. 3, inset). This suggests that $\alpha_1\beta$ expressing COS-7 cells reflect the behaviour of the entire (α_1, β, α_2-δ) membrane-bound channel with respect to Ca^{2+} binding at millimolar concentrations.

We summarize our data as follows:

(1) Co-expression of the β-subunit increases the affinity of the α_1-subunit for DHP binding without increasing the number of DHP binding sites. This result is supported by the analysis of α_1-mediated Ca^{2+} currents of cardiac α_1 expressed in *Xenopus* oocytes [11] or CHO cells [12], showing that the density of α_1-subunits is not altered by co-expression of β-subunit. Low affinity DHP binding to α_1-subunits expressed alone is in con-

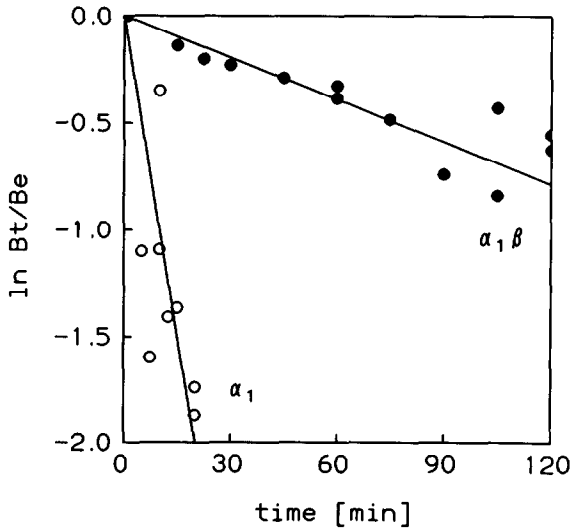

Fig. 2. Dissociation kinetics of (+)-[^{3}H]isradipine binding to membranes from COS-7 cells transfected with c_{60}CaCH2a cDNA alone or in combination with β_1 cDNA. After 60 min incubation with (+)-[^{3}H]isradipine the forward reaction was blocked by addition of 1 μM (±)-isradipine at the times indicated. The dissociation rate constants (k_{-1}), calculated as described in section 2, were 0.11 ± 0.01 min^{-1} for α_1 (○), and 0.0076 ± 0.0027 min^{-1} for $\alpha_1\beta$ (●). B_t = specifically bound ligand at dissociation time t, B_e = specifically bound ligand at equilibrium (t = 0). Each data point represents the mean of three independent determinations.

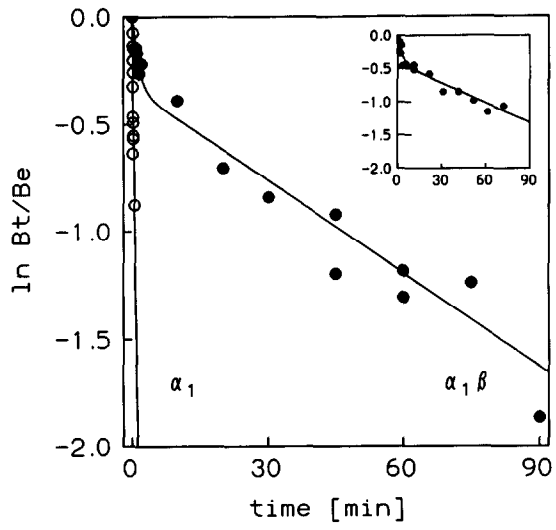

Fig. 3. Co-expression of the β-subunit increases the Ca^{2+} affinity of the α_1-subunit. After 60 min incubation with (+)-[^{3}H]isradipine reduction of total Ca^{2+} to < 10 nM was initiated by addition of 10 mM EDTA and specifically bound radioligand was measured at the times indicated. The conversion rate constant was 2.16 min^{-1} when α_1 was expressed alone (○). The conversion rate constants obtained for $\alpha_1\beta$ (●) were 0.73 min^{-1} (27%) and 0.014 min^{-1} (73%) for the fast and slow component, respectively. The lines represent logarithmic transformation of the curves obtained by nonlinear fitting. Inset: data obtained with guinea-pig cardiac membranes ($n = 2$) are depicted. Two components (0.43 min^{-1} (35%) and 0.014 min^{-1} (65%)) of EDTA-induced decay of (+)-[^{3}H]isradipine binding were observed. Each data point represents the mean of three independent determinations.

trast to the findings from cell lines stably transfected with various α_1-subunits [7,12,28–30]. This contradiction might arise from the intrinsic difference between stable and transient expression. In transient expression experiments a population of cells is examined whereas in an approach for stable expression individual clones are selected for specific channel properties, such as high affinity DHP binding. Endogenous expression of β-subunit, which is not detected by PCR, Northern- and Western-blot analysis (due to low density and/or sequence divergence with the probes used), could therefore become an involuntary selection criterion and account for the stabilization of a small portion of expressed α_1-subunits in the high affinity DHP binding state. Subsequent co-expression of β-subunits would then result in an increase of functional, high affinity DHP receptors (i.e. an increase in B_{max}).

(2) Based on the model of interdependence between Ca^{2+} and DHP binding [31] our results indicate that the β-subunit has a crucial influence on the affinity of Ca^{2+} sites coupled to the DHP binding domain. This is evident from the fact that co-expression of β-subunit restores the biphasic conversion behaviour of the membrane-bound channels from heart. At present we are unable to predict which of the two proposed Ca^{2+} binding sites of the α_1-subunit [32,33] are involved in this phenomenon. Site-directed mutagenesis will help to resolve this issue. The most likely candidate is the Ca^{2+} binding site near the DHP binding domain. This domain has been localized in the P-regions of repeats III and IV [34] and encompasses two of the four glutamate residues which form the Ca^{2+} coordination sites of the pore [33,35–37]. As a working hypothesis we would like to propose that the β-subunit conformationally stabilizes these Ca^{2+} coordination sites and, in turn, increases the affinity by which dihydropyridines can bind.

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