Coordination of Ca^{2+} by the Pore Region Glutamates Is Essential for High-Affinity Dihydropyridine Binding to the Cardiac Ca^{2+} Channel α_1 Subunit†

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ABSTRACT: The molecular determinants for Ca^{2+} modulation of dihydropyridine (DHP) binding to cardiac Ca^{2+} channels were identified by mutational neutralization of the glutamate residues that comprise the Ca^{2+} channel selectivity filter. The binding activity of the DHP (+)-[^{3}H]isradipine, monitored after expression of wild-type and mutant α_1 subunits in COS-7 cells, was markedly reduced in four single mutants and a double mutant. Evidence for decreased Ca^{2+} affinity was obtained for two single mutants in kinetic and equilibrium binding studies. Mutational destabilization of Ca^{2+} binding resulted in a concomitant decrease of (+)-[^{3}H]isradipine binding affinity. Recovery of (+)-[^{3}H]isradipine binding activity by the allosteric modulator (+)-tetrandrine in two single mutants was associated with a recovery of Ca^{2+} and DHP binding kinetics to wild-type values. Our findings demonstrate that high-affinity DHP binding is dependent on Ca^{2+} coordination by glutamate residues which form the selectivity filter of the channel pore.

Voltage-dependent L-type Ca^{2+} channels are heterooligomeric complexes, which control the contractility of heart and smooth muscle by selectively conducting Ca^{2+} ions across the cell membrane in response to membrane depolarization. The basic determinants of Ca^{2+} channel function are located within the α_1 subunit, which is composed of four homologous repeats (I−IV), each consisting of six transmembrane segments (S1−S6). A segment between S5 and S6 (P-regions) of each repeat forms part of the ion-selective pore [for review, see Catterall (1993), Hofmann et al. (1994), and Striessnig et al. (1993)]. Ca^{2+} selectivity and flux rates are determined by conserved glutamate residues within this pathway (Kim et al., 1993; Tang et al., 1993; Yang et al., 1993).

L-type Ca^{2+} channel function can be pharmacologically modulated by Ca^{2+} antagonists, such as dihydropyridines (DHP)[1] and benzothiazepines, which are used in the treatment of angina pectoris and hypertension. Although DHP binding regions have been identified within the α_1 subunit (Catterall & Striessnig, 1992; Striessnig et al., 1991), the molecular mechanism by which Ca^{2+} flux is blocked remains unknown. High-affinity DHP binding to L-type Ca^{2+} channels is dependent on the occupation of a yet unidentified Ca^{2+} binding site (Gould et al., 1982; Luchowski et al., 1984; Glossmann & Ferry, 1985a; Maan et al., 1986; Ebata et al., 1990; Knaus et al., 1992). The relevance of this Ca^{2+} binding site is underpinned by the recent finding that the increase of DHP affinity upon co-expression of the accessory Ca^{2+} channel β subunit is associated with an increase of Ca^{2+} binding affinity of the channel proteins (Mitterdorfer et al., 1994). Alterations of Ca^{2+} coordination to the channel via the reciprocal allosteric interaction between a Ca^{2+} binding site and the DHP binding domain could represent the essential mechanism of channel block by these drugs (Glossmann et al., 1985b). We therefore tried to identify amino acid residues within the α_1 subunit responsible for the Ca^{2+}-induced stabilization of high-affinity DHP binding. The close proximity of the glutamate residues, which comprise the Ca^{2+} channel selectivity filter, to the DHP binding region (Catterall & Striessnig, 1992; Striessnig et al., 1993) prompted us to investigate their role in the Ca^{2+} dependence of DHP binding. By combining site-directed mutagenesis, transient expression in COS-7 cells, and radioligand binding, we now demonstrate that replacement of the P-region glutamates in the repeats I (E393), II (E736), III (E1145), and IV (E1446) by glutamine decreases DHP binding affinity as a result of impaired Ca^{2+} coordination.

MATERIALS AND METHODS

Construction of Expression Plasmids and Transfection. Chimeric α_1 subunit c_{60}CaCH2a was constructed by replacing the first 144 amino-terminal residues of $\alpha_{1C\text{-}a}$ (Mikami et al., 1989) with the corresponding 60 residues from carp skeletal muscle α_1 (Grabner et al., 1991). When expressed in yeast, this construct yielded higher expression levels than $\alpha_{1C\text{-}a}$ cDNA (unpublished data). The single-channel conductance and DHP sensitivity of c_{60}CaCH2a, when expressed in *Xenopus laevis* oocytes, were indistinguishable from those of $\alpha_{1C\text{-}a}$ (Wang et al., 1995). For reasons of simplicity we refer to c_{60}CaCH2a as α_1 in the text. The 5′-noncoding region of PCR-amplified β subunit cDNA derived from rabbit skeletal muscle (β_{1a}) was modified as described (Mitterdorfer et al., 1994). Large-scale plasmid preparation and purification was performed using QIAGEN-tips (QIAGEN Inc.). COS-7 cells (ATCC CRL 1651) were transfected

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[1] Abbreviations: DHP, dihydropyridine; Tris, Tris(hydroxymethyl)-aminomethane; PMSF, phenylmethanesulfonyl fluoride; EDTA, (ethylenedinitrilo)tetraacetic acid, disodium salt dihydrate; PCR, polymerase chain reaction; SDS, sodium dodecyl sulfate.

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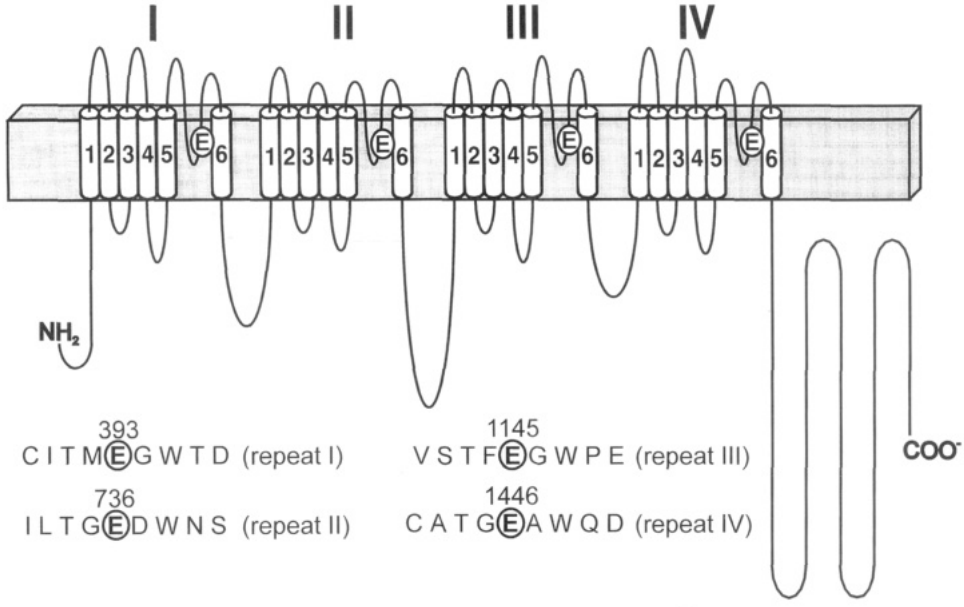

FIGURE 1: Proposed transmembrane topology of the α_1 subunit of voltage-dependent Ca^{2+} channels. The glutamate residues (E) forming the Ca^{2+} selectivity filter are indicated. Amino acid sequences in the S5−S6 linker of each repeat are shown below the diagram. The numbers above the glutamate residues correspond to their positions in $\alpha_{1c\text{-}a}$.

following a modified DEAE-Dextran protocol (Lopata et al., 1984) using 4 μg of α_1 and 1 μg of β_{1a} cDNAs per 100-mm dish.

Site-Directed Mutagenesis. Site-directed mutagenesis was performed on c_{60}CaCH2a using the Site-Directed Mutagenesis Kit (Boehringer Mannheim) or the Sculptor IVM system (Amersham). Residues are numbered according to $\alpha_{1C\text{-}a}$ (Mikami et al., 1989). The following mutagenic antisense primers were used: 5′-GGTCCAGCCCT**G**CATGGTGAT-3′ (nt 1168−1188, E393Q), 5′-TTCCAGTCCT**G**CCCGGT-CAGG-3′ (nt 2197−2215, E736Q), 5′-CTGGCCAGCCCT**G**-GAAGGTGG-3′ (nt 3425−3443, E1145Q), and 5′-CTGC-CAAGCCT**G**CCCCGTGGC-3′ (nt 4327−4345, E1446Q). Desired mutations were verified by sequence analysis. At least two independent mutant clones were used for expression studies.

Membrane Preparation and DHP Binding Assay. Membranes of transfected COS-7 cells were prepared as described (Mitterdorfer et al., 1994), except that $CoCl_2$ was ommitted from the lysis buffer. Binding assays were carried out in 50 mM Tris-HCl [pH 7.4 (25 °C)], 0.1 mM PMSF, and 1 mg/mL bovine serum albumin (binding buffer) in a final assay volume of 0.505 mL. For equilibrium binding and kinetic studies 0.5−2.5 nM (+)-[^{3}H]isradipine (83−88 Ci/mmol, New England Nuclear, Vienna, Austria) was incubated with 80−150 μg of membrane protein for 60−120 min at 25 °C in the absence (control) and presence of 1 μM (±)-isradipine. At K_d concentrations (wild type, 100 μg of membrane protein) specific binding was typically 15−20 pM. Ca^{2+} concentrations > 3 mM resulted in a linear increase of nonspecific binding from 120−150% (10 mM) to 160−200% (30 mM). Stock solutions of all drugs were prepared in Me_2SO. The final concentration of Me_2SO in the binding assay never exceeded 1%, and control experiments indicated that this concentration of solvent did not affect any of the binding interactions. Bound and free ligand were separated by filtration over GF/C glass fiber filters using 10% (w/v) polyethylene glycol 6000, 10 mM Tris-HCl, pH 7.4, and 10 mM $MgCl_2$ as the washing buffer. All experiments were performed at least three times, employing membranes from different transfections, and results are given as means ± standard deviations. Dissociation rate constants were determined by nonlinear curve fitting as described (Mitterdorfer et al., 1994).

Immunoblotting Analysis. Samples were reduced in the presence of 10 mM dithiothreitol, separated by electrophoresis through 8% SDS−polyacrylamide gel, and electroblotted to Immobilon-P membranes (Millipore) (Harlow & Lane, 1988). The membrane was blocked for 180 min with 0.05% gelatin and 0.25% nonfat milk in 20 mM Tris-HCl, pH 7.4, 150 mM NaCl, 0.1% Tween-20, and 0.5% Triton X-100. Binding of affinity-purified anti-CP(1382−1400) (Striessnig et al., 1990) was visualized using alkaline phosphatase-conjugated goat anti-rabbit immunoglobulins and nitroblue tetrazolium salt/5-bromo-4-chloro-3-indolyl phosphate (Harlow & Lane, 1988). Expression of wild-type and mutant α_1 subunits was quantified using a PhosphoImager (FUJIX BAS 1000) after immunoblots were incubated with 50 μCi [^{125}I]-protein A (8.5 μCi/μg, DuPont NEN, Vienna, Austria). Prestained molecular weight markers were from Bio-Rad.

RESULTS

E → Q Mutations Decrease (+)-[^{3}H]Isradipine Binding Activity. In Figure 1 the localization of the conserved P-region glutamates, which comprise the Ca^{2+} channel selectivity filter (Kim et al., 1993; Tang et al., 1993; Yang et al., 1993), is depicted. To investigate the role of these residues in the Ca^{2+} dependence of high-affinity DHP binding, we generated a series of single mutants and a double mutant by replacing the glutamate residues 393 (repeat I), 736 (repeat II), 1145 (repeat III), and 1446 (repeat IV) with glutamine. The single mutants were designated EIQ, EIIQ, EIIIQ, and EIVQ, and the double mutant, EI+IIQ. We have previously demonstrated that the β_{1a} subunit in our heterologous expression system stabilizes the DHP and Ca^{2+} binding properties of the α_1 subunit (Mitterdorfer et al., 1994). The β_{1a} subunit was therefore coexpressed with wild-type and mutant α_1 subunits.

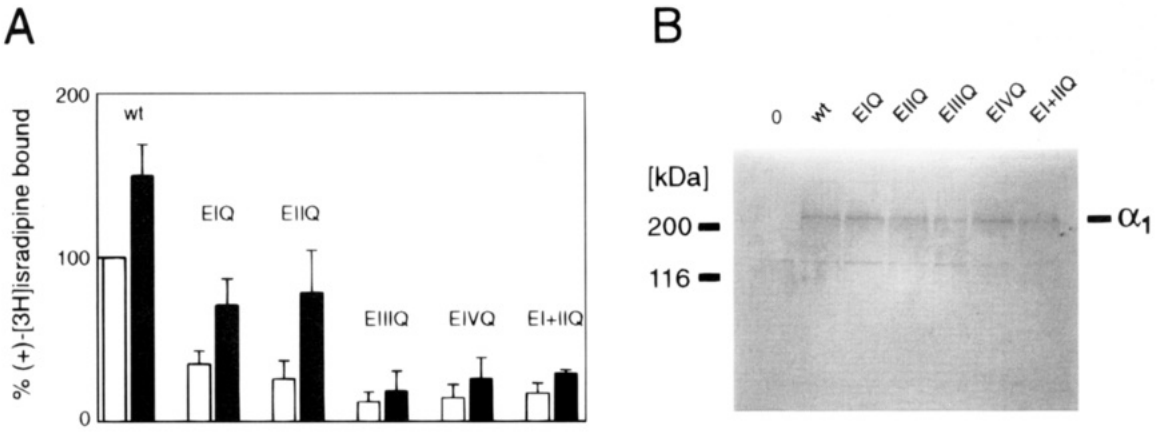

FIGURE 2: Mutational neutralization of the P-region glutamate residues reduces DHP binding activity. (A) DHP binding activity of wild-type and mutant α_1 subunits at 1 nM (+)-[^{3}H]isradipine and 1 mM Ca^{2+} in the absence (open bars) and presence (filled bars) of 3 μM (+)-tetrandrine. Data were normalized for each experiment with respect to (+)-[^{3}H]isradipine binding of wild type in the absence of (+)-tetrandrine (= 100%). Error bars indicate the SD of at least three separate transfections. (B) Immunoblot analysis of membranes from COS-7 cells transfected with vector cDNA (0, lane 1), wild type (wt, lane 2), EIQ (lane 3), EIIQ (lane 4), EIIIQ (lane 5), EIVQ (lane 6), and EI+IIQ (lane 7). Membrane proteins (35 μg each) were immunostained with the affinity-purified, sequence-directed antibody anti-CP(1382−1400). α_1 subunit staining was specific, because it was completely absent after preblocking anti-CP(1382−1400) with 1 μM antigenic peptide (not shown).

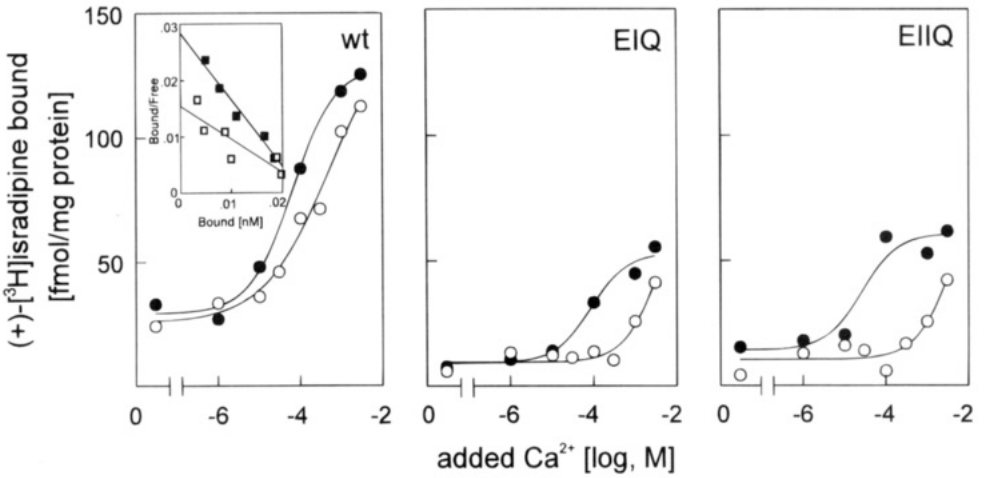

FIGURE 3: Ca^{2+} dependence of (+)-[^{3}H]isradipine binding to wild-type and mutant α_1 subunits EIQ and EIIQ. (+)-[^{3}H]Isradipine binding activity was monitored at increasing concentrations of added Ca^{2+} in the absence (○) and presence (●) of 3 μM (+)-tetrandrine. One of at least three experiments yielding similar results is shown in each panel. Inset: Modulation of (+)-[^{3}H]isradipine binding affinity by Ca^{2+}: Scatchard transformation of a representative (+)-[^{3}H]isradipine saturation analysis of wild-type $\alpha_1\beta$ at 0.1 (□) and 1 mM (■) added Ca^{2+}. K_d and B_{max} values for the experiment shown are given in the text.

Figure 2A shows the effect of the E → Q mutations on (+)-[^{3}H]isradipine equilibrium binding. At 1 mM Ca^{2+} and 1 nM ligand, the binding activity was reduced to 35 ± 8%, 26 ± 11%, 12 ± 6%, 14 ± 8%, and 17 ± 6% of wild type (= 100%) in EIQ, EIIQ, EIIIQ, EIVQ, and EI+IIQ, respectively. This was not due to a decreased protein expression level of mutant α_1 subunits (Figure 2B). Western blot analysis employing [^{125}I]protein A detection revealed that the density of α_1 mutants was 93 ± 14% (EIQ), 114 ± 2% (EIIQ), 92 ± 16% (EIIIQ), 157 ± 18% (EIVQ), or 127 ± 14% (EI+IIQ) of wild type (n = 3).

In electrophysiological experiments these mutations decrease divalent cation selectivity as a result of decreased divalent cation binding affinity (Yang et al., 1993). We therefore investigated whether the reduced DHP binding activity of the mutants was accompanied by a change in the sensitivity of Ca^{2+}. Due to the almost complete loss of DHP binding activity in EIIIQ, EIVQ, and the double mutant EI+IIQ, further analysis of the Ca^{2+} and DHP binding properties in equilibrium radioligand binding and kinetic experiments was restricted to EIQ and EIIQ.

Impaired Ca^{2+} Stimulation of (+)-[^{3}H]Isradipine Binding to EIQ and EIIQ. Figure 3 illustrates, that Ca^{2+} stimulates (+)-[^{3}H]isradipine equilibrium binding of wild-type α_1 subunits in a concentration-dependent manner up to a maximum of 2.4−4.2-fold at 3 mM Ca^{2+}. Ca^{2+} concentrations greater than 3 mM interfered with our assay by increasing nonspecific binding (see Materials and Methods). The Ca^{2+} stimulation of (+)-[^{3}H]isradipine binding is qualitatively similar to that observed in cardiac membranes, which were washed with a chelator. Under these conditions, a 3-fold stimulation of DHP binding with a $K_{0.5}$ value close to 10 μM was reported (Luchowski et al., 1984), whereas in wild-type $\alpha_1\beta$ the $K_{0.5}$ values were estimated to be 150−500 μM.

In our experimental system the enhancement of ligand binding at increasing Ca^{2+} concentrations is due to an increase in DHP binding affinity rather than an increase in B_{max} as found by saturation analysis of wild-type $\alpha_1\beta$. An

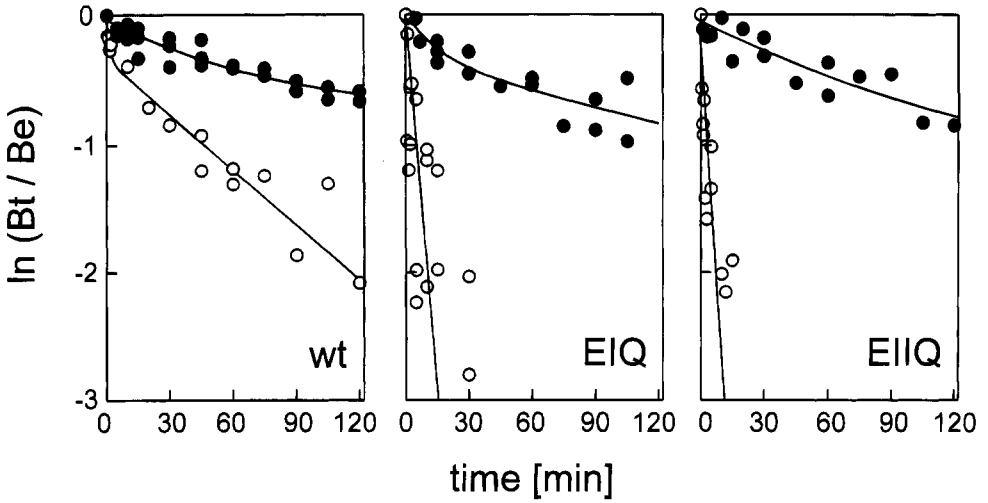

FIGURE 4: (+)-Tetrandrine reverts the decreased Ca^{2+} affinity of EIQ and EIIQ to wild type. After 60−120 min of incubation with (+)-[^{3}H]isradipine (1 mM Ca^{2+} added as $CaCl_2$) in the absence (○) and presence (●) of 3 μM (+)-tetrandrine, the reduction of total Ca^{2+} to <10 nM was initiated by addition of 10 mM EDTA, and specifically bound radioligand was measured at the times indicated. The lines represent the logarithmic transformation of the curves obtained by nonlinear fitting to a mono- or biexponential decay. Each data point represents the mean of three independent determinations. The statistics of the rate constants are given in the text. Bt = specifically bound ligand at time t; Be = specifically bound ligand at equilibrium (t = 0).

example is shown in Figure 3 (inset) where 0.1 or 1 mM Ca^{2+} was added (as $CaCl_2$). The K_d and B_{max} values here were 1.71 nM and 134 fmol/mg of protein at 0.1 mM Ca^{2+} and 0.84 nM and 121 fmol/mg of protein at 1.0 mM Ca^{2+}. The mean value of K_d reduction by increasing Ca^{2+} from 0.1 to 1 mM was 2.3 ± 0.4 (n = 3). As reported before (Mitterdorfer et al., 1994), the affinity of (+)-[^{3}H]isradipine for cardiac α_1, coexpressed with skeletal muscle β_{1a} subunit in COS-7 cells, is 1 order of magnitude lower than the affinity of cardiac membranes (K_d = 0.05−0.08 nM, not shown; Ptasienski et al., 1985) or intact cardiac myocytes (Kokubun et al., 1986). Likewise, the Ca^{2+} stimulatory effect required higher Ca^{2+} concentrations (see above) than in native cardiac membranes. The reasons for this are unknown.

In EIQ and EIIQ the ability to respond to Ca^{2+} with an increase in DHP binding seemed to be impaired when compared to wild type (Figure 3). $K_{0.5}$ values could not be accurately determined for the reasons outlined above (increase in nonspecific binding when the Ca^{2+} concentration was raised above 3 mM), but the stimulation curves were clearly shifted to the right (see Figure 3). Similar to wild-type $\alpha_1\beta$, stimulation of (+)-[^{3}H]isradipine binding at 3 mM Ca^{2+} was 2.0−3.1-fold for EIQ and 1.9−3.8-fold for EIIQ. However, the residual (+)-[^{3}H]isradipine binding activity (ligand concentration: 1.0−1.5 nM) without added Ca^{2+} seemed to be lower in both mutants than in wild type [EIQ, 8.8 ± 3.2; EIIQ, 13.2 ± 7.6; wt, 25.5 ± 12.6 (fmol/mg of protein)]. Accordingly, the maximal stimulation of (+)-[^{3}H]-isradipine binding at 3 mM Ca^{2+} was decreased 2−3-fold in the mutants when compared with wild type [EIQ, 24.7 ± 7.8; EIIQ, 39.7 ± 14.5; wt, 78.3 ± 31.1 (fmol/mg of protein)]. In these experiments effects of the E → Q mutations on either Ca^{2+} or DHP binding properties could not be distinguished. Therefore we next tested whether the impaired Ca^{2+} stimulation of the mutants was caused by a decrease in Ca^{2+} affinity.

Impaired Ca^{2+} Stimulation of (+)-[^{3}H]Isradipine Binding in EIQ and EIIQ Is a Consequence of Accelerated Ca^{2+} Dissociation. We performed kinetic experiments, where (+)-[^{3}H]isradipine dissociation was initiated by reducing the free Ca^{2+} concentration below 10 nM by the addition of 10 mM EDTA. From previous studies it is known that reduction of free Ca^{2+} below micromolar concentrations causes a destabilization of the DHP−channel complex, which is followed by rapid ligand dissociation. The rate-limiting step for EDTA-induced loss of bound ligand is the conversion to the low-affinity state rather than subsequent DHP dissociation (Knaus et al., 1992). Thus, the rate constant for EDTA-induced ligand loss is an estimate for the rate constant of Ca^{2+} dissociation from the channel complex (Staudinger et al., 1991). As shown in Figure 4, the characteristic biphasic decay of (+)-[^{3}H]isradipine binding (Mitterdorfer et al., 1994) with conversion rate constants of 0.73 min^{-1} (27%) and 0.014 min^{-1} (73%) was observed in wild-type membranes. In EIQ and EIIQ the Ca^{2+}-dissociation kinetics were dramatically altered. Both mutants exhibited a fast, monophasic conversion with rate constants of 0.48 min^{-1} (EIQ) and 0.71 min^{-1} (EIIQ). From these findings we conclude that the impaired Ca^{2+} sensitivity of EIQ and EIIQ, observed in the equilibrium binding experiments, is a result of decreased Ca^{2+} binding affinity.

EIQ Exhibits Increased (+)-[^{3}H]Isradipine Dissociation. The impaired Ca^{2+} affinity of the E → Q mutants studied predicts a decrease of DHP binding affinity, as Ca^{2+} and DHP binding were reported to be interdependent (Glossmann et al., 1985b). Due to the very low specific binding signal (see Figure 3) we were unable to address this question directly by saturation analysis with EIQ or EIIQ. Instead, we measured changes of the dissociation rate constant (k_{-1}) as a convenient parameter for changes of (+)-[^{3}H]isradipine binding affinity. EIQ and wild-type membranes were reversibly labeled with (+)-[^{3}H]isradipine, and dissociation was induced by the addition of unlabeled isradipine. Figure 5 illustrates that the monophasic dissociation from EIQ was about 3-fold faster (k_{-1} = 0.024 ± 0.007 min^{-1}) than for wild-type (0.0083 ± 0.0029 min^{-1}). Assuming no change in the association rate constant for (+)-[^{3}H]isradipine, the observed increase in k_{-1} reflects an about 3-fold decrease in binding affinity for EIQ. This would predict a reduction in

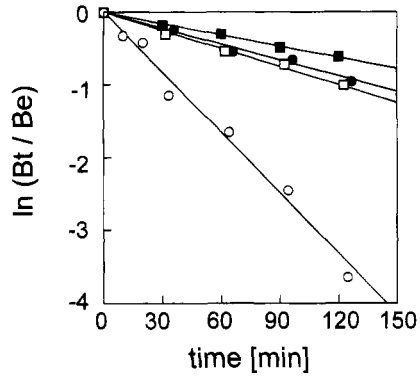

FIGURE 5: Dissociation kinetics of (+)-[^{3}H]isradipine binding to wild type (squares) and EIQ (circles) in the absence (open symbols) and presence (filled symbols) of 3 μM (+)-tetrandrine. Data points represent the mean of three separate experiments. The mean ± SD of the dissociation rate constants from the single experiments was 0.024 ± 0.007 min^{-1} (○), 0.0073 ± 0.0016 min^{-1} (●), 0.0083 ± 0.0029 min^{-1} (□), and 0.0052 ± 0.0014 min^{-1} (■).

receptor occupancy from 50% (wild-type control) to 25% (EIQ) under the experimental conditions used in Figure 2 and translates into a reduction to 50% of control binding. This is in good agreement with the 35 ± 8% value measured for EIQ (Figure 2). These findings suggest that the decreased DHP affinity of EIQ is a result of decreased Ca^{2+} affinity.

Modulation by (+)-Tetrandrine. The Ca^{2+} antagonist (+)-tetrandrine binds to the benzothiazepine site of L-type Ca^{2+} channels and thereby increases DHP affinity (King et al., 1988). It is well established that positive allosteric modulators like (+)-*cis*-diltiazem and (+)-tetrandrine stimulate DHP binding by increasing the affinity of Ca^{2+} for sites that are coupled to the DHP binding domain (Staudinger et al., 1991). We therefore tested whether (+)-tetrandrine was able to improve the impaired Ca^{2+} coordination of the mutant α_1 subunits and thereby overcome the reduction of DHP binding activity. As shown in Figure 2A, 3 μM (+)-tetrandrine indeed recovered (+)-[^{3}H]isradipine binding at 1 mM Ca^{2+} and 1 nM ligand to 71 ± 16% and 79 ± 26% of wild type in EIQ and EIIQ. Only comparably small recoveries to 19 ± 9%, 26 ± 13%, and 29 ± 1% of wild-type control binding were observed in EIIIQ, EIVQ, and EI+IIQ, respectively. The Ca^{2+} concentration dependence in the stimulation of DHP equilibrium binding (Figure 3) indicated that 3 μM (+)-tetrandrine simultaneously increased (+)-[^{3}H]isradipine binding activity and Ca^{2+} sensitivity of wild-type, EIQ, and EIIQ at radioligand concentrations of 1.0−1.5 nM (Figure 3). The stimulatory effect in the mutants, compared with wild type, was incomplete within the Ca^{2+} concentration range tested.

The (+)-tetrandrine-mediated increase in Ca^{2+} sensitivity was due to an increase in Ca^{2+} affinity, as evident from the Ca^{2+} dissociation kinetics of wild type, EIQ, and EIIQ (Figure 4). In wild-type, both components of channel conversion were slowed at least 40-fold in the presence of 3 μM (+)-tetrandrine (fast, 49%, k_{-1} = 0.017 min^{-1}; slow, 51%, no dissociation within 120 min, $k_{-1} \ll 0.001$ min^{-1}). Surprisingly, (+)-tetrandrine was able to restore the biphasic conversion reaction with rate constants close to wild type in EIQ and EIIQ. Again, an apparently EDTA-insensitive slow component (no dissociation within 120 min, $k_{-1} \ll 0.001$ min^{-1}) was observed for both mutants, and the rate constants of the fast components (EIQ, 0.032 min^{-1}, 53%; EIIQ, 0.011 min^{-1}, 66%) were similar to wild type in the (+)-tetrandrine-stabilized state. These data suggest that Ca^{2+} binding, destabilized by replacement of the glutamate residue in position 393 or 736 with glutamine, can be increased by (+)-tetrandrine.

Improved Ca^{2+} coordination in the (+)-tetrandrine-stabilized state should result in an increased DHP binding affinity, as suggested in a previous report (Staudinger et al., 1991). We therefore assessed the (+)-[^{3}H]isradipine dissociation rate constants of EIQ and wild type in the presence of (+)-tetrandrine (Figure 5). As observed for Ca^{2+} dissociation, the difference of (+)-[^{3}H]isradipine dissociation between EIQ and wild type was almost completely abolished in the presence of 3 μM (+)-tetrandrine (EIQ, k_{-1} = 0.0073 ± 0.0016 min^{-1}; wild-type, 0.0052 ± 0.0014 min^{-1}). These data are compatible with a decrease of (+)-[^{3}H]isradipine binding affinity in mutant EIQ, that can be compensated for by simultaneous binding of (+)-tetrandrine.

DISCUSSION

Reversible binding of Ca^{2+} channel antagonists to the α_1 subunit of L-type Ca^{2+} channels is modulated by Ca^{2+} (Glossmann et al., 1985b; Gould et al., 1982; Luchowski et al., 1984; Maan et al., 1986; Schneider et al., 1991; Sumimoto et al., 1988). Occupancy of a high-affinity Ca^{2+} binding site is required for high-affinity dihydropyridine and phenylalkylamine binding to neuronal and purified skeletal muscle channels [for a review, see Striessnig et al. (1993)]. In cardiac membranes Ca^{2+} stimulates DHP binding in the range 3−100 μM (Luchowski et al., 1984). Some Ca^{2+} channel drugs affect the affinity of Ca^{2+} for the channel and by this mechanism act as allosteric modulators for other Ca^{2+} channel blockers. This has been experimentally confirmed for positive allosteric regulators of DHP binding, like (+)-tetrandrine, (−)-BM20.1140, and (+)-*cis*-diltiazem. These drugs stimulate DHP binding by increasing the channel's affinity for Ca^{2+} (Staudinger et al., 1991). We have previously demonstrated that the increase of the DHP binding affinity upon coexpression of the β_{1a} subunit is accompanied by an increase in the Ca^{2+} binding affinity of an α_1 subunit derived from rabbit heart. Here we show that $\alpha_1\beta$ subunit complexes, when expressed in COS-7 cells, exhibit Ca^{2+} dependence of DHP binding. Saturation analysis (Figure 3, inset) indicated that a 10-fold increase in the concentration of Ca^{2+}, from 0.1 to 1 mM, results in a ~2-fold increase of DHP binding affinity. This confirms that DHP binding affinity is modulated by Ca^{2+}, although K_d and $K_{0.5}$ values are not identical to those of native heart membranes. We can also demonstrate that the P-region glutamate residues which comprise the Ca^{2+} channel selectivity filter play an important role in this phenomenon. Mutational neutralization of the P-region glutamate residues in repeats III and IV and in a double mutant (repeat I plus II) resulted in a nearly complete loss of DHP binding, which prevented further analysis in radioligand binding experiments. However, sufficient residual DHP binding activity of repeat I or II mutants was retained to allow a detailed study. In these mutants we observed an estimated 2−8-fold decrease in the Ca^{2+} sensitivity for (+)-[^{3}H]isradipine equilibrium binding. This reduction of Ca^{2+} sensitivity was due to a decrease in Ca^{2+} affinity, which was revealed by the kinetics of the EDTA-induced conversion of the channel to a low-affinity DHP binding state. If Ca^{2+} and DHP binding are interdependent, the decrease in Ca^{2+} affinity predicts a destabiliza-

tion of the DHP−channel complex. This, indeed, was the case as evident from an increase of (+)-[^{3}H]isradipine dissociation kinetics in EIQ. Our data therefore strongly suggest that the observed decrease in DHP binding affinity of mutants EIQ and EIIQ can be attributed to impaired Ca^{2+} coordination to the P-region glutamate residues which form the Ca^{2+} channel selectivity filter.

We obtained independent experimental support for this hypothesis in experiments with the alkaloid (+)-tetrandrine, which binds to the diltiazem-selective site of the channel (King et al., 1988) and allosterically modulates DHP binding affinity via Ca^{2+} (Staudinger et al., 1991). (+)-Tetrandrine increased (+)-[^{3}H]isradipine equilibrium binding in mutants EIQ and EIIQ but not in EIIIQ, EIVQ, or the double mutant EI+IIQ. Apparently the Ca^{2+} effect on DHP binding can be sustained by (+)-tetrandrine even if the repeat I or repeat II glutamate is neutralized. Why the almost complete restoration of dissociation kinetics did not result in a complete recovery of DHP equilibrium binding is not clear. It is possible either that the B_{max} is slightly decreased or that charge neutralization alters the DHP association kinetics. Neutralization of both glutamates (in the double mutant EI+IIQ) prevented the (+)-tetrandrine effect, suggesting that at least one of these two glutamates must be present to form a residual Ca^{2+} coordination site. Due to the almost complete loss of DHP binding affinity in the repeat III and IV mutants, which could not be recovered by (+)-tetrandrine, we cannot completely exclude that the P-region glutamates of repeats III and IV are directly involved in DHP binding. However, this interpretation seems unlikely for the following reason. There is striking homology between DHP-sensitive and -insensitive Ca^{2+} channels not only in the P-region glutamates (Ca^{2+} channel selectivity filter) but also with respect to the adjacent amino acid residues. More likely, the DHP binding domain is stabilized by coordination of Ca^{2+} to a site formed by the four P-region glutamates. Ca^{2+} binding to these residues is essential for divalent cation selectivity (Kim et al., 1993; Tang et al., 1993; Yang et al., 1993). By subtle conformational arrangements the P-region glutamates can accommodate either one Ca^{2+} ion with high affinity or two with low affinity (Yang et al., 1993; Yellen et al., 1993). Positive allosteric modulators of DHP binding like (+)-tetrandrine appear to modulate this Ca^{2+} binding site as revealed by the Ca^{2+} dissociation kinetics of wild type, EIQ, and EIIQ. From the inability of (+)-tetrandrine to compensate for mutational neutralization of repeat III and repeat IV glutamate residues, we conclude that Ca^{2+} coordination involving the P-region glutamates in the vicinity of the DHP binding pocket (Catterall & Striessnig, 1992; Striessnig et al., 1991) is essential for high-affinity DHP binding.

In summary, we identified the molecular determinants for Ca^{2+} modulation of DHP binding and demonstrated that (+)-tetrandrine stabilizes the DHP−channel complex by improving Ca^{2+} coordination to the P-region glutamates in repeats III and IV. Clearly, functional studies employing the E → Q mutants are required for further evidence. Nevertheless, our results support the hypothesis that dihydropyridine Ca^{2+} antagonists block the entry of Ca^{2+} through voltage-dependent L-type Ca^{2+} channels by enhancing its coordination within the channel pore (Glossmann et al., 1985b).

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REFERENCES

Catterall, W. A. (1993) *Trends Neurosci. 16*, 500−506.
Catterall, W. A., & Striessnig, J. (1992) *Trends Pharmacol. Sci. 13*, 256−262.
Ebata, H., Mills, J. S., Nemcek, K., & Johnson, J. D. (1990) *J. Biol. Chem. 265*, 177−182.
Glossmann, H., & Ferry, D. R. (1985) *Methods Enzymol. 109*, 513−550.
Glossmann, H., & Striessnig, J. (1990) *Rev. Physiol. Biochem. Pharmacol. 114*, 1−105.
Glossmann, H., Ferry, D. R., Goll, A., Striessnig, J., & Zernig, G. (1985) *Drug Res. 35*, 1917−1935.
Gould, R. J., Murphy, K. M. M., & Snyder, S. H. (1982) *Proc. Natl. Acad. Sci. U.S.A. 79*, 3656−3660.
Grabner, M., Friedrich, K., Knaus, H. G., Striessnig, J., Scheffauer, F., Staudinger, R., Koch, W. J., Schwartz, A., & Glossmann, H. (1991) *Proc. Natl. Acad. Sci. U.S.A. 88*, 727−731.
Harlow, E., & Lane, D. (1988) in *Antibodies: A Laboratory Manual*, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
Hofmann, F., Biel, M., & Flockerzi, V. (1994) *Annu. Rev. Neurosci. 17*, 399−418.
Kim, M.-S., Takashi, M., Sun, L.-X., Imoto, K., & Mori, Y. (1993) *FEBS Lett. 318*, 145−148.
King, V. F., Garcia, M. L., Himmel, D., Reuben, J. P., Lam, Y. T., Pan, J., Han, G., & Kaczorowski, G. J. (1988) *J. Biol. Chem. 263*, 2238−2244.
Knaus, H. G., Moshammer, T., Kang, H. C., Haugland, R. P., & Glossmann, H. (1992) *J. Biol. Chem. 267*, 2179−2189.
Kokubun, S., Prod Hom, B., Becker, C., Porzig, H., & Reuter, H. (1986) *Mol. Pharmacol. 30*, 571−584.
Lopata, M. A., Cleveland, D. W., & Sollner-Webb, B. (1984) *Nucleic Acids Res. 12*, 5707.
Luchowski, E. M., Yousif, F., Triggle, D. J., Maurer, S. C., Sarmiento, J. G., & Janis, R. A. (1984) *J. Pharmacol. Exp. Ther. 230*, 607−613.
Maan, A. C., Ptasienski, J., & Hosey, M. M. (1986) *J. Pharmacol. Exp. Ther. 239*, 768−774.
Mikami, A., Imoto, K., Tanabe, T., Niidome, T., Mori, Y., Takeshima, H., Narumiya, S., & Numa, S. (1989) *Nature 340*, 230−233.
Mitterdorfer, J., Froschmayr, M., Grabner, M., Striessnig, J., & Glossmann, H. (1994) *FEBS Lett. 352*, 141−145.
Ptasienski, J., McMahon, K. K., & Hosey, M. M. (1985) *Biochem. Biophys. Res. Commun. 129*, 910−917.
Schneider, T., Regulla, S., & Hofmann, F. (1991) *Eur. J. Biochem. 200*, 245−253.
Staudinger, R., Knaus, H. G., & Glossmann, H. (1991) *J. Biol. Chem. 266*, 10787−10795.
Striessnig, J., Glossmann, H., & Catterall, W. A. (1990) *Proc. Natl. Acad. Sci. U.S.A. 87*, 9108−9112.
Striessnig, J., Murphy, B. J., & Catterall, W. A. (1991) *Proc. Natl. Acad. Sci. U.S.A. 88*, 10769−10773.
Striessnig, J., Berger, W., & Glossmann, H. (1993) *Cell Physiol. Biochem. 3*, 295−317.
Sumimoto, K., Hirata, M., & Kuriyama, H. (1988) *Am. J. Physiol. 254*, 45−52.
Tang, S., Mikala, G., Bahinski, A., Yatani, A., Varadi, G., & Schwartz, A. (1993) *J. Biol. Chem. 268*, 13026−13029.
Wang, Z., Grabner, M., Berjukov, S., Savchenko, A., Glossmann, H., & Hering, S. (1995) *J. Physiol. (London)* (in press).
Yang, J., Ellinor, P. T., Sather, W. A., Zhang, J.-F., & Tsien, R. W. (1993) *Nature 366*, 158−161.
Yellen, G. (1993) *Nature 366*, 109−110.

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