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Subunit Composition Is a Major Determinant in High Affinity Binding of a Ca^{2+} Channel Blocker

HAEYOUNG SUH-KIM, XIANGYANG WEI, and LUTZ BIRNBAUMER

Department of Anatomy, Ajou University, Suwon 442–749, Korea (H.S.-K.), Institute for Molecular Medicine and Genetics, Medical College of Georgia, Augusta, Georgia 30912 (X.W.), and Departments of Anesthesiology and Biological Chemistry, University of California, Los Angeles, School of Medicine, Los Angeles, California 90024-1778 (L.B.)

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SUMMARY

Skeletal muscle L-type channels are the pharmacological receptors for Ca^{2+} channel antagonists, including dihydropyridines (DHPs). High affinity DHP binding to these channels in skeletal muscle membranes has been reported to be independent of Ca^{2+} addition and to become dependent on Ca^{2+} after solubilization. The channel is a multimeric complex composed of $\alpha 1$, β, γ, and $\alpha 2\delta$, of which $\alpha 1$ is the pore-forming and DHP-binding component. In this study we coexpressed non-$\alpha 1$ components with $\alpha 1$ in L and COS cells and investigated their roles in the regulation of high affinity DHP binding by Mg^{2+} and Ca^{2+}. No DHP binding to membranes of cells expressing $\alpha 1\beta$ alone was detected in the absence of Ca^{2+} or Mg^{2+}. Addition of Mg^{2+} revealed the presence of (+)-PN200-110 (DHP) binding sites with a K_d of 1 nM. This affinity was 4-fold lower than that of skeletal muscle membrane binding sites (K_d = 0.25 nM). Addition of Ca^{2+} increased the affinity for DHP in membranes from $\alpha 1\beta$-expressing cells to that seen in skeletal muscle membranes (K_d = 0.2–0.3 nM; EC_{50} of 0.2 μM). Ca^{2+} did not affect DHP binding to skeletal muscle membranes. Coexpression of all of the subunits completely recapitulated the high affinity DHP binding seen with skeletal muscle membranes in the absence of Ca^{2+} and Mg^{2+} (K_d = 0.15 nM). This affinity was unaffected by addition of Ca^{2+} or Mg^{2+}. Coexpression of $\alpha 1\beta$ with either $\alpha 2\delta$ or γ alone resulted in DHP binding intermediate between levels seen with $\alpha 1\beta$ and $\alpha 1\beta\alpha 2\delta\gamma$. Thus, this study demonstrates that $\alpha 2\delta$ and γ are essential for full reconstitution of the DHP binding characteristics of the skeletal muscle L-type Ca^{2+} channel/DHP receptor.

L-type voltage-dependent calcium channels are expressed in neurons, endocrine cells, and cardiac, smooth, and skeletal muscle cells. They are targets of a variety of clinically used Ca^{2+} channel antagonists, including DHPs, and are thus referred to as DHP receptors. High affinity binding of DHPs to these receptors is subject to complex allosteric regulation by other Ca^{2+} channel antagonists, such as phenylalkylamines and benzothiazepines, as well as by divalent cations. The allosteric regulation by divalent cations appears to vary with tissue and hence with the subtype of the L-type channels. Thus, in cardiac and brain membranes, DHP binding is inhibited by EDTA and restored by addition of Ca^{2+} or Mg^{2+} (1, 2). In skeletal muscle membranes, DHP binding is insensitive to EDTA or EGTA as long as the membranes are intact but becomes sensitive to inhibition by the chelators and dependent on addition of divalent cations after disruption of membrane integrity with agents such as the calcium ionophore A23187 or detergents such as digitonin or CHAPS (3). The skeletal muscle DHP receptors reside mainly in T-tubule membranes, which for the most part are isolated as sealed inside-out vesicles (4). The insensitivity of DHP binding to the addition of chelators and the resulting lack of requirement for divalent cations for high affinity DHP binding have been largely ascribed to the presence of entrapped Ca^{2+} interacting with a site located on the extracellular aspect of the receptor complex. As assessed in reconstituted digitonin vesicles, this extracellular site has an affinity in the 2–5 μM range (5). In those studies, the effects of Ca^{2+} on DHP binding to purified receptors or to the Ca^{2+}-depleted membranes were detected in Scatchard plots as changes in the total number of binding sites, without a change in affinity, indicating that Ca^{2+} stabilizes the high affinity form of the receptor.

The skeletal muscle DHP receptor was purified as a multicomponent complex composed of $\alpha 1$, $\alpha 2\delta$, β, and γ (6). It is now recognized that L-type Ca^{2+} channels in heart, smooth muscle, endocrine cells, and brain are composed of the same

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ABBREVIATIONS: DHP, dihydropyridine; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N,N,N′,N′*-tetraacetic acid; D600, 2-(3,4,5-trimethoxyphenyl)-2-isopropyl-5-[(3,4-dimethoxyphenyl)methylamino]valeronitrile hydrochloride; G418, geneticin sulfate; BAPTA, 1,2-bis(2-aminophenoxy)ethane-*N,N,N′,N′*-tetraacetic acid; MOPS, 3-(*N*-morpholino)propanesulfonic acid; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]propanesulfonic acid.

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$\alpha 2\delta$ subunit, one of several distinct but homologous $\alpha 1$ subunits, and one of several distinct β subunits. A γ subunit has thus far been detected only in skeletal muscle (7).

The skeletal muscle $\alpha 1$ alone constitutes both a Ca^{2+} channel pore and a receptor for DHPs and other Ca^{2+} channel antagonists (8, 9). However, both the kinetics of the Ca^{2+} channel currents and the allosteric regulation of DHP binding by a phenylalkylamine antagonist, (−)-D600, are abnormal in cells expressing $\alpha 1$ alone (10), indicating regulatory roles for the missing β, $\alpha 2\delta$ and γ components. Coexpression of β with $\alpha 1$ normalizes the activation kinetics of $\alpha 1$ (11). Here we investigated the allosteric regulation by Ca^{2+} of DHP binding in membranes from cells expressing $\alpha 1\beta$ and found that, in contrast to binding to T-tubule membranes, binding to $\alpha 1\beta$ is fully inhibited by divalent cation chelators and is absolutely dependent on the addition of divalent cations (Mg^{2+} or Ca^{2+}). As will be reported elsewhere, in membranes from cells expressing $\alpha 1\beta$ alone, DHP binding (K_d = 1 nM) was increased by addition of the phenylalkylamine (−)-D600, and this effect was occluded upon coexpression of all four components of the DHP receptor (11a). We found that coexpression of all of the skeletal muscle Ca^{2+} channel subunits also made high affinity DHP binding independent of divalent cation addition. This led us to study the divalent cation dependence of high affinity DHP binding as a function of subunit composition.

Materials and Methods

cDNAs and expression plasmids. Expression plasmids for skeletal muscle subunits $\alpha 1S$ (GenBank accession number M23919), $\beta 1A$ (GenBank accession number M25817), $\alpha 2\delta$ (a kind gift from Dr. Shosaku Numa, University of Tokyo, Japan), and γ (GenBank accession number M32231) and the pSVβGal plasmid have been described (11, 12). Negative control plasmids were prepared either by deleting their inserts or by subcloning the inserts in the antisense orientation.

Culture and transfection of L cells. L cells were grown in minimum essential medium α (GIBCO, Grand Island, NY) with 100 units/ml penicillin and 100 μg/ml streptomycin, in the presence of 10% fetal bovine serum, and were transfected by the calcium phosphate method, as described by Graham and Van der Eb (13). Ltk^- cells were transfected with the $\alpha 1$ expression vector containing the neomycin resistance gene and were plated in 96-well plates at a density that, on average, yielded one G418-resistant cell clone every two or three wells. After selection with 300–400 μg/ml G418, cells from single colonies were expanded and analyzed electrophysiologically for Ca^{2+} currents (13). LCaN$\alpha 1$ cells that gave consistent currents were chosen for the subsequent transfections. To obtain L cells expressing $\alpha 1$ and other Ca^{2+} channel subunits, the thymidine kinase-deficient LCaN$\alpha 1$ cells were transfected with expression plasmids for β in p91023(B) and γ in pcD, together with limiting amounts of the herpes simplex virus thymidine kinase gene in pHSV-106 (BRL, Grand Island, NY). Cell clones surviving in the presence of 100 μM hypoxanthine, 0.4 μM aminopterin, 160 μM thymidine, and 300–400 μg/ml G418 were expanded and subjected to Northern analysis for the expression of β and/or γ. At least two L cell lines, each derived from an independent transfection event as confirmed by Southern analysis, were isolated for each of the subunit combinations tested. Transfections of COS cells were carried out as described previously (12).

Preparation of membranes. L cells, grown to confluence in 100-mm dishes, or COS.M6 cells, 60 hr after transfection, were used to prepare crude membranes as described (12). Membranes enriched in T-tubules with a DHP receptor density between 3 and 6 pmol/mg of membrane protein were prepared at 4° from frozen rabbit skeletal muscle, as described (14).

(+)-[^{3}H]PN200-110 binding assays. Binding reactions were carried out in duplicate in a final volume of 1 ml, at pH 7.5, containing 800 μl of membrane suspension (100–200 μg of protein in 1.25 mM $MgCl_2$, 1.0 mM EGTA, 50 mM Tris·HCl), 100 μl of varying concentrations of $CaCl_2$ in 50 mM Tris·HCl, and 100 μl of (+)-[^{3}H]PN200-110 in 50 mM Tris·HCl. Binding to rabbit skeletal muscle membranes was determined under the same conditions, except that membrane protein was only 10–20 μg/assay. Nonspecific binding was determined in the presence of 2.5 μM unlabeled nitrendipine. Unless indicated otherwise, the binding reactions were initiated by addition of the membrane suspensions. Incubations were for 90 min at room temperature and were terminated by filtration though Whatman GF/B glass fiber filters using a Brandel cell harvester. Filters were washed four times with 5 ml of ice-cold 25 mM Tris·HCl, pH 7.5, and counted in 5 ml of scintillation cocktail in a liquid scintillation counter. Specific binding to membranes with an abundance of DHP binding sites (40–50 fmol/mg of protein) was typically 50% of total binding at 300–400 pM (+)-[^{3}H]PN200-110. For analysis of binding according to the method of Scatchard, "free" ligand was calculated as the difference between the amount of (+)-[^{3}H]PN200-110 added to each assay and the total (+)-[^{3}H]PN200-110 bound.

Preparation of Ca^{2+}/EGTA buffers. Free Ca^{2+} and Mg^{2+} concentrations were calculated using the apparent stability constants for Ca·EGTA and Mg·EGTA at pH 7.5 of 2.473×10^7 M^{-1} and 127 M^{-1}, respectively. Calculations were performed on an IBM-compatible desktop computer using the software described by Fabiato (15). The actual concentrations of free Ca^{2+} in the mixtures were confirmed spectrophotometrically at 254 nm with 50 μM levels of the calcium-sensitive indicator dye BAPTA (Sigma Chemical Co., St. Louis, MO), as described by Tsien (1980) (16), using as standards the $CaCl_2$/EGTA reference solutions made in 100 mM KCl, 50 mM MOPS, pH 7.4 (Molecular Probes, Eugene, OR).

Results

Stimulation by Ca^{2+} of DHP binding to $\alpha 1\beta$. The effect of Ca^{2+} on DHP binding to $\alpha 1\beta$ was investigated in the presence of 1 mM $MgCl_2$ in membranes from L cells stably transformed with the skeletal muscle $\alpha 1$ and β subunits (LCaN$\alpha 1\beta$ cells). Fig. 1 shows that DHP binding to $\alpha 1\beta$ was increased by Ca^{2+} in a concentration-dependent manner, whereas under the same conditions DHP binding to rabbit skeletal muscle membranes was hardly affected by addition of Ca^{2+}. The increase in binding produced by Ca^{2+} was saturable, with a half-maximal effect at 0.2 μM. In the absence of Ca^{2+}, specific DHP binding to $\alpha 1\beta$ was about 30% of the maximum. Fig. 2 shows the experiments carried out in a similar way except that Mg^{2+} was omitted from the reaction. Without both Ca^{2+} and Mg^{2+}, no significant DHP binding could be detected with LCaN$\alpha 1\beta$ membranes. Specific DHP binding was thus absolutely dependent on Ca^{2+}, which promoted half-maximal binding at concentrations not different from those seen in the presence of Mg^{2+}. The remainder of the experiments reported in this article were performed in the presence of 1.0 mM Mg^{2+} unless specifically indicated otherwise.

Identification of DHP binding parameters affected by divalent cations. Equilibrium binding assays were carried out without and with 0.2 mM Ca^{2+} and were subjected to Scatchard analysis. DHP binding to $\alpha 1\beta$ in L cell membranes (Fig. 3A) gave binding affinities (K_d) in the absence and presence of Ca^{2+} of 1.2 nM and 0.30 nM, respectively, a difference of 4-fold. In contrast, the affinity of the DHP receptor

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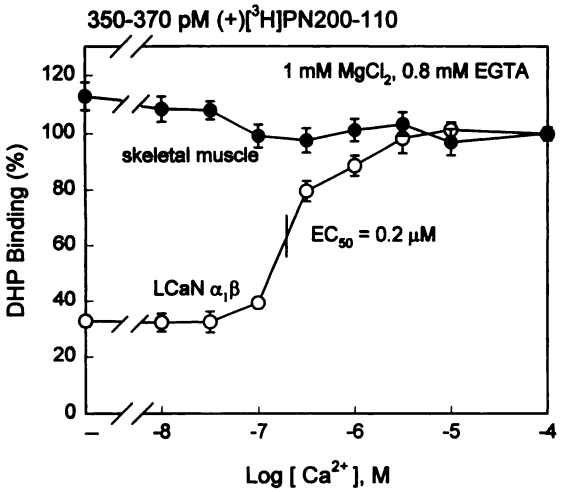

Fig. 1. Estimation of EC_{50} with which Ca^{2+} enhances the affinity of the high affinity state of the $\alpha 1\beta$ DHP binding complex. Binding assays were carried out in the presence of 1 mM $MgCl_2$, 0.8 mM EGTA, and varying concentrations of $CaCl_2$ to give the concentrations of free Ca^{2+} indicated. Data are presented as percentage of (+)-[^{3}H]PN200-110 bound at each of the concentrations of Ca^{2+}, relative to specific binding obtained at 0.1 mM Ca^{2+}. The data are mean ± standard error of three independent experiments.

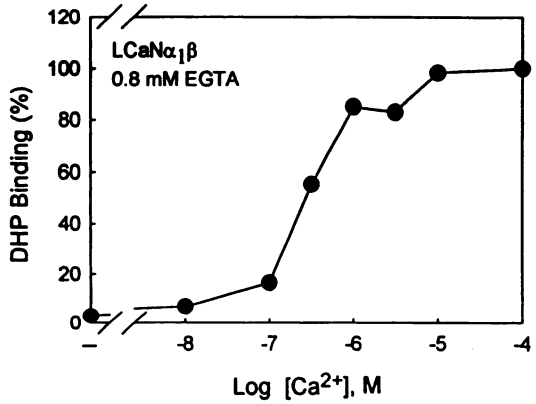

Fig. 2. Effect of Ca^{2+} on (+)-PN200-110 binding to membranes from $\alpha 1\beta$-expressing L cells in the absence of Mg^{2+}. Binding assays were carried out in the presence of 0.8 mM EGTA and varying concentrations of $CaCl_2$ to give the concentrations of free Ca^{2+} indicated. Data are presented as percentage of (+)-[^{3}H]PN200-110 bound at each of the concentrations of Ca^{2+}, relative to specific binding obtained at 0.1 mM Ca^{2+}. Results are means of two independent experiments.

in skeletal muscle membranes for (+)-PN200-110 was 0.27 nM in the absence of Ca^{2+} and was unaffected by addition of Ca^{2+}. The total number of binding sites in the $\alpha 1\beta$-expressing L cells was about 130 fmol/mg, compared with 3000 fmol/mg in skeletal muscle membranes, and this number was not significantly altered by Ca^{2+}. The results showed that the enhancement by Ca^{2+} of DHP binding observed at subsaturating concentrations of (+)-[^{3}H]PN200-110 in membranes from LCaN$\alpha 1\beta$ cells was the result of an increase in affinity, without significant changes in the total number of detectable binding sites, i.e., without apparent changes in the equilibrium between high (detectable) and low (undetectable) affinity states of the DHP-binding unit. These results also indicated that Ca^{2+} was required for high affinity DHP binding to $\alpha 1\beta$ and that Mg^{2+} alone only partially satisfied the divalent cation requirement for DHP binding. This inability

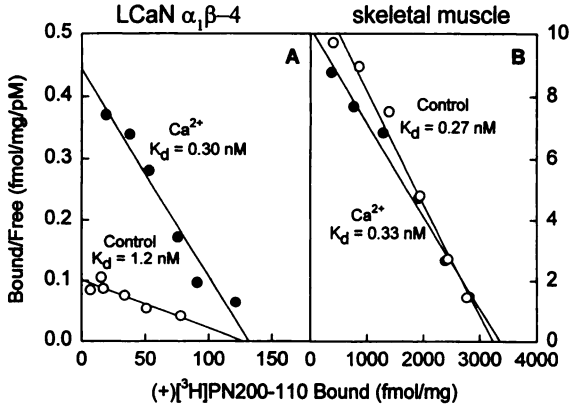

Fig. 3. Effect of Ca^{2+} on (+)-PN200-110 binding to membranes from $\alpha 1\beta$-expressing L cells (A) and to rabbit skeletal muscle microsomes (B). Assays were carried out without (*control*) or with 1 mM $CaCl_2$ (0.2 mM Ca^{2+}), in the presence of 1 mM $MgCl_2$ and 0.8 mM EGTA. The data are representative of similar results obtained for each type of membranes in three independent experiments.

of Mg^{2+} to fully induce the high affinity binding conformation of the $\alpha 1\beta$ complexes was overcome by Ca^{2+}.

The difference between LCaN$\alpha 1\beta$ membranes and skeletal muscle membranes may be explained in several ways. First, the skeletal muscle membranes may carry sufficient entrapped Ca^{2+} to induce the high affinity binding state of DHP receptors. Because the fibroblast-like L cells do not contain deep plasma membrane invagination, such entrapment would not be expected to occur, leading to dependence on Ca^{2+} addition. Second, the $\alpha 1\beta$ complex may have a conformation different from the $\alpha 1\beta\gamma\alpha 2\delta$ complex of the skeletal muscle receptor because of the lack of $\alpha 2\delta$ and γ. Third, the $\alpha 1\beta$ complex may be thermolabile, so that stimulation of binding could be a reflection of the stabilizing effect of Ca^{2+}. In this case the difference would not represent a regulatory effect of Ca^{2+} on DHP binding but would instead reflect an irreversible conformational change of the binding sites in the absence of Ca^{2+}. It has been reported that the purified skeletal muscle Ca^{2+} channels are irreversibly converted to a lower affinity state for DHP in the absence of Ca^{2+} at 30° (17).

To test the third possibility, we first omitted Ca^{2+} from the DHP binding assay, to allow putative thermal inactivation to occur, and then added Ca^{2+} and proceeded with the DHP binding reaction for an additional 1 hr. Addition of Ca^{2+} after a 60-min incubation of membranes without Ca^{2+} increased DHP binding to LCaN$\alpha 1\beta$ membranes to the same level obtained in control incubations to which Ca^{2+} had been added at time 0 (Fig. 4). Thus, the loss of stability in the absence of Ca^{2+} was not the reason for obtaining a higher affinity in the presence of Ca^{2+}. To test the second possibility, we first attempted to raise stable cell lines that would express all of the DHP receptor subunits, but we were unable to do so. Thus, we sought to gain additional information by analyzing the DHP-binding properties of cells expressing these subunits transiently, as seen in COS cells.

Stoichiometry of multicomponent complexes expressed in COS cells. COS cells are monkey kidney cells producing the large T antigen, which allows high expression of exogenous genes carried in plasmids containing the simian virus 40 viral origin of replication (18). When four separate plasmids are transiently transfected, heterogeneous pools of

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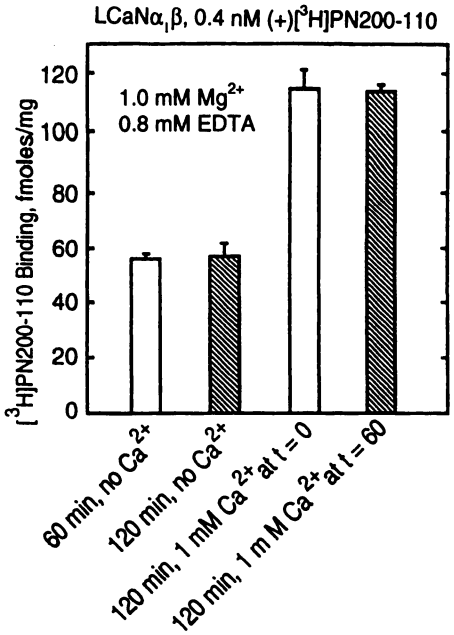

Fig. 4. Stimulation by Ca^{2+} of (+)-PN200-110 binding in LCaNα1β-4 cells. Assays were carried out in the presence of 1 mM $MgCl_2$ and 0.8 mM EGTA. The concentration of (+)-[^{3}H]PN200-110 was 0.4 nM. In this two-step binding assay with a total of 120 min of incubation, $CaCl_2$ (1 mM) was added at the beginning (*Ca^{2+} at t = 0*) or after a 60-min incubation without Ca^{2+} (*Ca^{2+} at t = 60*). Before the addition of Ca^{2+} at 60 min, an aliquot of the binding mixture was removed and the reaction was terminated (*60 min, no Ca^{2+}*), to serve as an internal control for Ca^{2+} at *t* = 60 min. Data are expressed as mean ± standard error of three experiments.

multicomponent complexes can form. To minimize the heterogeneity of the stoichiometry of the DHP-binding complexes, i.e., the complexes that contain α1, and to ensure the interaction of all of the peptides, we designed the experiments as follows. First, we limited the α1 expression plasmid (α1 DNA) to a subsaturating amount. Second, we used an excess of the plasmid carrying the β subunit cDNA (β DNA), in relation to α1 DNA, so that all α1 molecules would form complexes with β. We found that under our conditions 0.3 μg of α1 DNA and 1 μg of β DNA satisfied this condition (data not shown). Finally, to assess the interaction of γ and/or α2δ with α1β, we varied the amounts of both γ and α2δ DNA during the transfection.

Effects of γ and α2δ on the binding properties of the α1β complex. COS cells were transfected with 0.3 μg of α1 DNA, 1 μg of β DNA, and increasing amounts of α2δ DNA or γ DNA. Membranes were prepared 60 hr after transfection and DHP binding assays were performed with 300–350 pM (+)-[^{3}H]PN200-110 in the absence and presence of Ca^{2+}. The changes in regulation by Ca^{2+} of DHP binding are shown in Fig. 5 as the ratios of (+)-PN200-110 binding in the presence of Ca^{2+} to that in its absence. In interpreting the data, we assumed that a homogeneous pool of DHP-binding complexes would be assembled when additional increases in DNA at the time of transfection led to no additional changes in the variable that was being analyzed. Fig. 5 (*insets*) shows the absolute DHP binding values obtained with varying amounts of γ or α2δ DNA in the absence of Ca^{2+}. Both α2δ and γ increased DHP binding to COS cell membranes in a concentration-dependent manner. A decrease in the ratio indicates an occlusion of the stimulatory effect of Ca^{2+} on DHP binding despite the increase in the absolute binding (as shown in Fig. 5, *insets*).

When only α1 and β DNAs were used (Fig. 5), (+)-PN200-110 binding had the same characteristics as binding to membranes from α1β-expressing L cells. Ca^{2+} increased DHP binding to α1β by 2.5-fold. This ratio was markedly reduced by the additional expression of γ [compare binding ratios without and with 1 μg of γ DNA at 0 μg of α2δ DNA (Fig. 5B)]. This effect was dependent on the amount of γ DNA used in the transfection, reaching a plateau at 0.1 μg/dish (Fig. 5A). Like γ, α2δ partially reduced the stimulatory effect of Ca^{2+}. This effect was also dependent on the amount of α2δ DNA, reaching a plateau at 0.3 μg/dish.

In parallel assays with skeletal muscle membranes, the ratio was 1.0, confirming that Ca^{2+} does not affect DHP binding to these membranes. When all four cDNAs (α1, β, γ, and α2δ) were cotransfected, DHP binding with respect to its allosteric regulation by Ca^{2+} was most similar to that seen in skeletal muscle membranes. We noted that the presence of γ DNA lowered the amount of α2δ DNA required for normalization of binding and vice versa; less γ DNA was required to

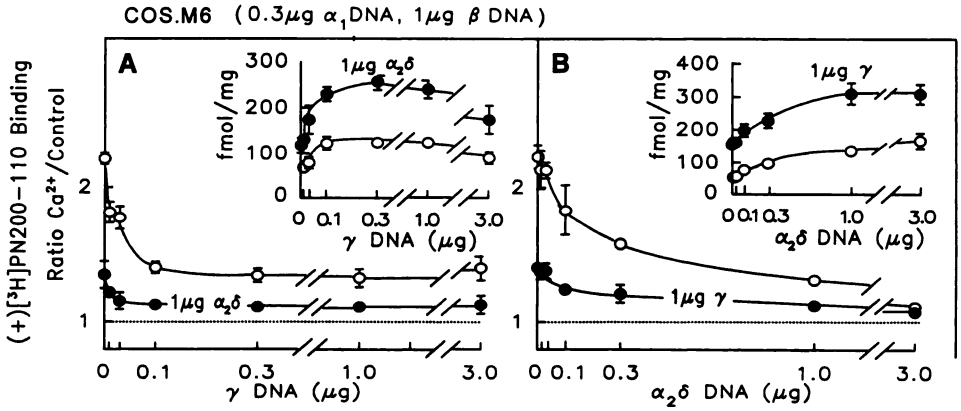

Fig. 5. Disappearance of Ca^{2+} dependence for high affinity binding by coexpression of α1β with γ and α2δ in COS cells. Shown are the effects of increasing amounts of γ DNA (A) or α2δ DNA (B) on the DHP binding to membranes from cells cotransfected with α1 plus β DNAs, either alone (○) or in combination with 1 μg of α2δ DNA (A, ●) or 1 μg of γ DNA (B, ●). *Insets*, changes in absolute (+)-[^{3}H]PN200-110 binding (fmol/mg of membrane protein) for control incubations, i.e., without Ca^{2+}. *Main panels*, changes in the ratios of DHP bound in the presence of 0.2 mM Ca^{2+} to control values. Assays were carried out in 1 mM $MgCl_2$, 0.8 mM EGTA, 50 mM Tris·HCl, pH 7.5, with 0.30–0.35 nM (+)-[^{3}H]PN200-110. The data are mean ± standard error of three independent experiments.

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obtain the same occlusion of the effect of Ca^{2+} in the presence of $\alpha 2\delta$ DNA. Transfection with the expression plasmids lacking the DHP receptor subunit cDNAs or with the plasmids carrying the cDNAs in the reverse orientation did not alter the binding ratio, indicating that the effects of γ or $\alpha 2\delta$ are specific (data not shown).

Stimulation by Ca^{2+} of DHP binding to $\alpha 1\beta$ in COS cells. To further substantiate the effect of γ and $\alpha 2\delta$ in the regulation of DHP binding, the binding assays were carried out with various concentrations of Ca^{2+} (Fig. 6). Ca^{2+} increased DHP binding to $\alpha 1\beta$ in COS cells in a concentration-dependent manner, as it had done in membranes from L cells expressing $\alpha 1\beta$. In the absence of Ca^{2+}, specific DHP binding to $\alpha 1\beta$ was about 50% of the maximum, and Ca^{2+} caused a half-maximal effect at 0.2 μM. Importantly, when all of the subunits were expressed, DHP binding was hardly affected by Ca^{2+}, as seen with skeletal muscle membranes. This result demonstrated that coexpression of γ and $\alpha 2\delta$ with $\alpha 1\beta$ eliminated the dependence of high affinity DHP binding on addition of micromolar concentrations of Ca^{2+}.

Binding parameters affected in COS cells by coexpression of all DHP receptor components. Equilibrium binding assays were carried out to determine whether the increase in binding caused by Ca^{2+} was the result of a change in the affinity of the binding site or the number of detectable binding sites (Fig. 7). Fig. 7A shows that $\alpha 1\beta$ expressed in COS cells has an affinity for DHP (K_d = 1.0 nM). Addition of 0.2 mM Ca^{2+} increased affinity by 3–4-fold (K_d = 0.3 nM). This affinity is close to that observed in skeletal muscle membranes. As was the case with membranes from $\alpha 1\beta$-expressing L cells, Mg^{2+} was only partially effective in inducing high affinity DHP binding to $\alpha 1\beta$ complexes in COS cell membranes. Thus, stimulation by Ca^{2+} at subsaturating concentrations of (+)-PN200-110 (as shown in Fig. 6) was the result of an increase in the affinity of the $\alpha 1\beta$ complex without significant changes in the total number of binding sites. In contrast, the $\alpha 1\beta\gamma\alpha 2\delta$ receptor complex expressed in COS cells showed a high affinity for DHP in the absence of added Ca^{2+} that was not increased by the addition of Ca^{2+}. A summary of the results obtained with various types of membranes is presented in Table 1. The data are the averages of three or four experiments with membranes from $\alpha 1\beta$-expressing L cells (LCaN$\alpha 1\beta$-4), rabbit skeletal muscle, COS cells transfected with only $\alpha 1$ and β DNAs (COS-$\alpha 1\beta$), and COS cells transfected with $\alpha 1$, β, γ, and $\alpha 2\delta$ DNAs (COS-$\alpha 1\beta\gamma\alpha 2\delta$). The similarity of the binding properties of membranes from COS cells transiently expressing $\alpha 1\beta$ to those of L cells expressing $\alpha 1\beta$ in a stable manner validates the use of transient expression to explore multicomponent interactions.

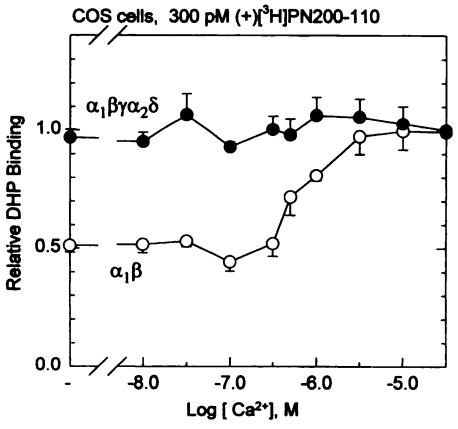

Fig. 6. Comparison of $\alpha 1\beta$ and $\alpha 1\beta\gamma\alpha 2\delta$ in COS cell membranes for the effect of Ca^{2+}. COS cells were transfected with 0.3 μg of $\alpha 1$ DNA and 1 μg each of β, γ, and $\alpha 2$ DNA. Binding assays were carried out in the presence of 1 mM $MgCl_2$, 0.8 mM EGTA, and varying concentrations of $CaCl_2$ to give the indicated concentrations of free Ca^{2+}. Data are presented as percent binding, relative to specific binding obtained at 0.1 mM Ca^{2+}. The data are mean ± standard error of three independent experiments.

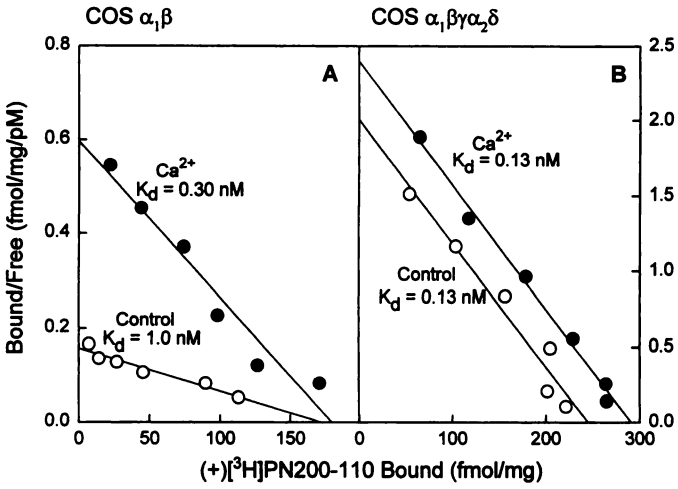

Fig. 7. Scatchard analysis of the effects of Ca^{2+} on DHP binding to $\alpha 1\beta$ (A) and $\alpha 1\beta\gamma\alpha 2\delta$ (B) in COS cell membranes. DNA amounts were 0.3 μg for $\alpha 1$ DNA and 1 μg each for β, γ, and $\alpha 2\delta$ DNAs. Binding assays were carried out in 1 mM $MgCl_2$, 0.8 mM EGTA, 50 mM Tris·HCl, pH 7.5. The data are representative of similar results from three independent experiments.

Conversion of $\alpha 1\beta$ to a low affinity form in the absence of Ca^{2+}. The results of Fig. 4 showed that the lower binding of DHP to $\alpha 1\beta$ in L cell membranes in the absence of Ca^{2+}, compared with its presence, was not the result of increased thermolability of the $\alpha 1\beta$ complex. To further test whether this is also the case in the context of COS cell membranes, binding studies were performed as a function of time, with preformed receptor-DHP complexes (Fig. 8). (+)-[^{3}H]PN200-110 at a subsaturating concentration was allowed to bind to the membranes from COS cells expressing $\alpha 1\beta$ or $\alpha 1\beta\gamma\alpha 2\delta$, in the presence of Ca^{2+} and Mg^{2+}, overnight at 4°. Dissociation was then initiated at room temperature by addition of 2.5 mM EGTA, and the reactions were terminated at the indicated times by dilution and filtration. In parallel assays, after 1 or 2 hr of incubation with 2.5 mM EGTA, 3 mM $CaCl_2$ was added and the reaction was allowed to continue for an additional 1 hr. The results are presented as binding at the indicated times, relative to binding at the time of EGTA addition (time 0). For COS cells expressing the $\alpha 1\beta$ complex, DHP dissociated from the complex upon addition of EGTA. Addition of Ca^{2+} allowed for rebinding without significant loss of sites. In contrast, for COS cells expressing $\alpha 1\beta\gamma\alpha 2\delta$, addition of EGTA did not result in a significant release of DHP from its receptor in the time studied. This result eliminates the possibility that the $\alpha 1\beta$ is irreversibly inactivated in the absence of Ca^{2+} ions and that the effect of Ca^{2+} is merely to prevent thermal inactivation. Instead, $\alpha 1\beta$ has a conformation from which both the Ca^{2+} ions and DHP can easily dissociate.

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TABLE 1

Summary of data on equilibrium binding of (+)-PN200-110 to membranes with partial and complete complements of skeletal muscle DHP receptor subunits

Values were derived from Scatchard analyses of equilibrium binding assays and represent the means ± standard errors of three independent experiments for each type of membrane. Binding assays were performed in 50 mM Tris · HCl, pH 7.5, 1 mM $MgCl_2$, 0.8 mM EGTA, in the absence or presence of 1 mM $CaCl_2$ (Ca^{2+} = 0.2 mM). L$\alpha1\beta$ denotes clone LCaN-$\alpha1\beta4$; COS cell transfections were with 0.3 μg of $\alpha1$ DNA and 1 μg each of β, γ, and $\alpha2\delta$ DNA. For additional details, see Figs. 3 and 7.

Binding parameter	Assay conditions	Source of membranes			
		L$\alpha1\beta$	Skeletal muscle	COS $\alpha1\beta$	COS $\alpha1\beta\gamma\alpha2\delta$
K_d (nM)	Control	0.94 ± 0.14[a]	0.25 ± 0.05	1.03 ± 0.13[a]	0.15 ± 0.02
	Ca^{2+}	0.22 ± 0.04[b]	0.23 ± 0.03	0.31 ± 0.02[b]	0.14 ± 0.02
B_{max} (fmol/mg)	Control	138 ± 23	2910 ± 446	157 ± 7	204 ± 31
	Ca^{2+}	128 ± 3	2940 ± 509	140 ± 32	228 ± 38

[a] Different from values obtained with membranes from either rabbit skeletal muscle or COS $\alpha1\beta\gamma\alpha2\delta$ cells, at a level of significance of at least $p < 0.005$.
[b] Different from the respective controls, at a significance level of at least $p < 0.01$.

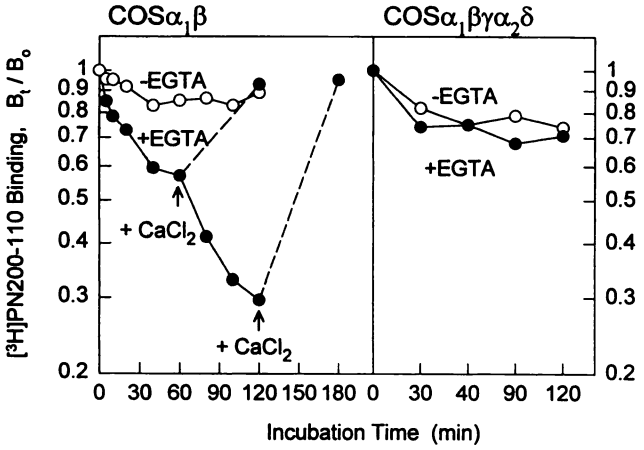

Fig. 8. Dissociation kinetics of DHP from $\alpha1\beta$ and $\alpha1\beta\gamma\alpha2\delta$ in COS cell membranes. COS cells were transfected with 0.3 μg of $\alpha1$ DNA and 1 μg each of β, γ, and $\alpha2$ DNA. Binding assays were carried out in the presence of 0.2 mM $CaCl_2$ and 0.8 mM $MgCl_2$ at 4° overnight. Dissociation was initiated by addition of 2.5 mM EGTA to the binding reaction, and mixtures were incubated at room temperature for the indicated periods of time. One and 2 hr later, 3 mM $CaCl_2$ was added to the reaction. Incubations were then continued for another 60 min and stopped. The bound radioactivity was measured after removal of free ligands. The results were normalized to the binding level at time 0. The data are representative of similar results from two independent experiments.

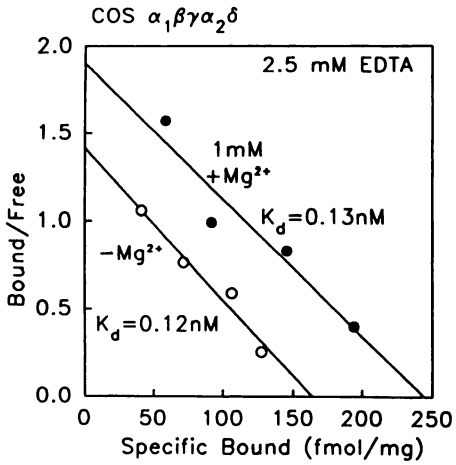

Fig. 9. Scatchard analysis of DHP binding to $\alpha1\beta\gamma\alpha2\delta$ in COS cell membranes in the absence of divalent cations. Amounts of DNA were 0.3 μg for $\alpha1$ DNA and 1 μg each for β, γ, and $\alpha2\delta$ DNAs. Membranes were prepared in 50 mM Tris, pH 7.5, 2.5 mM EDTA, and binding assays were carried out in 50 mM Tris, 2.5 mM EDTA, with or without 3.5 mM $MgCl_2$. The data are representative of similar results from two independent experiments.

Binding of (+)-PN200-110 in the absence of divalent cations. As mentioned above, the experiments described thus far were all performed in the presence of 1.0 mM Mg^{2+}, because omission of Mg^{2+} from binding assays with membranes from cells expressing $\alpha1\beta$ led to total loss of binding. In contrast, with membranes from COS cells expressing $\alpha1\beta\gamma\alpha2\delta$, omission of Mg^{2+} resulted in a loss of only 20–30% of the specific binding. As shown in Fig. 9, Scatchard analysis showed that this loss of binding was the result of a reduction in the B_{max} without a change in the affinity of the $\alpha1\beta\gamma\alpha2\delta$ complex for (+)-PN200-110.

Discussion

Purification of the skeletal muscle DHP receptor/Ca^{2+} channel yields an $\alpha1\beta\gamma\alpha2\delta$ complex. A complex of only $\alpha1\beta$ expressed in L cells yields Ca^{2+} currents that resemble those found in skeletal muscle, but with an allosteric regulation of its DHP binding that does not fully recapitulate that of intact skeletal muscle membranes, in that 1) DHP binding to isolated membranes is absolutely dependent on addition of a divalent cation (Mg^{2+} or Ca^{2+}); 2) the affinity of the DHP antagonist (+)-PN200-110 measured in the presence of Mg^{2+} is about 4-fold lower than that seen in skeletal muscle membranes; and 3) high, skeletal muscle-like affinity for DHP can be restored either by the phenylalkylamine (−)-D600 (11a) or by Ca^{2+}. The effects of (−)-D600 and Ca^{2+} are nonadditive and occlude each other, indicating that the binding sites for phenylalkylamines and Ca^{2+} are tightly coupled to regulate DHP binding.

In the present report, we tested whether the difference in dependence on divalent cations of DHP binding seen for skeletal muscle membranes and $\alpha1\beta$ complexes formed in L or COS cells by recombinant means is the result of lack of the other regulatory subunits, such as γ and $\alpha2\delta$, by studying the properties of complexes formed in COS cells expressing all subunits. As shown in Results, the $\alpha1\beta\gamma\alpha2\delta$ complexes formed in COS cells behaved like the receptors in skeletal muscle membranes, in that they adopted the high affinity DHP-binding conformation and were insensitive to omission (or chelation) of Ca^{2+}.

As shown in the DHP dissociation study carried out in the presence of Mg^{2+}, depletion of Ca^{2+} by addition of EGTA decreased DHP binding to $\alpha1\beta$, whereas it had a marginal effect on DHP binding to $\alpha1\beta\gamma\alpha2\delta$. The loss of DHP from $\alpha1\beta$

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was the result of conversion of the $\alpha 1\beta$ complex from a state with high affinity stabilized by Ca^{2+} to one with lower affinity stabilized by Mg^{2+}. The transition between these two states is reversible, because addition of Ca^{2+} restored DHP binding to the level observed before addition of EGTA. These results indicated that $\alpha 1\beta$ adopts a conformation from which Ca^{2+} can be easily removed by addition of EGTA. These results also showed that $\alpha 1\beta$ can distinguish between Ca^{2+} and Mg^{2+}, raising the possibility that the free cations may be interacting with different sides of $\alpha 1\beta$. In the absence of both divalent cations, DHP binding was not detectable. In contrast, in membranes with $\alpha 1\beta\gamma\alpha 2\delta$, the high affinity state of the DHP receptor formed spontaneously in the absence of divalent cations, and both Ca^{2+} and Mg^{2+} stabilized the same high affinity state of the receptor. Whether this is because the receptor is independent of divalent cations or because it has Ca^{2+} tightly bound to it is not known (see below).

Recently, Peterson and Catterall (19) reconstituted DHP receptors composed of $\alpha 1\beta\alpha 2\delta$ by transient expression in ts-A201 cells, a T antigen-expressing variant of human embryonic kidney 293 cells. Although the stoichiometry of expressed subunits was not specifically addressed in their experiments, the results obtained agreed with ours in that cells transfected with $\alpha 1$, β, and $\alpha 2\delta$ formed complexes requiring addition of Ca^{2+} to exhibit high affinity DHP binding. The requirement for Ca^{2+} (EC_{50} = 0.56 μM) was similar to values that we obtained with $\alpha 1\beta$ expressed in COS cell membranes. Analysis of point mutations of $\alpha 1$ located the Ca^{2+} binding site responsible for induction of high affinity DHP binding to the pore region of the channel.

As mentioned earlier, DHP binding to skeletal muscle membranes has been known to be insensitive to Ca^{2+} omission or addition of divalent cation-chelating agents. It was postulated that Ca^{2+} ions entrapped in inside-out sealed vesicles stabilized DHP receptors in the high affinity state and that disruption of membrane integrity released Ca^{2+} ions and caused DHP binding to be dependent on Ca^{2+} (3, 20). These results led to the conclusion that high affinity DHP binding to the DHP receptor in its natural environment is dependent on Ca^{2+}. This conclusion is at variance with what we would conclude from our data with membranes from COS cells expressing all DHP receptor subunits, in which we see no evidence for a requirement for Ca^{2+}. This lack of requirement for Ca^{2+} seems unlikely to be the result of entrapment of Ca^{2+} by COS cell membranes, because it seems unlikely that membranes from COS cells expressing only $\alpha 1\beta$ would not entrap Ca^{2+} and show a requirement for divalent cation addition for high affinity DHP binding, whereas membranes from COS cells with $\alpha 1\beta\gamma\alpha 2\delta$ would entrap the ion and render a receptor that does not require divalent cation addition. Thus, although it recapitulates most of the properties of the skeletal muscle DHP receptor, the $\alpha 1\beta\gamma\alpha 2\delta$ complex expressed in COS cells may differ in at least one aspect. It either retains Ca^{2+} bound to the channel itself, conferring to it high affinity for DHP, or it is still lacking another subunit or channel-interacting protein that is required for reconstitution of the Ca^{2+} dependence seen in permeabilized skeletal muscle membranes. The possibility of additional molecules interacting with and modulating voltage-dependent Ca^{2+} channels was distinctly raised by Catterall and colleagues (21), who showed that syntaxin, a presynaptic plasma membrane protein, has the ability to interact with a defined segment of the N-type Ca^{2+} channel $\alpha 1$ subunit. It is possible that the DHP receptor complexes, when expressed in nonexcitable COS cells, may undergo post-translational modifications different from those that occur in skeletal muscle, which may affect the destination and interactions of receptor subunits. The actual proportion of plasma membranes in crude membranes and the stoichiometry of DHP receptor complexes in plasma membranes were not tested in this study. On the other hand, the study in which the treatment of skeletal muscle membranes with the ionophore A23187 and EDTA rendered the DHP receptor dependent on Ca^{2+} for high affinity binding did not evaluate whether membrane structure and/or Ca^{2+} channel subunit interactions were altered by the treatment. Thus, it may be that the Ca^{2+} requirement for high affinity DHP binding after this treatment may have reflected disruption of normal subunit interactions.

To date, six $\alpha 1$ genes and four β genes have been cloned (22) and the regulatory roles of $\alpha 2\delta$ and γ in channel activity have been extensively studied with isoforms of non-skeletal muscle $\alpha 1$. Whereas $\alpha 2\delta$ has been detected in other excitable tissues, thus far the existence of γ has been reported only in skeletal muscle. Thus, the present study is the first to demonstrate a role of γ in reconstituting high affinity DHP binding of skeletal muscle DHP receptors.

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Send reprint requests to: Lutz Birnbaumer, Department of Anesthesiology, BH-612 CHS, UCLA School of Medicine, 10833 LeConte Ave., Los Angeles, CA 90095-1778.

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