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Roles of L-type Ca^{2+} Channel Subunits of Skeletal Muscle in Dihydropyridine Binding: Evidence for Functional Interaction among α_1, β, γ and $\alpha_2\delta$ Subunits

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Purified skeletal muscle 1,4-dihydropyridine (DHP) receptors are composed of five polypeptides termed α_1, β, γ, α_2, and δ. Among these, the α_1 subunit is known to be sufficient to function as a voltage-dependent Ca^{2+} channel and a DHP receptor. The α_1 alone exhibits very similar allosteric regulation of DHP binding to that found in skeletal muscle T-tubules. However, we previously showed that in the absence of Ca^{2+}, DHP binding to α_1 alone at subsaturating concentrations was reduced, which could be restored by the (−) stereoisomer of a phenylalkylamine, D600. We hypothesized that this difference is due to lack of other regulatory subunits, specifically the β, γ and $\alpha_2\delta$ components that copurify with α_1.

In order to test our hypothesis, we coexpressed the α_1 subunit with non-α_1 components in mouse fiboblasts, L cells, and monkey kidney cells, COS.M6 in various combinations and compared their DHP binding activity for the effect of (−)D600 in the absence of Ca^{2+}. Coexpression of β with α_1 did not normalize the abnormal enhancing effect of (−)D600 on DHP binding. Coexpression of either γ or $\alpha_2\delta$, partially reduced the enhancing effect of (−)D600. Importantly, coexpression of all the components, that is, when the receptor was composed of $\alpha_1\beta\gamma\alpha_2\delta$, completely abrogated the abnormal effect of (−)D600. Thus, our data clearly demonstrate that all the component copurifying with α_1 are essential to constitute the functional DHP receptor.

Key Words: Calcium channel, Dihydropyridine binding, Allosteric regulation, Skeletal muscle

INTRODUCTION

Dihydropyridine (DHP) binding to its skeletal muscle receptor is under complex allosteric regulation by calcium channel blockers such as phenylalkylamines and benzothiazepines. It has been thought that DHP receptors exist in two widely different affinity states for DHP, one detectable by saturation analysis with labeled DHP ligands and another with an affinity that is too low for detection. The shifts of equilibria between these two states are perceived upon Scatchard analysis as changes in the total number of high affinity binding sites. In rabbit skeletal muscle T-tubule membranes, formation of the high affinity state of the DHP receptor is dependent on the presence of either divalent cations (Mg^{2+} or Ca^{2+})[1], and phenylalkylamines such as verapamil and D600 decrease the apparent affinity of the high affinity form of the DHP receptor without affecting the equilibrium between the high and low affinity states (increase in K_D, without change in B_{max} of DHP binding). On the other hand, benzothiazepines appear to increase the proportion of total DHP binding sites in the high affinity state (increase in B_{max}, without change in K_D)[2].

The purified skeletal muscle DHP receptor is composed of five polypeptides termed α_1, β, γ, α_2, and δ, which are encoded in four genes, α_1, α_2, β, and γ[3~8]. We have previously shown that expression of α_1 alone in mouse

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fibroblast L cells has led to appearance of voltage gated Ca^{2+} channel currents and measurable high affinity DHP binding sites[9]. DHP binding in those α_1 expressing L cell lines (LCa cells) could be modulated by a benzothiazepine, diltiazem, and a phenylalkylamine, verapamil[10], as found in skeletal muscle T-tubules, with the following exceptions: ① the effect of verapamil to lower affinity was more marked in the LCa cells than in the T-tubules and ② in incubations from which Ca^{2+} had been omitted, binding of subsaturating concentrations of DHP was reduced and could be restored by the (−) isomer of D600 but not its (+) isomer[11]. The behavior exhibited by α_1 in LCa cells differed from that exhibited in T-tubules not only in this subtle "defects" in allosteric regulation of DHP binding, but also in its physiological response to depolarizing test potentials. Activation of skeletal muscle Ca^{2+} channel currents is complete within ca. 50～100 msec, but took as much as 6,000 msec in LCa cells. One reason for the different behavior of α_1 in L cells as compared to in T-tubules, could be that L cells are unable to properly process the molecule. Another reason could be that the different behavior is merely due to lack of regulatory subunits, specifically the β, γ and $\alpha_2\delta$ components that copurify with α_1.

We also reported previously that the rate of activation of Ca^{2+} channel currents in α_1-expressing L cells is accelerated to a level close to normalcy upon coexpression of the β component of the purified DHP receptor[12]. This indicated that the β component is a regulatory, if not also a structural subunit of the skeletal muscle Ca^{2+} channel, and prompted us to investigate the possible roles of not only the β component but also the γ and the $\alpha_2\delta$ components in the regulation of DHP binding to α_1.

In this article, we reconstitute DHP receptor complexes composed of α_1, $\alpha_1\beta$, $\alpha_1\beta\gamma$, $\alpha_1\beta\gamma\alpha_2\delta$ in L or COS cells. We compare those complexes for the effects of (−)D600 on their DHP binding activity in the absence of Ca^{2+}. We report that the allosteric regulation by (−)D600 is different between cells with α_1 alone, or with α_1 plus β, and skeletal muscle membranes, and that conversion of the DHP binding activity to that observed in skeletal muscle membranes requires the coexpression of all of the polypeptides found in the DHP binding complex purified from skeletal muscle T-tubules.

MATERIALS and METHODS

Complementary DNA's and expression plasmids

Expression plasmids, p91023(B)[13], was a gift from Dr. Randall Kaufman (Genetics Institute, Cambridge, MA); pKNH[14] and its derivative pKCR-$\alpha_2\delta$ containing the rabbit skeletal muscle $\alpha_2\delta$ cDNA (FnuDII(−5)/EcoRI(+3544) fragment with ORF of 3546 nt) from Dr. Shosaku Numa (University of Kyoto, Japan); pSkMCaChγ.3 containing the rabbit skeletal muscle γ cDNA (1208 nt full length cDNA with a 666 nt ORF in Okayama-Berg plasmid, pcD)[6] from Dr. Kevin Campbell (University of Iowa). The rabbit skeletal muscle α_1 (KpnI(−15)/KpnI(+5976) fragment)[10] was subcloned into the HindIII site of pKNH[12]. The rabbit skeletal muscle β (XmaIII(−20)/PflMI(+1608))[5] was subcloned into the EcoRI site of p91023(B) by blunt end ligation. Negative control plasmids were prepared either by deleting their inserts or by subcloning the inserts in the antisense orientation. A plasmid, pSV-βGal[15] containing the bacterial β-galactosidase gene in pSV2 plasmid, was a gift from Dr. Grant MacGregor (Institute of Molecular Genetics, Baylor College of Medicine). In all cases transfections were carried out with supercoiled plasmid DNA isolated from CsCl gradients.

Transfection and stable expression in L cells

L cells were grown in Minimum Essential Medium (MEM) α Medium (GIBCO, Grand Island, NY) with 100 units/ml penicillin and 100 μg/ml streptomycin in the presence of 10% fetal bovine serum (FBS), and were transfected with the indicated cDNA-containing vectors by the calcium phosphate method[16]. For transfection of Ltk^- cells with α_1 DNA containing neomycin resistant gene, transfected cells were plated in 96-well plates at densities such that after selection in MEM α Medium with 10% fetal FBS and 300～400 μg/ml geneticin sulfate (G418), and there were survivors in only about 50% of the wells[17]. Single colonies were expanded and analyzed electrophysiologically for Ca^{2+} current[12]. For coexpression of β and γ, transfections were carried out to the α_1-expressing L cells with 5 μg of corresponding expression vectors in the presence of limiting amounts of Herpes simplex virus thymidine kinase gene in pHSV-106 (BRL, Grand Island, NY), then selected in MEM α medium with 10% FBS,

100 μM hypoxanthine, 0.4 μM aminopterin, 160 μM thymidine, and 300～400 μg/ml G418. Cells from wells with single colony were expanded and subjected to an initial analysis for the presence of the desired mRNA.

Culture and transfection of COS.M6 cells

COS.M6 cells were grown in Dulbecco's Modified Eagle Medium (DMEM) with high glucose supplemented with 10% FBS, 100 units/ml penicillin, and 100 μg/ml streptomycin. Cells were transfected as previously described[18] with slight modifications. Briefly, one day before transfection, $2x10^6$ cells were plated per 100 mm dish. Cells were washed twice with Hank's balanced salt solution (HBSS) and overlaid with 4 ml 25 mM Tris-HCl (pH 7.5), 137 mM NaCl, 5.1 mM KCl, 1.4 mM Na_2HPO_4, 1.3 mM $CaCl_2$, 1.0 mM $MgCl_2$, and 10% NuSerum (Collaborative Research, Bedford, MA), containing 0.01～3 μg plasmid DNA and 0.144 mg/ml DEAE-dextran. After incubation for 4 hours at 37℃, this solution was replaced first with 5 ml 10% dimethylsulfoxide in HBSS for 2 min, and then with 10 ml DMEM with high glucose, 2% FBS, and 100 μM chloroquine for 3～4 hours. Thereafter, the chloroquine containing medium was discarded and the cells were first washed twice with HBSS, and then overlaid with 10 ml of DMEM with high glucose and 10% FBS and incubated at 37℃ for 60 hours.

The transfection efficiency of this protocol was tested by transfecting cells with 2 μg of the pSV-βGal indicator plasmid and testing cells histochemically for the functional expression of β-galactosidase gene as previously described[15]. Briefly, 60 hours post transfection, the transfected cells were rinsed with phosphate buffered saline, and overlaid with 1 ml of 0.6% glutaraldehyde in the same buffer for 10 min at room temperature. The cells were then rinsed twice with 50 mM Tris-HCl, pH 7.5 and 150 mM NaCl (TBS) and overlaid with 8 ml of TBS containing 0.5 mg/ml X-Gal, 5 mM potassium ferrocyanide/ 5mM potassium ferricyanide, and 2 mM $MgCl_2$. After an overnight incubation at 37°C, the proportion of cells developing blue color due to the β-galactosidase expression was determined with a phase contrast microscope at a magnification of 200X.

Preparation of crude membranes from transfected cells

All subsequent procedures were carried out at 4℃. L cells grown close to confluence or COS.M6 cells 60 hours after transfection were rinsed with and harvested in TBS using a rubber policeman. The cells were pelleted by centrifugation at 450 × g for 5 min and crude membranes fractions were as previously described[10]. Briefly, cells were resuspended in 50 mM Tris-HCl, pH 7.5 and 1 mM EDTA (TE) to lyse for 20 min and centrifuged for 10 min at 450 × g. The pellet was resuspended in TE and homogenized vigorously using a Brinkmann Polytron. The homogenates were centrifuged at 450 × g for 5 min, and the supernatant was collected and centrifuged at 200,000 × g for 45 min. The resulting pellet was resuspended in either buffer A (1 mM $CaCl_2$, 0.5 mM $MgCl_2$, and 50 mM Tris, pH 7.5) or buffer B (1.34 mM $MgCl_2$, 1 mM EGTA, and 50 mM Tris-HCl, pH 7.5). BC3H1 cells were grown to close to confluence exactly as COS cells in the presence of 10% fetal bovine serum. Seven days before harvesting they were induced to differentiate by reducing the serum to 0.5%.

Preparation of skeletal muscle microsomes enriched T-tubule membranes

Membranes enriched in T-tubules with a DHP receptor density between 3 and 6 pmol/mg membrane protein were prepared at 4°C from frozen rabbit skeletal muscle as described previously[19] with a slight modification. Briefly, about 80 g of frozen tissue (−70°C) was crushed into small pieces and homogenized with polytron in 400 ml of buffer C (20 mM Tris-HCl, pH 7.2, 0.3 M sucrose, 1.5 nM pepstatin A., 0.2 mM phenylmethylsulfonyl fluoride, 0.5 μg/ml leupeptin, 54 KIU/ml or 1 μM aprotinin). The homogenate was centrifuged at 3000 × g for 30 min and then the supernatant was filtered through cheese cloth to remove fat. The pellet was homogenized again in 250 ml buffer C and subjected to the same procedure as above. Potassium chloride was added to the supernatant to a final concentration of 0.5 M and incubated for 1 hour and centrifuged at 150,000 × g for 1 hour. The pellet was resuspended in 50 ml of Tris-HCl (pH 7.2) and 1 mM EDTA with all the protease inhibitors as mentioned above and centrifuged at 150,000 × g for 1 hour, and the final pellet was resuspended in 10 ml of 50 mM Tris-HCl, (pH 7.2) and protease inhibitors, and stored in aliquots at −70°C at a concentration of 17 mg protein per ml.

(+)[^{3}H]PN200-110 binding assays

Unless otherwise indicated the binding reactions were carried out in a final volume of 1 ml containing 900 μl of membrane suspensions in buffer A and 100 μl of (+)[^{3}H]PN200-110 (specific activity, 80~85 Ci/mmol; Amersham, Arlington Heights, IL) in 50 mM Tris-HCl, pH 7.5, to give final concentrations of (+)[^{3}H]300-350 pM PN200-110, 0.9 mM $CaCl_2$, 0.45 mM $MgCl_2$, 50 mM Tris-HCl, pH 7.5, and 100~200 μg membrane proteins.

To determine the concentration-dependent effects of (−)D600, binding reactions were performed in 1 ml containing 800 μl of membrane suspensions in buffer B, 100 μl of varying concentrations of (−)D600 in 50 mM Tris-HCl (pH 7.5) and 100 μl (+)[^{3}H]PN200-110 as above to give final concentrations of 0.8 mM EGTA, 1.07 mM $MgCl_2$, and 50 mM Tris-HCl, pH 7.5 and 100~200 μg membrane proteins. Binding to rabbit microsomes was determined under the same conditions as to membranes from L cells or COS cells, except that membrane protein was only 10~20 μg per assay. Reactions were initiated by addition of the membrane suspensions and incubated for 90 minutes at 22~24℃. Nonspecific binding was determined in the presence of 2.5 μM unlabeled nitrendipine. Reactions 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.

RESULTS

Stereospecific effects of D600 on (+)[^{3}H]PN200-110 binding to membranes from L cells with α_1 alone vs. skeletal muscle

Fig. 1 depicts the effects of diltiazem and phenylalkylamine amines on the binding of (+)PN200-110 to either membranes from the α_1-expressing LCa-11 cell or from rabbit skeletal muscle. It can be seen that allosteric regulation of DHP binding to L cell membranes resembles that of skeletal muscle with respect to the ability of diltiazem to increase the proportion of sites in the membranes in a (measurable) high affinity state. Verapamil increased the K_D value of [^{3}H](+)PN200-110 from 0.21 nM to 0.61 nM in rabbit skeletal muscle membranes. In L cells with only α_1, the effect of

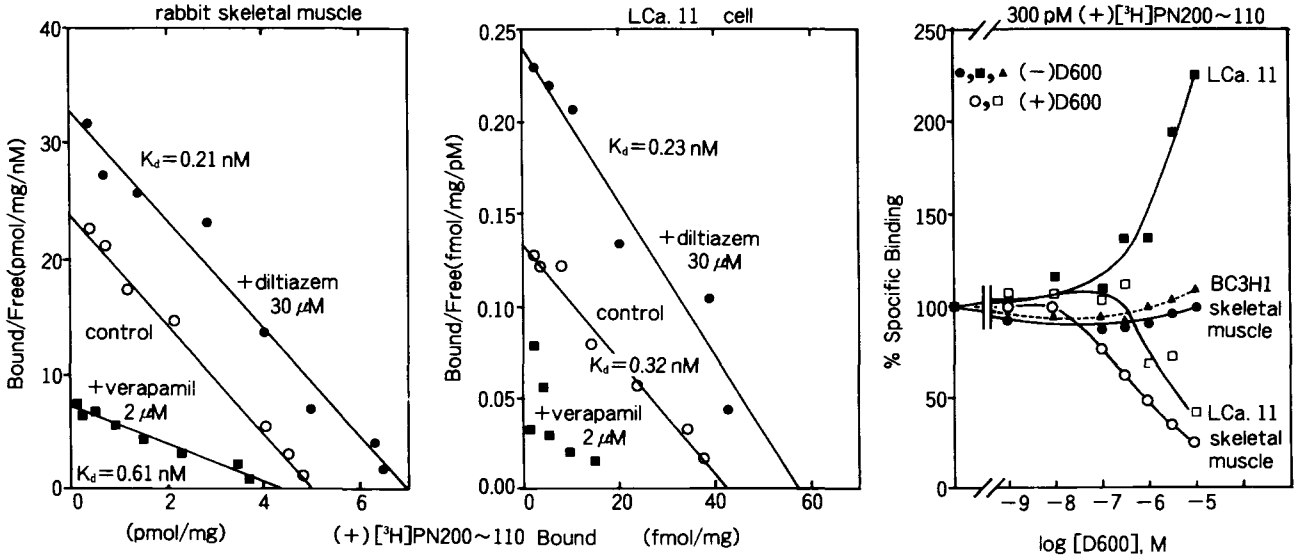

Fig. 1. Comparison of allosteric regulation of DHP binding by diltiazem and phenylalkylamines in α_1-expressing L cells (clone LCa-11) to those in rabbit skeletal muscle and BC3H1 cells. Left and center panel, Scatchard plots of (+)[^{3}H]PN200-110 to rabbit skeletal muscle and LCa-11 cell membranes; right panel, effects of stereoisomers of a phenylalkylamine, D600, on (+)[^{3}H]PN200-110 to rabbit skeletal muscle, BC3H1 cell, and LCa-11 cell membranes. The data are representative of similar results obtained in three independent experiments.

verapamil was difficult to analyze by Scatchard analysis due to low level of binding after being inhibited by verapamil. The center panel of Fig. 1 shows a representative of result obtained in the presence of 2 μM verapamil. To further analyze the allosteric interaction between DHP and phenylalkylamines, effects of (−)D600 and (+)D600 on (+)PN200-110 binding were determined in incubations with out added Ca^{2+} (right panel of Fig. 1). While under these conditions (−)D600 did not increase the binding of (+)[^{3}H]PN200-110 to rabbit skeletal muscle membranes, it enhanced the binding to LCa-11 cell membranes 2~3 fold[11]. (+)D600 decreased in a concentration dependent manner the binding of (+)PN200-110 to both types of membranes.

The lack of the (−)D600 effect on DHP binding to skeletal muscle T-tubule enriched membranes did not appear to be related to the T-tubule structure, for it also failed to affect DHP binding to membranes from BC3H1 cells, which are brain derived cells with skeletal muscle phenotype[20~22] and express all the known components of the DHP receptor (α_1, β, γ, and $\alpha_2\delta$)[12,23]. These cells while expressing all of the components of the DHP receptor complex, lack T-tubule-like structures[24].

We investigated whether the discrepancy between L cells and skeletal muscle membranes was due to lack of one or more of the components of the DHP receptor complex. As a first step we attempted to construct L cell lines expressing α_1 in various combinations with β, γ, and $\alpha_2\delta$. Although we were unable to obtain L cells expressing all of the DHP receptor components, we succeeded in obtaining cells that expressed α_1 in combination with β, and $\beta\gamma$ together, LCaN-α_1 β and LCaN-$\alpha_1\beta\gamma$ cells. Expression of β and γ mRNA in these cell lines was confirmed by Northern blot analysis, and for β it was further possible to confirm protein synthesis by Western blot analysis as reported recently[12]. Typically, the total number of DHP binding sites recovered in membranes from LCaN cells in a high affinity state increased 3-fold upon expression of which was not significantly altered by β (left panel of Fig. 2). Figure 2 (left panel) presents a Scatchard

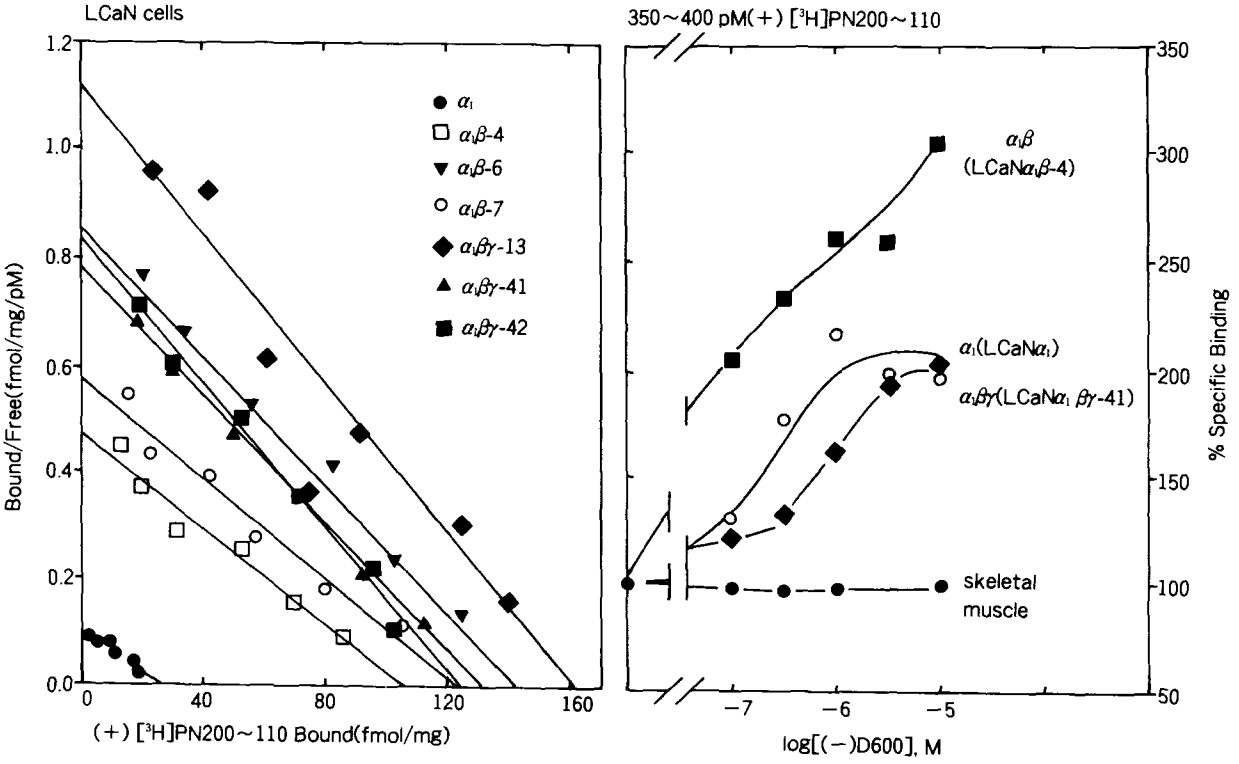

Fig. 2. Left panel, Scatchard plots of (+)[^{3}H]PN200-110 binding to membranes from α_1-expressing L cells (LCaN-α_1, clone 162), and derivatives stably transfected with β (LCaN-$\alpha_1\beta$ cells) and β plus γ (LCaN-$\alpha_1\beta\gamma$ cells); right panel, the effect of (-)D600 on (+)PN200-110 binding to representative L cell clones expressing α_1 , $\alpha_1\beta$, and $\alpha_1\beta\gamma$.

analysis of DHP binding to membranes from LCaN-α_1 cells as well as to membranes from several LCaN-$\alpha_1\beta$ and LCaN-$\alpha_1\beta\gamma$ clones. The right panel shows that expression of β did not normalize (−)D600 action on DHP binding to α_1, i.e., it did not lead to a ratio of (+)PN200-110 binding in the presence of (−)D600 relative to that in the absence of (−)D600 of approximately 1.0. In fact, coexpression with β rather than normalizing the effect of (−)D600, caused it to be more pronounced. Coexpression of γ together with β had a tendency to negate this β effect. Scatchard plot analysis showed that the enhancement of DHP binding by (−)D600 observed at subsaturating concentrations of (+) [^{3}H]PN200-110 in membranes from LCaN-$\alpha_1\beta$ cells was due to an increase in affinity without significant changes in the total number of detectable binding sites, i.e., without apparently changing the equilibrium between high and low affinity states of the DHP binding unit (data not shown). These results suggest that additional component(s) other than $\alpha_1\beta$ is (are) required to normalize DHP receptor function. In order to prove this hypothesis, it was necessary to express DHP receptor subunits in the same cell line and compare DHP binding to $\alpha_1\beta$ and $\alpha_1\beta\gamma\alpha_2\delta$. However, in view of our inability to obtain L cells expressing stably all of the protein components of the purified DHP receptor complex, we sought to gain further information by analyzing DHP binding properties of cells that would express these proteins transiently, as seen in COS cells.

Expression of the DHP receptor in COS.M6 cells

The skeletal muscle α_1 cDNA in pKNH vector (α_1 DNA) was transfected alone and in various combination with β, γ, and $\alpha_2\delta$ expression plasmids into COS.M6 cells using the transfection protocol outlined in Fig. 3. In these experiments all cells were transfected with 2 μg each of plasmid DNA's and binding was performed at 300~350 pM (+)[^{3}H] PN200-110, which, because of its subsaturating nature, allowed for detection of effects on either binding affinity or on the total number of the binding sites in the high affinity state in the absence or presence of (−)D600. Thus, a decrease in the ratio indicates a decrease in the stimulatory effect of (−)D600 on(+)PN200-110 binding despite the changes in absolute binding values. The efficiency of the transfections, estimated in parallel assays using pSV-βGal and quantifying the proportion of cells developing a blue color at the end of a 15 hour incubation at 37℃, was such that 30

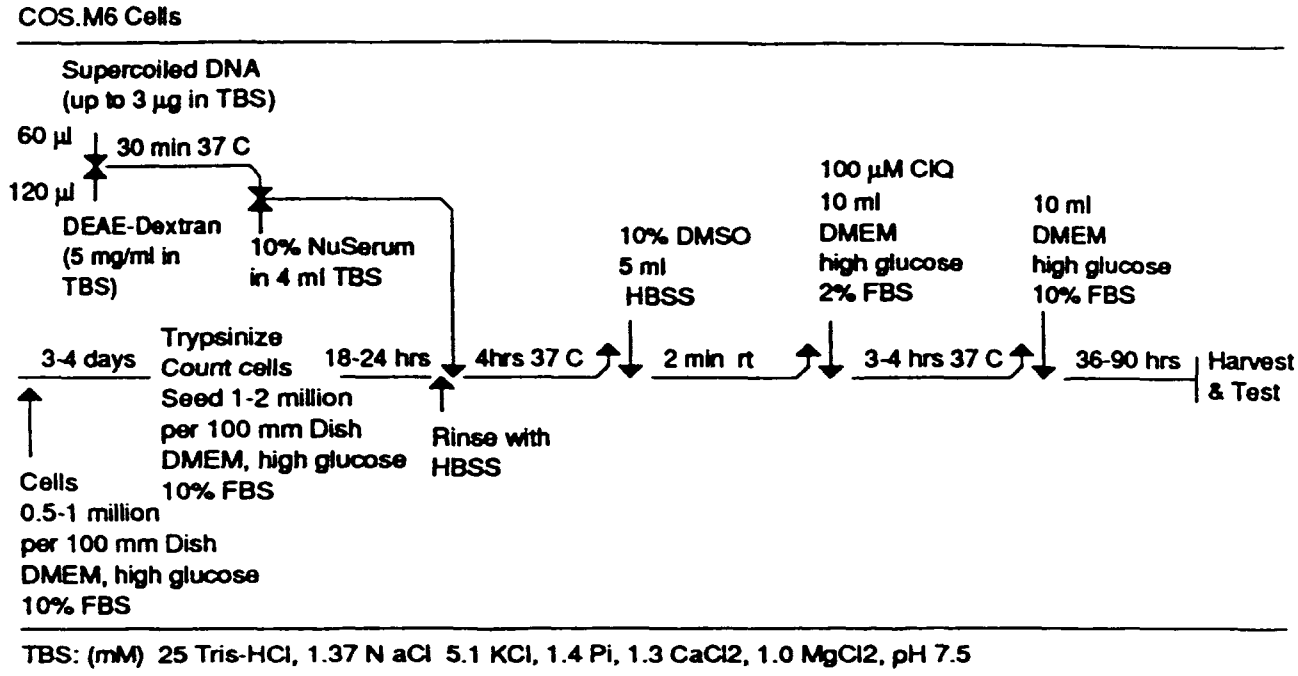

Fig. 3. Schematic presentation of transient transfection of COS cells using DEAE-dextran as a DNA carrier.

Table 1. Effects of (-)D600 on the DHP binding to the receptor complexes expressed in COS cells

Cell type	Receptor complex	DHP binding (−)D600/control
COS cells	$\alpha_1\beta$	3.0±0.3
	$\alpha_1\beta\gamma$	1.3±0.1
	$\alpha_1\beta\alpha_2\delta$	1.8±0.4
	$\alpha_1\beta\gamma\alpha_2\delta$	1.0±0.1
Skeletal muscle	$\alpha_1\beta\gamma\alpha_2\delta$	1.0±0.1
BC3H1	$\alpha_1\beta\gamma\alpha_2\delta$	1.1±0.1

DHP binding to membranes from COS cells transfected with α_1 alone and in combination with β, γ, and/or $\alpha_2\delta$. Input DNA was 2 μg each of the plasmids with the corresponding DHP receptor complex cDNA's. DHP binding was performed using 430 pM of (+)[^{3}H]PN200-110. The data are shown as ratios of PN200-110 binding in the presence of 10^{-5}M (−)D600 relative to in its absence (means ±SEM of at least three independent experiments).

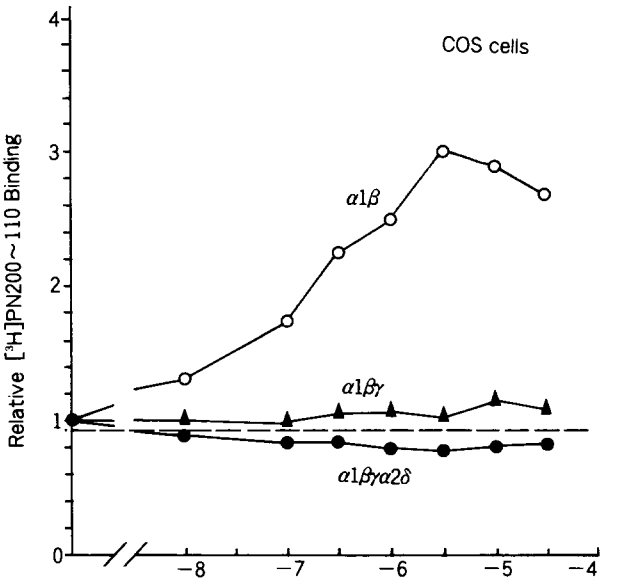

Fig. 4. Comparison of $\alpha_1\beta$, $\alpha_1\beta\gamma$, and $\alpha_1\beta\gamma\alpha_2\delta$ in COS cell membranes for the effect of (-)D600. COS cells were transfected with 0.3 μg α_1 DNA and 1 μg each of β, γ, and α_2 DNA. Binding assays were carried out in the presence of 1 mM $MgCl_2$, 0.8 mM EGTA and indicated concentrations of (-)D600. Data are presented as ratios of specific binding in the presence of (-)D600 relative to in its absence. The data are representative of similar results for each type of membranes obtained in three independent experiments.

to 40% of the cells had taken up and expressed the β -galactosidase gene (data not shown).

Table 1 shows that in COS cells, DHP binding to $\alpha_1\beta$ was increased about 3.3 times by (−)D600, and coexpression of γ lowered this ratio to 1.3. Coexpression of all the components further lowered the ratio to 1.0, which is very similar to that obtained with skeletal muscle or BC3H1 cell membranes. This result clearly demonstrates that γ and $\alpha_2\delta$ account for the discrepancies between $\alpha_1\beta$-expressing L-cells and skeletal muscle T-tubules for the effect of (−)D600. Neither cotransfection of plasmid pKNH without $\alpha_2\delta$, nor pcD without the γ cDNA, nor p91023(B) with the β cDNA in the antisense orientation altered the (−)D600/control (+)PN200-110 binding ratios (data not shown). This indicated that the effects of $\alpha_2\delta$ and γ are specific.

In order to determine whether expression of all the subunits leads to disappearance of the stimulatory effect of (−)D600 and to further substantiate the effect of γ and $\alpha_2\delta$ on the binding properties of α_1, assays were carried out with indicated concentrations of (−)D600. Membranes were prepared from COS cells transfected with 0.3 μg α_1 and 1 μg each of β, γ and $\alpha_2\delta$ DNA's. The amounts of DNA allowed saturation of all the α_1 molecules with β and subsequently with γ and $\alpha_2\delta$ (unpublished data). Therefore, all the DHP binding activity is derived from the pseudohomogeneous population of receptors, either $\alpha_1\beta$ or $\alpha_1\beta\gamma\alpha_2\delta$. Results are shown as ratios to the PN200-110 binding level obtained in the absence of (−)D600. (−)D600 stimulated DHP binding to $\alpha_1\beta$ in COS cells in a concentration dependent manner. While coexpression of γ with $\alpha_1\beta$ in COS cells increased absolute DHP binding (data not shown), it abrogated the effect of (−)D600 and lowered the (−)D600/control ratio close to 1.0 even at 10^{-5} M. This is consistent with the results obtained with LCaN$\alpha_1\beta$ and LCaN$\alpha_1\beta\gamma$ clones as shown in Fig.1 except that the effect of γ is more pronounced to negate the effect of β. Under the same condition, (−)D600 did not stimulate PN200-110 binding to membranes from COS$\alpha_1\beta\gamma\alpha_2\delta$, COS cells transfected with all the DNA's. These data clearly indicate that $\alpha_1\beta$ in L or COS cells has such a conformation that (−)D600 can stimulate DHP binding. These data also strongly suggest that lack of stimulation by (−)D600 in skeletal muscle membranes might be due to a different conformation derived from different subunit

composition

DISCUSSION

The skeletal muscle DHP receptor/Ca^{2+} channel complex was isolated in a highly purified state from rabbit skeletal muscle T-tubules and found to contain five polypeptides, α_1, α_2, β, γ, and δ. These polypeptides are encoded in the α_1, β, γ, and $\alpha_2\delta$ genes[25,26]. Transient expression in α_1-deficient skeletal muscle cells[27] and stable expression in non-muscle L cells lacking β, γ, and $\alpha_2\delta$[9] showed that the Ca^{2+} channel function of the DHP receptor complex resides in α_1. The aims of the present as well as previous studies from our laboratory are to establish whether or not β, γ, and $\alpha_2\delta$ are obligatory subunits of the skeletal muscle DHP receptor/Ca^{2+} channel, or whether the physical association of one or the other of the components with α_1 is a fortuitous occurrence induced by the solubilization procedures that are necessary during the analytical processes used to establish physical association.

The roles of a subunit can be described in terms of quantitative and qualitative effects. The quantitative effects can be exemplified by appearance of increased number of DHP binding sites. However, it may not always be possible to discern simple scaling effects. An increase in the apparent B_{max} of DHP binding could be due to an increase in the number of mature sites on the cell surface (a quantitative effect), or a shift in the equilibrium between the (measurable) high affinity binding state of the channel and its (undetectable) low affinity state without real change in total amount of mature protein (a qualitative effect).

In the present study, we analyzed the effects of skeletal muscle β, $\alpha_2\delta$, and γ on α_1 in two systems: stable expression in L-cells and transient expression in COS cells. The major observations are 1) when α_1 or $\alpha_1\beta$ are expressed the receptor exhibits different allosteric regulations of DHP binding by the phenylalkylamine (−)D600 from those seen with skeletal muscle membranes. 2) These differences are partially corrected by $\alpha_2\delta$ or γ, and completely corrected when both $\alpha_2\delta$ and γ are coexpressed. Importantly, this qualitative alteration of $\alpha_1\beta$ with γ and $\alpha_2\delta$ can be only detected with pharmacological tools used in the present experiment, since electrophysiological studies showed that the $\alpha_1\beta$ is sufficient to function as a normal channel. Our data strongly suggest that all the biochemically identified subunits are essential for forming a functional complex of DHP receptor/calcium channel in skeletal muscle and support the idea that biochemically copurifying these components are the true subunits of the skeletal muscle DHP receptor. Although the stoichiometry of the reconstituted receptor in COS cells have not been tested, the validity of the transient expression in these cells was shown by obtaining consistent result with the γ subunits as found in L cells. Interestingly, enhancement of DHP binding by (−)D600 can be observed only in the absence of Ca^{2+}. Further studies are needed to elucidate the relationship of the allosteric regulation by phenylalkylamines and divalent cations.

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