THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 270, No. 45, Issue of November 10, pp. 27106–27111, 1995

Printed in U.S.A.

Molecular Determinants of Cardiac Ca^{2+} Channel Pharmacology

SUBUNIT REQUIREMENT FOR THE HIGH AFFINITY AND ALLOSTERIC REGULATION OF DIHYDROPYRIDINE BINDING*

(Received for publication, June 23, 1995, and in revised form, August 24, 1995)

Xiangyang Wei‡§¶, Su Pan‖, Wenhua Lang‡, Haeyoung Kim‖, Toni Schneider, Edward Perez-Reyes‡‡, and Lutz Birnbaumer§§**

*From ‡Institute for Molecular Medicine and Genetics, and the §Department of Pharmacology and Toxicology, Medical College of Georgia, Augusta, Georgia 30912, the ‖Department of Cell Biology, Baylor College of Medicine, Houston, Texas 77030, the **Institute of Neurophysiology, University of Koeln, Germany, the ‡‡Department of Physiology, Loyola University Medical Center, Maywood, Illinois 60153, and the §§Department of Anesthesiology, UCLA School of Medicine, Los Angeles, California 90024*

Cardiac L-type Ca^{2+} channels are multisubunit complexes composed of α_{1C}, $\alpha_2\delta$, and β_2 subunits. We tested the roles of these subunits in forming a functional complex by characterizing the effects of subunit composition on dihydropyridine binding, its allosteric regulation, and the ability of dihydropyridines to inhibit channel activity. Transfection of COS.M6 cells with cardiac $\alpha_{1C\text{-}a}$ (α_1) led to the appearance of dihydropyridine ([^{3}H]PN200-110) binding which was increased by coexpression of cardiac β_{2a} (β), $\alpha_2\delta_a$ (α_2), and the skeletal muscle γ. Maximum binding was achieved when cells expressed α_1, β, and α_2. Cells transfected with α_1 and β had a binding affinity that was 5–10-fold lower than that observed in cardiac membranes. Coexpression of α_2 normalized this affinity. (−)-D600 and diltiazem both partially inhibited PN200-110 binding to cardiac microsomes, but stimulated binding in cells transfected with α_1 and β. Again, coexpression of α_2 normalized this allosteric regulation. Therefore coexpression of $\alpha_1\beta$ and α_2 completely reconstituted high affinity dihydropyridine binding and its allosteric regulation as observed in cardiac membranes. Skeletal muscle γ was not required for this reconstitution. Expression in *Xenopus* oocytes demonstrated that coexpression of α_2 with $\alpha_1\beta$ increased the potency and maximum extent of block of Ca^{2+} channel currents by nisoldipine, a dihydropyridine Ca^{2+} channel antagonist. Our results demonstrate that α_2 subunits are essential components of the cardiac L-type Ca^{2+} channel and predict a minimum subunit composition of $\alpha_{1C}\beta_2\alpha_2\delta$ for this channel.

Voltage-gated L-type Ca^{2+} channels are a key element in the excitation-contraction coupling in cardiac muscle. These channels are the molecular target for many drugs including the clinically useful nifedipine, verapamil, and diltiazem. Physiologically they represent one of the sites where β-adrenergic stimulation regulates cardiac function. Thus, understanding the molecular structure of cardiac L-type Ca^{2+} channels, including their subunit composition and the structure-function relationship of each subunit, is of fundamental importance. Biochemical purification of L-type Ca^{2+} channels from skeletal muscle has revealed that, in this tissue, the Ca^{2+} channel complex consists of five subunits (1), which are encoded in four genes (2–6). Functional expression of α_1 in cells lacking endogenous Ca^{2+} channel subunits has established that the α_1 subunit is by itself able to form a functional channel (7), although the activation kinetics of the channel formed by α_1 alone is abnormal. Coexpression of the skeletal muscle β subunit normalizes the activation kinetics (8), suggesting that β is an essential component of the skeletal muscle L-type channel.

Definitive identification of the subunit composition of the cardiac L-type Ca^{2+} channel has been hampered by the low abundance of dihydropyridine binding sites in cardiac muscle. Biochemical studies have demonstrated the existence of α_1, β, and α_2-δ in heart (9–12). This has been further confirmed by molecular cloning approaches (3, 13–15). Cardiac α_1 and its splice variants, collectively referred to as α_{1C} (16), have been studied extensively by expression in *Xenopus* oocytes and mammalian cells. Coexpression studies have suggested crucial physiological roles for the β subunit in the functioning of the cardiac Ca^{2+} channel as in the case of the skeletal muscle channel. In these studies, various β subunits are found to increase peak current, accelerate activation kinetics, and shift the voltage dependence of activation to more hyperpolarized potentials (15, 17–19). The mechanism by which β increases current density is ascribed to a facilitated coupling between the movement of voltage sensor and pore opening without affecting the number of channels being expressed (20). β subunits have also been found to facilitate dihydropyridine binding to the channel by increasing the B_{max} of receptor sites or the affinity for ligands (21, 22). In contrast, the effect of α_2 on cardiac α_1 has not been consistent, although stimulation of peak current (13, 23) and changes in inactivation kinetics (17, 21) have been reported. One problem in dealing with these variable effects is that there have been no good criteria as to whether these effects are required for the physiological function of the channel or they are artifactual effects observed only in heterologous expression systems. A similar confusion surrounds the role of the skeletal muscle γ, which is able to affect the cardiac isoforms of α_1 (17, 18) although its expression has been detected only in skeletal muscle (5, 6).

In this article, we describe an analysis of the functional importance of cardiac Ca^{2+} channel subunits by characterizing their effects on the ligand binding properties of the complex. By transiently expressing a cardiac α_1 in various combinations with a cardiac β, $\alpha_2\delta$, and the skeletal muscle γ in COS.M6 cells, we demonstrate that high affinity dihydropyridine bind-

* This work was supported by American Heart Association (National Center) Grant-In-Aid 93-1202 (to X. W.) and National Institutes of Health Grant AR43411 (to L. B.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

¶ To whom correspondence and reprint request should be addressed: Institute for Molecular Medicine and Genetics, Medical College of Georgia, Augusta, GA 30907. Tel.: 706-721-0688; Fax: 706-721-7915; E-mail: cwei@mail.mcg.edu.

ing and its allosteric regulation are identical to those in cardiac membranes only when α_1, β, and $\alpha_2\delta$ are expressed, and that $\alpha_2\delta$ plays a key role in the formation of cardiac L-type Ca^{2+} channel complex.

EXPERIMENTAL PROCEDURES

Complementary DNAs and Other Materials—For transfection of COS.M6 cells, the cDNA encoding a rabbit cardiac Ca^{2+} channel $\alpha_{1C\text{-}a}$ subunit (18) was cloned into the expression plasmid pKNH. pKNH and its derivative pKCR-$\alpha_2\delta$ containing the rabbit skeletal muscle $\alpha_2\delta_a$ cDNA (*Fnu*DII(−5)/*Eco*RI(+3544) fragment) were gifts from Dr. Tsutomu Tanabe (Yale University). The β subunit cDNA encoded the rat β_{2a} isoform (14, 15). It was subcloned into the expression plasmid p91203(B), which was a gift from Dr. Randall Kaufman (Genetics Institute, Cambridge, MA). The expression plasmid pSkMCaChγ.3 containing the rabbit skeletal muscle γ cDNA (5) was a gift from Dr. Kevin Campbell (University of Iowa). All DNAs used for transfection were prepared by the CsCl gradient centrifugation method. The $\alpha_{1C\text{-}a}$ used for expression in *Xenopus* oocytes was ΔN60, which is an N-terminal deletion mutant of the rabbit cardiac $\alpha_{1C\text{-}a}$ (24). β_2 and $\alpha_2\delta$ were subcloned into pBluescript and pGEM-3, respectively. T7 RNA polymerase was used for *in vitro* synthesis of cRNAs. In this report we will refer to the cardiac $\alpha_{1C\text{-}a}$ clones as α_1, β_{2a} as β, and $\alpha_2\delta$ as α_2.

(+)-[^{3}H]PN200-110 (isopropyl-4-(2,1,3-benzoxadiazol-4-yl)-1,4-dihydro-5-methoxycarbonyl-2,6-dimethyl-3-pyridinecarboxylate), specific activity 80–85 Ci/mmol, was purchased from Amersham. Nisoldipine was a kind gift from Dr. David J. Triggle (State University of New York at Buffalo). Nitrendipine, (−)-D600, and diltiazem were purchased from CalBiochem. Tissue culture media and sera were purchased from BRL/Life Technologies, Inc. NuSerum was purchased from Collaborative Research (Bedford, MA). T7 RNA polymerase and other chemicals for *in vitro* transcription were obtained from Boehringer Mannheim.

Culture and Transfection of COS.M6 Cells—COS.M6 cells were grown in Dulbecco's modified Eagle's medium with high glucose. The medium was supplemented with 10% fetal bovine serum, 100 units/ml penicillin, and 100 μg/ml streptomycin. Cells were transfected using a DEAE-dextran method described by Luthman and Magnusson (25) with modifications. Briefly, 1 day before transfection, cells were plated at a density of 2×10^6 cells/100-mm dish. At the time of transfection, cells were washed twice with 10 ml of Hank's balanced salt solution and overlaid with 4 ml of a solution containing 25 mM Tris (pH 7.5), 137 mM NaCl, 5.1 mM KCl, 1.4 mM Na_2HPO_4, 1.3 mM $CaCl_2$, 1.0 mM $MgCl_2$, 10% NuSerum (Collaborative Research), 144 μg/ml DEAE-dextran, and plasmid DNA. α_1 DNA was used at 1 μg/100-mm dish, and all other DNAs were used at 2 μg/dish. Cells were incubated for 4 h at 37 °C in an atmosphere of 95% air and 5% CO_2. After the incubation, the DNA containing solution was removed and cells incubated first with 5 ml of 10% dimethyl sulfoxide in Hank's balanced salt solution for 2 min at room temperature, and then with 10 ml of Dulbecco's modified Eagle's medium with high glucose, 2% fetal bovine serum, and 100 μM chloroquine for 3–4 h at 37 °C. Thereafter, the chloroquine containing medium was discarded and the cells were first washed twice with Hank's balanced salt solution, and then overlaid with 10 ml of Dulbecco's modified Eagle's medium with high glucose and 10% fetal bovine serum and incubated at 37 °C for 60 h.

The transfection efficiency of this procedure 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 as described by MacGregor *et al.* (26). Briefly, 60 h after transfection, the cells in a 100-mm dish were rinsed with 10 ml of phosphate-buffered saline, and overlaid with 1 ml of 0.6% glutaraldehyde in phosphate-buffered saline for 10 min at room temperature. The cells were then rinsed twice with 10 ml of 50 mM Tris (pH 7.5) and 150 mM NaCl (TBS) and overlaid with 8 ml of TBS containing 0.5 mg/ml 5-bromo-4-chloro-3-indoyl α-D-galactoside, 5 mM potassium ferrocyanide, 5 mM potassium ferricyanide, and 2 mM $MgCl_2$. After an overnight incubation at 37 °C, the proportion of cells developing blue color due to β-galactosidase expression was determined with a phase-contrast microscope at a magnification of 200 ×. The transfection efficiency thus tested was between 30 and 40%.

Preparation of Crude Membranes from Transfected COS.M6 Cells—Approximately 60 h after transfection, COS.M6 cells were rinsed with ice-cold TBS, and harvested in 20 ml of ice-cold TBS using a rubber policeman. The cells were pelleted by centrifugation at 450 × *g* for 5 min at 4 °C. Cells from one 100-mm dish were resuspended in 5 ml of 50 mM Tris (pH 7.5) and 1 mM EDTA and lysed by repeated vigorous vortexing over 20 min. The crude particulate fraction was then pelleted by centrifugation for 10 min at 450 × *g* at 4 °C. This fraction was resuspended in 4 ml of ice-cold buffer containing 0.5 mM $MgCl_2$ and 50 mM Tris (pH 7.5) for dihydropyridine binding assays. Protein concentration was determined by the Lowry method.

Preparation of Microsomal Membranes from Cardiac Muscle—Microsomal membranes from rabbit cardiac muscle were prepared as described previously (27). The tissue was placed in ice-cold phosphate-buffered saline and minced with scissors, and then homogenized in 20 ml of 50 mM Tris (pH 7.5) per gram of tissue using a Polytron homogenizer. The homogenate was centrifuged at 3,000 × *g* for 10 min at 4 °C. The pellet was discarded and the supernatant was further centrifuged at 45,000 × *g* for 45 min. The pellet was suspended in ice-cold buffer containing 0.5 mM $MgCl_2$ and 50 mM Tris (pH 7.5) for dihydropyridine binding assays. Protein concentration was determined by the method of Lowry.

(+)-[^{3}H]PN200-110 Binding Assays—Binding assays were carried out in a final volume of 1 ml. Reactions contained 50 mM Tris (pH 7.5), 0.4 mM $MgCl_2$, 100–200 μg of membrane protein, and various concentrations of (+)-[^{3}H]PN200-110 and other drugs. The concentration of (+)-[^{3}H]PN200-110 was 0.4 nM in single concentration assays and from 0.01 to 1 nM for saturation assays. Nonspecific binding was determined in the presence of 2.5 μM unlabeled nitrendipine. $CaCl_2$ was added to a final concentration of 1 mM in assays with added Ca^{2+}. Incubations were carried out for 90 min at room temperature (22–24 °C). The reactions were terminated by filtration through Whatman GF/B glass fiber filters using a Brandel cell harvester. The filters were washed four times each with 5 ml of ice-cold 25 mM Tris (pH 7.5) and were then extracted in 5 ml of scintillation mixture for at least 2 h. Radioactivity was determined by liquid scintillation spectrometry.

Expression in Xenopus Oocytes and Electrophysiological Recordings—cRNAs for α_1, β, and α_2 were synthesized *in vitro* as described previously (18) and suspended in water for injection. The final concentration for all cRNAs was 100 ng/μl. 50 nl of cRNA was injected per oocyte using a Drummond Nanojet automatic oocyte injector. Oocytes were maintained at 19 °C on a rotating platform (20 rpm) in a medium containing 100 mM NaCl, 2 mM KCl, 1.8 mM $CaCl_2$, 1 mM $MgCl_2$, 2.5 mM pyruvic acid, and 5 mM HEPES (pH 7.6). Either 50 μg/ml gentamicin or 100 units/ml penicillin plus 100 μg/ml streptomycin was added to the medium. Voltage clamp recording was performed approximately 7 days after cRNA injection.

Currents were recorded using the cut-open oocyte vaseline-gap voltage clamp technique (28) with a CA-1 amplifier from Dagan Corp. An oocyte was placed in triple Perspex chambers that isolated a portion (approximately one-sixth to one-fifth) of the oocyte surface for current recording. Oocyte membrane exposed to the bottom compartment was permeabilized by a brief treatment with 0.1% saponin to allow low access resistance to the interior of the oocyte. The voltage pipettes, filled with a solution containing 2.7 M sodium methanesulfonate, 10 mM EGTA, and 10 mM NaCl, had tip resistance of 300 to 400 kΩ. Data acquisition and analysis were performed using the pCLAMP system (Axon Instruments). Currents were induced by 250-ms depolarization steps from a holding potential of −40 mV. Linear components were subtracted using P/−4 protocol. Current signals were filtered at 0.5 kHz and digitized at 2 kHz.

All experiments were performed at room temperature (22–24 °C). Each oocyte was injected with 50 nl of 50 mM BAPTA(Na)$_4$[1] prior to recording to minimize the contamination by the oocyte endogenous Cl^- currents, which can be activated by the influx of Ba^{2+} as well as of Ca^{2+} (29). The external recording solution contained 10 mM Ba^{2+}, 96 mM Na^+, and 10 mM HEPES, pH adjusted to 7.4 with methanesulfonic acid. The internal solution contained 120 mM potassium glutamate, 10 mM EGTA, and 10 mM HEPES, pH adjusted to 7.3 with KOH. Nisoldipine was diluted in the external solution so that 5% of the top chamber volume was replaced to achieve the desired final concentration. Before an oocyte was subjected to nisoldipine, it was observed for 10–15 min with repeated stimulation to ensure a stable current amplitude. Under our conditions, currents usually remained stable for at least 1 h. Rundown occurred only in oocytes which were severely damaged and these oocytes were discarded. In order to obtain cumulative dose-response relationships for nisoldipine, an oocyte was stimulated by a 250-ms depolarization step to +10 mV repeated every 10 s. A higher drug concentration was applied after the response to the previous concentration stabilized, usually within 20 s.

[1] The abbreviation used is: BAPTA, 1,2-bis(*O*-aminophenoxy)ethane-*N*,*N*,*N*′,*N*[*prime*]-tetraacetic acid.

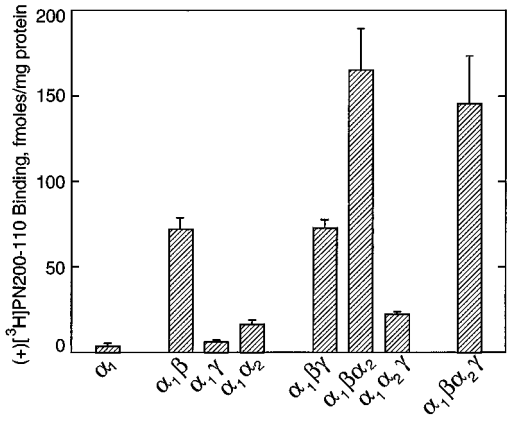

FIG. 1. **Comparison of specific (+)-[^{3}H]PN200-110 binding to membranes from COS cells transfected with Ca^{2+} subunits in various combinations.** Binding assays were performed using 0.4 nM (+)-[^{3}H]PN200-110, 0.4 mM Mg^{2+}, and 1 mM Ca^{2+}. *Bars* indicate standard error of mean (n = 3–4).

RESULTS

Effects of Subunit Combination on (+)-[^{3}H]PN200-110 Binding—COS cells were transfected with α_1 alone or with β, α_2, and skeletal muscle γ plasmids in various combinations. Dihydropyridine binding was tested at 0.4 nM (+)-[^{3}H]PN200-110. In preliminary experiments with α_1 alone, maximum binding was achieved when 4 μg of plasmid DNA was transfected per 100-mm dish. Binding was approximately half-maximal at 1 μg of α_1 DNA/dish. The effects of β, α_2, or γ were clearly detectable when 2 μg each of the plasmids were co-transfected with α_1, and the effects did not increase further when the amount of plasmid DNA was 5 μg. Therefore in all the experiments, the amounts of plasmid DNA were 1 μg for α_1 and 2 μg for all other subunits. Using submaximal amounts of α_1 and saturating amounts of other subunits would minimize the potential formation of heterogeneous populations of complexes. Transfection of COS cells with β, α_2, and γ without α_1 did not lead to the appearance of dihydropyridine binding (data not shown).

Fig. 1 shows (+)-[^{3}H]PN200-110 binding to membranes from COS cells transfected with various combinations of subunits. The results represent the average from four experiments. Cells transfected with α_1 only had a measurable binding of 3.8 ± 1.5 fmol/mg of protein. Thus, the level of binding achieved in cells transfected with α_1 alone was very low and close to the limit of detection. Coexpression of α_1 with β, α_2, or γ increased the amount of (+)-[^{3}H]PN200-110 binding. The most effective subunit was β, which stimulated binding 19-fold. In comparison, α_2 stimulated binding only 4.4-fold. γ appeared to have a small effect (an 1.5-fold increase) although this effect may not be significant due to the sensitivity of the binding assay. Co-transfection of α_1 with combinations of β, α_2, and γ revealed interesting properties in terms of the effects of these subunits in stimulating binding. First, maximum PN200-110 binding was obtained when α_1, β, and α_2 were coexpressed; binding reached 165.4 ± 8.0 fmol/mg of protein which represents a 44-fold stimulation. This degree of stimulation by β and α_2 together is much greater than predicted by the sum of the effects of each subunit alone, suggesting that β and α_2 functioned synergistically. Second, coexpression of α_1 with β and γ stimulated binding by 19-fold, coexpression with $\alpha_2\gamma$ stimulated binding by 5.8-fold, and coexpression with $\beta\alpha_2\gamma$ stimulated binding by 39-fold. These results suggest that γ did not have a significant effect on dihydropyridine binding when either β or α_2 or both were coexpressed with α_1.

The stimulation of dihydropyridine binding to α_1 by other subunits may be due to an increase in the affinity for ligand or due to an increase in the number of receptors. In order to investigate these possibilities, (+)-[^{3}H]PN200-110 binding was measured at various concentrations of the ligand and Scatchard analyses were performed. COS cells expressing α_1 alone, α_1 with α_2 and/or γ but without β had amounts of PN200-110 binding that were too low to allow for Scatchard analysis. Therefore binding in cells expressing $\alpha_1\beta$ was used to determine the effects of other subunits. Binding to cardiac microsomal membranes was carried out in parallel as control. Binding was also compared in the absence and presence of 1 mM added Ca^{2+} in all membrane preparations to determine the dependence on divalent cations. All data represent the average from three to four experiments each performed in duplicate. [^{3}H]PN200-110 binding to rabbit cardiac microsomal membranes had a K_D of 38 ± 4 pM (n = 3) and 33 ± 6 pM (n = 3) in the presence and absence of Ca^{2+}, respectively. The B_{max} values were 213 ± 14 and 176 ± 11 fmol/mg of protein in the presence and absence of Ca^{2+}, respectively (Table I). The K_D values obtained in the present study were similar to those reported previously for cardiac myocytes (27). The results demonstrated that high affinity dihydropyridine binding to cardiac membranes did not require added Ca^{2+}. In contrast, PN200-110 binding to membranes from COS cells expressing $\alpha_1\beta$ was different in two ways. First, in the presence of Ca^{2+}, the K_D of $\alpha_1\beta$ expressing cells was 175 ± 11 pM; this reflects a 5-fold lower affinity than that in cardiac membranes. Second, the affinity was still lower in the absence of added Ca^{2+}. Both of these differences suggest that additional subunits may be required to reconstitute the high affinity dihydropyridine binding seen in cardiac membranes.

Fig. 2 shows representative Scatchard plots of binding in the presence of Ca^{2+} in COS cells expressing either $\alpha_1\beta$, $\alpha_1\beta\alpha_2$, $\alpha_1\beta\gamma$, or $\alpha_1\beta\alpha_2\gamma$. In the presence of Ca^{2+}, coexpression of α_2, γ, or both with $\alpha_1\beta$ increased the affinity of PN200-110 binding in comparison with cells expressing $\alpha_1\beta$ only. The K_D values (Table I) were very similar to that in cardiac microsomal membranes. However, in the absence of added Ca^{2+} only in cells expressing $\alpha_1\beta\alpha_2$ and $\alpha_1\beta\alpha_2\gamma$, the affinity of PN200-110 resembled that in cardiac membranes. Binding affinity in cells expressing $\alpha_1\beta\gamma$ was 6.5-fold lower than that in cardiac membranes. Thus, coexpression of γ with $\alpha_1\beta$ failed to correct the divalent cation dependence, although it restored the affinity of PN200-110 binding in the presence of Ca^{2+}. The B_{max} in $\alpha_1\beta$ expressing cells was close to that in cardiac membranes and was not altered by the coexpression of α_2 or $\alpha_2\gamma$ (Table I). However, B_{max} was lower in cells expressing $\alpha_1\beta\gamma$ both in the absence and presence of Ca^{2+} (Table I). That transient expression of Ca^{2+} channel subunits in COS cells led to receptor densities close to that in cardiac muscle demonstrates that COS cells are suitable for studying ligand binding to Ca^{2+}

TABLE I

Comparison of K_D and B_{max} values of (+)-[^{3}H]PN200–110 binding to membranes from cardiac muscle and from COS cells transfected with cloned Ca^{2+} channel subunits

COS cells were transfected with cDNAs encoding Ca^{2+} channel subunits in the combination indicated. Saturation binding assays of (+)-[^{3}H]PN200–110 on the membrane preparations were carried out both in the presence and in the absence of 1 mM $CaCl_2$. K_D and B_{max} values were derived from Scatchard analysis of saturation binding data and presented as mean ± S.E.

Membranes	K_D, pM		B_{max}, fmol/mg protein	
	$+Ca^{2+}$	$-Ca^{2+}$	$+Ca^{2+}$	$-Ca^{2+}$
COS cell, $\alpha_1\beta$	175 ± 11	387 ± 44	191 ± 23	159 ± 17
COS cell, $\alpha_1\beta\alpha_2$	41 ± 4	35 ± 5	189 ± 31	167 ± 32
COS cell, $\alpha_1\beta\gamma$	49 ± 6	215 ± 41	125 ± 27	129 ± 25
COS cell, $\alpha_1\beta\alpha_2\gamma$	36 ± 3	34 ± 3	181 ± 35	161 ± 30
Rabbit heart	38 ± 4	33 ± 6	213 ± 14	176 ± 11

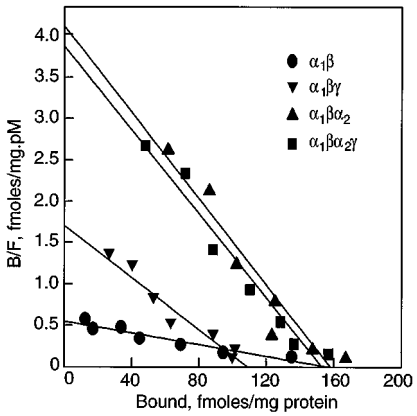

FIG. 2. **Representative Scatchard plots of specifically bound (+)-[^{3}H]PN200-110 in membranes from COS cells transfected with either $\alpha_1\beta$, $\alpha_1\beta\alpha_2$, $\alpha_1\beta\gamma$, or $\alpha_1\beta\alpha_2\gamma$.** Only the data obtained in the presence of 1 mM Ca^{2+} were shown. The parameters of Scatchard analyses of binding data both in the presence and absence of Ca^{2+} are shown in Table I.

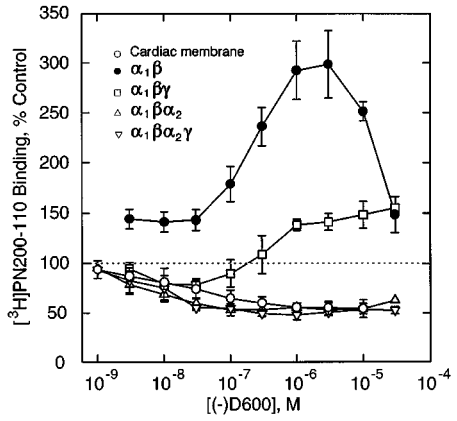

FIG. 3. **Effects of (−)-D600 on (+)-[^{3}H]PN200-110 binding in membrane preparations from rabbit cardiac muscle and COS cells transfected with Ca^{2+} channel subunits.** The combinations of subunit are illustrated in the figure. Binding assays were carried out using 0.4 nM (+)-[^{3}H]PN200-110, in the presence of 0.4 mM Mg^{2+} and in the absence of added Ca^{2+}. Data are normalized against the binding in the absence of (−)-D600. Control binding was 6.4 ± 2.1 fmol/mg of protein in COS cells with $\alpha_1\beta$, 74.7 ± 21.2 fmol/mg of protein in $\alpha_1\beta\alpha_2$, 9.8 ± 2.2 fmol/mg of protein in $\alpha_1\beta\gamma$, 76.9 ± 36.1 fmol/mg of protein in $\alpha_1\beta\alpha_2\gamma$, and 126.8 ± 23.6 fmol/mg in cardiac muscle. These values were lower than those in Fig. 1 because binding assays in the present figure were performed in the absence of added Ca^{2+}. Data are mean ± S.E., $n = 4$.

channels. These results indicate that the complex formed by $\alpha_1\beta\alpha_2$ is the minimum subunit structure which qualitatively resembles native cardiac Ca^{2+} channels in terms of dihydropyridine binding.

Effects of Subunits on Allosteric Regulation of Dihydropyridine Binding—(−)-D600 and diltiazem are allosteric regulators of dihydropyridine binding (30, 31). The regulatory effects of (−)-D600 and diltiazem are thought to be coupled to or mediated by Ca^{2+} binding sites in L-type Ca^{2+} channels (32, 33). In previous studies, we demonstrated that the effect of (−)-D600 differed in skeletal muscle and in cells expressing the skeletal muscle α_1 subunit (34). In the present study we tested the effect of (−)-D600 on binding of [^{3}H]PN200-110 (0.4 nM) to membranes from transfected COS cells in the absence of added Ca^{2+} and investigated how the (−)-D600 effect was modulated by the subunit composition. In cardiac microsomal membranes, (−)-D600 partially inhibited binding of PN200-110 in a concentration-dependent manner (Fig. 3); the maximum extent of inhibition was 46%. In contrast, (−)-D600 significantly enhanced PN200-110 binding at all concentrations tested in COS cells expressing $\alpha_1\beta$ (Fig. 3). The stimulatory effect of (−)-D600 was observed clearly at 3 nM, and reached maximum at 1 μM where PN200-110 binding was stimulated 3-fold. The dose-response relationship of (−)-D600 was biphasic, with less stimulatory effect observed at concentrations greater than 3 μM. Therefore, the allosteric regulation of dihydropyridine binding by (−)-D600 was opposite in membranes from cardiac muscle and from COS cells expressing the α_1 and β subunits of cardiac L-type Ca^{2+} channels.

Coexpression of γ subunit with $\alpha_1\beta$ markedly attenuated the stimulatory effect of (−)-D600 (Fig. 3). At concentrations between 3 and 100 nM, (−)-D600 had a slight inhibitory effect on PN200-110 binding, which was similar to the response obtained in cardiac membranes. However, at concentrations above 1 μM, (−)-D600 stimulated PN200 binding with the maximum stimulation of approximately 55% observed at 30 μM. In contrast, when α_2 was coexpressed with $\alpha_1\beta$, (−)-D600 partially inhibited PN200-110 binding to COS cells in exactly the same manner as in cardiac microsomal membrane, as illustrated in Fig. 3. Coexpression of γ with $\alpha_1\beta_2\alpha_2$ did not cause a further change in the allosteric effect of (−)-D600. These results once again suggest that the complex of $\alpha_1\beta\alpha_2$ is the minimum combination of subunits that resemble cardiac Ca^{2+} channels in dihydropyridine binding.

The effect of diltiazem was tested using similar assays in the presence of added Ca^{2+}. Diltiazem has been reported to increase, decrease, or have no effect on dihydropyridine binding. Its effect is dependent on factors such as temperature, divalent cations, and membrane potential. It is thus important to define carefully the experimental conditions under which diltiazem affects dihydropyridine binding. In the present study, diltiazem was tested in binding assays performed at room temperature (22–24 °C), in the presence of 0.4 mM Mg^{2+}, 1 mM Ca^{2+}, and 0.4 nM [^{3}H]PN200-110. Under these conditions, diltiazem had a slight inhibitory effect on PN200-110 binding to cardiac microsomal membranes; the maximum inhibition was less than 15% (Fig. 4). However, in COS cells expressing $\alpha_1\beta$, diltiazem stimulated PN200-110 binding in a concentration-dependent manner. Binding was 270% of control in the presence of 30 μM diltiazem. This discrepancy in the effects of diltiazem between cardiac muscle and COS cells expressing $\alpha_1\beta$ is similar although not identical to that observed with the effects of (−)-D600. Coexpression of α_2, γ, or both, eliminated this discrepancy. As shown in Fig. 4, diltiazem slightly inhibited PN200-110 binding in COS cells transfected with $\alpha_1\beta\alpha_2$, $\alpha_1\beta\gamma$, or $\alpha_1\beta\alpha_2\gamma$; the concentration-response relationships in these cells were very similar to that in cardiac membranes.

Inhibition of Ca^{2+} Channel Currents by Nisoldipine and Its Modulation by α_2—Our binding results indicated that $\alpha_1\beta$ had a lower affinity for dihydropyridine Ca^{2+} channel antagonists than $\alpha_1\beta\alpha_2$. To determine if subunit composition altered the pharmacological sensitivity of Ca^{2+} channel currents, we tested the ability of nisoldipine to inhibit currents from *Xenopus* oocytes expressing $\alpha_1\beta$ and $\alpha_1\beta\alpha_2$. Currents were recorded using the cut-open oocyte voltage clamp technique in 10 mM Ba^{2+}. Oocytes were held at −40 mV and stimulated by repeated depolarization to +10 mV for 250 ms. Nisoldipine was added cumulatively and inhibited currents in oocytes injected with $\alpha_1\beta$ and $\alpha_1\beta\alpha_2$ (Fig. 5*A*). The cumulative dose-response curves for nisoldipine are shown in Fig. 5*B*, which indicates that nisoldipine was more potent in oocytes injected with $\alpha_1\beta\alpha_2$ than in those with $\alpha_1\beta$. For example at 0.1 nM, nisoldipine inhibited 23% of the currents from oocytes injected with $\alpha_1\beta\alpha_2$, while it had no significant inhibition on currents from oocytes injected with $\alpha_1\beta$. The IC_{50} values of nisoldipine was 2.2 and

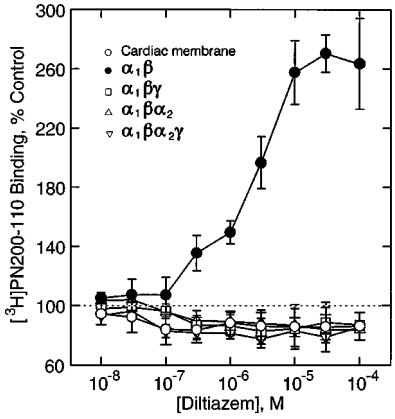

FIG. 4. **Effects of diltiazem on (+)-[^{3}H]PN200-110 binding in membrane preparations from rabbit cardiac muscle and COS cells transfected with Ca^{2+} channel subunits.** Subunit combination for each curve is illustrated in the figure. Binding assays were carried out using 0.4 nM (+)-[^{3}H]PN200-110, in the presence of 0.4 mM Mg^{2+} and 1 mM Ca^{2+}. Data are normalized against the binding in the absence of diltiazem. Control binding was 23.3 ± 8.1 fmol/mg of protein in $\alpha_1\beta$, 95.7 ± 26.2 fmol/mg of protein in $\alpha_1\beta\alpha_2$, 43.0 ± 7.5 fmol/mg of protein in $\alpha_1\beta\gamma$, 103.3 ± 3.4 fmol/mg of protein $\alpha_1\beta\alpha_2\gamma$, and 135.2 ± 32.7 fmol/mg of protein in cardiac membranes. Data are mean ± S.E., $n = 4$.

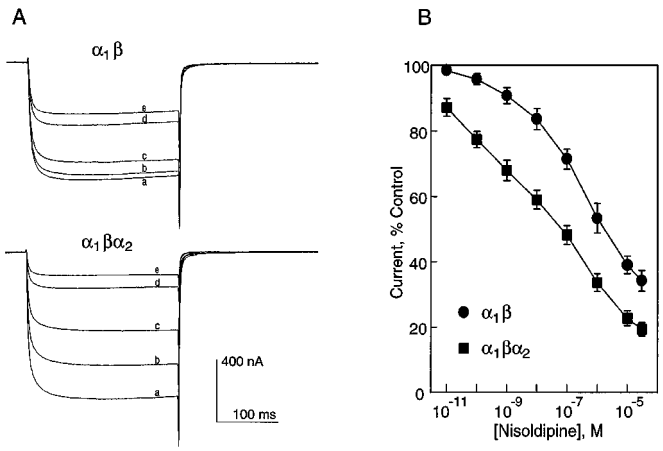

FIG. 5. **Inhibition of Ca^{2+} channel currents by Ca^{2+} channel antagonist nisoldipine.** Ca^{2+} channel subunits were expressed in *Xenopus* oocytes and currents recorded in 10 mM Ba^{2+} using the cut-open oocyte voltage clamp technique. *A*, currents induced by repeated depolarization to +10 mV from −40 mV holding potential in oocytes injected with $\alpha_1\beta$ and $\alpha_1\beta\alpha_2$. Nisoldipine was added in a cumulative manner to the bath (the top chamber). The *lower-case letters* beside the traces indicate currents in the absence (*a*) and presence of 0.1 nM (*b*), 10 nM (*c*), 1 μM (*d*), and 30 μM (*e*) nisoldipine. *B*, cumulative dose-response relationship of nisoldipine in oocytes injected with $\alpha_1\beta$ ($n = 8$) and $\alpha_1\beta\alpha_2$ ($n = 9$). Data are percentage of control currents and shown as mean ± S.E.

0.084 μM in oocytes injected with $\alpha_1\beta$ and $\alpha_1\beta\alpha_2$, respectively. This represents a 26-fold shift in the half-maximum concentration for inhibition. At the maximum concentration tested (30 μM), nisoldipine inhibited 66 and 81% of currents in oocytes injected with $\alpha_1\beta$ and $\alpha_1\beta\alpha_2$, respectively.

Current-voltage relationships in the presence and absence of nisoldipine are shown in Fig. 6. Nisoldipine inhibited currents at all potentials tested. It inhibited peak currents to a greater extent in oocytes injected with $\alpha_1\beta\alpha_2$ (Fig. 6*B*) than in oocytes injected with $\alpha_1\beta$ (Fig. 6*A*). The position and shape of the current-voltage relationships were not modified by nisoldipine. The percentage of inhibition by nisoldipine at potentials where inward currents can be reliably measured is plotted as a function of membrane potential in Fig. 6*C*. Thus, when the membrane was depolarized to potentials ranging from −30 to +30

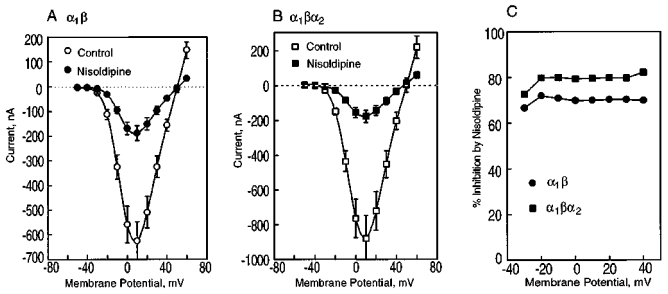

FIG. 6. **Current-voltage relationships from oocytes injected with $\alpha_1\beta$ (*A*) and $\alpha_1\beta\alpha_2$ (*B*) in the absence and presence of 30 μM nisoldipine.** Currents were recorded during voltage steps over the range of −50 to +60 mV in 10 mV increments from −40 mV holding potential. Data represent mean ± S.E. The percentages of inhibition of inward currents between −30 to +40 mV are plotted as a function of membrane potential (*C*).

mV, currents were inhibited by approximately 70% in oocytes injected with $\alpha_1\beta$ and by approximately 80% in those with $\alpha_1\beta\alpha_2$. The relative effectiveness of nisoldipine was not affected by the amplitude of the step pulse.

DISCUSSION

Cloning and functional expression of cardiac and several other L-type Ca^{2+} channel α_1 subunits have firmly established that α_1 is by itself sufficient to form the voltage-gated ion-conducting pore and that α_1 has the receptor sites for all major chemical classes of ligands, such as dihydropyridines, phenylalkylamines, and benzothiazepines (7, 13, 23, 35). It has also been generally accepted that the β subunit is an essential component of Ca^{2+} channels, including the cardiac L-type channel, since β has consistently been observed to modulate significantly the biophysical characteristics of α_1 (8, 15, 17–19). However, the functional role of α_2 in cardiac L-type Ca^{2+} channels has not been clearly defined, although its presence in cardiac muscle has been demonstrated by biochemical and molecular means (3, 36, 37). The most consistent effect of α_2 has been an approximately 2-fold increase in current density over α_1 alone when expressed in oocytes (13, 23), although effects on channel inactivation have been reported (17, 21). Due to the lack of measurable criteria, it is difficult to conclude whether these effects of α_2 are necessary for normal channel function.

In the present study, we examined the role of subunits, particularly α_2, on the ligand binding properties of expressed cardiac L-type Ca^{2+} channel in two systems: transient expression in COS.M6 cells in which dihydropyridine binding and its allosteric regulation by Ca^{2+}, (−)-D600, and diltiazem were studied, and expression in *Xenopus* oocytes where the sensitivity of Ca^{2+} channel currents to an antagonist nisoldipine was examined. Our results demonstrate for the first time that α_2 is essential for the reconstitution of both high affinity dihydropyridine binding and its allosteric regulation. Thus the Ca^{2+} channel complex formed by $\alpha_1\beta$, in comparison to that in cardiac membranes, has several defects: 1) its affinity for PN200-110 was severalfold lower; 2) its affinity was dependent on added Ca^{2+}; and 3) its allosteric regulation by (−)-D600 and diltiazem is abnormal. Coexpression of α_2 corrected all the defects associated with $\alpha_1\beta$. Furthermore, consistent with the finding that α_2 increased the affinity of Ca^{2+} channel for antagonists, coexpression of α_2 with $\alpha_1\beta$ in oocytes greatly increased the potency of the antagonist nisoldipine in blocking Ca^{2+} channel currents. These results clearly demonstrate that α_2 is an essential functional component of the cardiac L-type Ca^{2+} channel, and that $\alpha_1\beta\alpha_2$ is the minimum subunit combination of a complex that has pharmacological properties iden-

tical to those of the native cardiac L-type Ca^{2+} channel. Lack of α_2 in the complex may cause conformational changes that are not readily detected by analyzing its biophysical properties, but that substantially alter its ligand binding ability.

In analyzing the effect of each subunit on ligand binding to α_1, we found that the cardiac β (β_2) alone stimulated PN200-110 binding by 19-fold. The mechanism of this stimulation was not examined in this study. By expression in Chinese hamster ovary cells, a skeletal β subunit, β_1, and another widely expressed β, β_3, increased the B_{max} of PN200-110 binding to α_1 without an effect on affinity (21, 38). In these studies the K_D of PN200-110 was between 130 and 150 pM, similar to that in $\alpha_1\beta$ expressing COS cells in the present study. The increase in B_{max} may suggest an increase in Ca^{2+} channel expression. However, such a conclusion would be contradicted by the observation that β did not increase the amount of α_1 protein detected on immunoblots (22, 39). In contrast, Mitterdorfer *et al.* (22) reported that β_1 increased 35-fold the affinity of PN200-110 to a modified cardiac α_1 due to a decrease in the rate the dissociation (22). The discrepancy may be explained by a shift from a low affinity state (fast k_{off}) to a high affinity state (slow k_{off}), if the lower affinity state cannot be readily detected, thus leading to an apparent increase in B_{max}.

The present study clearly indicates that the skeletal muscle γ was capable of interacting with cardiac $\alpha_1\beta$. In the study γ showed three effects on dihydropyridine binding to cells expressing $\alpha_1\beta$: it increased binding to $\alpha_1\beta$ by increasing affinity both in the presence and in the absence of Ca^{2+}, it attenuated the stimulatory effect of (−)-D600, and it completely eliminated the stimulatory effect of diltiazem. However, γ failed to correct the divalent cation dependence of dihydropyridine binding and it did not correct the anomalous effect of (−)-D600. The effect of γ was relatively small compared to those of β and α_2, and when α_1 was coexpressed with β and α_2, γ had no additional effect. It appears that γ is not necessary for the formation of a complex that resembles the cardiac Ca^{2+} channel in terms of ligand binding properties. Thus, demonstration that γ interacts with the cardiac $\alpha_1\beta$ (or α_1) in this and previous studies (17, 18) is not sufficient evidence for the existence of a cardiac homolog of the γ subunit. The ability of γ to interact with cardiac $\alpha_1\beta$ is not surprising given that skeletal muscle α_1 and cardiac α_1 share a high degree of sequence homology (2, 13).

The present study demonstrates that by expressing the cloned α_{1C}, β_2, and $\alpha_2\delta$ in COS cells, a Ca^{2+} channel complex is obtained that had ligand binding affinity and allosteric regulation identical to those in cardiac membranes. The density of dihydropyridine receptors obtained in this transient expression system was also close to that in cardiac membranes. Our results suggest that transient expression of Ca^{2+} channels in COS cells provides a useful model for studying the functional, particularly the pharmacological, properties of Ca^{2+} channels and the underlying structural basis for Ca^{2+} channel function.

To date six α_1 genes have been cloned as recently reviewed by Perez-Reyes and Schneider (40). Attempts to correlate these cloned α_1 subunits with endogenous Ca^{2+} channel types have relied on the expression of these clones followed by electrophysiological and pharmacological characterization. Discrepancies between the potency of ω-agatoxin-IVa and ω-conotoxin-MVIIC to block P-type currents in cerebellar Purkinje neurons and α_{1A}-induced currents have led to the suggestion that α_{1A} does not encode the P-type current (41). The present study indicates a vital role of subunit composition in determining the pharmacological properties of a cloned channel. Numerous studies have also shown that subunit composition can alter the biophysical properties, such as kinetics and voltage-dependence, of cloned channels. Therefore caution must be taken in interpreting apparent differences in the pharmacology and electrophysiology between cloned channels and their *in vivo* counterparts.

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