

Normalization of current kinetics by interaction between the α_1 and β subunits of the skeletal muscle dihydropyridine-sensitive Ca^{2+} channel

Antonio E. Lacerda*, Haeyoung S. Kim†, Peter Ruth‡§,
 Edward Perez-Reyes*, Veit Flockerzi‡,
 Franz Hofmann‡§, Lutz Birnbaumer*†||
 & Arthur M. Brown*

Departments of *Molecular Physiology & Biophysics and
 † Cell Biology and the ||Division of Neuroscience,
 Baylor College of Medicine, Houston, Texas 77030, USA
 ‡ Institute of Physiological Chemistry, Faculty of Medicine,
 University of Saarland, D-6650 Homburg/Saar, Saarland, Germany
 § Present address: Institute of Pharmacology, Technical University of
 Munich, D-8000 Munich, Germany

PURIFICATION of skeletal muscle dihydropyridine binding sites has enabled protein complexes to be isolated from which Ca^{2+} currents have been reconstituted. Complementary DNAs encoding the five subunits of the dihydropyridine receptor, α_1 , β , γ , α_2 and δ (ref. 1), have been cloned²⁻⁶ and it is now recognized that α_2 and δ are derived from a common precursor^{7,8}. The α_1 subunit can itself produce Ca^{2+} currents, as was demonstrated using mouse L cells lacking $\alpha_2\delta$ (refs 9, 10), β (ref. 10) and γ (our unpublished results). In L cells, stable expression of skeletal muscle α_1 alone was sufficient to generate voltage-sensitive, high-threshold L-type Ca^{2+} channel currents which were dihydropyridine-sensitive and blocked by Cd^{2+} , but the activation kinetics were about 100 times slower than expected for skeletal muscle Ca^{2+} channel currents. This could have been due to the cell type in which α_1 was being expressed or to the lack of a regulatory component particularly one of the subunits that copurifies with α_1 . We show here that coexpression of skeletal muscle β with skeletal muscle α_1

generates cell lines expressing Ca^{2+} channel currents with normal activation kinetics as evidence for the participation of the dihydropyridine-receptor β subunits in the generation of skeletal muscle Ca^{2+} channel currents.

We previously described the construction of the LCa.11 cell line that resulted from transfecting pDHPR- α_1 into L(tk⁻) cells^{9,10}. pDHPR- α_1 is an expression vector based on pEV142 in which the dihydropyridine receptor (DHPR) α_1 subunit cDNA is under the control of a metallothionein promoter. These cells expressed DHP binding and Ca^{2+} currents but the binding and current density were low. To increase expression we prepared new cell lines by transfecting murine L(tk⁻) cells with pKNH-DHPR- α_1 as before⁹. The expression construct was engineered by subcloning the rabbit skeletal muscle DHPR α_1 subunit^{2,8} into the HindIII site of the pKNH expression vector, in which the inserted cDNA is under the control of the simian virus 40 (SV40) enhancer/promoter (for details, see ref. 11). G418-resistant cells, designated LCaN- α_1 cells, were selected and 49 clones screened initially for L-type Ca^{2+} channel currents in the presence of 0.5 μ M Bay K8644.

Ca^{2+} channel currents in LCaN- α_1 cells were similar to those obtained in LCa.11 cells⁹. The currents were unaffected by changes in the holding potential between -50 and -90 mV, were stimulated by Bay K 8644, blocked by cadmium or cobalt and clearly belonged to the high-threshold or L category. Unfortunately the current densities remained low and were not different from those obtained with LCa.11 cells (Table 1). Thirty-three per cent (48/145) of the LCaN- α_1 cells from 49 clones having input resistances of about 10⁹ ohm gave rise to detectable tail currents in the presence of 0.5 μ M Bay K8644; 52% (25/48) of these cells gave inward currents >5pA. This level was selected because it was more than twice the maximum inward current produced by errors associated with our linear correction procedure in control L cells. Just as for LCa.11 cells, the rate of current activation was very slow (Table 2), indicating that the slowness was not the result of induction of the metallothionein promoter with Zn²⁺ or the type of expression vector used previously. Activation time constants of Ca^{2+} channel currents in LCa.11 and LCaN- α_1 cells measured at test potentials of +30 or +40 mV were about 738 and 552 milliseconds, respectively

FIG. 1 a, Expression of α_1 mRNA in recipient LCaN- α_1 (clone 162) cells and of α_1 and β mRNAs in LCaN- α_1 derived cell lines after cotransfection with DHPR β in p91023(B) (p9-DHPR- β) and herpes simplex virus thymidine kinase gene in pHSV-106 (HSV-tk). The pKNH (ref. 11) and p91023(B) (ref. 12) vectors were gifts from S. Numa and R. J. Kaufman, respectively. Ten cell lines resistant to 400 μ g ml⁻¹ G418 (selective pressure for conservation of α_1 DNA sequences) and able to grow in HAT medium (selection for expression of thymidine kinase and coexpression of β) were cloned, examined and tested for the presence of α_1 and β mRNA. Six out of the ten G418- and HAT-resistant cell lines expressed both DHPR components. Probes α_1 and β were labelled and blots were treated as described¹⁰. BC3H1 is a mouse cell line with a skeletal muscle phenotype. Migration positions of 28S and 18S ribosomal RNA markers are indicated. b, Specific DHP binding to membranes from cells expressing skeletal muscle DHPR α_1 before and after transfection with DHPR β . The cell lines were the same as analysed for α_1 and β mRNA in a. Top, specific binding at 0.32 nM [³H]-(+)-PN 200-110 to different LCaN- α_1 transfectants. Non-specific binding was determined with 10 μ M unlabelled nitrendipine. Bottom, Scatchard analysis of DHP-binding sites in cell membranes of LCaN- α_1 clone 162 (K_d = 0.14 nM; open circles) and in cell membranes of one of its transfected DHPR β positive derivatives, LCaN- $\alpha_1\beta$ clone 4 (K_d = 0.23 nM; filled circles).

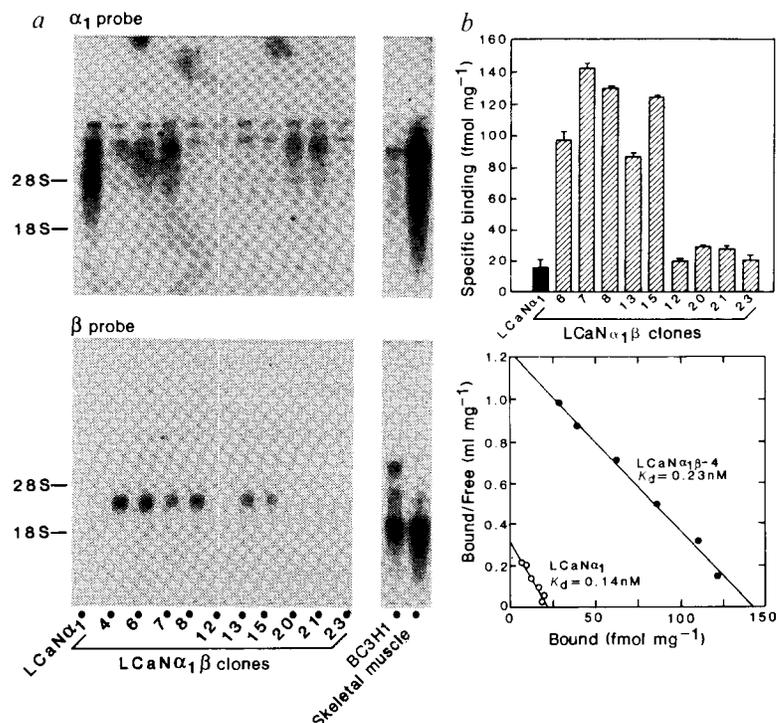


TABLE 1 DHP binding and current density characteristics of murine L cells transfected with the α_1 and β components of the rabbit skeletal muscle DHP receptor complex

Cell line	Component(s) expressed	Expression vector	Selection	DHP binding ([³ H]PN200-110)*		Current density†
				K_d (nM)	B_{max} (fmol mg ⁻¹)	
LCa.11	α_1	pEV-DHPR. α_1	HAT (tk ⁺)	0.38	61	0.73 ± 0.72 (22)
LCaN- α_1	α_1	pKNH-DHPR. α_1	G418	0.16	21	0.63 ± 0.45 (25)
LCaN- $\alpha_1\beta$ clone 13	$\alpha_1\beta$	+p9-DHPR. β	G418 plus HAT	0.17	145	0.74 ± 0.31 (7)
LCaN- $\alpha_1\beta$ clone 4	$\alpha_1\beta$	+p9-DHPR. β	G418 plus HAT	0.23	160	0.14 (1)
LCaN- $\alpha_1\beta$ clone 7	$\alpha_1\beta$	+p9-DHPR. β	G418 plus HAT	0.22	123	1.41 (1)‡

* Average from two separate Scatchard analysis with numbers agreeing within 10% of the mean. For LCaN- α_1 , binding was performed on membranes from clone 162. The total number of binding sites (B_{max}) of LCa.11 differs from that of clone 162 at a significance level of $P < 0.001$.

† Average of densities exhibited by cells with net inward currents at +20 to +40 mV (0.5 μ M Bay K8644) of >5 pA, using 115 mM Ba²⁺ as the charge carrier. For LCaN- α_1 , current density measurements are from 1 to 2 cells each of 13 independent cell clones, including clone 162.

‡ Current density (pA/pF ± s.d. (cells measured)) was recorded using Ba²⁺ as charge carrier at 40 mM instead of 115 mM.

(Table 2). LCaN- α_1 clone 162, which expressed about one third as many specific DHP binding sites as the LCa.11 cell (Table 1), seemed to produce currents most frequently (Fig. 2a) and was used for further transfection studies.

For expression of the rabbit skeletal muscle DHPR- β subunit, its cDNA⁴ was subcloned into the *Eco*RI site of p91023(B) (ref. 12) in which the DHPR- β cDNA is under the control of the adenovirus major late promoter. The plasmid was designated p9-DHPR. β and was transfected with the herpes simplex virus thymidine kinase gene^{9,13} into clone 162 cells. Cells potentially expressing both DHPR α_1 and β messenger RNAs were designated LCaN- $\alpha_1\beta$ cells or $\alpha_1\beta$ cells.

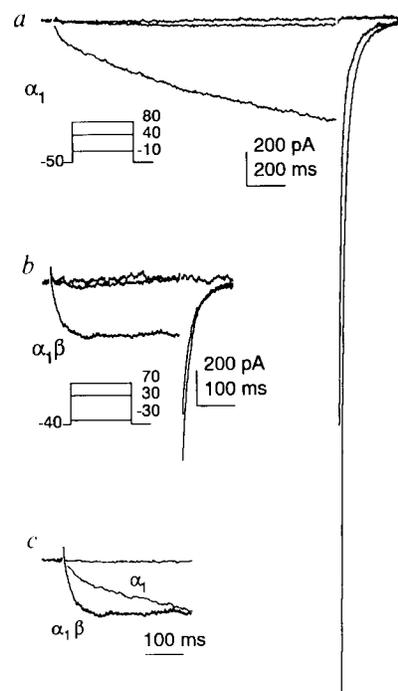
Transformants expressing thymidine kinase and neomycin resistance were selected by growing cells in hypoxanthine/aminopterin/thymidine (HAT) medium with G418 and were cloned using standard techniques. Individual cell clones

were then tested for DHPR- β expression in three assays: northern blot hybridization analysis to detect DHPR β mRNA, specific binding of DHP to their membranes to measure possible effects on the DHP receptor activity of α_1 , and whole-cell patch-clamp measurements¹⁴ to test for possible effects of β on the Ca²⁺ currents produced by α_1 .

Northern blot hybridization of total RNA derived from ten independent neomycin-resistant, thymidine kinase-positive clones revealed that six of the clones were expressing both α_1 and β (Fig. 1a). Coincident with expression of DHPR β mRNA, binding assessed with 0.32 nM [³H](+)-PN200-110, a dihydropyridine Ca²⁺ channel antagonist, was increased by a factor of four to five (Fig. 1b). Scatchard analysis of DHP binding to assess the kinetic basis of this effect revealed that DHPR β increases the maximal number of detectable binding sites without altering their affinity.

FIG. 2 Comparison of Ca²⁺ currents of an α_1 cell (a, LCaN- α_1 clone 162) and from an $\alpha_1\beta$ cell (b, LCaN- $\alpha_1\beta$ clone 7). Note change in timescale between α_1 (a, 200 ms) and $\alpha_1\beta$ (b, 100 ms) records. For both cells the currents were produced by voltage steps to the potentials indicated in the voltage protocol. The potentials correspond to potentials at threshold, maximum inward current and strong depolarization. Currents for α_1 were obtained with the 115 mM Ba²⁺ bath solution and for $\alpha_1\beta$ with 40 mM Ba²⁺ solution. The lower record (c) shows on the same timescale currents from the α_1 cell elicited by potential steps to -10 and 40 mV superimposed on the current from the $\alpha_1\beta$ cell at a step potential of 30 mV. Current amplitudes were normalized to the current at the end of the step from the $\alpha_1\beta$ cell.

METHODS. Cells were grown under standard conditions in 35-mm Falcon Petri dishes on 10-mm square glass coverslips coated with poly-D-lysine in α MEM supplemented with 10% fetal calf serum in the presence of HAT and/or 400 μ g ml⁻¹ G418 as indicated. Seeding was at 5,000 to 10,000 cells per ml and recordings were obtained 3 days afterwards. In the case of LCa.11 cells, 100 μ M ZnSO₄ was added 15 h before use. Coverslips with attached cells to be patched were broken into fragments and the fragments kept at room temperature in Tyrode's solution until used (<1 h). The coverslip fragments were then placed into a static bath containing 0.5 ml of bath solution (composition in mM: BaCl₂ 115, HEPES 10, EGTA 1, pH 7.4, adjusted with *N*-methyl-D-glucamine (NMG)) with 0.5 μ M racemic Bay K 8644. In a few cases a Cl⁻-free bath solution was used (composition in mM: BaOH 40, tetraethylammonium 30, 4-aminopyridine 5, HEPES 10, pH 7.4, adjusted with methanesulphonic acid containing 0.5 μ M racemic Bay K 8644). This did not significantly reduce leak currents and its use was discontinued. Coverslip fragments were discarded after 15 min in bath solution. The pipette filling solution was usually (in mM): HEPES 150, EGTA 20, NMG 110, MgCl₂ 2, pH 7.3, adjusted with NMG. In some experiments a pipette-filling solution of composition (in mM): NMG 130, EGTA 15, BAPTA 5, HEPES 10, MgCl₂ 11.5, CaCl₂ 8, Na₂ATP 3, Na₂GTP 0.1, pH 7.3, adjusted with methanesulphonic acid, was used. There was no consistent difference between currents obtained with these solutions and results have been pooled. Whole-cell patch clamp recordings¹⁴, were performed with a LIST EPC-7 amplifier. Patch clamp electrodes were made from 8161 glass tubing (Garner Glass) and had resistances of 2 M Ω or less. Series resistance error was reduced by adjusting the series resistance compensation circuit of the EPC-7 to just



below the point of oscillation. Whole-cell patch-clamp current data were analysed with pCLAMP software and hardware (Axon Instruments) installed in an IBM-compatible computer. Data were recorded at various digitization rates and filtered with an eight-pole Bessel filter at 0.2 times the digitization frequency, except when data were digitized at two frequencies in the same record. Cell capacitance was estimated by applying symmetric linear positive and negative potential ramps under voltage clamp and measuring the capacitive current produced by the ramps.

Stable coexpression of the β component of relative molecular mass 55,000 (M_r 55K) of the rabbit skeletal muscle DHP binding complex resulted in a dramatic increase in the rate of activation of α_1 -mediated currents produced by voltage clamp depolarization (Fig. 2) in three $\alpha_1\beta$ clones selected for electrophysiological studies on the basis of DHP binding (Table 1). Thus, we observed in all three of the selected $\alpha_1\beta$ clones that depolarizations from holding potentials between -50 and -40 mV to test potentials between 10 and 60 mV produced activation rates that were about 100 times faster than those for recipient clone 162, in which α_1 alone expressed Ca^{2+} currents. Despite the large difference in their absolute values, the voltage-dependence of the two sets of rate constants was similar. Deactivation rates measured at the holding potentials were also significantly increased but only by a factor of two (Table 2), far less than the differences observed at depolarized potentials.

We also found slowly activating currents in clone 23 of the LCaN- $\alpha_1\beta$ series. This cell line had undergone the same transfection, selection and cloning procedures as clones 4, 7 and 13, but did not express detectable DHPR β mRNA (Fig. 1). This result makes it unlikely that the accelerated response to depolarization was due to insertional mutagenesis or to non-specific activation of the murine β gene. Using a monoclonal antibody raised against purified rabbit DHPR- β (P.R. and F.H., unpublished results), we found that all clones positive for β mRNA were also positive for expression of an immunoreactive DHPR β protein with the expected apparent M_r of 55K when electrophoresed on polyacrylamide gels (data not shown).

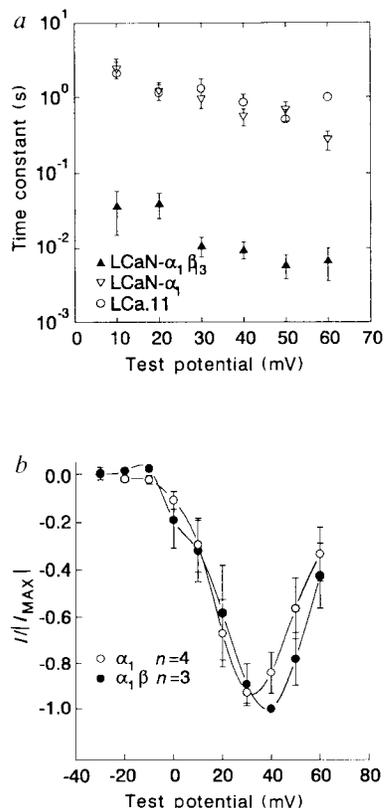


FIG. 3 *a*, Activation time constants for α_1 and $\alpha_1\beta$ currents compared at test potentials between 10 and 60 mV. Activation was fitted to a monoexponential function using nonlinear least-squares optimization. Data were from LCa.11, LCaN- α_1 and LCaN- $\alpha_1\beta$ clone 13. Values at each point are averages from 2–5 cells (LCa.11), 5–11 cells (LCaN- α_1) and 2–4 cells (LCaN- $\alpha_1\beta$). *b*, Comparison of peak current–voltage relationships for α_1 and $\alpha_1\beta$ currents. Peak currents in each cell were normalized by the magnitude of the maximum inward current recorded in the cell. For α_1 currents the potential at which the peak current occurred varied slightly. Both sets of data were measured in 115 mM Ba^{2+} .

TABLE 2 Activation and deactivation times (τ) of murine L cells transfected with the α_1 and β components of the rabbit skeletal muscle DHP receptor complex

Cell line	Deactivation τ (-50 mV) (ms \pm s.d.)	Activation τ (40 mV) (ms \pm s.d.)
LCa.11	59 ± 14 ($n=5$)	738 ± 266 ($n=5$)
LCaN- α_1 *	63 ± 9 ($n=5$)	552 ± 431 ($n=10$)
LCaN- $\alpha_1\beta$, clone 13	18 ± 4 ($n=9$)	8 ± 1 ($n=5$)
LCaN- $\alpha_1\beta$, clone 4	24 ± 7 ($n=5$, -40 mV)	5
LCaN- $\alpha_1\beta$ †, clone 7	25 ± 3 ($n=2$)	13 ± 1 ($n=2$)

* Kinetic measurements are from 1 to 2 cells, each of 9 independent cell clones, including clone 162.

† Kinetic measurements were obtained using Ba^{2+} as charge carrier at 40 mM instead of 115 mM.

thermore, both the recipient clone 162 cell and transfected cell clones that were negative for β mRNA expression were also negative for expression of immunoreactive material.

Although the normalization of activation produced by $\alpha_1\beta$ is clear, the $\alpha_1\beta$ currents were detected less frequently. Gigaseals were more difficult to obtain, and of those that were successful only 28% (32/121) had detectable tail currents. Of this group, only 9/32 showed inward currents of 5 pA or more, even in the presence of Bay K8644 at 0.5 μ M. For this reason the results for α_1 and $\alpha_1\beta$ were all obtained using the same concentration of Bay K8644. Under the same conditions, steady-state activation of the coexpressed $\alpha_1\beta$ currents was unchanged from that of the currents expressed by α_1 alone. In two experiments, the potential dependence of steady-state activation was compared between α_1 and $\alpha_1\beta$ using tail current amplitude measurements. The Boltzmann fits to the normalized data had slope factors of 5.2 and 7.0, indicating no change in voltage sensitivity. More compelling was a comparison of the peak current–voltage relationships for four α_1 cells and three $\alpha_1\beta$ cells. The normalized peak current–voltage relationships were similar in the two cases (Fig. 3*b*). As the peak currents correlate with steady-state activation in these non-inactivating currents, we conclude that activation gating is the same for α_1 and $\alpha_1\beta$ cells.

On the basis of the almost 100-fold increase in the rate of activation following its coexpression with α_1 , we propose that the β subunit of the DHPR combines with α_1 as a true subunit of the skeletal muscle Ca^{2+} channel. Such a large effect is unlikely to be due to the presence of Bay K8644. This compound is known to enhance activation and inactivation kinetics of Ca^{2+} currents, possibly by shifting gating to more negative potentials. It also slows deactivation¹⁵ and produces a similar panoply of effects on skeletal muscle Ca^{2+} currents^{16,17}. These effects are much smaller, however, and cannot explain the very large differences in activation rates. Moreover, the β effect on the rate of activation was selective, being much larger at depolarized potentials. As in this case, the deactivation rate for Ca^{2+} currents measured at potentials below threshold is faster than the activation rates measured at depolarized potentials (Table 2). The deactivation rate is mainly voltage-independent¹⁸, which is consistent with the idea that the final closed–open transition in voltage-sensitive Na^+ , K^+ and Ca^{2+} channels is voltage-independent. Thus one possibility is that the β subunit has an unusually pronounced effect on voltage-dependent transitions among earlier closed states of the channel; perhaps this is related to the lower current densities produced in the $\alpha_1\beta$ cells.

At present we cannot explain the discrepancy between the increased DHP binding capacity of $\alpha_1\beta$ cells and the lack of significant change in current densities. A similar discrepancy has been noted before for DHPR in skeletal muscle¹⁹. It may be that inactivation of channels formed by $\alpha_1\beta$ is increased, although 30-s hyperpolarizing prepulses to -100 mV did not

affect test current amplitudes. These prepulses might not have been sufficient to correct an ultraslow inactivation process. Another factor could be a reduction in mean open time. It may also be that increased DHP binding occurs in cells where $\alpha_1\beta$ does not reach functional maturity as an ion channel, and hence that changes in DHP binding at the cell population level do not reflect the binding properties that exist in the individual cells from which Ca^{2+} channel currents are recorded.

Our experiments not only demonstrate the functional interaction between α_1 and β , but they also raise questions concerning the roles of γ and $\alpha_2\delta$ in Ca^{2+} channel function, and the extent to which these are tissue-specific. Several α_1 genes with varying tissue-specific expression, each giving rise to more than one alternatively spliced product, have been identified²⁰. Likewise, northern analysis indicates the existence of more than one form of the β subunit^{4,10}. No γ -like mRNA has been found in non-skeletal muscle tissues^{7,8} and $\alpha_2\delta$ is expressed in all tissues that express α_1 (ref. 3). The γ subunit may be a skeletal muscle-specific component responsible for the high levels of expression of DHP binding sites in this tissue. Also, Mikami *et al.*²¹ have found that co-injection of mRNA encoding cardiac α_1 with mRNA encoding skeletal muscle $\alpha_2\delta$ into *Xenopus* oocytes results in a roughly twofold increase in current densities over those obtained with α_1 mRNA alone. It will be of interest to see whether stable expression of all skeletal muscle DHP

components in L cells produces current densities much larger than have so far been obtained with α_1 or with α_1 plus β . □

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