Recovery of Ca^{2+} Current, Charge Movements, and Ca^{2+} Transients in Myotubes Deficient in Dihydropyridine Receptor β_1 Subunit Transfected with β_1 cDNA

Maryline Beurg,* Manana Sukhareva,* Caroline Strube,* Patricia A. Powers,# Ronald G. Gregg,# and Roberto Coronado*

*Department of Physiology, University of Wisconsin School of Medicine, and #Waisman Center, University of Wisconsin, Madison, Wisconsin 53706 USA

ABSTRACT The Ca^{2+} currents, charge movements, and intracellular Ca^{2+} transients of mouse dihydropyridine receptor (DHPR) β_1-null myotubes expressing a mouse DHPR β_1 cDNA have been characterized. In β_1-null myotubes maintained in culture for 10–15 days, the density of the L-type current was ~7-fold lower than in normal cells of the same age (I_{max} was 0.65 ± 0.05 pA/pF in mutant versus 4.5 ± 0.8 pA/pF in normal), activation of the L-type current was significantly faster (τ activation at +40 mV was 28 ± 7 ms in mutant versus 57 ± 8 ms in normal), charge movements were ~2.5-fold lower (Q_{max} was 2.5 ± 0.2 nC/μF in mutant versus 6.3 ± 0.7 nC/μF in normal), Ca^{2+} transients were not elicited by depolarization, and spontaneous or evoked contractions were absent. Transfection of β_1-null cells by lipofection with β_1 cDNA reestablished spontaneous or evoked contractions in ~10% of cells after 6 days and ~30% of cells after 13 days. In contracting β_1-transfected myotubes there was a complete recovery of the L-type current density (I_{max} was 4 ± 0.9 pA/pF), the kinetics of activation (τ activation at +40 mV was 64 ± 5 ms), the magnitude of charge movements (Q_{max} was 6.7 ± 0.4 nC/μF), and the amplitude and voltage dependence of Ca^{2+} transients evoked by depolarizations. Ca^{2+} transients of transfected cells were unaltered by the removal of external Ca^{2+} or by the block of the L-type Ca^{2+} current, demonstrating that a skeletal-type excitation-contraction coupling was restored. The recovery of the normal skeletal muscle phenotype in β_1-transfected β-null myotubes shows that the β_1 subunit is essential for the functional expression of the DHPR complex.

INTRODUCTION

Excitation-contraction (EC) coupling is the process whereby muscle cell depolarization increases the Ca^{2+} permeability of the sarcoplasmic reticulum (SR) resulting in a transient increase in myoplasmic Ca^{2+}. Two molecular complexes involved in this transduction are the dihydropyridine receptor (DHPR), which functions as a voltage sensor in the transverse tubular membrane, and the ryanodine receptor (RyR), which releases Ca^{2+} from the SR. The DHPR of skeletal muscle is composed of α_{1S}, β_1, α_2/δ, and γ subunits. The α_1 subunit contains the binding site for the DHPs, the Ca^{2+} pore, and the voltage sensor (Hofmann et al., 1994). EC coupling is thought to be controlled by tetrads of four DHPRs juxtaposed to tetrads of four RyRs (Flucher and Franzini-Armstrong, 1996).

The β subunit of the DHPR is an ~55 to 65-kDa cytoplasmic protein which interacts with the α_1 subunit (Pragnell et al., 1994). β subunits are encoded by four different genes and several splice variants have been described. The predominant isoform of adult skeletal muscle is β_{1a}, herein called β_1 (Ruth et al., 1989; Pragnell et al., 1991). The properties of L-type Ca^{2+} channels determined by the interaction of β and α_1 subunits have been investigated in heterologous systems. When β_1 and α_{1S} were expressed in L-cells, the activation kinetics of the coexpressed current was faster than that found in clonal cell lines expressing α_1 alone (Lacerda et al., 1991; Varadi et al., 1991; Lory et al., 1992). Furthermore, a higher DHP binding site density was consistently observed in L-cells expressing both subunits (Lacerda et al., 1991; Varadi et al., 1991). In contrast, increases in Ca^{2+} current density or shifts in voltage dependence were not observed in all cloned cell lines expressing α_{1S} and β_1 subunits. Coexpression of the cardiac isoform α_{1C} and β_1 either stably in L-cells (Lory et al., 1993), or transiently in oocytes (Singer et al., 1991; Wei et al., 1991), CHO cells (Nishimura et al., 1993) and HEK cells (Perez-Garcia et al., 1995; Kamp et al., 1996) invariably resulted in Ca^{2+} currents with densities much higher than those of control cells expressing α_{1C} alone. These results show that DHPR β subunits contribute to fundamental electrophysiological properties of Ca^{2+} channels.

The participation of the α_1 subunit in EC coupling has been investigated in dysgenic myotubes that lack the α_{1S} subunit and also lack EC coupling (Tanabe et al., 1988; Powell et al., 1996; Flucher and Franzini-Armstrong, 1996). Expression of α_{1S} restores the L-type Ca^{2+} current and the electrically evoked cell contraction. EC coupling persists in the absence of external Ca^{2+}, consistent with the properties of EC coupling in normal skeletal muscle. These results and others involving expression of α_{1C} have solidified the view that α_1 determines to a large extent the type of EC coupling expressed in the muscle cell (Adams et al., 1990; Tanabe et al., 1990).

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Address reprint requests to R. Coronado, Dept. of Physiology, University of Wisconsin, 1300 University Ave., Madison, WI 53706. Tel.: 608-263-7487; Fax: 608-265-5512; E-mail: blmlab@vms2.macc.wisc.edu.

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The involvement of the β subunit in EC coupling has been examined in β_1-null cells from knock-out mice carrying a null mutation in the β_1 gene (Gregg et al., 1996; Strube et al., 1996). Intercostal β_1-null myotubes, like dysgenic myotubes, fail to contract in response to electrical stimulation despite the presence of normal action potentials, SR Ca^{2+} storage and caffeine-induced SR Ca^{2+} release capacities. Strube et al. (1996) showed that β_1-null cells have a low density of charge movements and do not generate Ca^{2+} transients in response to depolarization. Evidently, β_1-null cells fail to transduce depolarization into SR Ca^{2+} release due either to the low density of voltage sensors or to the specific absence of β_1 from the voltage sensor. Gregg et al. (1996) suggested that the β_1 subunit may have additional roles related to the targeting of α_{1S} subunits to the transverse tubular membrane, since β_1-null cells are deficient in α_{1S} subunits, whereas dysgenic cells are not deficient in β_1 subunits.

In the present study, we investigated EC coupling in β_1-null cells after transfection with a cDNA encoding the missing β_1 subunit. Expression of β_1 in β_1-null myotubes results in a quantitative recovery of the Ca^{2+} current density seen in normal cells of the same age, the intramembrane charge movement density, the amplitude and voltage dependence of intracellular Ca^{2+} transients, and an extracellular-Ca^{2+}-independent skeletal-type EC coupling within 4 to 13 days following transfection. Part of these results appeared in abstract form (Beurg et al., 1997).

MATERIALS AND METHODS

Primary cultures of mouse myotubes

Primary cultures were prepared from hindlimbs of 18-day-old mouse fetuses as described elsewhere, with some changes (Takekura et al., 1994). Homozygotes (*cchb1*$^{-/-}$) for the β_1-null mutation, hereafter called β_1-null or mutant, were recognized as described (Gregg et al., 1996). Controls, hereafter called normals, were either heterozygotes (*cchb1*$^{+/-}$) or wild type (+/+). Dissected muscles were incubated for 9 min at 37°C in Ca^{2+}/Mg^{2+}-free Hanks balanced salt solution (136.9 mM NaCl, 3 mM KCl, 0.44 mM KH_2PO_4, 0.34 mM $NaHPO_4$, 4.2 mM $NaHCO_3$, 5.5 mM glucose, pH 7.2) containing 0.25% (w/v) trypsin and 0.05% (w/v) pancreatin (Sigma, St. Louis, MO). After centrifugation, mononucleated cells were resuspended in plating medium containing 78% Dulbecco's modified Eagle's medium with low glucose (DMEM, Gibco BRL, Gaithersburg, MD), 10% horse serum (HS, Sigma), 10% fetal bovine serum (FBS, Sigma), 2% chicken embryo extract (CEE, Gibco) and plated on plastic culture dishes coated with gelatin at a density of $\sim 1 \times 10^4$ cells per dish. Cultures were grown at 37°C in 8% CO_2. After the fusion of myoblasts (7 days), the medium was replaced with a FBS-free medium (88.75% DMEM, 10% horse serum, 1.25% CEE) and cells were incubated in 5% CO_2. All media contained 0.1% v/v penicillin and streptomycin (Sigma).

Transfection of cultured myoblasts

β_1-null cells were transfected with an expression plasmid encoding the mouse β_{1a} subunit of the DHPR. A full-length mouse β_{1a} cDNA was subcloned into the pSG5 expression plasmid (Stratagene Inc., La Jolla, CA) containing the early simian virus-40 (SV40) promoter. Transfections were performed by lipid-mediated DNA transfer using a mixture of DOTAP (*N*-[1-(2,3-dioleoyloxy)propyl]-*N,N,N*-trimethylammonium methyl sulfate) and DOPE (dioleoyl phosphatidylethanolamine) provided by the Vector Core Laboratory at the Waisman Center, University of Wisconsin. Cells were exposed for 2–3 h to a transfection solution prepared by mixing DOTAP and DNA in a ratio of 20 μg lipid to 5 μg plasmid DNA per each 35-mm plate of cells.

Ca^{2+} current and charge movements

Whole-cell recordings were performed as described previously (Strube et al., 1996). We used an Axopatch 1D amplifier and a headstage with a 50 MΩ feedback resistor (Axon Instruments, Foster City, CA). Linear capacitance and leak currents were compensated with an analog circuit. Effective series resistance was compensated up to the point of amplifier oscillation with the Axopatch circuit. All experiments were performed at room temperature. The external solution was (in mM) 130 TEA (tetraethylammonium) methanesulfonate, 10 $CaCl_2$, 1 $MgCl_2$, 10^{-3} TTX (tetrodotoxin), and 10 HEPES titrated with TEA(OH) to pH 7.4. The pipette solution consisted of (in mM) 140 Cs aspartate, 5 $MgCl_2$, 0.1 EGTA (when Ca^{2+} transients were recorded) or 5 EGTA (all other recordings), and 10 MOPS titrated with CsOH to pH 7.2. Patch pipettes had a resistance of 2–5 MΩ when filled with the pipette solution. For recordings of charge movement, the external solution was supplemented with 0.5 mM $CdCl_2$ and 0.1 mM $LaCl_3$ to block the ionic Ca^{2+} currents. A prepulse protocol similar to that described by Adams et al. (1990) was used to measure the immobilization-resistant component of charge movement. This protocol was the same as protocol B of Strube et al. (1996). Voltage was first stepped from holding potential -80 mV to -20 mV for 1 s, then to -50 mV for 5 ms, then to test potential P for 25 ms, then to -50 mV for 30 ms and finally to the -80 mV holding potential. Subtraction of linear components was assisted by a P/4 procedure following the test pulse. P/4 pulses were in the negative direction, had a duration of 25 ms, and were separated by 500 ms. Currents were sampled at 200–250 μs per point, filtered with a low-pass Bessel filter at 2 KHz, and analyzed with pClamp (Axon Instruments) and Sigmaplot-Jandel (San Rafael, CA) softwares.

Intracellular Ca^{2+} transients measurement

Intracellular Ca^{2+} was monitored with the fluorescent Ca^{2+} indicator fluo-3 (Molecular Probes, Eugene, OR) in a setup described previously (Strube et al., 1996). We used an inverted microscope equipped with a monochromator for probe excitation and photomultiplier tube for epifluorescent emission detection. Cells were loaded with 1 μM fluo-3 AM (Molecular Probes) for 20–30 min at room temperature. Fluorescence excitation was set at 488 nm. A dichroic mirror centered at 505 nm and an emission barrier filter centered at 535 nm were used to separate excitation from emission. During voltage-clamp protocols, the emission sampling frequency was 200 Hz. Background fluorescence from a field without cells was subtracted from the sampled emission.

External stimulation

The cell culture media was replaced with Krebs solution consisting (in mM) of NaCl (136), KCl (5), $CaCl_2$ (2), $MgCl_2$ (1), HEPES (10), and titrated with NaOH to pH 7.4. Cells were placed between two platinum wires separated at the tip by a distance of ~ 100 μm. A single test pulse of fixed duration (5 ms) and intensity (>20 V) was applied with a Grass stimulator (Model S48, W. Warwick, RI). Cell movement was visualized under phase contrast at $\sim 100\times$ magnification. To establish the yield of rescued cells in dishes of β_1-transfected β_1-null cells, we determined the voltage threshold for observing a twitch in cultures of normal cells run in parallel. The test voltage for dishes of transfected cells was set $\sim 10\%$ above the threshold estimated for normal cells.

TABLE 1 Contractile response of normal, β_1-null, and β_1-transfected myotubes

	Cell Culture (Days)				
	8–9	10–11	12–13	14–15	16–17
Normal					
Contracting cells/tested cells	81/149	157/284	278/410	165/185	20/25
% Contracting cells	54.3	55.3	67.8	89.2	80
Total cultures	3	6	7	5	1
β_1-null					
Contracting cells/tested cells	0/240	0/200	0/460	0/210	0/90
% Contracting cells	0	0	0	0	0
Total cultures	6	7	11	4	2
β_1-transfected					
Contracting cells/tested cells	17/218	26/280	101/472	63/234	21/75
% Contracting cells	7.8	9.3	21.4	26.9	28
Total cultures	6	7	11	4	2

Cell contraction was confirmed by visual inspection. Transfection was performed on day 3 or 4 of cell culture. Each cell culture was prepared from a separate litter of mouse embryos.

Chemicals

Deionized glass-distilled water was used in all solutions. All salts were reagent grade. Nifedipine and Bay K 8644 were made as 10-mM and 5-mM stocks in absolute ethanol. Nifedipine and TTX were from Sigma. Bay K 8644 was from Calbiochem (La Jolla, CA).

Curve fitting

For each cell, the voltage dependence of charge movements (Q), Ca^{2+} conductance (G), and peak intracellular Ca^{2+} (F/Fo) was fitted according to a Boltzmann equation, $A = A_{max}/\{1 + \exp[-(V_. - V_{½})/k]\}$ as described previously (Strube et al., 1996). A_{max} was either Q_{max}, G_{max}, or $F/Fo_{(max)}$,

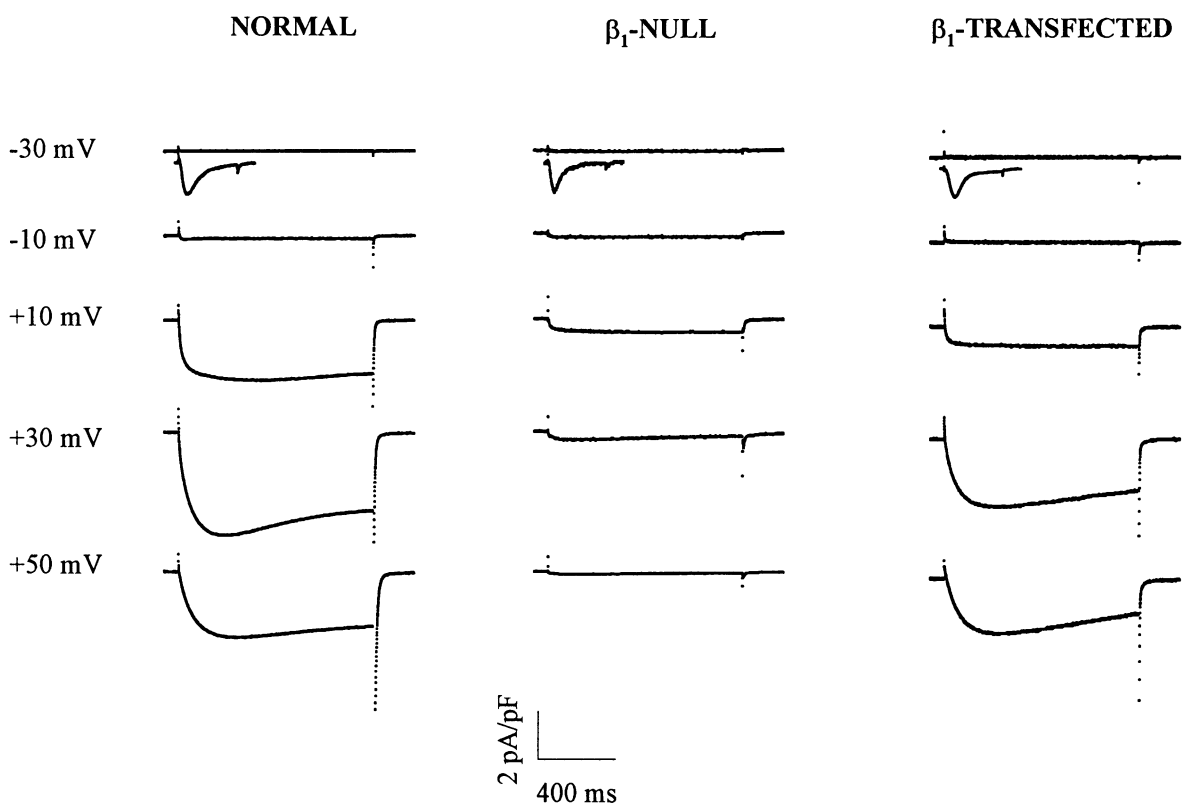

FIGURE 1 Ca^{2+} currents from a normal, β_1-null and β_1-transfected myotube in response to a 1-s depolarizing voltage step from a holding potential of −40 mV to the indicated potential. The lower trace at −30 mV shows the Ca^{2+} current in the same cell in response to a 300-ms voltage step from a holding potential of −80 mV. Cell capacitance was 540, 395, and 323 pF for the normal, β_1-null, and β_1-transfected cells, respectively.

$V_{½}$ is the potential at which $A = A_{max}/2$, and k is the slope factor. The time constant τ_1, describing activation of Ca^{2+} current, was obtained from a fit of the pulse current at each voltage according to $I(t) = K[1 - (\exp -t/\tau_1)] \exp -t/\tau_2$, where K is constant and τ_2 describes inactivation. All averages are presented as mean ± SE.

RESULTS

Previous observations showed that neither muscles (Gregg et al., 1996) nor single myotubes (Strube et al., 1996) from end-gestation β_1-null mice were able to contract in response to external electrical stimulation or during depolarizing potentials under whole-cell clamp. The reappearance of the electrically evoked twitch was therefore conveniently used to identify individual cells in dishes of β_1-transfected cells that were successfully rescued. Table 1 shows the percentage of myotubes in a culture that transiently contract in response to external stimulation. As expected, the number of normal cells responding to the stimulation increased with the age of the culture, reflecting the stage of maturation of the myotubes. After a week of culture, contractions were seen in ~50% of normal myotubes. After 14 or 15 days, ~90% of normal cells responded to the stimulation. Similar results have been described by others (Grouselle et al., 1991; Romey et al., 1989). In β_1-null myotubes, contraction was not observed at any stage of cell culture, indicating that the missing subunit is essential for normal myotube function. In cells transfected with the β_1 cDNA, there was recovery of EC coupling beginning at day 8 or 9 of cell culture (3 or 4 days following lipofection). In young transfected cells, mechanical activity was often slow and localized. The percentage of contracting cells increased with the age of cultures and reached between 21 and 28% after 2 weeks. In older transfected cells, twitches were usually fast and global. In 2-week-old cultures, the percentage of mutant cells rescued by the transfection, relative to the activity of normal cells of the same age, was ~30% (26.9/89.2). All cells identified below as β_1-transfected were selected on the basis of their ability to contract in response to external stimulation.

Fig. 1 shows whole-cell Ca^{2+} currents in response to 1-s depolarizing test pulses from a holding potential of −40 mV in a normal, β_1-null, and β_1-transfected myotube at day 12 to 15 of culture. The charge carrier was 10 mM Ca^{2+} throughout this work. In all whole-cell recordings of normal (50 cells) and rescued (29 cells) myotubes, we consistently observed a high-voltage-activated sustained Ca^{2+} current, typical of skeletal muscle previously identified in normal myotubes in culture as the L-type Ca^{2+} current (Cognard et al., 1986; Beam and Knudson, 1988). The sustained Ca^{2+} current of β_1-null myotubes was considerably smaller than that of normal cells (54 of 69 cells) and in some cells, this current was undetectable (15 of 69 cells). The low density of the sustained Ca^{2+} current in β_1-null myotubes agreed with previous results (Strube et al., 1996). When the holding potential was −80 mV, we observed in all cases a low-voltage-activated T-type Ca^{2+} current. These are shown in Fig. 1 in response to a shorter test pulse (300 ms) to −30 mV. Robust T-type Ca^{2+} currents were seen in ~40% of normal, 70% of β_1-null, and 50% of transfected cells. In order to compare cells from different cultures, we investigated the extent to which the Ca^{2+} current density varied with cell age. Fig. 2 shows Ca^{2+} current densities of normal, β_1-null, and β_1-transfected myotubes kept in culture for up to 16 days. On average, the maximal current density remained unchanged after 8 days of cell culture. Based on these results, the data from cells kept in culture between 8 to 16 days were pooled and averaged.

Fig. 3 *A* shows Ca^{2+} current-voltage relationships in normal, β_1-null, and β_1-transfected myotubes. Measurements correspond to the peak current and current at the end of the 300-ms pulse from a holding potential of −80 mV. In all cell types, the T-type current activated at ~−40 mV and had a maximum density at −20 mV, and the L-type current

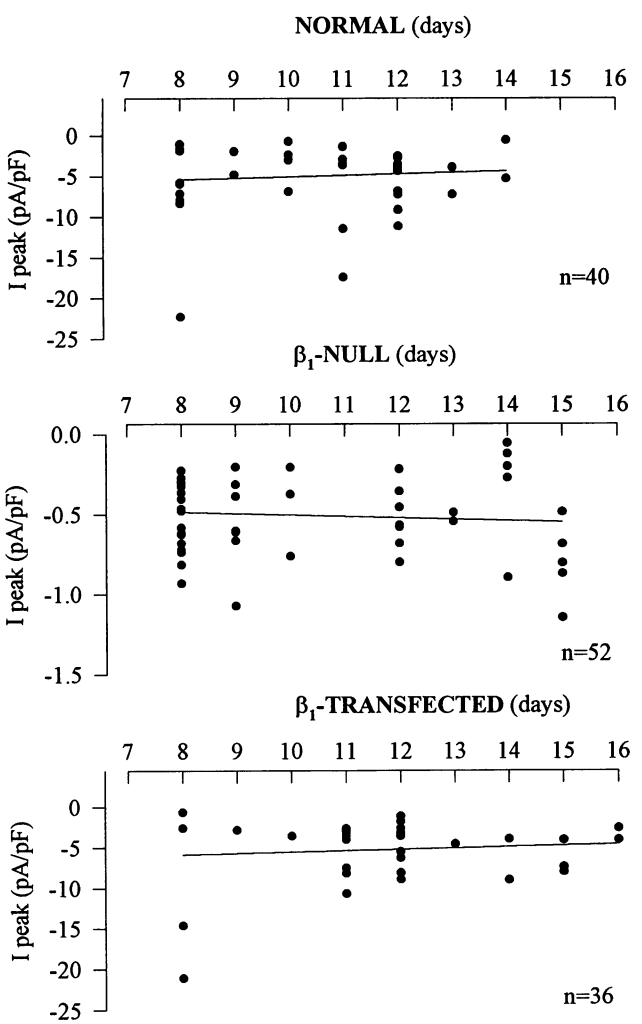

FIGURE 2 Maximum density of Ca^{2+} current at different days in cell culture. Current was measured at +20 mV from a holding potential of −40 mV in the indicated number of cells, each represented by a single dot. A linear regression of the maximum Ca^{2+} current density is shown by the line.

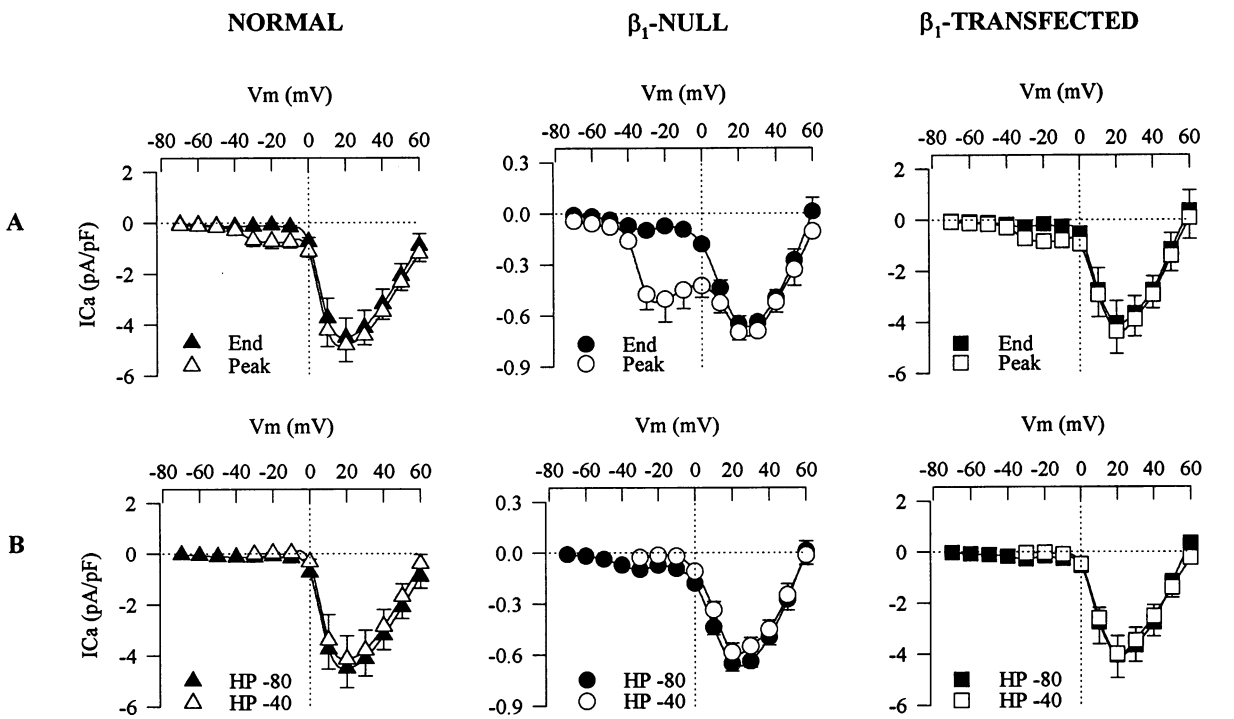

FIGURE 3 Voltage dependence of the average Ca^{2+} current in normal (13 cells), β_1-null (12 cells), and β_1-transfected (12 cells) myotubes. Curves in (*A*) are in response to voltage steps of 300 ms from a holding potential of −80 mV for the peak Ca^{2+} current and the current at the end of the pulse. Curves in (*B*) are for the same voltage step from a holding potential of −80 mV or −40 mV for the current at the end of the pulse. The maximum Ca^{2+} current density in (*B*) is 4.5 ± 0.7 pA/pF in normal, 0.65 ± 0.05 pA/pF in mutant myotubes, and 4.0 ± 0.9 pA/pF in β_1-transfected cells.

activated at ~−10 mV and had a maximum density at +20 mV. Normal and rescued cells had T-type current densities lower than those of β_1-null cells. This reflected the lower percentage of normal and rescued cells with measurable T-type currents. Fig. 3 *B* shows the voltage dependence of the end-pulse current from a holding potential −80 mV or −40 mV. The sustained L-type Ca^{2+} current was invariant with holding potential, indicating there was no contamination of the end-pulse current with T-type Ca^{2+} current. Consequently, the current at the end of a 300-ms pulse was used to estimate the density of the sustained Ca^{2+} current in normal, β_1-null, and β_1-transfected cells. Normal myotubes had a large end-pulse Ca^{2+} current with a maximum density of 4.5 ± 0.7 pA/pF (13 cells) at +20 mV. In β_1-null cells the density was 0.65 ± 0.05 pA/pF (12 cells), a reduction of ~7-fold. In β_1-transfected cells, the end-pulse Ca^{2+} current recovered to a maximum density of 4 ± 0.9 pA/pF (12 cells). This value was statistically indistinguishable from that of normal cells (unpaired *t*-test, $p < 0.05$). The reduction in end-pulse current of β_1-null cells was significantly less than the ~13-fold reduction seen in myotubes from end-gestation β_1-null embryos (Strube et al., 1996). Fig. 4 shows Ca^{2+} conductance-voltage (*G-V*) curves computed by extrapolation of currents to the reversal potential. The lines correspond to a Boltzmann fit to the population average. The fitted *G-V* curve of β_1-transfected cells was similar to that of normal cells, whereas that of β_1-null cells was less steep and was shifted ~9 mV toward positive potentials. Averages of Boltzmann parameters fitted to each cell separately are shown in Table 2. From these results we conclude that the functional expression of the β_1 subunit in

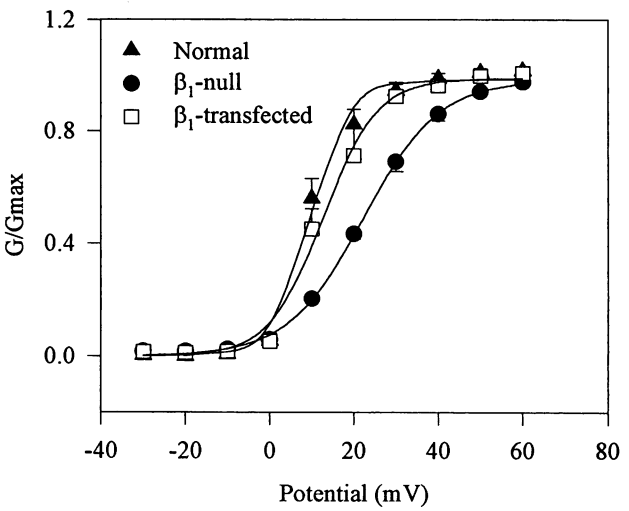

FIGURE 4 Voltage dependence of the average Ca^{2+} conductance in normal (13 cells), β_1-null (12 cells), and β_1-transfected myotubes (12 cells). Conductance was normalized according to the mean maximum conductance (G_{max}) of each group of cells. Curves correspond to a Boltzmann fit of the population mean *G-V* curve. Parameters of the fit were $V_{½}$ = 11.0 mV, 20.3 mV, and 11.1 mV; k = 4.1 mV, 8 mV, and 4.71 mV for normal, β_1-null, and β_1-transfected myotubes, respectively.

TABLE 2 Boltzmann parameters of Ca^{2+} conductance, charge movements, and Ca^{2+} transients in normal, β_1-null, and β_1-transfected myotubes

	G-V			*Q-V*			*F/Fo-V*		
	G_{max} (pS/pF)	$V_{1/2}$ (mV)	k (mV)	Q_{max} (nC/μF)	$V_{1/2}$ (mV)	k (mV)	$F/Fo_{(max)}$	$V_{1/2}$ (mV)	k (mV)
Normal	98.2 ± 13.2 (13)	11 ± 2.4	4.1 ± 0.6	6.3 ± 0.7 (10)	3.8 ± 3	13.7 ± 0.8	1.5 ± 0.1 (14)	−2.6 ± 1.8	5 ± 0.6
β_1-null	22 ± 3.6 (12)	20.3 ± 1.3	8 ± 0.7	2.5 ± 0.2 (13)	−12.3 ± 4.2	11.4 ± 1	—	—	—
β_1-transfected	109.4 ± 7.8 (12)	11.1 ± 1.8	4.7 ± 0.6	6.7 ± 0.4 (8)	1.9 ± 4.5	13 ± 0.7	1.4 ± 0.1 (17)	0.7 ± 1.6	6.3 ± 0.8

Boltzmann parameters G_{max}, $V_{1/2}$, and k (mean ± SE) were fitted to the *G-V*, *Q-V*, and *F/Fo-V* curves of each cell. The number of cells is shown in parentheses.

β_1-null cells is sufficient to rescue the density and the voltage dependence of the L-type Ca^{2+} current.

We verified that the rescued Ca^{2+} current had the pharmacological characteristics of an L-type current by determining the increase in end-pulse current when cells were exposed to the DHP agonist Bay K 8644. Fig. 5 shows that 5 μM Bay K8644 produced a characteristic increase in maximum Ca^{2+} current and a moderate negative shift of the *I-V* curve in the three cell types. The percentage of stimulation of the end-pulse current measured at the peak of the *I-V* curve was 36 ± 11% (6 cells) in normal cells, 38 ± 14% (6 cells) in rescued cells, and 135 ± 21% in β_1-null cells (13 cells). The stimulation of the rescued Ca^{2+} current by Bay K 8644 and the inhibition by 5 μM nifedipine (data not shown) showed that the Ca^{2+} current of β_1-transfected cells was sensitive to DHPs to the same extent as the normal L-type Ca^{2+} current.

To further verify that the L-type Ca^{2+} current in normal and transfected myotubes is identical, we compared the kinetics of activation. We used test pulses of 1 s from a holding potential of −40 mV to fit the entire activation phase of the L-type current and to suppress the T-type current if present. Fig. 6 *A* shows scaled traces of L-type current at +20 mV in normal, β_1-null, and β_1-transfected myotubes. The Ca^{2+} current activated much slower in the normal and β_1-transfected myotubes than in the β_1-null myotube. Furthermore, inactivation was clearly present in the normal and transfected cell but not in the β_1-null cell. The pulse current was fit in each case with $I(t)$, described in Materials and Methods. This equation was derived from a linear kinetic scheme consisting of three states: closed, open, and inactive. A fit of $I(t)$ to the pulse current is shown by the continuous curve superimposed on the digitized data. In all cell types, there was a good agreement between the fit and the data, indicating that the three-state model was sufficient to represent the L-type Ca^{2+} current in the range of potentials analyzed. Fig. 6 *B* shows the time constant of activation of the L-type Ca^{2+} current obtained from the fit of $I(t)$ in the range of 0 mV to +40 mV. The time constant of activation of the β_1-null current was significantly smaller than that of normal or β_1-transfected Ca^{2+} cells at the same potential (unpaired *t*-test, $p < 0.05$). Furthermore, the acti-

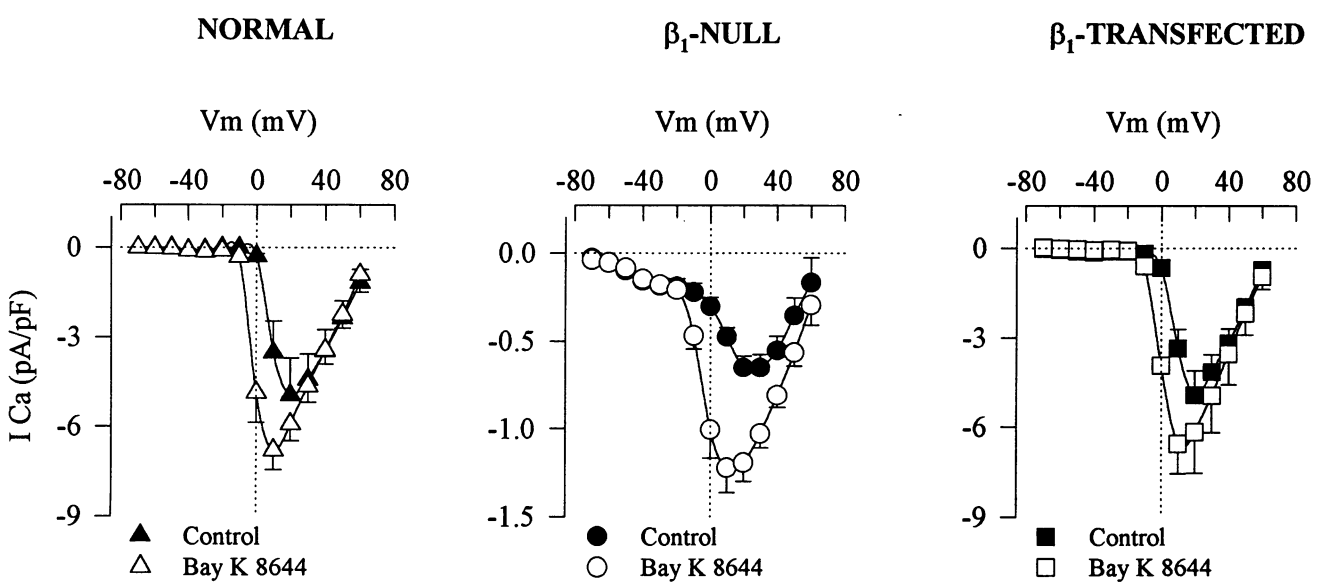

FIGURE 5 The stimulation of the average Ca^{2+} current by Bay K 8644 is shown in normal (6 cells), β_1-null (13 cells), and β_1-transfected (6 cells) myotubes. Ca^{2+} current was measured at the end of a voltage step of 300-ms during a control period and 10 min after addition of 5 μM Bay K 8644.

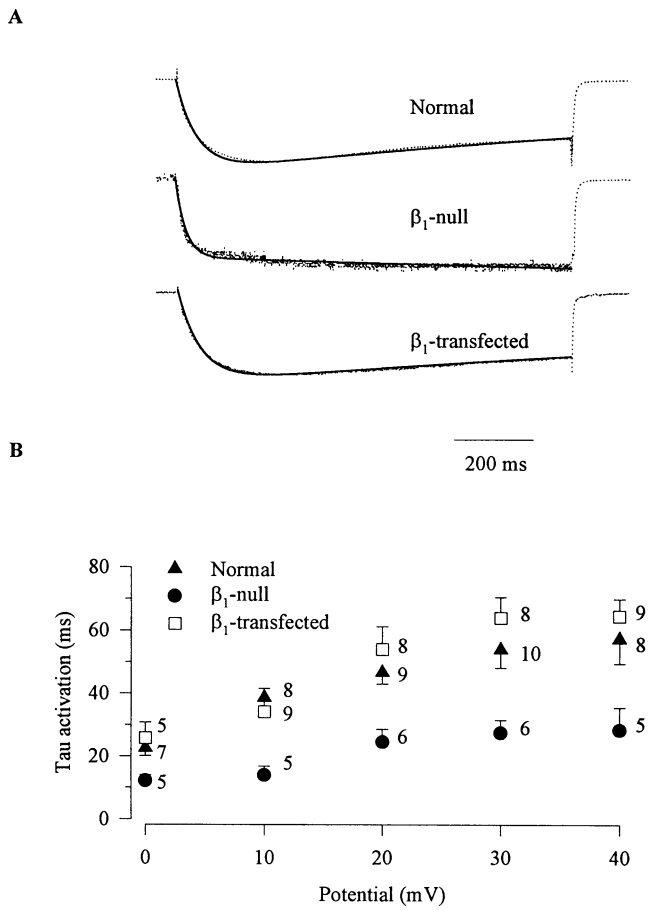

FIGURE 6 Scaled traces of Ca^{2+} current in response to a 1-s depolarizing voltage step from a holding potential of −40 mV to +20 mV are shown in (*A*). Curves correspond to a fit of the current at +20 mV using the equation $I(t) = K[1 - (\exp -t/\tau_1)] \exp -t/\tau_2$ with parameters $K = -0.95$, $\tau_1 = 52.8$ ms, $\tau_2 = 2068$ ms for normal; $K = -0.83$, $\tau_1 = 25.7$ ms, $\tau_2 = 8230$ ms for β_1-null; and $K = -1.1$, $\tau_1 = 57.3$ ms, $\tau_2 = 2742$ ms for β_1-transfected myotubes. (*B*) shows the time constant of activation of the Ca^{2+} current obtained from a fit of $I(t)$ for the indicated number of cells in response to a 1-s depolarization voltage step in the range of 0 mV to +40 mV from a holding potential of −40 mV.

vation time constants of normal and β_1-transfected myotubes at the same potential were not significantly different. We also compared the inactivation time constant, τ_2, in normal and transfected cells at a test potential of +20 mV (not shown). The inactivation time constant was 4.6 ± 1.5 s for normal myotubes (7 cells) and 4.5 ± 1.3 s for β_1-transfected myotubes (6 cells). These results demonstrated that the kinetics of the L-type current in normal and rescued cells were indistinguishable.

The recovery of the L-type Ca^{2+} current in β_1-transfected myotubes was paralleled by a similar recovery of charge movements. Fig. 7 *A* shows recordings of nonlinear charge movements in normal, β_1-null, and β_1-transfected myotubes. In normal cells, the onset of ON and OFF components of charge movements occurred at ~−40 mV and increased with voltage until a plateau was reached at potentials more positive than +40 mV. In β_1-null cells, the amplitude of the charge movement was severely depressed at all positive potentials, in agreement with previous results (Strube et al., 1996). In β_1-transfected cells, the amplitude of charge movements was similar to that of normal cells. In Fig. 7 *B*, Q_{max} was calculated by integration of the ON component and was plotted as a function of test potential. The Q_{max} of normal myotubes was ~6.5 nC/μF, in agreement with previous determinations (Garcia et al., 1994; Strube et al., 1996). The population Q_{max} of β_1-transfected myotubes was the same as that of normal cells and, furthermore, the averages of Boltzmann parameters fitted separately to each cell were indistinguishable (Table 2).

To establish if charge movements in rescued cells were significant for EC coupling, we investigated the voltage dependence of intracellular Ca^{2+} transients. Fig. 8 shows Ca^{2+} transients monitored by fluo-3 fluorescence in cells stimulated by a 50-ms test pulse from a holding potential of −40 mV. The fluorescence intensity ratio *F/Fo* corresponds to the cell fluorescence normalized according to the resting fluorescence. In normal myotubes, Ca^{2+} transients were produced by depolarizations more positive than −10 mV. The onset of the Ca^{2+} transient was simultaneous with the onset of the pulse, which is indicated by P in the traces of fluorescence. The amplitude of the Ca^{2+} transient increased with the pulse potential and reached a plateau at potentials more positive than +10 mV. As shown in the middle panel, no Ca^{2+} transients were produced in the β_1-null cell. Increasing the duration of the pulse to 500 ms also failed to produce a Ca^{2+} transient (not shown). In β_1-transfected cells, Ca^{2+} transients had the same threshold as in normal cells, the onset was fast, and the amplitude at each voltage was within the normal range. For the test pulse shown in Fig. 8 to +30 mV, the time to the peak of the Ca^{2+} transient was ~70 ms in normal and transfected cells. The decay time constants at the same potential were fit to a single exponential and were 297 ms and 310 ms, respectively. Such values are in agreement with studies in normal rat and mouse myotubes (Grouselle et al., 1991; Garcia and Beam, 1994). The right panel of Fig. 8 shows Ca^{2+} currents in the same β_1-transfected cell simultaneous with the cell fluorescence. A decrease in the Ca^{2+} current was obvious at +50 and +70 mV. However, no decline was observed in the amplitude of the Ca^{2+} transient at these potentials. The same observation was made in normal cells (not shown). The voltage dependence of the fluorescence ratio *F/Fo* measured at the peak of the Ca^{2+} transient is shown in Fig. 9 *A* for normal and β_1-transfected cells. The curves correspond to a fit of the population average *F/Fo-V* curve according to a Boltzmann equation. In both cell types, *F/Fo* increased in a sigmoidal manner, reaching a maximum at depolarizations more positive than +30 mV. In β_1-transfected cells, F/Fo_{max} was slightly lower than in normal cells (Table 2), but this difference was not statistically significant. Averages of Boltzmann parameters fitted separately to each cell are shown in Table 2. These results are in agreement with previous studies using fluo-3 (Garcia et al., 1994). For the

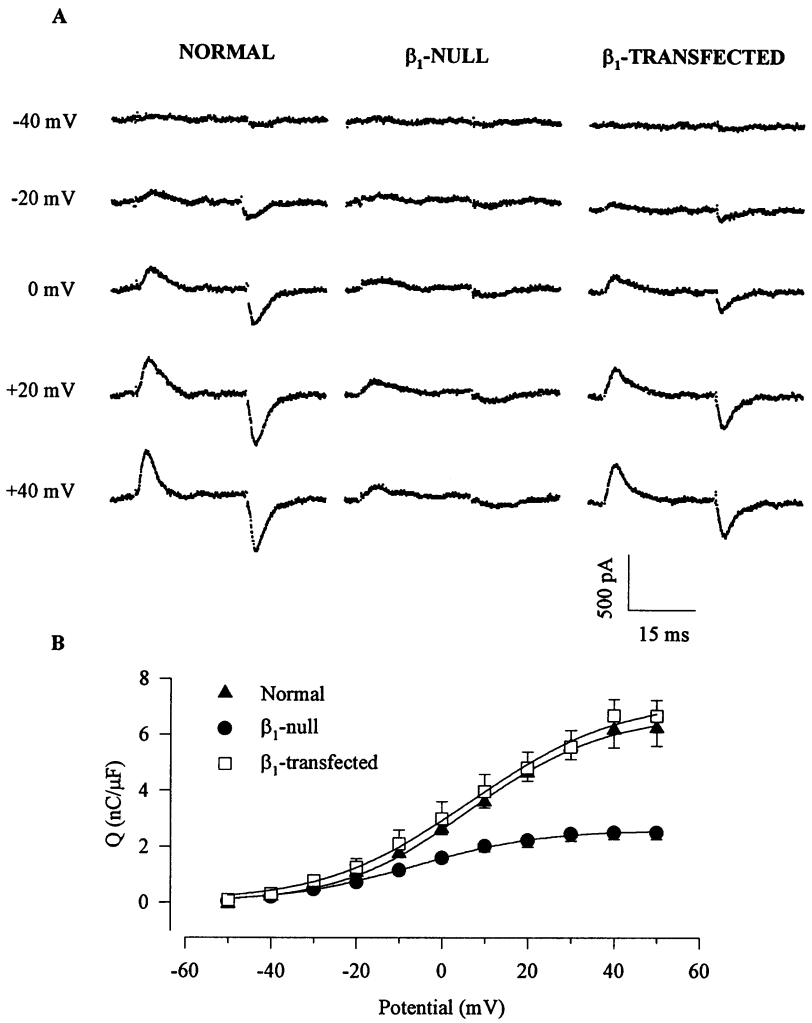

FIGURE 7 Asymmetric currents produced by intramembrane charge movement are shown (*A*) in a normal, β_1-null, and β_1-transfected myotubes. Currents are during the test potential P of protocol B. Cell capacitance was 288, 312, and 348 pF for the normal, β_1-null, and β_1-transfected cells, respectively. The voltage dependence of the average charge movements are shown (*B*) in normal (10 cells), β_1-null (13 cells), and β_1-transfected (8 cells) myotubes. Curves correspond to a Boltzmann fit of the population mean *Q-V* curve. Parameters of the fit were Q_{max} = 6.3, 2.5, and 6.7 nC/μF; $V_{1/2}$ = 3.8, −12.3, and 1.9 mV; k = 13.7, 11.4, and 13 mV for normal, β_1-null, and β_1-transfected, respectively.

same cells, Fig. 9 *B* shows the voltage dependence of the Ca^{2+} current measured at the end of the 50-ms pulse used to stimulate the Ca^{2+} transient, and Fig. 9 *C* shows the voltage dependence of the total Ca^{2+} entry during the 50-ms pulse. Ca^{2+} currents and Ca^{2+} entry decreased to almost undetectable levels for the test depolarization to +70 mV. However, the amplitude of the Ca^{2+} transient at this potential was maximum. This observation confirmed that Ca^{2+} transients in β_1-transfected cells, like those in normal cells, are entirely controlled by voltage without participation of the Ca^{2+} current.

In principle, a component of the intracellular Ca^{2+} transient triggered by Ca^{2+} entry into the cell could have been inactivated or damaged when the whole-cell configuration was established. To rule out this possibility, we subjected cells to external stimulation without establishing a whole-cell patch. As shown in Fig. 10 *A*, removal of external 2 mM Ca^{2+} from the same normal or transfected myotube did not alter the intracellular Ca^{2+} transient. Controls indicated that Ca^{2+} transients were sensitive to TTX thus resulting from cell depolarization (not shown). Often, the amplitude of Ca^{2+} transients evoked in cells by external stimulation were larger than those evoked in cells by depolarizations under voltage-clamp. This could be due to the cell size and degree of maturation, since for patch recording we preferred smaller cells, whereas the larger cells were easier to stimulate by external electrodes. Fig. 10 *B* shows Ca^{2+} transients in 2 mM external Ca^{2+} followed by addition of 0.1 mM $LaCl_3$ to the external solution to block the Ca^{2+} current. The blocker had no effect on the amplitude of the subsequent Ca^{2+} transient in either cell type. Controls indicated that 0.1 mM $LaCl_3$ entirely blocked Ca^{2+} currents from a holding potential of −80 mV or −40 mV (not shown). Furthermore, if a Ca^{2+} current persisted in the presence of 0.1 mM $LaCl_3$, the OFF component of the charge movement would have been contaminated by the presumed $LaCl_3$-resistant tail current. The ON and OFF components of charge movements recorded in the presence of $LaCl_3$ were always similar. Thus, Ca^{2+} entry under these conditions was extremely unlikely. Finally, Fig. 10 *C* shows that Ca^{2+} transients stimulated externally in both cell types resulted from a release of Ca^{2+} from stores sensitive to thapsigargin (TG), consistent with the identification of the Ca^{2+} stores as the sarcoplasmic reticulum. These data dem-

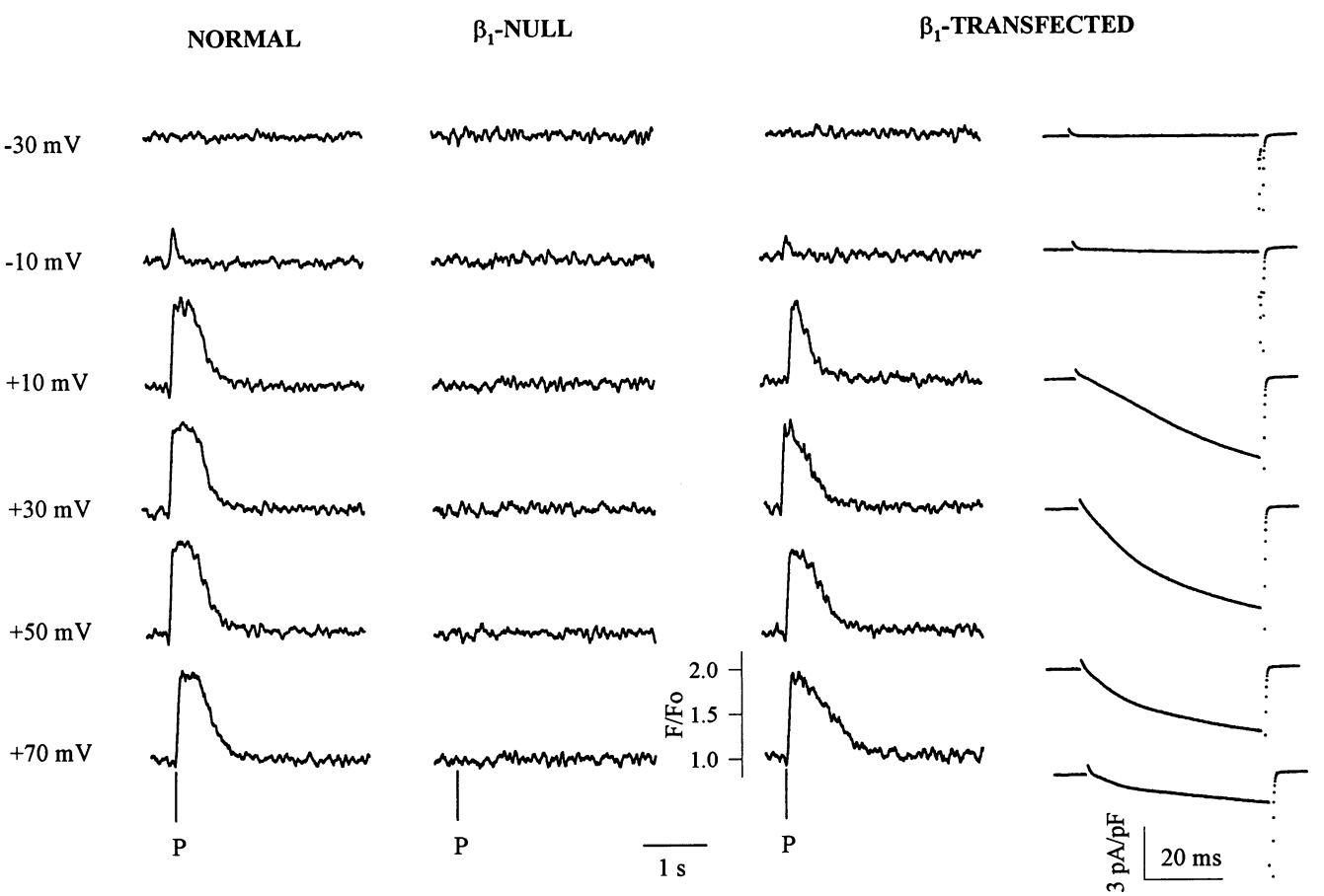

FIGURE 8 Intracellular Ca^{2+} transients under whole-cell clamp in normal, β_1-null, and β_1-transfected myotubes. Ca^{2+} currents are for the same β_1-transfected myotube. Ca^{2+} transients were elicited by voltage steps of 50 ms from a holding potential of −40 mV to the indicated potential. *P* indicates the onset of the voltage pulse. Fluorescence was normalized according to the fluorescence *Fo* immediately before the pulse.

onstrated that β_1-transfected cells displayed EC coupling typical of skeletal muscle cells.

DISCUSSION

The development of a skeletal muscle expression system for the β subunit of the DHPR is essential for understanding the participation of this subunit in muscle cell-specific functions such as EC coupling and transverse-tubule targeting of other DHPR subunits. Also, the mutant myotube may prove ideal for understanding the modulation of the L-type Ca^{2+} current by β subunits in a homologous expression system. The present results show that β_1-null cells cultured for up to 3 weeks maintain the EC uncoupled phenotype. Expression of β_1 in these cells resulted in a rapid and complete recovery of the normal phenotype in 3 to 4 days after transfection. The phenotype of the rescued cells was one expected of skeletal muscle in which contractions were observed in the absence of Ca^{2+} entry into the cell. Furthermore, the density of the rescued Ca^{2+} current and charge movements were indistinguishable from those of normal cells, strongly suggesting that functional DHPR complexes were expressed at normal levels in the rescued cells. Based on the contraction evoked by external stimulation, the yield of rescued cells was ~30%. This value may represent a lower limit because at early stages of normal myotube development in culture, the Ca^{2+} transient is present but the resultant mechanical activity is not obvious under microscopic observation (Grouselle et al., 1991). As a function of time in culture, the contractions observed in rescued cells involved increasing amounts of cell volume, beginning with highly localized twitches after 2 days of transfection. This pattern is similar to that described in rescued dysgenic myotubes (Courbin et al., 1989; Franzini-Armstrong et al., 1991) and may be due to the expression of β_1 by only a few nuclei in the rescued β_1-null cell. Since protein expression in the myoplasm of differentiating myotubes is controlled locally by each nucleus (Pavlath et al., 1989), the expression of β_1 and the assembly of Ca^{2+} release sites along the myotube may be heterogeneous.

Understanding how the β_1 subunit may modulate the functional expression of the L-type current in the β_1-null myotube requires that we establish whether the L-type current of β_1-null cells originates from DHPR complexes of α_{1S} without the β_1 subunit or from another α_1 subunit that could be expressed in the β_1-null cell. Strube et al. (1997)

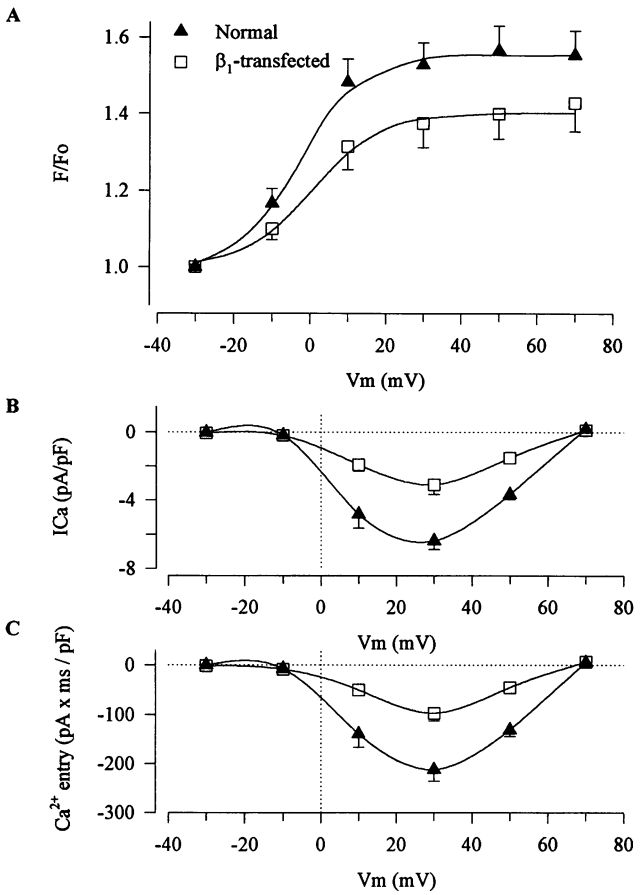

FIGURE 9 Voltage dependence of the Ca^{2+} transient and Ca^{2+} current in normal and β_1-transfected myotubes. (*A*) shows peak fluorescence increase during the Ca^{2+} transient as a function of the step potential in normal (14 cells) and β_1-transfected (17 cells) myotubes under whole-cell clamp. Ca^{2+} transients were elicited by voltage steps of 50 ms from a holding potential of −40 mV. Curves correspond to Boltzmann fit of the population mean peak fluorescence ratio. Parameters of the fit were F/Fo_{max} = 1.5 and 1.4; $V_{½}$ = −2.6 and 0.4 mV; k = 8.2 mV and 8.4 mV for normal and β_1-null, respectively. For the same cells, (*B*) shows the voltage dependence of the peak Ca^{2+} current, and (*C*) shows the integral of the Ca^{2+} current normalized according to the cell capacitance.

compared I_{dys}, the background L-type Ca^{2+} current of α_{1S}-deficient dysgenic skeletal muscle (Adams and Beam, 1989), and the Ca^{2+} current of β_1-null myotubes, herein called $I_{\beta null}$. In cultures of the same age, $I_{\beta null}$ and I_{dys} were expressed at similar densities. However, I_{dys} activated much faster and at more negative potentials than $I_{\beta null}$, and Bay K 8644 stimulated I_{dys} more strongly than $I_{\beta null}$. Also, when 10 mM Ca^{2+} was replaced by 10 mM Ba^{2+}, the Ba^{2+}/Ca^{2+} peak current ratio for I_{dys} was ~2 (Adams and Beam, 1989) whereas that for $I_{\beta null}$ is closer to 1 (unpublished results). These observations suggest that $I_{\beta null}$ and I_{dys} have different molecular compositions. $I_{\beta null}$ could originate from β-less Ca^{2+} channel complexes involving α_{1S} and possibly γ and α_2/δ subunits. However, our results do not discard the possibility that $I_{\beta null}$ may represent a mixture of I_{dys} and current from skeletal-type DHPR complexes without the β_1 subunit.

In the following, we compare our results to observations made previously in L-cells (Lacerda et al., 1991; Varadi et al., 1991; Lory et al., 1992) under the assumption that a skeletal-type DHPR complex without β_1 is responsible for the bulk of $I_{\beta null}$. Bay K8644 produced a strong stimulation of $I_{\beta null}$ and a much weaker stimulation of the rescued and normal Ca^{2+} currents. These observations would be consistent with results in L-cells showing that Bay K 8644 produced a stronger stimulation of the Ca^{2+} current of cells expressing α_{1S} alone than the Ca^{2+} current of cells expressing α_{1S} and β_1 (Varadi et al., 1991, Lory et al., 1992). On the other hand, expression in L-cells of the α_{1S} subunit without the β_1 subunit results in Ca^{2+} currents with activation kinetics slower than those of L-cells, expressing both α_{1S} and β_1 subunits (Lacerda et al., 1991; Varadi et al., 1991). In skeletal myotubes, the β_1-null Ca^{2+} current was found to be faster than the rescued Ca^{2+} current. Thus it appears that in our case the β_1 subunit slows rather than accelerates Ca^{2+} current activation. It may be possible that the slowing of the Ca^{2+} current produced by the β_1 subunit may be a manifestation of a muscle-specific event in which the β_1 subunit might play a permissive or a structural role. There are many known cases in which properties of expressed Ca^{2+} current varies depending on the expression system. For example, a moderate shift in the current-voltage curve of the coexpressed $\alpha_{1C}\beta_1$ Ca^{2+} current, amounting to ~10 mV in the negative direction, was observed in oocytes (Singer et al., 1991; Wei et al., 1991) but not in HEK cells (Perez-Garcia et al., 1995; Kamp et al., 1996). Negative shifts in the current-voltage curve of the Ca^{2+} current generated by the α_{1C} subunit have also been reported following coexpression with β_2, β_3, or β_4 subunits in oocytes (Perez-Reyes et al., 1992; Neely et al., 1993; Castellano et al., 1993). However, this was not the case when α_{1C} and β_3 were coexpressed in HEK cells (Josephson and Varadi, 1996). Taken together, these results suggest that different host cells may process Ca^{2+} channel subunits differently. Furthermore, β subunits may perform multiple functions, which are perhaps manifested differently, depending on the host cell.

The Q_{max} of normal cells measured in this study (6.3 ± 0.7 nC/μF) agreed with measurements in dissociated rat myotubes (Beam and Knudson, 1988) and dissociated or cultured mouse myotubes (Garcia et al., 1994; Strube et al., 1996). In β_1-null cells, the total charge movement was reduced ~2.5-fold. The lower density of charge movements in the mutant cell may be insufficient to initiate a transduction signal between the DHPR and the RyR channel, and could possibly be one factor determining the EC uncoupling. However, this may not be the only explanation because young normal myotubes with comparable charge movements often display Ca^{2+}-independent EC coupling (Strube et al., 1994). Transfection with β_1 cDNA restored the normal charge movement density and rescued the functional state of the voltage sensor. It is possible that the recovery of the L-type Ca^{2+} current and the recovery of EC coupling may both require the assembly of DHPRs into

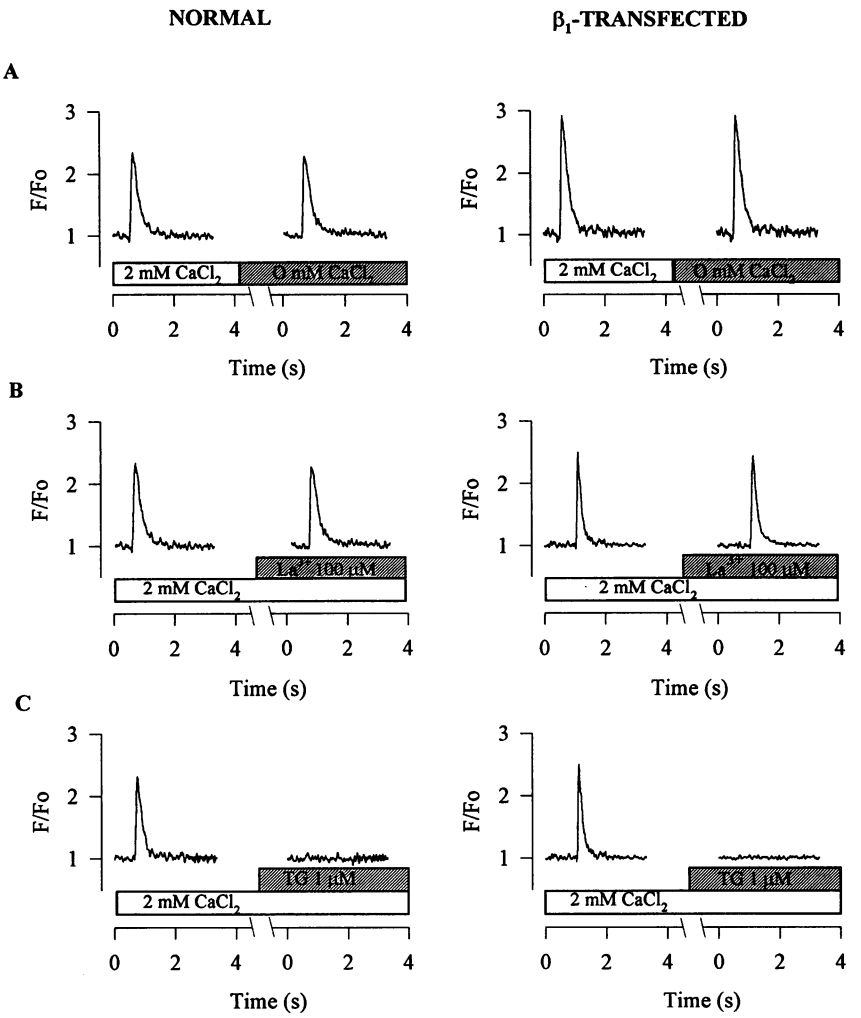

FIGURE 10 Ca^{2+} transients elicited by extracellular stimulation in normal and β_1-transfected myotubes. (*A*) shows the same myotube in Krebs solution and Krebs solution without $CaCl_2$. (*B*) shows a different myotube in Krebs solution before and after supplementation with 100 μM $LaCl_3$. (*C*) shows a different myotube in Krebs solution before and after supplementation with 1 μM TG.

tetrads and the establishment of functional interactions between DHPRs and RyRs. Nakai et al. (1996) showed in dyspedic skeletal muscle (RyR1-deficient), that Ca^{2+} currents and formation of tetrads are reduced. Thus, the β_1 subunit may be required for the assembly of DHPR complexes into tetrads or may be a critical component of the transduction itself. Both roles may be different from the proposed role of β subunits in the membrane targeting of the α_1 subunit (Chien et al., 1995).

Molecular interactions of DHPRs and RyRs result in local releases of Ca^{2+} or sparks from individual triads that have been measured by confocal microscopy (Tsugorka et al., 1995; Klein et al., 1996). Conklin et al. (1997) recently showed that Ca^{2+} sparks in β_1-null cells are kinetically different from those of normal cells. Sparks in mutant cells are significantly larger in diameter and last longer than their counterparts in normal cells. Thus, the β_1 subunit may be a critical element controlling the kinetics of Ca^{2+} sparks. The β_1 expression system now appears indispensable for testing numerous hypotheses regarding the functions proposed for the β subunit in assembly of DHPR-RyR complex and functional aspects of EC coupling.

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