A Novel Calcium Current in Dysgenic Skeletal Muscle

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ABSTRACT The whole-cell patch-clamp technique was used to study voltage-dependent calcium currents in primary cultures of myotubes and in freshly dissociated skeletal muscle from normal and dysgenic mice. In addition to the transient, dihydropyridine (DHP)-insensitive calcium current previously described, a maintained DHP-sensitive calcium current was found in dysgenic skeletal muscle. This current, here termed I_{Ca-dys}, is largest in acutely dissociated fetal or neonatal dysgenic muscle and also in dysgenic myotubes grown on a substrate of killed fibroblasts. In dysgenic myotubes grown on untreated plastic culture dishes, I_{Ca-dys} is usually so small that it cannot be detected. In addition, I_{Ca-dys} is apparently absent from normal skeletal muscle. From a holding potential of −80 mV, I_{Ca-dys} becomes apparent for test pulses to ∼−20 mV and peaks at ∼+20 mV. The current activates rapidly (rise time ∼5 ms at 20°C) and with 10 mM Ca as charge carrier inactivates little or not at all during a 200-ms test pulse. Thus, I_{Ca-dys} activates much faster than the slowly activating calcium current of normal skeletal muscle and does not display Ca-dependent inactivation like the cardiac L-type calcium current. Substituting Ba for Ca as the charge carrier doubles the size of I_{Ca-dys} without altering its kinetics. I_{Ca-dys} is ∼75% blocked by 100 nM (+)-PN 200-110 and is increased about threefold by 500 nM racemic Bay K 8644. The very high sensitivity of I_{Ca-dys} to these DHP compounds distinguishes it from neuronal L-type calcium current and from the calcium currents of normal skeletal muscle. I_{Ca-dys} may represent a calcium channel that is normally not expressed in skeletal muscle, or a mutated form of the skeletal muscle slow calcium channel.

INTRODUCTION

Vertebrate cell membranes contain a variety of voltage-gated calcium channels that are categorized according to voltage dependence, kinetics, and pharmacology (Bean, 1989). To date, skeletal muscle has been shown to express three distinct types of calcium currents. These are (*a*) a fast-activating, transient current that is insensitive to 1,4-dihydropyridine (DHP) derivatives (Beam et al., 1986; Cognard et al., 1986*a;* Beam and Knudson, 1988; Gonoi and Hasegawa, 1988); (*b*) a fast-activating, maintained current that is also DHP-insensitive (Cota and Stefani, 1986;

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Arreola et al., 1987; Garcia et al., 1988); and (*c*) a slowly-activating current that is DHP-sensitive (Donaldson and Beam, 1983; Beam et al., 1986; Cognard et al., 1986*a* and *b;* Beam and Knudson, 1988; Gonoi and Hasegawa, 1988). In this paper, we have adopted the following nomenclature for these different currents. The fast-activating, transient current is termed $I_{Ca\text{-}ft}$; this current is found in developing skeletal muscle, and resembles the T-type (Nowycky et al., 1985) calcium current reported for many different cell types (Bean, 1989). The fast-activating, maintained current is termed $I_{Ca\text{-}fm}$; this current has been described for frog and mammalian skeletal muscle, and does not fit easily into the T, L or N categories of Nowycky et al. (1985). The slowly-activating current is termed $I_{Ca\text{-}s}$; this current is a distinctive form of L-type calcium current characteristic of skeletal muscle.

$I_{Ca\text{-}s}$ is the dominant calcium current in normal skeletal muscle. However, in skeletal muscle from mice with muscular dysgenesis, $I_{Ca\text{-}s}$ is absent (Beam et al., 1986; Rieger et al., 1987) and excitation-contraction (E-C) coupling is nonfunctional (Powell and Fambrough, 1973; Klaus et al., 1983). Genetic analysis indicates that the muscular dysgenesis mutation alters the structural gene for the alpha_1 subunit of the skeletal muscle DHP receptor; injecting cDNA for this subunit into developing dysgenic myotubes restores both $I_{Ca\text{-}s}$ and E-C coupling (Tanabe et al., 1988). These results indicate that the alpha_1 subunit of the DHP receptor is a necessary component of both the slow calcium channel and the E-C coupling mechanism.

Grown under standard tissue culture conditions, myotubes from dysgenic mice express only a single prominent calcium current, $I_{Ca\text{-}ft}$ (Beam et al., 1986; Rieger et al., 1987). However, in dysgenic skeletal muscle that has developed in vivo or under special culture conditions, we have observed an additional calcium current. This current, which we term $I_{Ca\text{-}dys}$, is distinct, both kinetically and pharmacologically, from $I_{Ca\text{-}s}$ and also from the other calcium currents found in skeletal muscle ($I_{Ca\text{-}ft}$, $I_{Ca\text{-}fm}$). $I_{Ca\text{-}dys}$ does not appear to be present in normal skeletal muscle.

In this paper we describe the kinetics, voltage dependence, and pharmacology of $I_{Ca\text{-}dys}$ in dysgenic skeletal muscle. In addition, we compare the properties of $I_{Ca\text{-}dys}$ with those of other known calcium currents, especially those found in normal skeletal muscle. A preliminary account of some of the results presented here has appeared previously (Adams and Beam, 1989).

METHODS

Voltage Clamp

Calcium currents were recorded using the whole-cell variant of the patch-clamp technique (Hamill et al., 1981). Pipettes were fabricated from borosilicate glass and filled with an internal solution containing (millimolar) 140 Cs-aspartate, 5 $MgCl_2$, 10 Cs_2EGTA, 10 Hepes, pH 7.4 with CsOH. The resistance of the pipettes varied from 1.3 to 2.5 M-ohm. For each cell, the linear capacitative and leakage currents were measured for a depolarizing or hyperpolarizing control pulse of 10–20 mV from a holding potential of -80 mV. The area beneath the capacitative transient and the time constant of the transient's decay were used to calculate the cell's linear capacitance and the series resistance associated with the pipette (Matteson and Armstrong, 1984). Electronic compensation was used to reduce the effective series resistance, generally to a value <1.5 M-ohm. The control current described above was scaled appropriately and used to correct test currents for linear components of capacitative and leakage currents. Test pulses were separated by an interval of at least 5 s. To allow comparison of test currents recorded from different cells, the current recorded from each cell was normalized by that cell's linear capacitance (current expressed as picoamperes per picofarad). In many of the illustrated traces, the first several data points after a change in potential have been blanked. Data were filtered at 500 Hz and sampled at 1,000 Hz. Experiments were conducted at room temperature (20–22°C). Average numerical values are presented in the text and figures as mean ± SEM.

External Solutions

Calcium channel currents were recorded while the cells were bathed in an external solution containing (millimolar) 145 TEA-Cl, 10 $CaCl_2$ [or 10 $BaCl_2$], 10 Hepes, pH 7.4 with CsOH. In addition, all external solutions contained 2–10 μM TTX, as required, to block voltage-gated sodium channels.

Cell Culture

Primary cultures of myoblasts were prepared from skeletal muscles of late-term fetal or newborn mice. The muscles were minced and then incubated at 37°C for 40–60 min in Ca, Mg-free Ringer (155 mM NaCl, 5 mM KCl, 10 mM Hepes, pH 7.4 with NaOH) containing 0.25% (wt/vol) crude trypsin (1:250, Difco Laboratories Inc., Detroit, MI). After filtration and centrifugation to remove large debris, the cell suspension was plated at a density of 10^5 cells/dish onto 35-mm Primaria dishes (Becton Dickinson Labware, Lincoln Park, NJ) containing an attached layer of killed fibroblasts (see below). The plating medium contained (vol/vol) 80% Dulbecco's modified Eagle's medium with 4.5 g/liter glucose (DMEM) and 20% fetal bovine serum. After 3–5 d, the plating medium was replaced with maintenance medium: 90% DMEM and 10% horse serum. All culture media contained penicillin (100 U/ml) and streptomycin (100 μg/ml). Cultures were maintained at 37°C in a 95% air/5% CO_2, water-saturated atmosphere.

The substrate of killed fibroblasts was created by first growing fibroblasts to confluence on untreated culture dishes. The culture medium was then removed and the fibroblasts were exposed to deionized water for 1 min, followed by complete dessication under ultraviolet light for 15 min. The resultant dishes, containing a layer of lysed and dessicated fibroblasts, were then stored at 4°C until use. Fibroblasts from three different sources (NIH 3T3 cell line; skin fibroblasts from dysgenic mice and their nondysgenic littermates) were used for this purpose.

Acute Isolation of Muscle Cells

Single skeletal muscle fibers were dissociated from the plantar muscles (i.e., all the muscles on the sole of the hind foot) of fetal (day 15–20) or neonatal (1–2-d-old) mice following the procedure of Beam and Knudson (1988).

Application of Drugs

Concentrated (1 or 10 mM) stock solutions of the DHP compounds (+)-PN 200-110 (kindly provided by Dr. A. Lindenmann and Dr. E. Rossi of Sandoz Ltd., Basel, Switzerland) and racemic Bay K 8644 (kindly supplied by Dr. A. Scriabine, Miles Laboratories, Inc., New Haven, CT) were prepared by dissolving the drugs in 100% ethanol. The stock solutions were stored in the dark at −20°C. Test concentrations were prepared just before use by dilution with the external saline used for current measurement (final ethanol concentration, <0.01%). The microscope light was turned off and the room was darkened during application of these test solutions.

The effects of pharmacological agents or ionic substitution were examined by bulk exchange of the external medium with 10–20 times the bath volume of new medium. Because we found that simply replacing the bathing solution with a fresh, but identical solution tended to potentiate calcium currents, the following procedure was adopted. After stable calcium currents had been recorded from a given myotube or muscle fiber, the bath solution was replaced with identical external saline and currents were recorded both immediately and 5 min after this first "control" solution exchange. Next, the test solution (containing Cd, Ba, or drugs) was perfused through the chamber, and calcium currents were again measured immediately and after 5 min. Finally, the test solution was washed out of the chamber and calcium currents were once more recorded immediately and 5 min afterwards.

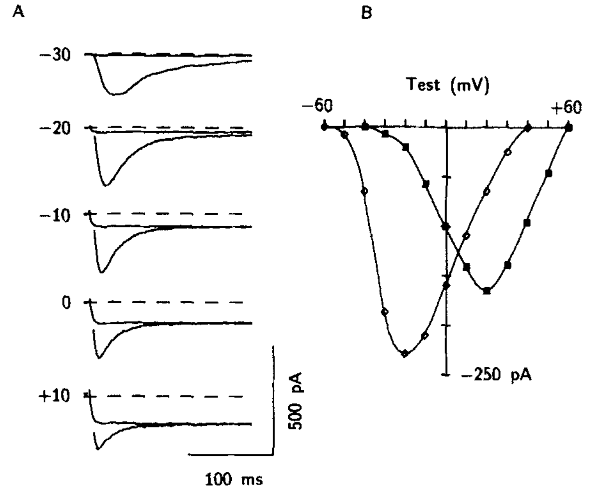

FIGURE 1. Two components of inward calcium current in a dysgenic skeletal muscle fiber. (*A*) Calcium currents were elicited by test pulses to the indicated potentials from a holding potential (HP) of −80 mV; the HP was then changed to −40 mV for 100 s and a second series of currents was obtained. A portion of the activation phase of the currents elicited from HP = −80 mV has been blanked to reveal the kinetics of current elicited from HP = −40 mV. (*B*) Current-voltage relations of $I_{Ca\text{-}ft}$ and $I_{Ca\text{-}dys}$ in dysgenic skeletal muscle. (*Open symbols*) $I_{Ca\text{-}ft}$, the difference between transient and maintained components of current elicited from HP = −80 mV, (*Solid symbols*) $I_{Ca\text{-}dys}$, current elicited from HP = −40 mV. Cell B00M69, acutely dissociated from the plantar muscles of a fetal day 15 dysgenic mouse; capacitance (*C*) = 153 pF. 10 mM Ca^{2+} + 2 μM TTX.

RESULTS

Two Calcium Currents Are Present in Dysgenic Skeletal Muscle

Fig. 1 illustrates the presence of two distinct calcium currents in a freshly dissociated muscle fiber from a fetal (day 15) dysgenic mouse. Fig. 1 *A* shows calcium currents elicited from holding potentials of −80 and −40 mV. From a holding potential of −80 mV, two components of inward current were observed, a transient component and a maintained component. Changing the steady holding potential from −80 to −40 mV eliminated the transient component and left the maintained component unaltered. For the traces shown, the holding potential was changed from −80 to −40 mV for 100 s; however, only brief prepulses (e.g., 1 s to −30 mV) were required to inactivate the transient component (Beam and Knudson, 1988). The transient component corresponds to a calcium current ($I_{Ca\text{-}ft}$) previously described for developing skeletal muscle from both normal and dysgenic mice (Beam et al., 1986; Beam and Knudson, 1988; Gonoi and Hasegawa, 1988). In contrast, the maintained component (here termed $I_{Ca\text{-}dys}$) does not correspond to any previously described calcium current of normal or dysgenic skeletal muscle. Fig. 1 *B* shows the peak current-voltage relations for the calcium currents $I_{Ca\text{-}ft}$ and $I_{Ca\text{-}dys}$ illustrated in Fig. 1 *A*. It is clear from the plot that the two currents are activated over different ranges of transmembrane voltage.

$I_{Ca\text{-}dys}$ Is Mediated by Calcium Channels

$I_{Ca\text{-}dys}$ is evidently a calcium current because with our recording conditions Ca was the only inorganic ion available to carry inward current at depolarized test potentials. This interpretation is further supported by the observation that $I_{Ca\text{-}dys}$ was reversibly reduced in amplitude by lowering the external Ca concentration (Fig. 2 *A*). In three dysgenic myotubes, the peak $I_{Ca\text{-}dys}$ measured in 2 mM Ca was only 52 ± 3% of that measured in 10 mM Ca. In 2 mM external Ca the peak current-voltage relation of $I_{Ca\text{-}dys}$ was shifted by ~ −20 mV compared to that in 10 mM external Ca. In addition, $I_{Ca\text{-}dys}$ was almost completely blocked by the application of 100 μM Cd (Fig. 2 *B*). The effect of this concentration of Cd was completely reversible ($n = 3$; washout not shown). Furthermore, as discussed below, $I_{Ca\text{-}dys}$ is quite sensitive to modulation by DHP compounds (Fig. 6).

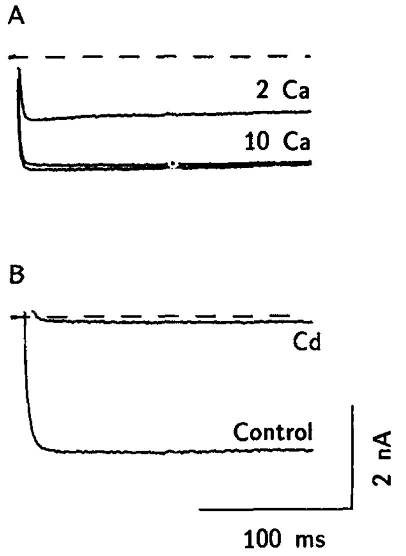

FIGURE 2. (*A*) The amplitude of $I_{Ca\text{-}dys}$ depends upon external calcium concentration. Shown are the peak calcium currents recorded in external solution containing 2 or 10 mM Ca plus 2 μM TTX. Steady HP = −80 mV; brief (1 s) prepulses to −60 mV (2 Ca) or −30 mV (10 Ca) were used to inactivate $I_{Ca\text{-}ft}$. Test pulses −10 mV (2 Ca) or +20 mV (10 Ca). Cell B00F49, dysgenic myotube, 12 d in culture; C = 450 pF. (*B*) $I_{Ca\text{-}dys}$ is blocked by 100 μM external cadmium (Cd). Cell B00E71, dysgenic myotube, 9 d in culture; C = 1450 pF. HP = −80 mV. Test pulses to +20 mV. 10 mM Ca^{2+} + 4 μM TTX.

$I_{Ca\text{-}dys}$ Is Larger when Barium Carries Charge

Fig. 3 demonstrates the relative effectiveness of Ba and Ca as charge carriers for $I_{Ca\text{-}dys}$. Substituting 10 mM Ba for 10 mM Ca in the external solution doubled the size of $I_{Ca\text{-}dys}$ without significantly altering its kinetics. This effect was very consistent and readily reversible for individual myotubes (Fig. 3 shows Ca currents obtained before and after switching to external Ba). Altogether, in five dysgenic myotubes the peak current in 10 mM Ba was 203 ± 13% of its magnitude in 10 mM Ca. In 10 mM

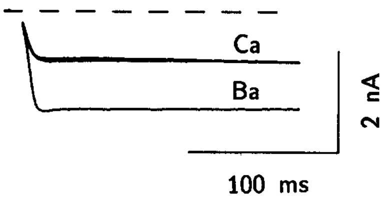

FIGURE 3. $I_{Ca\text{-}dys}$ is larger with Ba as charge carrier. Shown are the peak currents recorded with external Ca (test pulses to +20 mV) and external Ba (test pulse to 0 mV). The external solution contained 4 μM TTX + 10 mM Ca or 10 mM Ba. Cell B00B98, dysgenic myotube, 18 d in culture; C = 680 pF. HP = −80 mV.

external Ba solution the peak current-voltage relation for $I_{Ca\text{-}dys}$ was shifted negatively 10–20 mV compared to that measured in 10 mM external Ca.

$I_{Ca\text{-}dys}$ Is Distinct from the Slow Calcium Current of Normal Muscle

How is this new current ($I_{Ca\text{-}dys}$) related to the slow calcium current ($I_{Ca\text{-}s}$) of normal muscle? $I_{Ca\text{-}dys}$ and $I_{Ca\text{-}s}$ have markedly different kinetics. Fig. 4 compares the kinetics of $I_{Ca\text{-}dys}$ recorded from a dysgenic myotube with those of $I_{Ca\text{-}s}$ recorded from a normal myotube. The currents shown were elicited from steady holding potentials of −40 or −50 mV; thus, $I_{Ca\text{-}ft}$ is inactivated and $I_{Ca\text{-}dys}$ and $I_{Ca\text{-}s}$ are shown in isolation. Characteristically, $I_{Ca\text{-}dys}$ activates with rapid time-course (time-to-peak 5–10 ms) and displays little or no inactivation for test pulses lasting up to 200 ms (Fig. 4 *A*). In contrast, $I_{Ca\text{-}s}$ activates very slowly, with a time-to-peak of 200–300 ms (Fig. 4 *B*).

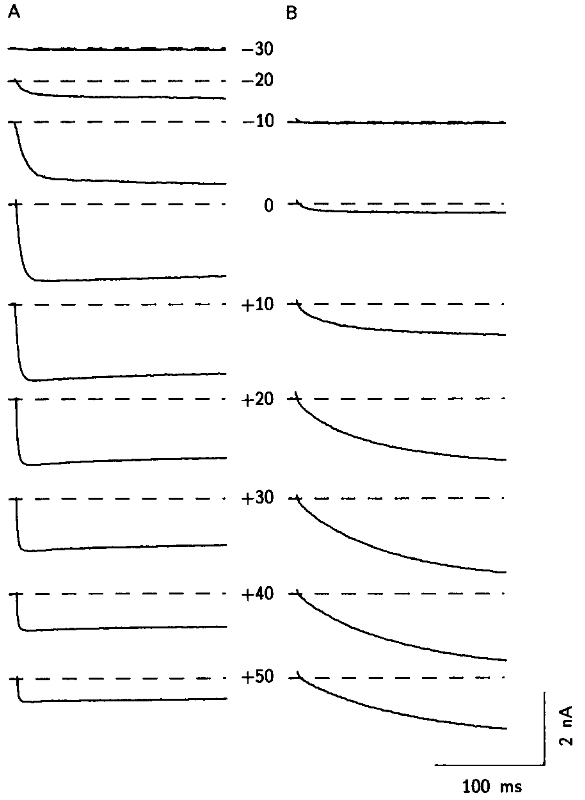

FIGURE 4. $I_{Ca\text{-}dys}$ and $I_{Ca\text{-}s}$ are kinetically distinct and also differ in voltage-dependence of activation. (*A*) $I_{Ca\text{-}dys}$ recorded from a dysgenic myotube. Cell B00C90, 8 d in culture; C = 260 pF. HP = −40 mV. (*B*) $I_{Ca\text{-}s}$ recorded from a normal myotube. Cell B00B57, 11 d in culture; C = 260 pF. HP = −50 mV.

Fig. 5 illustrates average current-voltage relations for $I_{Ca\text{-}dys}$ and $I_{Ca\text{-}s}$. The plot summarizes data obtained from 13 dysgenic and 12 normal myotubes. From a holding potential of −80 mV, $I_{Ca\text{-}dys}$ first becomes apparent for test pulses to ~−30 mV. In contrast, $I_{Ca\text{-}s}$ first appears for test pulses to ~−10 mV. $I_{Ca\text{-}dys}$ peaks near +15 mV, whereas $I_{Ca\text{-}s}$ peaks near +25 mV. The voltage-dependence of inactivation also differs between $I_{Ca\text{-}dys}$ and $I_{Ca\text{-}s}$. From a steady holding potential of −80 mV, 30-s prepulses to −30, −20, −10, and 0 mV inactivated $I_{Ca\text{-}s}$ by 21 ± 6, 49 ± 7, 74 ± 6, and 88 ± 3% respectively (*n* = 12 normal neonatal fibers), but only inactivated $I_{Ca\text{-}dys}$ by 11 ± 3, 22 ± 5, 44 ± 8, and 68 ± 10%, respectively (*n* = 6 dysgenic neonatal fibers). Thus, $I_{Ca\text{-}dys}$ activates over a more negative range of membrane potentials (Figs. 4 and 5) and inactivates over a more positive range of membrane potentials than does $I_{Ca\text{-}s}$.

It has previously been shown that $I_{Ca\text{-}s}$ is blocked or potentiated by various DHP compounds (Cognard et al., 1986*b;* Lamb and Walsh, 1987; Beam and Knudson, 1988). To assess the relative DHP-sensitivity of $I_{Ca\text{-}dys}$, calcium currents were recorded from normal and dysgenic myotubes in the absence and presence of (+)-PN 200-110, an inhibitor of calcium currents, and racemic Bay K 8644, a potentiator of calcium currents.

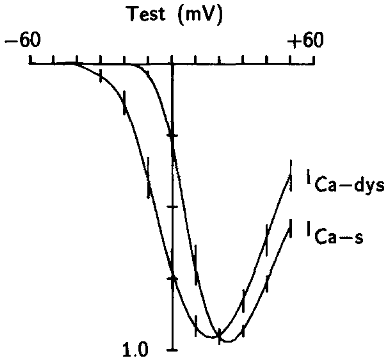

FIGURE 5. Comparison of peak current-voltage relations for $I_{Ca\text{-}dys}$ and $I_{Ca\text{-}s}$. The data shown are averages of normalized peak I-V relationships from 13 dysgenic and 12 normal myotubes. The normalized peak I-V for an individual myotube was obtained by dividing the peak calcium current measured at each test potential by the maximum calcium current recorded in that cell. The error bars represent ±SEM. For all myotubes, HP = −80 mV. 10 mM Ca^{2+} + 4 μM TTX.

$I_{Ca\text{-}dys}$ is considerably more sensitive to block by (+)-PN 200-110 than is $I_{Ca\text{-}s}$ (Fig. 6, *A–C*). Altogether, in 10 dysgenic myotubes held at −80 mV, 100 nM (+)-PN 200-110 reduced peak $I_{Ca\text{-}dys}$ by 76 ± 3%. In contrast, in 11 normal myotubes also held at −80 mV, this same concentration of (+)-PN 200-110 only reduced peak $I_{Ca\text{-}s}$ by 27 ± 2%. The responses of $I_{Ca\text{-}dys}$ and $I_{Ca\text{-}s}$ to (+)-PN 200-110 are summarized by Fig. 6 *C*. The effect of 100 nM (+)-PN 200-110 was completely reversible; washout of the drug-containing solution restored $I_{Ca\text{-}dys}$ or $I_{Ca\text{-}s}$ to within 10% of its initial value (data not shown). Higher concentrations of (+)-PN 200-110 caused more complete block of both currents; $I_{Ca\text{-}dys}$ and $I_{Ca\text{-}s}$ were reduced by >90% after exposure to 0.5 and 1 μM (+)-PN 200-110, respectively.

$I_{Ca\text{-}dys}$ is also more sensitive to potentiation by Bay K 8644 than is $I_{Ca\text{-}s}$ (Fig. 6, *D–F*). Altogether, in four dysgenic myotubes held at −80 mV, 500 nM Bay K 8644 increased peak $I_{Ca\text{-}dys}$ to 318 ± 62% of control values. In contrast, in nine normal myotubes, also held at −80 mV, this same concentration of Bay K 8644 only increased peak $I_{Ca\text{-}s}$ by 24 ± 4%. The differential responses of $I_{Ca\text{-}dys}$ and $I_{Ca\text{-}s}$ to Bay K

8644 are summarized in Fig. 6 *F*. Washout of 500 nM Bay K 8644 resulted in complete recovery of $I_{Ca\text{-}dys}$ or $I_{Ca\text{-}s}$ (data not shown). For both $I_{Ca\text{-}dys}$ and $I_{Ca\text{-}s}$, Bay K 8644 shifted the peak current-voltage relation negatively by ~10 mV. The effects of higher concentrations of Bay K 8644 were not systematically examined; however, 1 or 5 μM Bay K 8644 did not appear to cause greater potentiation of $I_{Ca\text{-}dys}$ or $I_{Ca\text{-}s}$ than 500 nM Bay K 8644.

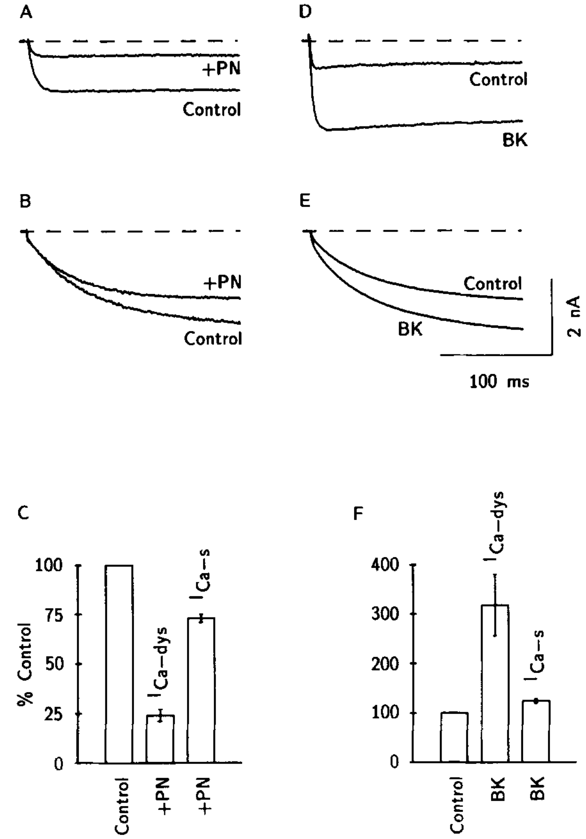

FIGURE 6. $I_{Ca\text{-}dys}$ is more sensitive to DHPs than $I_{Ca\text{-}s}$. $I_{Ca\text{-}ft}$ was absent from the currents illustrated in this figure; thus the kinetics of the currents shown reflect only those of $I_{Ca\text{-}dys}$ or $I_{Ca\text{-}s}$. (*A*) 100 nM (+)-PN 200-110 blocks $I_{Ca\text{-}dys}$ by ~75%. Test pulses to +10 mV. (*B*) 100 nM (+)-PN 200-110 blocks $I_{Ca\text{-}s}$ by ~25%. Test pulses to +30 mV. (*C*) Average responses of $I_{Ca\text{-}dys}$ and $I_{Ca\text{-}s}$ to 100 nM (+)-PN 200-110. Data from 10 dysgenic and 11 normal myotubes. Error bars indicate ±SEM. (*D*) 500 nM Bay K 8644 potentiates $I_{Ca\text{-}dys}$ about threefold. Shown are the largest currents recorded in the absence (test pulse to +30 mV) and presence (test pulse to +10 mV) of the drug. (*E*) 500 nM Bay K 8644 potentiates $I_{Ca\text{-}s}$ by ~25%. Shown are the largest currents recorded in the absence (+30 mV) and presence of the drug (+20 mV). The current calibration bar corresponds to 5 nA for (*E*) only. (*F*) Average responses of $I_{Ca\text{-}dys}$ and $I_{Ca\text{-}s}$ to 500 nM Bay K 8644. Data from four dysgenic and nine normal myotubes. Error bars indicate ±SEM. In all cases, HP = −80 mV. 10 mM Ca^{2+} + 2–4 μM TTX. (*A*) Cell B00E05, dysgenic myotube, 10 d in culture; *C* = 330 pF. (*B*) Cell B00E15, normal myotube, 15 d in culture; *C* = 370 pF. (*D*) Cell B00E23, dysgenic myotube. 9 d in culture; C = 200 pF. (*E*) Cell B00E25, normal myotube, 9 d in culture; *C* = 300 pF.

From the results presented above we conclude that $I_{Ca\text{-}dys}$ and $I_{Ca\text{-}s}$ are distinct calcium currents having different kinetics, voltage dependence, and pharmacological properties. Table I summarizes the distinguishing characteristics of $I_{Ca\text{-}dys}$ and the other known calcium currents of skeletal muscle.

Expression of $I_{Ca\text{-}dys}$ under Different Conditions

In dysgenic myotubes grown directly upon untreated culture dishes, $I_{Ca\text{-}dys}$ is usually so small that it cannot be easily detected. Measuring $I_{Ca\text{-}dys}$ in these cells requires that the leak conductance be very low and that voltage-dependent outward currents be completely blocked. Even with such ideal recording conditions, we found that $I_{Ca\text{-}dys}$ could be detected in only 50% (16/32) of dysgenic myotubes grown on untreated culture dishes. In these 32 cells (from two different cultures) the average (± SEM) peak current density of $I_{Ca\text{-}dys}$ was 0.48 ± 0.12 pA/pF (range, 0–2.22), whereas the average peak density of $I_{Ca\text{-}ft}$ was 2.46 ± 0.32 pA/pF (range, 0–7.4). The level of expression of $I_{Ca\text{-}dys}$ appears to vary widely among different cultures of dysgenic myotubes, and also appears to vary with the age of the culture. This variable, low-level expression of $I_{Ca\text{-}dys}$ in dysgenic myotubes grown on untreated culture dishes may reflect the variable presence of contaminating fibroblasts in our primary cultures (see below).

TABLE I

Calcium Currents of Skeletal Muscle

Name (*)	Kinetics	DHP sensitivity	Where found	References
$I_{Ca\text{-}ft}$ (I_{fast})	Fast-activating, transient	Very low	Normal, dysgenic	2, 3, 4, 8
$I_{Ca\text{-}fm}$ ($I_{Ca\text{-}f}$)	Fast-activating, maintained	Very low	Normal	1, 5, 7
$I_{Ca\text{-}s}$ (I_{slow})	Slowly activating, maintained	High	Normal	1–8
$I_{Ca\text{-}dys}$	Fast-activating, maintained	Very high	Dysgenic	This study

*Alternative nomenclature shown in parentheses. References: 1. Arreola et al., 1987. 2. Beam and Knudson, 1988. 3. Beam et al., 1986. 4. Cognard et al., 1986*a*. 5. Cota and Stefani, 1986. 6. Donaldson and Beam, 1983. 7. Garcia et al., 1988. 8. Gonoi and Hasegawa, 1988.

$I_{Ca\text{-}dys}$ was more frequently observed in freshly dissociated muscle fibers from fetal or neonatal dysgenic mice (e.g., Fig. 1). Out of 43 fibers examined, 35 possessed measureable $I_{Ca\text{-}dys}$. In these 43 fibers the average (± SEM) peak density of $I_{Ca\text{-}dys}$ was 0.61 ± 0.08 pA/pF (range, 0–2.12), and the average peak density of $I_{Ca\text{-}ft}$ was 2.76 ± 0.32 pA/pF (range, 0.7–8.82). We found that dysgenic myotubes grown on a substrate of killed fibroblasts (see *Methods*) frequently expressed $I_{Ca\text{-}dys}$, sometimes at fairly high density. In 86 dysgenic myotubes grown on killed fibroblasts (from nine different cultures) the average peak density of $I_{Ca\text{-}dys}$ was 1.22 ± 0.19 pA/pF (range, 0–8.86). In these same myotubes, the average peak density of $I_{Ca\text{-}ft}$ was 1.62 ± 0.17 pA/pF (range, 0–7.24). Of the 86 myotubes examined, 13 expressed only $I_{Ca\text{-}dys}$ and 24 expressed only $I_{Ca\text{-}ft}$.

The type of fibroblasts used to create the culture substrate did not seem to be important. We used fibroblasts obtained from three different sources: NIH 3T3 cell line, phenotypically normal littermates of dysgenic mice, and dysgenic mice. The average peak current densities of $I_{Ca\text{-}dys}$ in myotubes grown upon killed fibroblasts from these different sources were 1.49 ± 0.42 pA/pF (n = 10 randomly selected myotubes), 1.48 ± 0.51 (pA/pF (n = 5), and 2.35 ± 0.89 pA/pF (n = 9), respectively.

Is $I_{Ca\text{-}dys}$ Present in Normal Muscle?

An important question is whether $I_{Ca\text{-}dys}$ is present in normal developing skeletal muscle, where it could easily escape detection due to the obscuring presence of $I_{Ca\text{-}s}$.

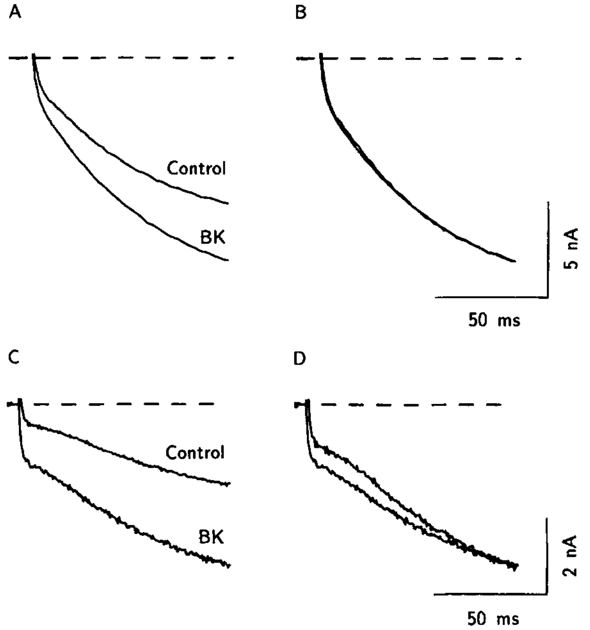

FIGURE 7. $I_{Ca\text{-}dys}$ may not be present in normal muscle. (*A*) Calcium currents recorded from a normal myotube in the absence (control) and presence (BK) of 0.5 μM Bay K 8644. In both cases, HP = −80 mV and test pulse = +20 mV. (*B*) The control current is shown scaled and superimposed upon the current potentiated by Bay K 8644. The scaling factor was determined as the ratio (potentiated current)/(control current), measured at the end of the test pulse. (*C*) Calcium currents in a dysgenic myotube, 4 d after injection of an expression plasmid (pCAC6) carrying cDNA for the rabbit skeletal muscle DHP receptor (Tanabe et al., 1988). Shown are the unpotentiated current (control), and the current after addition (BK) of 0.5 μM Bay K 8644. Both $I_{Ca\text{-}dys}$ and $I_{Ca\text{-}s}$ are present. HP = −80 mV, test pulses to +50 mV. (*D*) The control current illustrated in *C* was scaled as described in *B* and superimposed upon the current potentiated by 0.5 μM Bay K 8644. The nonsuperimposability of the traces is expected if the total calcium current contained a fast-activating component (corresponding to $I_{Ca\text{-}dys}$) that was potentiated more by Bay K 8644 than the slowly-activating component (corresponding to $I_{Ca\text{-}s}$). (*A* and *B*) Cell B00E42, normal myotube, 11 d in culture, grown on killed fibroblasts; C = 415 pF. 10 mM Ca^{2+} + 3 μM. (*C* and *D*) Cell A00J02, dysgenic myotube, 11 d in culture, injected with rabbit skeletal muscle DHP receptor cDNA on day 7; C = 700 pF. 10 mM Ca^{2+} + 3 μM TTX.

Because $I_{Ca\text{-}dys}$ activates much faster than $I_{Ca\text{-}s}$, its presence in normal muscle should cause the activation of inward current to display a fast initial phase followed by a slower phase due to $I_{Ca\text{-}s}$. We did observe such kinetics in calcium currents recorded from normal fetal and neonatal muscle fibers and from normal cultured myotubes, but the fast initial phase could always be removed with a brief (e.g., 1 s) prepulse to −30 mV, indicating that it was due to the presence of $I_{Ca\text{-}ft}$. Still, the possibility remained that $I_{Ca\text{-}dys}$ was present in normal myotubes at too low a density to be detected without potentiation. Therefore, we looked for $I_{Ca\text{-}dys}$ in the presence of racemic Bay K 8644. The following approach was employed. Calcium currents were recorded from normal myotubes grown on a substrate of killed fibroblasts. After recording control calcium currents, 0.5 or 1 μM Bay K 8644 was added to the external solution (Fig. 7 *A*). Because $I_{Ca\text{-}dys}$ is greatly potentiated by this compound and $I_{Ca\text{-}s}$ is not (Fig. 6 *F*), currents recorded after the addition of Bay K 8644 should have altered activation kinetics if $I_{Ca\text{-}dys}$ were present at significant levels. Such an alteration in activation kinetics should be revealed by the nonsuperimposability of scaled control and potentiated currents. However, application of this test to eight different normal myotubes showed that the scaled control current superimposed accurately upon the current potentiated by Bay K 8644 (Fig. 7 *B*).

As described by Tanabe et al. (1988), injection of an expression plasmid (pCAC6) carrying cDNA that encodes the rabbit skeletal muscle DHP receptor into developing dysgenic myotubes restores both $I_{Ca\text{-}s}$ and E-C coupling. In one instance, such an injected dysgenic myotube expressed both $I_{Ca\text{-}s}$ and a prominent $I_{Ca\text{-}dys}$ (Fig. 7 *C*). Even in the absence of Bay K 8644, $I_{Ca\text{-}dys}$ imparted a rapid initial phase to the current that could not be removed by a brief prepulse. In the presence of Bay K 8644, $I_{Ca\text{-}dys}$ was potentiated to a greater degree than $I_{Ca\text{-}s}$, and the rapid initial phase became even more prominent. The predicted change in activation kinetics (discussed above) became very apparent once the control current was scaled and superimposed upon the potentiated current (Fig. 7 *D*).

How small would $I_{Ca\text{-}dys}$ have to be to escape detection in normal muscle? To address this question, we modeled calcium currents that contained two components, one having time course and sensitivity to Bay K 8644 corresponding to $I_{Ca\text{-}dys}$, the other corresponding to $I_{Ca\text{-}s}$. This analysis indicated that altered activation kinetics could be easily detected if $I_{Ca\text{-}dys}$ composed only 5% of the current (which in these myotubes averaged 17.62 pA/pF) recorded before exposure to Bay K 8644. Thus, to have escaped detection in normal muscle, $I_{Ca\text{-}dys}$ would have had to be present at an average density of <0.88 pA/pF, which is lower than the average density of $I_{Ca\text{-}dys}$ (1.22 pA/pF) measured in dysgenic myotubes grown on killed fibroblasts. Although these observations are not conclusive, they suggest that $I_{Ca\text{-}dys}$ is absent from normal skeletal muscle, or at least present at lower density than in dysgenic skeletal muscle.

Relative Calcium Current Densities in Dysgenic and Normal Muscle

As mentioned above, in 43 dysgenic (fetal or neonatal) muscle fibers the average peak density of $I_{Ca\text{-}dys}$ was 0.61 ± 0.08 pA/pF, and the average peak density of $I_{Ca\text{-}ft}$ was 2.76 ± 0.32 pA/pF. For comparison, in 35 normal (fetal or neonatal) fibers the average peak density of $I_{Ca\text{-}s}$ was 6.30 ± 0.57 pA/pF (range, 2.33–17.83) and the average peak density of $I_{Ca\text{-}ft}$ was 2.24 ± 0.25 pA/pF (range, 0.47–6.35). Thus, for cells that have developed entirely in vivo, $I_{Ca\text{-}dys}$ in dysgenic muscle is present at roughly 10-fold lower density than is $I_{Ca\text{-}s}$ in normal muscle. In contrast, the densities of $I_{Ca\text{-}ft}$ are nearly equal in freshly dissociated dysgenic and normal muscle.

In 86 dysgenic myotubes grown on killed fibroblasts the average peak density of $I_{Ca\text{-}dys}$ was 1.22 ± 0.19 pA/pF and the average peak density of $I_{Ca\text{-}ft}$ was 1.62 ± 0.17

pA/pF. Again for comparison, in 31 normal myotubes (from six different cultures grown on killed fibroblasts) the average peak density of $I_{\text{Ca-s}}$ was 14.24 ± 1.08 pA/pF (range, 3.47–26.13) and the average peak density of $I_{\text{Ca-ft}}$ was 3.70 ± 0.82 pA/pF (range, 0–18.82). Thus, for cells that have developed in vitro, $I_{\text{Ca-dys}}$ is present at roughly 12-fold lower density than is $I_{\text{Ca-s}}$. This result is in good agreement with the 10-fold difference that was found for freshly dissociated muscle fibers.

DISCUSSION

Comparison of $I_{\text{Ca-dys}}$ with Other Calcium Currents

The present results characterize a previously undescribed calcium current in dysgenic skeletal muscle. This current, $I_{\text{Ca-dys}}$, is kinetically and pharmacologically distinct from the L-type calcium current ($I_{\text{Ca-s}}$) of normal skeletal muscle: it has faster activation kinetics, it activates over a more negative range of membrane potentials, inactivates over a more positive range of membrane potentials, and it is severalfold more sensitive to DHP compounds than $I_{\text{Ca-s}}$. In addition, the permeability characteristics of the calcium channel responsible for $I_{\text{Ca-dys}}$ are different from those of the channel responsible for $I_{\text{Ca-s}}$, because the amplitude of $I_{\text{Ca-dys}}$ is doubled by substituting Ba for Ca in the external medium (Fig. 3), while the amplitude of $I_{\text{Ca-s}}$ is affected little or not at all (Donaldson and Beam, 1983; Beam and Knudson, 1988).

$I_{\text{Ca-dys}}$ has kinetics that are very similar to a calcium current ($I_{\text{Ca-fm}}$) previously described for frog skeletal muscle fibers (Cota and Stefani, 1986), and recently reported for rat and rabbit skeletal muscle fibers (Garcia et al., 1988). However, these two calcium currents respond very differently to dihydropyridine drugs. $I_{\text{Ca-dys}}$ of dysgenic skeletal muscle is very sensitive to DHPs (Fig. 6), whereas $I_{\text{Ca-fm}}$ of normal skeletal muscle is insensitive to DHP calcium channel blockers (Arreola et al., 1987; Garcia et al., 1988). Thus, based on their different pharmacological properties, we conclude that $I_{\text{Ca-dys}}$ and $I_{\text{Ca-fm}}$ are distinct currents.

Taken together, the pharmacological and kinetic data support the idea that at least three different calcium currents ($I_{\text{Ca-ft}}$, $I_{\text{Ca-fm}}$, and $I_{\text{Ca-s}}$) are expressed in normal skeletal muscle. Additional support for this conclusion is provided by the results of Caffrey et al. (1987, 1988) for clonal cell lines derived from rodent muscle. Further, our present data indicate that dysgenic skeletal muscle expresses a fourth calcium current ($I_{\text{Ca-dys}}$) that is distinct from $I_{\text{Ca-ft}}$, $I_{\text{Ca-fm}}$, and $I_{\text{Ca-s}}$ (Table I).

What type of channel mediates $I_{\text{Ca-dys}}$? In many respects $I_{\text{Ca-dys}}$ resembles the L-type calcium current of cardiac muscle (Bean, 1985). $I_{\text{Ca-dys}}$ and cardiac L-current have similar kinetics, voltage dependence, and sensitivity to DHPs. Both pass Ba about twice as well as Ca. The primary difference between $I_{\text{Ca-dys}}$ and cardiac L-current is that $I_{\text{Ca-dys}}$ does not display prominent Ca-dependent inactivation. Thus, even with Ca as the charge carrier, $I_{\text{Ca-dys}}$ decays little during a 200 ms test pulse, and the kinetics of the current are unchanged upon replacement of Ca with Ba (Fig. 3). These results suggest that the channel mediating $I_{\text{Ca-dys}}$ is distinct from the cardiac L-type channel. However, the inactivation properties of the cardiac channel might be altered if it were expressed in skeletal muscle.

The kinetics and voltage-dependence of $I_{\text{Ca-dys}}$ also strongly resemble those of neuronal L-type calcium current (Nowycky et al., 1985; Fox et al., 1987). However, unlike $I_{Ca\text{-}dys}$, neuronal L-current elicited from a holding potential of −80 mV is not much affected by DHP calcium channel blockers (Fox et al., 1987; Bean, 1989; McCobb et al., 1989). A calcium current in fibroblasts that is potentiated by the DHP (+)202-791 (Chen et al., 1988) also has kinetics resembling $I_{Ca\text{-}dys}$. It cannot be stated whether the fibroblast current shows other similarities to $I_{Ca\text{-}dys}$ because Chen et al. (1988) did not report whether this current shows calcium-dependent inactivation or is sensitive to DHP calcium channel blockers at negative holding potentials.

Previous experiments have shown that E-C coupling and $I_{Ca\text{-}s}$ can be restored in dysgenic myotubes by co-culture with pure populations of living, nondysgenic fibroblasts; dysgenic fibroblasts are ineffective (Courbin et al., 1989). This "rescue" of dysgenic muscle appears to require the fusion of living fibroblasts with cultured myotubes (Chaudhari et al., 1988). As a control for these co-culture experiments, dysgenic myotubes were grown on a layer of killed fibroblasts. As reported here, the expression of $I_{Ca\text{-}dys}$ is enhanced in dysgenic myotubes grown on substrates of killed fibroblasts from either normal or dysgenic mice. It should be emphasized that dysgenic myotubes grown on killed fibroblasts do not exhibit restored E-C coupling (see below) or the $I_{Ca\text{-}s}$ characteristic of normal muscle.

The mechanism by which a substrate of killed fibroblasts enhances the expression of $I_{Ca\text{-}dys}$ remains uncertain. Presumably the fibroblasts provide an extracellular matrix resembling that present during muscle development in vivo because $I_{Ca\text{-}dys}$ is frequently present in acutely dissociated dysgenic muscle fibers. A similar extracellular environment may be present to a lesser extent in conventional cultures (grown directly on plastic dishes) in which dysgenic fibroblasts have proliferated; such an occurrence may explain the report of Bournaud et al. (1988) of "partial restoration of the slow calcium current" in aged cultures of dysgenic myotubes, and may also explain why we occasionally recorded $I_{Ca\text{-}dys}$ from dysgenic myotubes grown on untreated culture dishes. Beam et al. (1986) did not describe a calcium current in dysgenic myotubes corresponding to $I_{Ca\text{-}dys}$, probably because their myotubes were grown directly on untreated culture dishes in the absence of fibroblasts. Experiments are currently underway to further clarify the relationship between developmental substrate and the expression of $I_{Ca\text{-}dys}$.

Biological Implications of $I_{Ca\text{-}dys}$

Recent experiments have shown that E-C coupling and $I_{Ca\text{-}s}$ can be restored in dysgenic skeletal muscle by injecting, into myotubes in culture, cDNA for the normal skeletal muscle DHP receptor (Tanabe et al., 1988). These results support the hypothesis (Rios and Brum, 1987) that this DHP receptor has a dual function, serving both as the $I_{Ca\text{-}s}$ calcium channel and as the voltage sensor for E-C coupling. Because $I_{Ca\text{-}dys}$ presumably reflects the presence of an L-type calcium channel, in the same class as the channel that mediates $I_{Ca\text{-}s}$, it is reasonable to ask whether the channel responsible for $I_{Ca\text{-}dys}$ could also serve in skeletal muscle E-C coupling. Several lines of evidence suggest that it cannot. The presence of $I_{Ca\text{-}dys}$ is not correlated with the restoration of E-C coupling in dysgenic skeletal muscle. Dysgenic myotubes may express $I_{Ca\text{-}dys}$ (this study), yet do not have functional E-C coupling (Powell and Fambrough, 1973; Klaus et al., 1983). Furthermore, dysgenic myotubes grown on killed fibroblasts sometimes express $I_{Ca\text{-}dys}$ at high densities (up to 8.86 pA/pF, well within the range of magnitudes observed for $I_{Ca\text{-}s}$ in normal myotubes; Fig. 4 *A*) yet these same myotubes lack normal E-C coupling. The presence of $I_{Ca\text{-}dys}$ in dysgenic skeletal muscle, in the absence of E-C coupling, suggests that not all L-type calcium channels are equivalent in their ability to couple skeletal muscle excitation to contraction. E-C coupling in skeletal muscle apparently requires the presence of the specific DHP receptor that is associated with, and perhaps mediates, $I_{Ca\text{-}s}$.

One possibility raised by our results is that the channels underlying $I_{Ca\text{-}dys}$ constitute the DHP-binding sites reported for dysgenic skeletal muscle. Although fivefold lower than for normal muscle, the number of high-affinity DHP-binding sites in dysgenic muscle is still substantial (Pincon-Raymond et al., 1985). Previous to the discovery of $I_{Ca\text{-}dys}$, it had seemed puzzling that dysgenic skeletal muscle, which completely lacks $I_{Ca\text{-}s}$, could still bind DHPs.

The genetic origin of $I_{Ca\text{-}dys}$ remains unclear. One possibility is that the $I_{Ca\text{-}dys}$ channel is the product of a gene that is silent in normal skeletal muscle but becomes expressed as a result of the muscular dysgenesis mutation. For example, the presence of $I_{Ca\text{-}s}$ might suppress the expression of $I_{Ca\text{-}dys}$. The simultaneous presence of both $I_{Ca\text{-}dys}$ and $I_{Ca\text{-}s}$ in the pCAC6-injected dysgenic myotube shown in Fig. 7 *C* does not necessarily eliminate this as a possibility. Even if $I_{Ca\text{-}s}$ does suppress the expression of $I_{Ca\text{-}dys}$, there might not have been a sufficient length of time after the injection to have eliminated preexisting $I_{Ca\text{-}dys}$ channels. Alternatively, $I_{Ca\text{-}dys}$ may represent expression of a mutated gene for the skeletal muscle DHP receptor. Restriction-fragment analysis indicates that the structural gene for this protein is altered in muscular dysgenesis; in addition, a low level of mRNA related to the skeletal muscle DHP receptor may be present in dysgenic muscle (Tanabe et al., 1988). Whatever the source of $I_{Ca\text{-}dys}$, it will be important to determine the primary sequence of the protein responsible for this current. Because E-C coupling is nonfunctional in dysgenic muscle, differences in the primary sequences of the $I_{Ca\text{-}dys}$ and $I_{Ca\text{-}s}$ calcium channels may reveal those regions of the protein necessary for E-C coupling.

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