

Contractions of Dysgenic Skeletal Muscle Triggered by a Potentiated, Endogenous Calcium Current

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ABSTRACT The dihydropyridine (DHP) receptor of normal skeletal muscle is hypothesized to function as the voltage sensor for excitation–contraction (E-C) coupling, and also as the calcium channel underlying a slowly activating, DHP-sensitive current (termed I_{Ca-s}). Skeletal muscle from mice with muscular dysgenesis lacks both E-C coupling and I_{Ca-s} . However, dysgenic skeletal muscle does express a small DHP-sensitive calcium current (termed I_{Ca-dys}) which is kinetically and pharmacologically distinct from I_{Ca-s} . We have examined the ability of I_{Ca-dys} , or the DHP receptor underlying it, to couple depolarization and contraction. Under most conditions I_{Ca-dys} is small (~ 1 pA/pF) and dysgenic myotubes do not contract in response to sarcolemmal depolarization. However, in the combined presence of the DHP agonist Bay K 8644 (1 μ M) and elevated external calcium (10 mM), I_{Ca-dys} is strongly potentiated and some dysgenic myotubes contract in response to direct electrical stimulation. These contractions are blocked by removing external calcium, by adding 0.5 mM cadmium to the bath, or by replacing Bay K 8644 with the DHP antagonist (+)-PN 200-110. Only myotubes having a density of I_{Ca-dys} greater than ~ 4 pA/pF produce detectable contractions, and the strength of contraction is positively correlated with the density of I_{Ca-dys} . Thus, unlike the contractions of normal myotubes, the contractions of dysgenic myotubes require calcium entry. These results demonstrate that the DHP receptor underlying I_{Ca-dys} is unable to function as a “voltage sensor” that directly couples membrane depolarization to calcium release from the sarcoplasmic reticulum.

INTRODUCTION

The sarcolemma of normal skeletal muscle contains molecules that bind 1,4-dihydropyridine (DHP) derivatives with high affinity (Fosset et al., 1983). These DHP receptors are hypothesized to perform two distinct functions, serving both as ion channels that mediate a slowly activating calcium current (I_{Ca-s}), and also as “voltage sensors” for excitation-contraction (E-C) coupling (Rios and Brum, 1987; Tanabe et al., 1987, 1988). In their latter role, the DHP receptors are postulated to undergo a voltage-drive conformational change that is somehow communicated to the sarco-

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plasmic reticulum (SR), resulting in calcium release and contraction. An important feature of E-C coupling in skeletal muscle is that entry of external calcium is not required (Armstrong et al., 1972; Tanabe et al., 1988, 1990*b*). Thus, it is the voltage-sensing, and not the calcium-conducting property of the skeletal muscle DHP receptor that is responsible for triggering contraction.

In skeletal muscle from mice with muscular dysgenesis, depolarization of the sarcolemma does not normally cause contraction (Powell and Fambrough, 1973; Klaus et al., 1983; Tanabe et al., 1988). A variety of evidence indicates that the failure of E-C coupling in dysgenic muscle results from absence of the DHP receptors that predominate in normal skeletal muscle. For example, dysgenic skeletal muscle has a greatly reduced capacity to bind DHP compounds (Pincon-Raymond et al., 1985) and lacks the slowly activating, DHP-sensitive calcium current (I_{Ca-s}) associated with the normal skeletal muscle DHP receptor (Beam et al., 1986). In addition, genetic studies have shown that the gene encoding the skeletal muscle DHP-binding polypeptide is mutated in dysgenic mice, and the corresponding mRNA is strongly reduced or absent (Tanabe et al., 1988). Polyclonal antibodies directed against the DHP-binding polypeptide of skeletal muscle fail to detect this protein in dysgenic muscle (Knudson et al., 1989). Furthermore, expression of cDNA encoding the skeletal muscle DHP receptor restores both E-C coupling and I_{Ca-s} in dysgenic skeletal muscle (Tanabe et al., 1988, 1990*b*).

We have recently described an endogenous, DHP-sensitive calcium current (I_{Ca-dys}) in dysgenic skeletal muscle, and have shown this current to be distinct from I_{Ca-s} of normal skeletal muscle (Adams and Beam, 1989). Because of the similarities between I_{Ca-dys} and other calcium currents associated with E-C coupling in cardiac and normal skeletal muscle, we performed experiments to test whether I_{Ca-dys} , or the DHP receptor underlying it, could ever couple depolarization to contraction. We have found that I_{Ca-dys} can indeed mediate depolarization-induced contractions in dysgenic skeletal muscle, but only under conditions in which this inward calcium current is potentiated. Significantly, the contractions mediated by I_{Ca-dys} are like those of cardiac muscle, and unlike those of normal skeletal muscle, in that they require the entry of external calcium.

METHODS

Primary cultures of myotubes were prepared from skeletal muscle of newborn dysgenic mice and their phenotypically normal littermates using methods previously described (Beam and Knudson, 1988; Adams and Beam, 1989). In all cases, the myotubes were grown on a substrate of killed and desiccated fibroblasts (Adams and Beam, 1989). This culture condition was used because it increases, via an unknown mechanism, the density at which I_{Ca-dys} is expressed in dysgenic myotubes (Adams and Beam, 1989). 7–20 d after plating, myotubes were tested for the ability to contract in response to focal extracellular stimulation via an agar/saline-filled micropipette (2- μ m tip diameter) placed within 100 μ m of the cell surface. The intensity of the stimulation pulse (60–80 V, 5 ms duration) was adjusted to be suprathreshold for evoking contractions of normal myotubes. Contractions were monitored optically and recorded as the voltage drop across a small photoresistor placed at the edge of each myotube's image on a video monitor. The output of the photoresistor was amplified, filtered at 5–10 Hz, and stored on computer.

Strength of contraction was evaluated qualitatively (i.e., weak, medium, or strong). Weak

contractions were those that were just visibly detectible; intermediate contractions were pronounced but slow in onset and relaxation; strong contractions were forceful and brisk, resembling the twitches of normal myotubes. The location of each contracting myotube was recorded so that the same cells could later be relocated for voltage-clamping.

Voltage clamp data were obtained using the whole-cell configuration of the patch clamp technique (Hamill et al., 1981) as previously described (Beam and Knudson, 1988; Adams and Beam, 1989). The pipettes had resistances of 1.3–2.3 M Ω . Electronic compensation was used to minimize the effective series resistance (generally to <1 M Ω) and the time required to charge the cell capacitance (generally to <1 ms). Whole-cell currents were measured during 200-ms depolarizing test pulses administered from a steady holding potential of –80 mV. For each cell, linear capacitance and leakage currents were measured for 8–10 “control” voltage steps between –80 and –100 mV; these control currents were averaged, scaled appropriately, and used to correct test currents for linear components of capacitative and leakage currents. To allow comparison of test currents recorded from different cells, test currents recorded from each cell were normalized by that cell’s linear capacitance (current expressed as picoamperes per picofarad).

Evoked contractions were recorded while the myotubes were bathed in a solution containing (in mM) 145 NaCl, 10 CaCl₂, 10 HEPES, 5 KCl, and 0.001 Bay K 8644, pH 7.4 with NaOH. Calcium currents were measured while the myotubes were bathed in a solution containing (in mM) 145 TEACl, 10 CaCl₂, 10 HEPES, 0.003 TTX, and 0.001 Bay K 8644, pH 7.4 with CsOH. The patch pipettes were filled with a solution containing (in mM) 140 Cs-Aspartate, 5 MgCl₂, 10 Cs₂-EGTA, and 10 HEPES, pH 7.4 with CsOH. The 1,4-DHP derivatives (\pm)-Bay K 8644 (gift of Dr. A. Scriabine, Miles Laboratories, Inc., New Haven, CT) and (+)-PN 200-110 (gift of Dr. A. Lindenmann and Dr. E. Rossi, Sandoz Ltd., Basel, Switzerland) were added to the external solution just before use from stock solutions (10 mM in ethanol) which were stored in the dark at –20°C. All experiments were performed at room temperature (20–22°C). Values are presented in the text as mean \pm SEM with the number of observations in parentheses.

RESULTS

Depolarization-evoked Contractions of Dysgenic Skeletal Muscle

Previous studies (Powell and Fambrough, 1973; Tanabe et al., 1988) have shown that dysgenic myotubes bathed in physiological saline (containing ~2 mM calcium) are incapable of contracting either spontaneously or in response to electrical stimulation. However, the recent discovery of a DHP-sensitive calcium current (I_{Ca-dys}) in dysgenic skeletal muscle (Adams and Beam, 1989; Bournaud et al., 1989) led us to re-examine whether dysgenic myotubes could ever display E-C coupling under less physiological conditions. Specifically, we tested whether contractions could be triggered by calcium entry through the I_{Ca-dys} channels, or through a more direct interaction of the DHP receptor underlying I_{Ca-dys} with the SR.

To this end, we electrically stimulated dysgenic myotubes first while they were bathed in saline containing elevated (10 mM) calcium, and then again after the addition of 1 μ M Bay K 8644. No dysgenic myotubes were observed to contract in response to electrical stimulation while bathed in the high calcium saline that did not contain Bay K 8644 (>1,000 myotubes from three different muscle cultures were individually tested). However, in the combined presence of 10 mM calcium and 1 μ M Bay K 8644 (which should potentiate I_{Ca-dys} approximately sixfold; Adams and Beam, 1989) many of these same myotubes produced twitchlike contractions. For example,

in one culture of dysgenic muscle, 35 of 300 myotubes tested could contract after the addition of Bay K 8644. In a separate dysgenic muscle culture, 40 of 100 dysgenic myotubes tested could contract after the addition of Bay K 8644.

What mechanism accounts for the ability of dysgenic myotubes to contract in response to depolarization? One possibility is that in the combined presence of elevated external calcium and Bay K 8644, calcium entry through the I_{Ca-dys} channels becomes sufficient to activate directly the contractile proteins, or to trigger calcium-induced calcium release from the SR as in cardiac-type E-C coupling. Another possibility is that under physiological conditions the DHP receptor underlying I_{Ca-dys} is in an "inactivated" state, but in the combined presence of high calcium and Bay K 8644 this DHP receptor becomes "activated," enabling it to mediate direct, skeletal-type E-C coupling. This latter possibility is suggested by results demonstrating that the voltage sensor for E-C coupling in normal skeletal muscle is influenced both by external calcium concentration (Brum et al., 1988*a, b*) and DHP compounds (Pizarro et al., 1988). To distinguish between these two possibilities, we performed the experiments described below.

The Role of Calcium Entry

Several experimental manipulations that block calcium currents were used to investigate the role of calcium entry in triggering the evoked contractions of dysgenic myotubes. These included removing calcium from the external solution (10 mM magnesium was substituted), adding 0.5 mM cadmium to the external solution, and replacing Bay K 8644 in the external solution with the DHP calcium channel antagonist (+)-PN 200-110.

Fig. 1 illustrates the effects of these different manipulations. In three of three dysgenic myotubes tested, removal of external calcium abolished (reversibly) evoked contractions (Fig. 1*A, top*). In parallel with its effect on contraction, removing external calcium reduced the peak I_{Ca-dys} (measured during the test pulse) by $94 \pm 3\%$ ($n = 3$; Fig. 1*B, top*). The effect of calcium removal on I_{Ca-dys} was completely reversible (recovery not shown).

The evoked contractions of dysgenic myotubes were also abolished by the external application of 0.5 mM cadmium ($n = 4$; Fig. 1*A, middle*). As expected, 0.5 mM cadmium produced strong yet reversible block of I_{Ca-dys} , reducing the peak current during the test pulse by $92 \pm 1\%$, ($n = 5$; Fig. 1*B, middle*). As described by Swandulla and Armstrong (1989) for chicken dorsal root ganglion cells, cadmium reduced but did not completely block the calcium channel tail current.

The effect of substituting the DHP calcium channel antagonist (+)-PN 200-110 (2 μ M) for Bay K 8644 (1 μ M) in the external solution was also examined. In three of three myotubes tested, this substitution diminished but did not completely abolish evoked contractions of dysgenic myotubes (Fig. 1*A, bottom*). Parallel effects were seen on I_{Ca-dys} , where the peak calcium current was decreased by $46 \pm 6\%$ ($n = 3$; Fig. 1*B, bottom*). During these experiments, a large volume (~ 50 ml) of solution containing (+)-PN 200-110 (without Bay K 8644) was washed through the recording chamber (volume ~ 1 ml); the lack of complete blockade of contraction and current is possibly due to a higher affinity of the I_{Ca-dys} channel for Bay K 8644 than for (+)-PN 200-110.

For comparison, identical experiments were performed on normal myotubes. As

shown in Fig. 2A, evoked contractions of normal myotubes bathed in saline containing 10 mM calcium and 1 μ M Bay K 8644 were unaffected by replacing external calcium with magnesium ($n = 6$), by adding 0.5 mM cadmium to the external solution ($n = 6$), or by replacing 1 μ M Bay K 8644 with 2 μ M (+)-PN 200-110 ($n = 6$). However, these same treatments did produce strong block of I_{Ca-s} (Fig. 2B). Replacing external calcium with magnesium reduced peak I_{Ca-s} by $98 \pm 0.5\%$ ($n = 3$); addition of 0.5 mM cadmium reduced peak I_{Ca-s} by $87 \pm 1\%$ ($n = 3$);

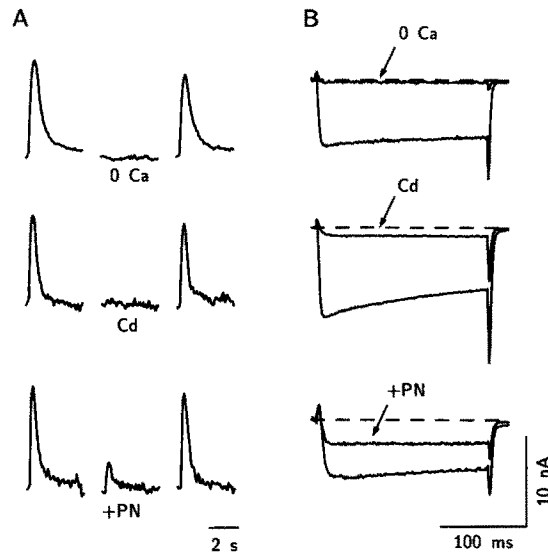


FIGURE 1. Contractions of dysgenic myotubes require calcium entry. (A) Evoked contractions and (B) inward calcium currents of dysgenic myotubes recorded in the presence of 10 mM external calcium and 1 μ M Bay K 8644. (A) Contractions are shown under control conditions (left), under experimental conditions (middle), and after washout (right). The contractions of dysgenic myotubes were abolished by removing external calcium (top) or by adding 0.5 mM cadmium (middle), and they were reduced in amplitude by substituting 2 μ M (+)-PN 200-110 for Bay K 8644 in the external solution (bottom).

(B) I_{Ca-dys} was blocked by replacing external calcium with 10 mM magnesium (top) or by external application of 0.5 mM cadmium (middle), and was reduced in amplitude by substituting 2 μ M (+)-PN 200-110 for Bay K 8644 in the external solution (bottom). The above effects on I_{Ca-dys} were completely reversible (washout not shown). Illustrated currents were elicited by test depolarizations to 0 mV, which evoked maximal I_{Ca-dys} . For the calcium currents shown in the middle panel of B, the calibration bar corresponds to 20 nA. The vertical scale for contractions is arbitrary. (A) Top, Cell B00E97, dysgenic myotube, 12 d in culture. Middle, Cell B00E92, dysgenic myotube, 18 d in culture. Bottom, Cell B00E94, dysgenic myotube, 18 d in culture. (B) Top, Cell B00G93, dysgenic myotube, 8 d in culture. Linear cell capacitance (C) = 345 pF. Middle, Cell B00G96, dysgenic myotube, 9 d in culture. C = 705 pF. Bottom, Cell B00G97, dysgenic myotube, 9 d in culture. C = 850 pF.

and substituting (+)-PN 200-110 for Bay K 8644 in the external solution reduced peak I_{Ca-s} by $90 \pm 1\%$ ($n = 3$). It is noteworthy that this last treatment caused a much stronger block of I_{Ca-s} than of I_{Ca-dys} (compare Fig. 1B, bottom).

The present results clearly demonstrate that calcium entry is required for the evoked contractions of dysgenic myotubes. In contrast, calcium entry is not required for evoked contractions of normal myotubes (Tanabe et al., 1990b; Fig. 2A). Furthermore, the results obtained above with (+)-PN 200-110 strongly suggest that

the calcium that triggers contraction of dysgenic myotubes enters through the DHP-sensitive calcium channels underlying I_{Ca-dys} .

Dependence of Contraction Strength on the Density of I_{Ca-dys}

If the contractions of dysgenic myotubes are triggered by calcium entry through the I_{Ca-dys} channels, then the strength of contraction should be related to the density of I_{Ca-dys} . In support of this idea, we found that contracting dysgenic myotubes possessed substantially higher densities of I_{Ca-dys} than noncontracting myotubes. In 19 dysgenic

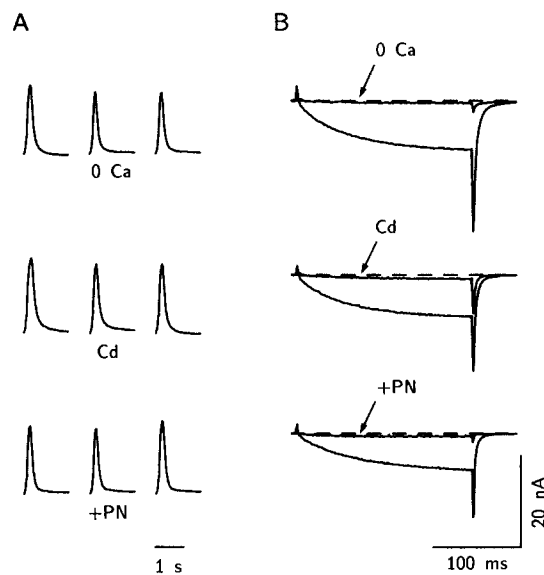


FIGURE 2. Contractions of normal myotubes do not require calcium entry. (A) Evoked contractions and (B) calcium currents of normal myotubes in the presence of 10 mM Ca and 1 μ M Bay K. (A) Contractions are shown under control conditions (left), under experimental conditions (middle), and after washout (right). The contractions of normal myotubes were unaffected by removing external calcium (top), by adding 0.5 mM cadmium (middle), or by substituting 2 μ M (+)-PN 200-110 for Bay K 8644 in the external solution (bottom). (B) I_{Ca-s} was strongly blocked by replacing external calcium with

10 mM magnesium (top), by external application of 0.5 mM cadmium (middle), or by substituting 2 μ M (+)-PN 200-110 for Bay K 8644 in the external solution (bottom). The above effects on I_{Ca-s} were completely reversible (washout not shown). Illustrated currents were elicited by test depolarizations to +20 mV, which evoked maximal I_{Ca-s} . For the currents shown in the middle panel of B, the calibration bar corresponds to 8 nA. The vertical scale for contractions is arbitrary. (A) Cell B00H50, normal myotube, 8 d in culture. (B) Top, Cell B00H45, normal myotube, 7 d in culture, C = 305 pF. Middle, Cell B00H42, normal myotube, 6 d in culture, C = 110 pF. Bottom, Cell B00H46, normal myotube, 7 d in culture, C = 200 pF.

myotubes that produced evoked contractions, the peak density of I_{Ca-dys} (measured in the presence of 10 mM external calcium and 1 μ M Bay K 8644) was 15 ± 2 pA/pF (range 4–30 pA/pF). In contrast, in 12 dysgenic myotubes that could not contract (these cells were from the same culture and were tested at the same time), the peak density of I_{Ca-dys} was only 0.7 ± 0.2 pA/pF (range 0–3 pA/pF). In addition, there was a positive correlation between the strength of contraction and the density of I_{Ca-dys} . For example, in five dysgenic myotubes that contracted weakly, average peak I_{Ca-dys} was only 5.6 ± 0.5 pA/pF (range 4–7 pA/pF), whereas in seven dysgenic myotubes that contracted strongly, average peak I_{Ca-dys} was 23 ± 2 pA/pF (range 15–30 pA/pF).

In seven dysgenic myotubes that contracted with intermediate strength, average peak I_{Ca-dys} was 14 ± 0.6 pA/pF (range 12–17 pA/pF). This relationship between contraction strength and the density of I_{Ca-dys} is illustrated in Fig. 3.

Both normal and dysgenic myotubes can express a T-type calcium current (I_{Ca-ft}) that is insensitive to DHP compounds (Cognard et al., 1986; Beam and Knudson, 1988). There was no correlation between the density of I_{Ca-ft} and the strength of contraction (Fig. 3). In 19 contracting dysgenic myotubes, average peak I_{Ca-ft} was 1.2 ± 0.4 pA/pF (range 0–8.2 pA/pF), a value indistinguishable from that measured in 12 noncontracting dysgenic myotubes (1.1 ± 0.6 pA/pF; range 0–7.4 pA/pF) from the same muscle culture. Interestingly, one noncontracting dysgenic myotube that lacked measurable I_{Ca-dys} did possess a relatively high density of I_{Ca-ft} (7.4 pA/pF); this density of I_{Ca-ft} is higher than the minimum density of I_{Ca-dys} found in weakly contracting dysgenic myotubes (~ 4 pA/pF).

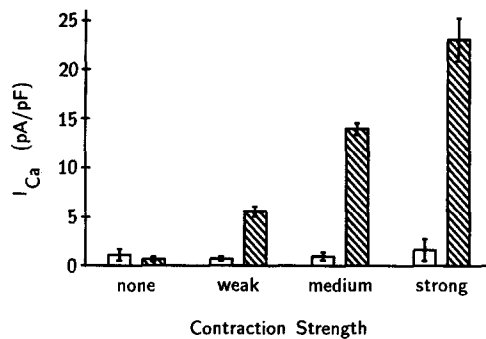


FIGURE 3. The strength of evoked contraction in dysgenic myotubes is positively correlated with the density of I_{Ca-dys} , but is not correlated with the density of I_{Ca-ft} . Maximal I_{Ca-dys} and I_{Ca-ft} were both measured in the presence of 10 mM Ca and 1 μ M Bay K 8644, using 200-ms test depolarizations (to $\sim +10$ mV for I_{Ca-dys} and to ~ -10 mV for I_{Ca-ft}) administered from a steady holding potential of -80 mV. Hatched bars represent

I_{Ca-dys} ; open bars represent I_{Ca-ft} . Average current densities were calculated for 5–12 myotubes per group. The error bars represent \pm SEM.

DISCUSSION

This paper describes the ability of an endogenous, DHP-sensitive calcium current, I_{Ca-dys} , to trigger contractions in dysgenic myotubes. Because the density of this calcium current is typically quite low (~ 1 pA/pF; Adams and Beam, 1989), contractions were not observed unless I_{Ca-dys} was first potentiated with Bay K 8644.

The evoked contractions of dysgenic myotubes were blocked or reduced by manipulations (removing external calcium, adding cadmium to the bath, or substituting (+)-PN 200-110 for Bay K 8644) that block or reduce I_{Ca-dys} . Furthermore, the intensity of contraction was positively correlated with the density of this calcium current. Thus, it seems clear that the evoked contractions of dysgenic myotubes depend upon calcium entry through the DHP-sensitive channels that underlie I_{Ca-dys} .

Depolarization–contraction coupling in cardiac muscle also requires calcium entry (Näbauer et al., 1989). Because of this similarity, we suggest that potentiated I_{Ca-dys} effects a “cardiac-type” of E-C coupling in dysgenic myotubes. By way of contrast, the contractions of normal myotubes (Fig. 2A), or of dysgenic myotubes that express the normal skeletal muscle DHP receptor, as a result either of incorporation of normal fibroblast nuclei (Chaudhari et al., 1989) or of cDNA injection (Tanabe et al., 1988,

1990b) do not require calcium entry. From these results we conclude that the type of E-C coupling mediated by the DHP receptor that underlies I_{Ca-dys} is fundamentally different from that mediated by the normal skeletal muscle DHP receptor. Thus, the DHP receptor underlying I_{Ca-dys} appears unable to couple sarcolemmal depolarization directly to SR calcium release in the absence of calcium entry. This result further strengthens the findings of Tanabe et al. (1990a, b) that not all types of DHP receptors expressed in skeletal muscle are able to directly trigger calcium release from the SR.

As shown in Fig. 2C, the evoked twitches of normal myotubes were unaffected when 2 μ M (+)-PN 200-110 was substituted for 1 μ M Bay K 8644 in the external solution. In other experiments (unpublished observations), we found that higher concentrations (up to 10 μ M) of (+)-PN 200-110 or nifedipine also had no significant effect on twitches of normal myotubes. In contrast, Cognard et al. (1990) reported that 5 μ M nifedipine strongly reduced contractions of rat myoballs. However, it should be noted that they studied contractions elicited by depolarizations applied to cells under whole-cell voltage clamp, whereas we studied contractions elicited by focal, extracellular stimulation of intact myotubes. Moreover, the effects of dihydropyridines on contraction of skeletal muscle seem quite variable. For example, Gallant and Goettl (1985) and Dulhunty and Gage (1988) both found that nifedipine *potentiated* the twitches of small bundles of rodent muscle fibers.

It is interesting that dysgenic myotubes that do not contract under the conditions used here sometimes express I_{Ca-ft} at higher density than that density of I_{Ca-dys} (~ 4 pA/pF) needed to support contraction. This observation suggests that I_{Ca-ft} alone cannot mediate depolarization–contraction coupling in dysgenic myotubes. Perhaps I_{Ca-ft} is less effective in triggering contractions due to its kinetic properties (slower activation, faster inactivation than I_{Ca-dys}). Another possibility is that the channels responsible for I_{Ca-ft} may be located more distantly from the SR calcium release channels than the channels responsible for I_{Ca-dys} .

Recently, the cDNA encoding the cardiac DHP receptor was cloned and expressed in *Xenopus* oocytes (Mikami et al., 1989). This same cardiac DHP receptor was also expressed in dysgenic myotubes, where it produces an L-type calcium current and cardiac-type E-C coupling, which requires the entry of external calcium (Tanabe et al., 1990b). The inward current mediated by the cardiac DHP receptor expressed in dysgenic myotubes resembles I_{Ca-dys} in voltage dependence, kinetics, and pharmacology. However, a major difference between these two calcium currents is that the exogenous cardiac current is expressed at much higher densities (12–56 pA/pF, measured in the presence of 10 mM external calcium and in the absence of Bay K 8644; Tanabe et al., 1990b) than I_{Ca-dys} and can thus easily trigger contraction without further potentiation.

An interesting question raised by the present study is whether internal calcium stores participate in the contractions triggered by I_{Ca-dys} . In dysgenic myotubes expressing the cardiac DHP receptor cDNA, calcium-entry–induced release of internal calcium does seem to be important. After caffeine treatment of such myotubes, contractions are initially small but then increase in size with repeated stimuli, even though the amplitude of the cardiac L-type calcium current remains constant (Tanabe et al., 1990b; see also Nábauer et al., 1989). This result suggests

that internal calcium stores initially depleted by the caffeine treatment become refilled and thus make an increasing contribution to contraction. It seems likely that the same internal calcium stores also participate in the contractions of dysgenic myotubes triggered by I_{Ca-dys} .

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