

**ABSTRACT:** Human normal (RCMH) and Duchenne muscular dystrophy (RCDMD) cell lines, as well as newly developed normal and dystrophic murine cell lines, were used for the study of both changes in inositol 1,4,5-trisphosphate (IP<sub>3</sub>) mass and IP<sub>3</sub> binding to receptors. Basal levels of IP<sub>3</sub> were increased two- to threefold in dystrophic human and murine cell lines compared to normal cell lines. Potassium depolarization induced a time-dependent IP<sub>3</sub> rise in normal human cells and cells of the myogenic mouse cell line (129CB3), which returned to their basal levels after 60 s. However, in the human dystrophic cell line (RCDMD), IP<sub>3</sub> levels remained high up to 200 s after potassium depolarization. Expression of IP<sub>3</sub> receptors was studied measuring specific binding of <sup>3</sup>H-IP<sub>3</sub> in the murine cell lines (normal 129CB3 and dystrophic *mdx* XLT 4-2). All the cell lines bind <sup>3</sup>H-IP<sub>3</sub> with relatively high affinity (K<sub>d</sub>: between 40 and 100 nmol/L). IP<sub>3</sub> receptors are concentrated in the nuclear fraction, and their density is significantly higher in dystrophic cells compared to normal. These findings together with high basal levels of IP<sub>3</sub> mass suggest a possible role for this system in the deficiency of intracellular calcium regulation in Duchenne muscular dystrophy.

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## **DIFFERENCES IN BOTH INOSITOL 1,4,5-TRISPHOSPHATE MASS AND INOSITOL 1,4,5-TRISPHOSPHATE RECEPTORS BETWEEN NORMAL AND DYSTROPHIC SKELETAL MUSCLE CELL LINES**

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**T**he role of inositol 1,4,5-trisphosphate (IP<sub>3</sub>) as a second messenger in signal transduction has been well established in many cell types. However, conflicting reports have led to a controversy regarding the role of inositol 1,4,5-trisphosphate signaling in skeletal muscle. Indeed expression of the inositol 1,4,5-trisphosphate receptor has been difficult to

quantify in skeletal muscle.<sup>16,29</sup> More recent investigations suggest that an elevation in myoplasmic IP<sub>3</sub> may be secondary to the primary signal for sarcoplasmic reticulum calcium release during contractile activation.<sup>13,25</sup> However, we have recently found that cultured myotubes have both a high IP<sub>3</sub> mass, modulated by membrane potential, and an important amount of IP<sub>3</sub> receptors associated with cell nuclei.<sup>17</sup> Furthermore, potassium depolarization elicited two types of intracellular calcium signals in cultured myotubes: a fast one, associated with contraction and a slow one, associated with increased nucleoplasmic calcium. The slow calcium signal possibly follows IP<sub>3</sub>

**Key words:** Duchenne muscular dystrophy; calcium channels; inositol trisphosphate; inositol trisphosphate mass; human cell lines  
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mass increases.<sup>17,20</sup> The inositol polyphosphates may have a modulatory function on sarcoplasmic reticulum (SR) Ca<sup>2+</sup> transients and other Ca<sup>2+</sup>-dependent processes of muscle metabolism, which are associated with the contractile cycle,<sup>9</sup> but may also be playing important roles in both ligand and membrane potential signaling to the nuclei.<sup>1,26,34</sup>

The muscular dystrophies comprise a heterogeneous group of heredity myopathies characterized by muscle degeneration followed by replacement of muscle with connective tissue and fat. The most severe and most common form of dystrophy is Duchenne muscular dystrophy (DMD), an X-linked disorder in which progressive proximal weakness starts in childhood and usually leads to death in the second or third decade of life. The severe muscle destruction observed in DMD patients is attributed to the absence of the protein dystrophin, and 427-kD protein encoded by a 14-kb transcript.<sup>15</sup> This deficiency results in the absence of a function associated with the protein at the cell membrane level, with subsequent thin filament-membrane impairments, increased intracellular Ca<sup>2+</sup> concentrations, and elevated protein degradation.<sup>28,32</sup> Our goal in this work was to investigate whether elements important in IP<sub>3</sub>-mediated processes, such as the total mass of IP<sub>3</sub> and the level of IP<sub>3</sub> receptors, are altered in dystrophin-deficient muscle cells.

## MATERIALS AND METHODS

**Primary Cultures.** Primary cultures were done according to previously established protocols.<sup>8</sup> Mice (*mdx* in C57BL-10 strain) came from permanent colonies at Smith College. Muscle tissue from hindlimbs was washed, rinsed in calcium magnesium free (CMF) balanced salt solution, and then dissociated in a solution of 0.125% trypsin, 0.05% pancreatin in CMF. Trypsinization was halted with 10% horse serum (HS) (Sigma, St. Louis, MO) solution. Cells were centrifuged and the pellet resuspended in complete medium [10% HS, 10% fetal bovine serum (FBS) (Life Technologies, Inc. Grand Island, NY), 2% chick embryo extract (CEE) (from 10-day embryos) in Dulbecco's Modified Eagle Medium (DMEM) (Life Technologies, Inc.)]. After 1 h CO<sub>2</sub> incubation at 37°C for enrichment of myoblasts, cells were plated at 1 × 10<sup>4</sup> cells/35-mm, 1% gelatin-coated primaria dish in complete medium, and placed in a 10% CO<sub>2</sub>, 37°C incubator. Near confluency, cells were treated with differentiation medium (10% HS, 1.25% CEE in DMEM).

**Generation of Cell Lines.** Primary normal (+/+) and dystrophic (*mdx*) dissociated cells were prepared

as noted above, plated at 2.0–2.5 × 10<sup>5</sup> cells/60-mm culture dish, and transfected using a modification of the Ca<sup>2+</sup> phosphate method.<sup>12</sup> Briefly, cells were allowed to grow to 50–80% confluency, the medium was changed from complete to growth medium (20% FBS in DMEM), and the cells were incubated for 24 h in the transfection solution containing 2 µg/mL of the plasmid pRBK (Invitrogen, San Diego, CA), which contains the BK Large T antigen as well as ampicillin and hygromycin B resistant sequences. After 34–40 h the cells were fed with growth medium. After a subsequent 24-h incubation in growth medium the cells were split 1:4 or 1:10 and plated in growth medium containing 100–200 µg/mL hygromycin B (Calbiochem, La Jolla, CA) for the selection of transfected cells.

Several hygromycin-resistant cell lines have been characterized. The mutant cell line was called XLT for *mdx*Line transfected with the Large T antigen. The specific line that differentiated the best was XLT 4-2. Passage numbers refer to the number of passages following the first transfer from a cloned ring culture to a 25-cm<sup>2</sup> flask. We estimated each passage (once a week) represents approximately 10 generations of cell division. Testing for optimal differentiation condition was performed on passages 2–5. All characteristics reported here are from cell cultures at passages 7 or higher. Cells were plated and proliferated in growth medium containing 10% FBS and 10% HS, and differentiated in fusion (differentiation) medium containing 2% horse serum in DMEM. For immunofluorescence labeling, cells were plated at 7 × 10<sup>3</sup> per 35-mm culture dish. Human normal (RCMH) and Duchenne muscular dystrophy (RCDMD) immortal cell lines were cultured as described,<sup>6,7</sup> and the normal murine cell line (129CB3)<sup>30</sup> cells were used as a control.

**Fluorescence Immunocytology.** Cultures were treated according to previously established protocols.<sup>8</sup> Briefly, cells were fixed with methanol at –20°C for 10 min and rinsed with phosphate-buffered solution (PBS) or CMF. Plates were treated with 10% normal goat serum, 1% bovine serum albumin (BSA), 0.02% Na azide in 0.1 mol/L PBS or CMF, and primary antibodies used were anti α-actinin (Sigma) at 1:200 and antidystrophin (gift of Dr. S.C. Froehner<sup>33</sup>) at 1:100. After rinsing with 1% BSA in PBS or CMF, the secondary antibody (goat anti-mouse immunoglobulinG fluorescein, Cappel Products, Malvern, PA) was applied at 1:200 for 1–2 h at room temperature. The nuclear staining with #33342 Hoechst (Polyscience, Inc., Warrington, PA)

was carried out either prior to fixing or added to the secondary antibody preparation.

**Binding of  $^3\text{H-IP}_3$  and  $^3\text{H-Ryanodine}$ .** The binding of  $^3\text{H-IP}_3$  to cultures of the mouse cell lines was determined under the following conditions. Confluent plates of XLT 4-2 cells were washed three times with PBS, and they were homogenized with Ultrasonic-Homogenizer for 10–15 s. They were then incubated in a medium that contains 50 mmol/L Tris-HCl pH 8.4, 1 mmol/L edetic acid 1 mmol/L 2 Me-EtOH, and different concentrations (10–200 nmol/L) of  $^3\text{H-IP}_3$  (D-[2- $^3\text{H}$ ]-*myo*-inositol 1,4,5-trisphosphate, specific activity 21.0 Ci/mmol, NEN-Dupont, 800–1000 cpm/pmol) to 4°C for 30–40 min. After the incubation the reaction was stopped by centrifugation at 10,000 *g* for 10 min (Heraus Biofuge 13), the supernatant was aspirated, and the pellets were washed with PBS, and dissolved in 1 mol/L NaOH to measure the radioactivity. The nonspecific binding was determined in the presence of 2  $\mu\text{mol/L}$   $\text{IP}_3$  (Sigma Chemical Co., St. Louis, MO).  $^3\text{H-Ryanodine}$  binding was measured in XLT 4-2 cell homogenates as described by Jaimovich et al.<sup>19</sup> The incubation medium contained 0.5 mol/L KCl, 0.1 mmol/L  $\text{CaCl}_2$ , 20 mmol/L HEPES-Tris pH 7.1, and 1 mmol/L 5'-adenylylimidodiphosphate or 0.5 mmol/L adenosine triphosphate; samples were incubated with  $^3\text{H-ryanodine}$  (5–100 nmol/L) for 90 min at 37°C, in the presence or absence of cold ryanodine (10  $\mu\text{mol/L}$ ) for nonspecific binding. These same markers were also measured in several crude fractions obtained from differential centrifugation of homogenates of XLT 4-2 cells.

**Mass of  $\text{IP}_3$  in Cell Cultures Determined by Radioreceptor Assay.** Confluent plates of cell cultures were washed with PBS and incubated in a control medium that contained: 4 mmol/L KCl, 62.5 mmol/L NaCl, 2.5 mmol/L  $\text{CaCl}_2$ , 1.2 mmol/L  $\text{MgSO}_4$ , 0.5 mmol/L edetic acid, 1.2 mmol/L  $\text{NaH}_2\text{PO}_4$ , 20 mmol/L LiCl, 10 mmol/L glucose, 20 mmol/L Tris-HEPES pH 7.4 for 30 min at 4°C. Then, the control medium was aspirated and replaced with a high potassium solution for specified times. The reaction was stopped by adding 5% (vol/vol) perchloric acid, and the plates were quickly immersed in liquid nitrogen for a brief time. Then the cells were harvested and centrifuged at 10,000 *g* for 10 min (Heraus Biofuge 13), the pellets were dissolved in NaOH for protein determination, and the supernatant was aspirated and quickly neutralized with a solution that contained: 2 mol/L KOH, 1 mmol/L edetic acid, 20 mmol/L trimethylaminoethane sul-

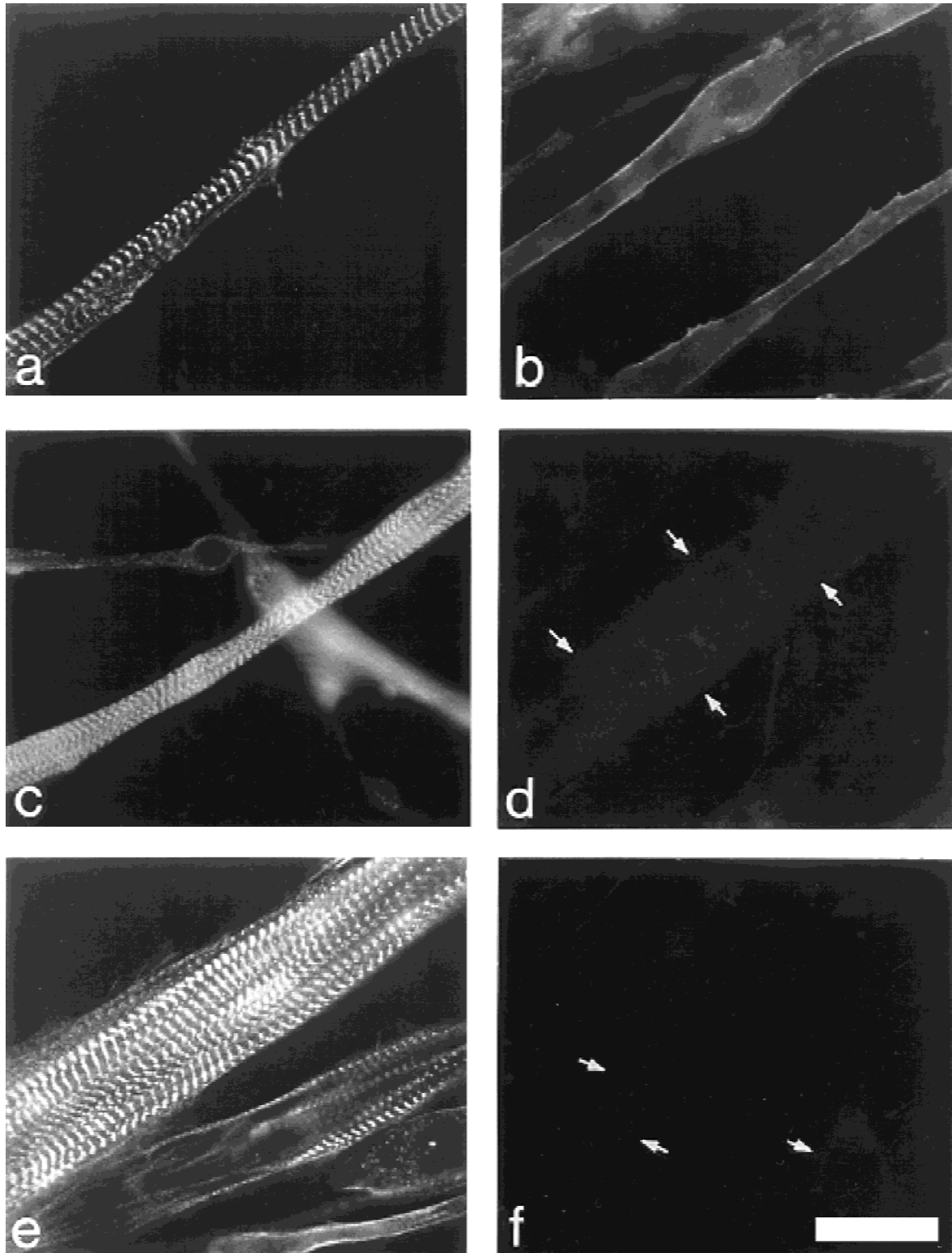
fonic acid, and then centrifuged again for 10,000 *g* for 10 min. The supernatants were stored for a mass of  $\text{IP}_3$  determination by radioreceptor assay.

**$\text{IP}_3$  Radioreceptor Assay.** The mass of endogenous  $\text{IP}_3$  was determined by radioreceptor assay.<sup>2,14</sup> The samples were extracted with perchloric acid and sedimented, and the extracts obtained were neutralized with a buffer containing 2 mol/L KOH.<sup>35</sup> The radioreceptor assay is based on the displacement of bound  $^3\text{H-IP}_3$  to membranes obtained from rat cerebella.<sup>2</sup> Briefly, these membranes were incubated for 20 min at 4°C in 0.5 mL of a solution containing 50 mmol/L Tris/HCl, pH 8.4, 1 mmol/L edetic acid, 1 mmol/L  $\beta$ -mercaptoethanol, 1.6 nmol/L  $^3\text{H-IP}_3$ , and variable concentrations of  $\text{IP}_3$  (1–120 nmol/L) or neutralized extracts. The reaction was stopped by centrifugation, and the radioactivity associated with the pellet was measured. The nonspecific binding was determined in the presence of 2  $\mu\text{mol/L}$   $\text{IP}_3$ .

## RESULTS

The level of differentiation in normal primary (Fig. 1a), dystrophic primary (Fig. 1c), and dystrophic cell line, XLT 4-2 (Fig. 1e) cultures is similar, as indicated by myofibrillar organization. Distinct cross-striated patterns were plainly visible in both normal and dystrophic myotubes with  $\alpha$ -actinin staining. Antidystrophin antibody labeled myotubes continuously on the surface of the sarcolemma in normal primary myotubes (Fig. 1b) and in normal cell line myotubes (results not shown), whereas in the dystrophic cultures, dystrophin immunolabeling resulted in negative staining in both the primary cultures (Fig. 1d) and in XLT 4-2 line cultures (Fig. 1f). In both normal primary and normal cell line cultures, dystrophin labeling increased with maturation of the myotubes. In some rare cases, patchy sarcolemmal staining was found in dystrophic primary and cell line cultures. The only difference observed between the normal and dystrophic cells was the lack of continuous sarcolemmal staining in the latter. In the dystrophic cell line, XLT 4-2, myotubes were similar to the dystrophic primary myotubes.

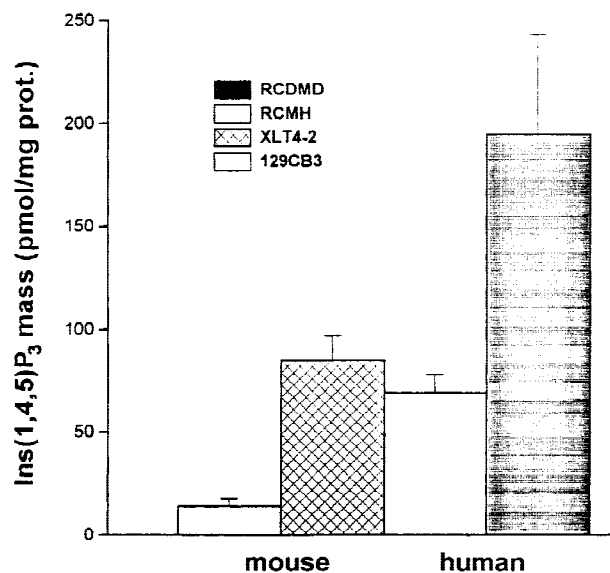
**Mass of  $\text{IP}_3$  in Dystrophic Cells.** The mass of  $\text{IP}_3$  was measured (by a radioreceptor assay) in both normal and dystrophic cell lines; normal human (RCMH) and normal mouse (129CB3) cells were compared to human Duchenne muscular dystrophy (RCDMD) and dystrophic *mdx* (XLT 4-2) cell lines. Basal levels of  $\text{IP}_3$  (Fig. 2) ranged between  $14.2 \pm 3.8$  pmol/mg protein for normal mouse muscle and  $69.2 \pm 8.7$  pmol/mg protein for normal human cells; in dystro-



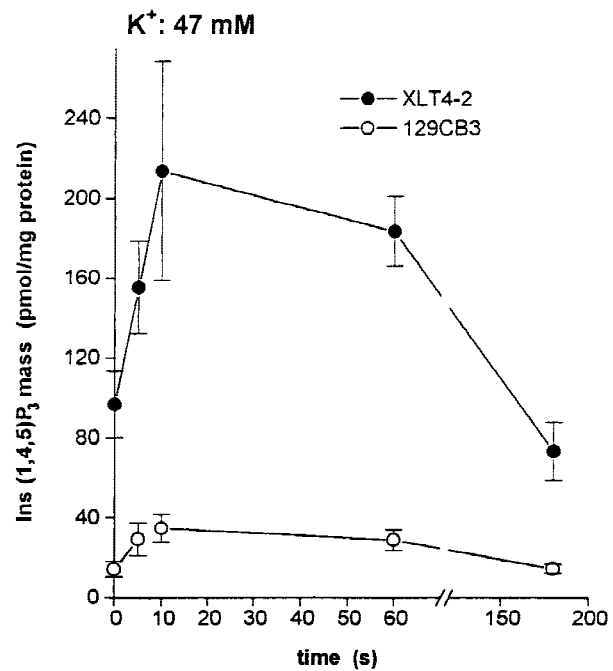
**FIGURE 1.** Immunolabeling of primary normal muscle, primary dystrophic (*mdx*) muscle, and cell line (XLT 4-2) dystrophic muscle. Distinct cross striations are visualized by  $\alpha$ -actinin labeling (**a**, **c**, and **e**). Myofibrillar organization in primary normal muscle (**a**) is not distinguishable from primary dystrophic (**c**) and cell line (**e**) dystrophic muscle. Antidyostrophin staining reveals continuous sarcolemmal localization throughout normal myotubes (**b**). In primary (**d**) and cell line (**f**) dystrophic muscle, negative staining for dystrophin is observed. Arrows indicate the edges of dystrophic myotubes, negatively labeled for dystrophin. Bar = 25  $\mu$ m.

phic cells, the basic levels were three- to sixfold higher than in their normal counterparts, reaching  $96.6 \pm 16.6$  pmol/mg protein in mouse cells and  $194.4 \pm 48.3$  pmol/mg protein in human cells. The possibility that these differences could be attributed to differences in protein content between normal and dystrophic cells can be disregarded, since a comparison between normal 129CB3 cells and dystrophic XLT 4-2 cells gives figures that differ by several fold when they are expressed either as pmol IP<sub>3</sub> per million of cells counted ( $9 \pm 2$  vs.  $35 \pm 5$ ) or as pmol IP<sub>3</sub> per  $\mu$ mol total membrane cholesterol content ( $39 \pm 10$  vs.  $275 \pm 41$ ). The level of IP<sub>3</sub> in cultured muscle cells appears to be regulated by membrane potential, and transient increases in the mass of IP<sub>3</sub> are elicited by potassium-induced depolarization.<sup>20</sup> When both normal and dystrophic cell lines were incubated in high potassium containing solutions (Figs. 3 and 4), we can see that all of the cell lines responded to potassium rise with an increase in the mass of IP<sub>3</sub> from their basal value, reaching a peak value two- to threefold the basal level between 5 and 10 s after depolarization. It is interesting to note that in the human RCDMD cell line (Fig. 4), IP<sub>3</sub> levels remain high up to 200 s after potassium depolarization.

**IP<sub>3</sub> Receptors.** Expression of IP<sub>3</sub> receptors was studied measuring specific binding of <sup>3</sup>H-IP<sub>3</sub> in the



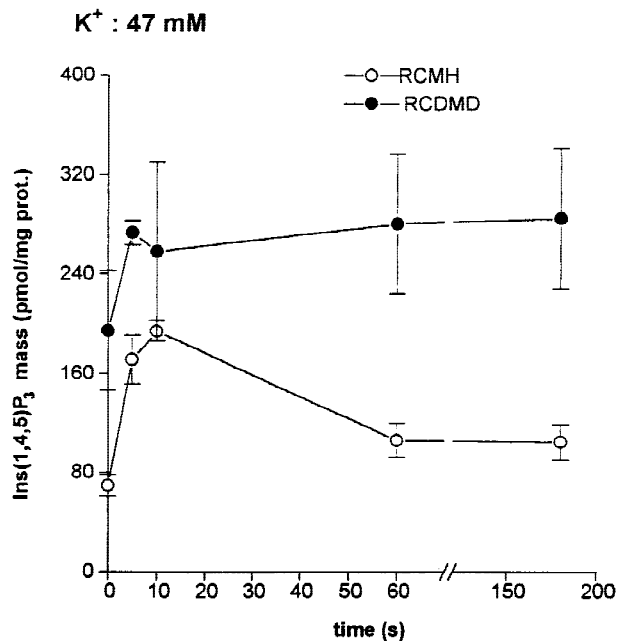
**FIGURE 2.** Basal levels of IP<sub>3</sub> in normal and dystrophic cell lines from humans and mice. Confluent plates of various muscle cell lines in culture were incubated 10 min in resting solution, the reaction was stopped with 0.8 mol/L perchloric acid, and the neutralized extracts were analyzed by radioreceptor assay for IP<sub>3</sub>. Each bar represents the mean  $\pm$  SD of triplicate samples of three independent experiments.



**FIGURE 3.** Time course of K<sup>+</sup>-induced changes in the mass of IP<sub>3</sub> in mice muscle cells. Confluent plates of XLT 4-2 cells were washed three times with PBS, and they were incubated during the times indicated with 47 mmol/L K<sup>+</sup>. The mass of IP<sub>3</sub> in the extract once neutralized was measured by radioreceptor assay. Filled circles XLT 4-2 cell line; open circles, 129CB3 cell line (control). Values were expressed as the mean  $\pm$  SD of at least three independent experiments.

mouse cell lines, normal 129CB3 and dystrophic *mdx* XLT 4-2 (Fig. 5A).

All the cell lines bind <sup>3</sup>H-IP<sub>3</sub> with relatively high affinity ( $K_d$ , between 40 and 100 nmol/L;  $B_{max}$ , between 1 and 5 pmol/mg protein in homogenates). Binding curves can be fitted to a single type of receptor; an example of such a curve is shown in Fig. 5B. The amount of IP<sub>3</sub> receptors is significantly higher in dystrophic cells compared to normal (5 vs. 1 pmol/mg protein in XLT 4-2 vs. 129CB3, Fig. 5B). As indicated in Table 1, a nuclei-enriched fraction shows a threefold higher binding of <sup>3</sup>H-IP<sub>3</sub> as compared with a microsomal fraction; furthermore, more than 60% of the total number of receptors initially found in the homogenate were recovered in the nuclei-enriched fraction, and only about 6% of the IP<sub>3</sub> receptors appear in the light, microsomal fraction (Table 1). On the other hand, the binding of <sup>3</sup>H-ryanodine was significantly higher (77% of the total) in the microsomal fraction, a fact that suggests a distinct distribution of the two different intracellular calcium release channels in cultured muscle cells.



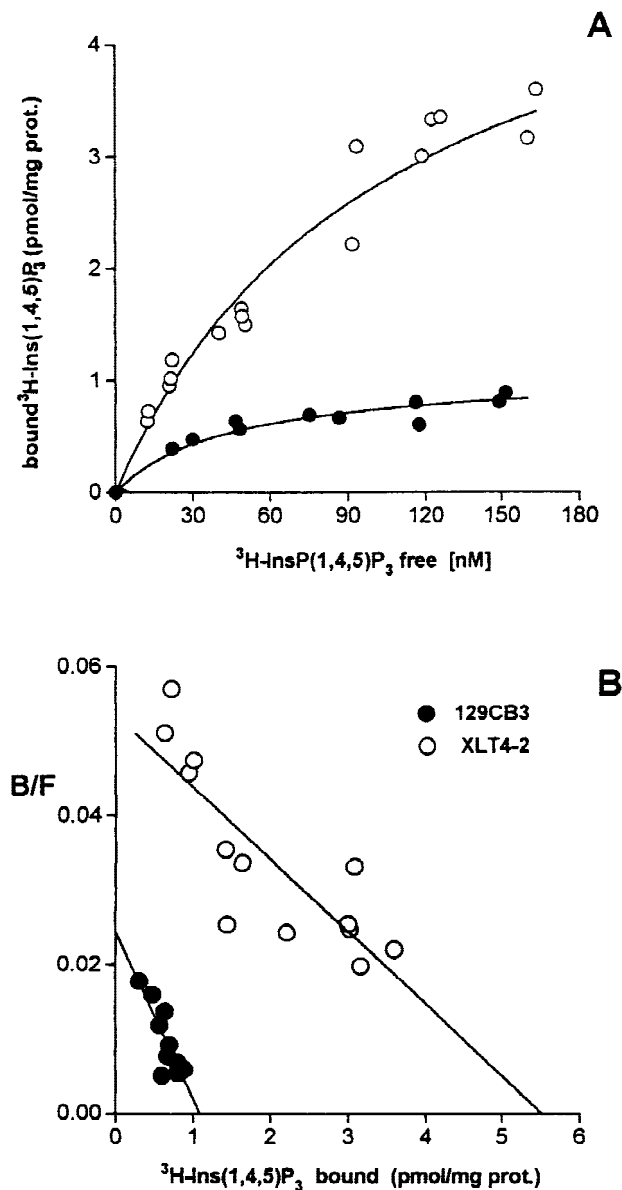
**FIGURE 4.** IP<sub>3</sub> mass changes upon KCl depolarization in normal (RCMH) and dystrophic (RCDMD) human cell lines. Confluent plates of cells were washed three times with PBS, and they were incubated during the times indicated with 47 mmol/L K<sup>+</sup>. The mass of IP<sub>3</sub> in the extract once neutralized was measured by radioreceptor assay. Open circles, RCMH normal human cell line; filled circles, RCDMD dystrophic human cell line. Values were expressed as the mean ± SD of at least three independent experiments.

### DISCUSSION

In this work we used two dystrophic cell lines derived in our laboratories; a cell line (RCDMD), derived from a muscle biopsy taken from a 7-year-old patient with Duchenne muscular dystrophy, was immortalized *in vitro*.<sup>6</sup> Unlike other cell lines established by the same procedure,<sup>7</sup> RCDMD cells were highly refractory to transformation, and the resulting cell line grew slowly, with a doubling time of approximately 48 h. Some characteristics of the cell line include the presence of ion currents and receptors for the dihydropyridine PN200-110 and for  $\alpha$ -bungarotoxin.<sup>6</sup> Unlike the human counterpart, the murine cell line described here has a tendency to form large myotubes with distinct myofibrillar organization upon differentiation; both dystrophic cell lines lack positive staining with antidystrophin antibodies.

A fairly elevated mass of resting IP<sub>3</sub> seems characteristic both for adult muscle<sup>14,27</sup> and for cultured muscle cells<sup>5</sup>; nevertheless, when we compare normal and dystrophic cell lines, we see that the basal levels of IP<sub>3</sub> ranged between 10 and 60 pmol/mg protein in normal cells, but dystrophic cells have basal IP<sub>3</sub> levels two- to threefold higher than their

normal counterparts. These figures do not vary significantly when results are calculated either per  $\mu$ mol of membrane cholesterol or per million of cells, meaning that these IP<sub>3</sub> mass differences are not a consequence of altered cell composition.



**FIGURE 5.** (A) Binding at equilibrium of <sup>3</sup>H-IP<sub>3</sub> to 129CB3 (control) and XLT 4-2 (dystrophic) cell homogenates in complete medium. Confluent plates from control (filled circles) and dystrophic (open circles) cells were washed three times with PBS, homogenized, and incubated in the presence of <sup>3</sup>H-IP<sub>3</sub> (5–200 nmol/L) for 30–40 min. The specific binding was fitted to a single type of receptor in both cases. (B) The Eadie–Scatchard analysis can be fitted to only one type of receptor; the curves were fitted according to the following parameters: K<sub>d</sub> = 103.5 ± 20.1 nmol/L and B<sub>max</sub> = 5.5 ± 0.6 pmol/mg protein for dystrophic cells, and K<sub>d</sub> = 44.7 ± 10.4 nmol/L, B<sub>max</sub> = 1.1 ± 0.1 pmol/mg protein for control cells. B/F, bound–free.

**Table 1.** Binding of  $^3\text{H-IP}_3$  and  $^3\text{H-ryanodine}$  in several XLT 4 cell fractions.

Cell fraction	$^3\text{H-Ins}(1,4,5)\text{P}_3$			$^3\text{H-Ryanodine}$		
	Binding (pmol/mg protein)	$K_d$ (nmol/L)	Total receptors (pmol)	Binding (pmol/mg protein)	$K_d$ (nmol/L)	Total receptors (pmol)
Homogenate	$5.5 \pm 0.7$	$103 \pm 20$	162	$1.1 \pm 0.3$	$20 \pm 1.3$	20.4
nuclear	$3.7 \pm 0.2$	$40 \pm 5$	99	$0.6 \pm 0.1$	$16 \pm 5.5$	3.7
microsomal	$1.2 \pm 0.1$	$44 \pm 7$	10	$1.7 \pm 0.1$	$17 \pm 2.8$	15.8

Confluent plates of XLT 4-2 cells (3–5 days), were rinsed with PBS, harvested with a rubber policeman, and homogenized with Dounce. The homogenate was centrifuged at 1000 g for 10 min (nuclear fractions), and the supernate was centrifuged at 5000 g for 10 min. Finally the supernate was centrifuged to >90,000 g for 90 min. The pellet obtained was called the microsomal fraction. The binding of  $^3\text{H-IP}_3$  and  $^3\text{H-ryanodine}$  was measured in all fractions, in the presence of both 2  $\mu\text{mol/L IP}_3$  and 10  $\mu\text{mol/L}$  of ryanodine for nonspecific binding. The values are expressed as means  $\pm$  SD of at least three different preparations.

We report a significant increase in  $\text{IP}_3$  mass shortly after exposure of muscle cell lines to depolarizing agents. These results agree with earlier reports linking  $\text{IP}_3$  metabolism to muscle electrical activity.<sup>13,36</sup> The level of  $\text{IP}_3$  in cultured muscle cells appears to be regulated both by membrane potential, through the  $\alpha$ -1 subunit of the dihydropyridine receptor,<sup>20</sup> and by muscarinic acetylcholine receptors.<sup>31</sup> In dystrophic cell lines the kinetics of the  $\text{IP}_3$  rise response to depolarization is similar to that of their normal counterparts, starting, however, in the dystrophic cells from a higher basal level. The falling phase of the  $\text{IP}_3$  transient is nevertheless slower in dystrophic cells, especially in the RCDMD cell line.

The present results raise the possibility that  $\text{IP}_3$  could induce an increase in intracellular calcium in muscle cells, provided that  $\text{IP}_3$ -sensitive calcium stores are present. A high content of  $\text{IP}_3$  receptors was reported in cultured rat muscle<sup>17</sup> and, in the present study, even higher levels of  $\text{IP}_3$  receptors were evident in dystrophic cells (5 vs. 1 pmol/mg protein in dystrophic, XLT 4-2 vs. normal, 129CB3 cells). The presence of  $\text{IP}_3$  receptors in skeletal muscle cells has been shown only recently,<sup>17,24,29</sup> and their precise role has not been established. Higher levels of  $\text{IP}_3$  receptors when compared to ryanodine receptors suggest that they may be involved in important cellular functions; when comparing normal and dystrophic mice cells, several results are worth noticing. First, binding curves can be fitted equally well assuming either one or two types of receptors. If  $\text{IP}_3$  receptors are heterogeneous in nature (as have been described in other tissues<sup>10</sup>), their dissociation constants will not differ by a factor higher than 3 or 4.<sup>16,21</sup> In fact, dissociation constants described for  $\text{IP}_3$  receptors in the literature are similar to those found in this work, and their value for a given type of receptor can vary depending on external conditions.<sup>37</sup> Receptors were preferentially

found in a heavy nuclear fraction, a location clearly different from that of ryanodine receptors, which were concentrated in a light microsomal fraction. Considering both the procedure used (we recovered only 68% of the receptors), and the fact that the specific binding activity in the homogenate is equal to or higher than that of the richest fraction, we cannot rule out the presence of receptors in either a soluble fraction or hidden after sealing of membrane vesicles in the partly purified fractions tested.

In many cell types the binding of  $\text{IP}_3$  to specific receptors opens  $\text{Ca}^{2+}$ -channels and causes  $\text{Ca}^{2+}$  release from intracellular  $\text{Ca}^{2+}$  stores.<sup>3</sup> However, the role of this mechanism in the fast calcium transients needed for twitch skeletal muscle excitation-contraction coupling remains controversial.<sup>18</sup> We have proposed a role for slow  $\text{IP}_3$  transients in regulating nucleoplasmic calcium in muscle cells.<sup>17</sup> We have shown<sup>17</sup> both the occurrence of slow calcium transients associated with cell nuclei and (see also the present results) the presence of  $\text{IP}_3$  receptors in nuclei-enriched fractions in cultured muscle cells. Intracellular calcium controls a number of cell functions, and it is particularly important during cell differentiation. There is good evidence that the control of nucleoplasmic calcium concentration may be associated with gene regulation.<sup>4,11,22</sup> The fact that both the total mass of  $\text{IP}_3$  and the number of  $\text{IP}_3$  receptors are significantly higher in dystrophic myotubes when compared to normal cells opens the possibility that the  $\text{IP}_3$  receptor-mediated calcium regulation in dystrophic muscles may be altered. A feedback mechanism that would imply down-regulation of receptors upon increased levels of ligand (free  $\text{IP}_3$ ) is apparently absent in dystrophic cells and could be the cause of altered calcium levels. Regulation of intracellular calcium has been reported to be altered in dystrophic muscle cells both *in vivo*<sup>23</sup> and *in vitro*<sup>28</sup>, if diseased cells have a defec-

tive intranuclear calcium regulation, it could be a sign (either the cause or the consequence) of abnormal gene expression activity, an important issue in understanding the evolution of damage and regeneration in dystrophin-defective cells. Should the IP<sub>3</sub> signaling pathway be implicated in the pathophysiology of Duchenne muscular dystrophy, it will allow completely new therapeutic approaches to be developed.

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