Involvement of Inositol 1,4,5-Trisphosphate in Nicotinic Calcium Responses in Dystrophic Myotubes Assessed by Near-plasma Membrane Calcium Measurement*

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In skeletal muscle cells, plasma membrane depolarization causes a rapid calcium release from the sarcoplasmic reticulum through ryano dine receptors triggering contraction. In Duchenne muscular dystrophy (DMD), a lethal disease that is caused by the lack of the cytoskeletal protein dystrophin, the cytosolic calcium concentration is known to be increased, and this increase may lead to cell necrosis. Here, we used myotubes derived from control and mdx mice, the murine model of DMD, to study the calcium responses induced by nicotinic acetylcholine receptor stimulation. The photoprotein aequorin was expressed in the cytosol or targeted to the plasma membrane as a fusion protein with the synapto-some-associated protein SNAP-25, thus allowing calcium measurements in a restricted area localized just below the plasma membrane. The carbachol-induced calcium responses were 4.5 times bigger in dystrophic myotubes than in control myotubes. Moreover, in dystrophic myotubes the carbachol-mediated calcium responses measured in the subsarcolemmal area were at least 10 times bigger than in the bulk cytosol. The initial calcium responses were due to calcium influx into the cells followed by a fast refilling/release phase from the sarcoplasmic reticulum. In addition and unexpectedly, the inositol 1,4,5-trisphosphate receptor pathway was involved in these calcium signals only in the dystrophic myotubes. This surprising involvement of this calcium release channel in the excitation-contraction coupling could open new ways for understanding exercise-induced calcium increases and downstream muscle degeneration in mdx mice and, therefore, in DMD.

Duchenne muscular dystrophy (DMD) is an X-linked disease that affects about 1 in 3500 males. DMD results in progressive muscle degeneration (1) that ultimately leads to premature death by respiratory or cardiac failure during the third decade. Pharmacological interventions such as glucocorticoids improve the quality of life of patients (2). DMD is caused by the absence of dystrophin, a 427-kDa protein localized under the plasma membrane (3). In normal skeletal muscle fibers, dystrophin is associated with a glycoprotein complex and provides a linkage between the extracellular matrix and the cytoskeleton. This complex tends to stabilize the plasma membrane (4). Numerous studies have shown that the absence of dystrophin in DMD impairs the stability of the plasma membrane, resulting in a greater fragility toward mechanical stress and increased permeability to calcium (Ca$^{2+}$) (5–9). Indeed, it has been proposed that an alteration of Ca$^{2+}$ homeostasis might be responsible for the muscle degeneration that occurs in muscle fibers from DMD patients or in those of the mouse model of DMD, the mdx mouse (10–12). Elevations of cytosolic or near-plasma membrane Ca$^{2+}$ concentrations in myotubes and skeletal muscle fibers from DMD patients or mdx mice have been reported (13, 14). How this increased entry of Ca$^{2+}$ affects the local concentration of Ca$^{2+}$ in subcellular compartments and whether this process is involved in the development of the disease is still unclear (15). However it has already been suggested that altered Ca$^{2+}$ homeostasis and fiber degeneration in mdx mice is “use-dependent” (16–18). In skeletal muscle cells and myotubes, it has been shown that activation of nicotinic receptors leads to plasma membrane depolarization, which triggers a voltage-gated Ca$^{2+}$ channel opening. Contraction is then triggered by the release of Ca$^{2+}$ from the sarcoplasmic reticulum through the opening of ryanodine receptors, which are activated by voltage-gated Ca$^{2+}$ channels (19). However, although the role of ryanodine receptors and voltage-gated Ca$^{2+}$ channels are well established in excitation-contraction coupling, the skeletal muscle sarcoplasmic reticulum possesses another type of Ca$^{2+}$ release channel, IP$_3$ receptors (20–22). In many cells, increases in intracellular Ca$^{2+}$ are mediated by the inositol 1,4,5-trisphosphate pathway in the following manner. The activation of numerous G-protein-coupled receptors triggers hydrolysis of phosphatidylinositol 4,5-bisphosphate to generate IP$_3$, which triggers the opening of IP$_3$ receptors, and intracellular Ca$^{2+}$ increases (23). Furthermore, IP$_3$ receptors may also be involved in the control of Ca$^{2+}$ influx through plasma membrane store-operated Ca$^{2+}$ channels (24–27).

In skeletal muscle cells, IP$_3$ receptors are localized in the plasma membrane store-operated Ca$^{2+}$ channels; PSS, physiological salt solution; SNAP-25, synaptosome-associated protein of 25 kDa.
nuclear envelope as well as in the sarcomplasmic reticulum (20, 22). In addition, it has been suggested that the intracellular messenger IP₃ may contribute to excitation-contraction coupling (28). Indeed, it has been shown that IP₃ can potentiate depolarization-induced Ca²⁺ release or produce the contraction of intact or skinned skeletal muscle fibers (29–31). These results suggest that IP₃ may play a role in excitation-contraction coupling in skeletal muscle cells. Furthermore, it has also been shown that the plasma membrane depolarization that activates voltage-gated Ca²⁺ channels can induce IP₃ production and IP₃ receptor activation in myotubes with a mechanism involving a G-protein (20, 32, 33).

The mechanisms involved in Ca²⁺ responses during physiological stimulation (using nicotinic receptor agonists) have not been studied in dystrophic myotubes to date. Here, we have studied the IP₃ receptor pathway in dystrophic myotubes using plasma membrane-targeted aequorin (SNAP-25 aequorin) for measuring the near-plasma membrane Ca²⁺ concentration. We show here that SNAP-25 aequorin is a reliable tool to measure Ca²⁺ increases occurring just below the plasma membrane. We also show that nicotinic receptor stimulation triggers near-plasma membrane Ca²⁺ increases that depend on IP₃ receptor activation in myotubes derived from mdx mice only, thus showing a non-common pathway for excitation-contraction coupling.

**EXPERIMENTAL PROCEDURES**

**Cell Culture**—Cultures of purified myoblasts were prepared in Petri dishes (Falcon; BD Sciences) and maintained at 37 °C in a water-saturated atmosphere of 95% air and 5% CO₂.

The cell cultures were obtained as described previously (34) with minor modifications. Briefly, 3-week-old mdx C57Bl/10 and control C57Bl/10 mice were killed by cervical dislocation, extensor digitorum longus (EDL) muscle was aseptically removed bilaterally, cleaned of its tendinous ends and adhering connective tissue, and minced into small pieces of ~1 mm³. Individual muscles from two mice were pooled. After the muscle tissues were rinsed in DMEM (Invitrogen), digestion was performed by three successive rounds of incubation in 10 ml of DMEM supplemented with 10 mM HEPES (pH 7.4), 10% fetal calf serum (Invitrogen), and 0.15% Pronase (Roche Applied Science) at 37 °C. After the first incubation (5 min), the tissues were triturated with a serological pipette, and the supernatant, containing predominantly small cells and connective debris, was discarded. The second and third incubations lasted 20 min each with frequent triturations. The supernatants were collected, pooled, filtered through a 40-µm mesh cell strainer (Falcon; BD Biosciences), and centrifuged at 300 × g for 10 min. The cell pellets were resuspended in growth medium (see below), and the suspensions were plated onto collagen type I-coated (1 µg/ml; Sigma) 100-mm diameter Petri dishes, one dish per original muscle. Growth medium was composed of a 1:1 (v/v) mixture of DMEM and MCDB202 (CryoBioSystem) supplemented with 20% fetal calf serum, 2% Ultrasor® SP (Biosepra SA), antibiotics (Ciprinax; Bayer), and NaHCO₃ from concentrated solution (Invitrogen) to get 2.6 g/liter in complete medium. Myoblasts were plated on coated dishes and grown for ~2 days in growth medium. For the experiments, cells were transfected (see below) and induced to fuse by changing the growth medium to differentiation medium (DMEM supplemented with 1.7% fetal calf serum, 3.3% horse serum (Sigma), 10 µg/ml insulin (Fluka), and Ciprinax).

**Transfection**—Myoblasts were plated at 12,000 cells/cm² on 13-mm Thermonax coverslips (Naige Nunc International) in four-well plates. When 80–90% confluent, growth medium was removed and replaced with a serum-free medium, Opti-MEM I (Invitrogen). Cells were transfected overnight using LipofectAMINE 2000 (Invitrogen) at a ratio of 1 µg of DNA per 2 µl of transfection reagent. The DNA-LipofectAMINE 2000 complex was prepared in Opti-MEM I medium. After overnight incubation, this medium was replaced by a differentiation medium. Myotubes were used 3 or 4 days after differentiation.

**Plasmids**—The aequorin plasmids were gifts from T. Pozzan, Padova, Italy. Cells were transfected with a pcDNAI expression vector containing a cDNA encoding either green fluorescent protein (GFP) for localization or wild type aequorin for Ca²⁺ measurement, both fused with the SNAP-25 targeting sequence to measure subsarcomembranous calcium (pmCa²⁺) (35) or with cytosolic aequorin to measure cytosolic calcium concentrations ([Ca²⁺]ᵣ) (13). The IP₃, cPspase plasmid was a gift from H. L. Roderick and M. D. Bootman, Cambridge, United Kingdom.

Cells were co-transfected with a pcDNAI expression vector (Microbiex Biosystems Inc.) containing a cDNA encoding enhanced GFP, the high affinity IP₃, sponge (36), and a pcDNAI expression vector containing a cDNA encoding the SNAP-25 aequorin.

**Confocal Microscopy**—Mdx myotubes were grown in plastic culture dishes and transfected with a pcDNAI expression vector containing the cDNA encoding a GFP-tagged SNAP-25 targeting sequence. Confocal imaging was performed on a living myotube on a LSM 510 Meta confocal scanner mounted on an upright Axioskop 2 FS microscope (Carl Zeiss). Fluorescence was excited at 488 nm, and emission was detected at 505–530 nm using a 40 × 0.8 numerical aperture water immersion objective (Achroplan, Carl Zeiss). Simultaneous differential interference contrast images of cells were recorded on the transmitted light detector with the polarized 488-nm light used for GFP fluorescence excitation.

**Intracellular Calcium Measurement**—After 3 or 4 days of differentiation, subsarcomembral Ca²⁺ in counts per minute was determined in a population of myotubes as described previously with minor modifications (37). Briefly, the SNAP-25 aequorin was reconstituted in a Ca²⁺-free physiological salt solution (PSS) (145 mM NaCl, 5 mM KCl, 1 mM MgCl₂, 5 mM HEPES, and 10 mM glucose, pH 7.6) containing 0.1 mM EGTA and 5 µM coelenterazine (Calbiochem) for 1 h before the experiment to decrease the [Ca²⁺]ᵣ within the cell. Cells were superfused at a rate of 1 ml/min in a custom-made 0.5-ml chamber thermostated at 37 °C (MegaTest, Geneva, Switzerland). Emitted luminescence was detected at 466 nm with a photomultiplier apparatus (EMI 9789A, Electron Tubes Limited, United Kingdom) and recorded every second using a computer photon-counting board (EMI C660) as described previously (38). The relationship between recorded counts and [Ca²⁺]ᵣ is shown in Equation 1 (39),

$$[Ca^{2+}]_r (\text{nM}) = \frac{\text{ratio}}{1 + \text{ratio} \times K_R} - 1$$

where $L$ represents the recorded photons/s and $L_{max}$ represents the remaining photons that correspond to the total light output during the whole experiment minus the photons emitted up to the measured point. $K_R$ and $K_a$ are the parameters of the aequorin and $n$ is the number of Ca²⁺ binding sites. At 37 °C, $K_{a} = 120, K_R = 1.01E+07 M^{-1}$, and $n = 2.99$. Total light output was obtained by exposing cells to 10 mM CaCl₂ after permeabilization with 100 µM digitonin to consume all the aequorin.

**45Ca²⁺ Influx Measurement—**Influx measurement was performed using 45Ca²⁺ (5–50 µCi/ml; PerkinElmer Life Sciences) as described with minor modifications (40). Briefly, myoblasts were washed twice and preincubated at 37 °C for 10 min in 250 µl of the PSS solution described above also containing 0.12 mM CaCl₂ and, eventually, the indicated inhibitor. 45Ca²⁺ influx was initiated by incubation with 200 µl of PSS containing 1.2 mM CaCl₂ alone or with 100 µM carbachol (CCh) and 0.4 nCi of 45Ca²⁺ for 10 min at 37 °C. The cells were subsequently washed four times with ice-cold PSS containing no CaCl₂ but 0.1 mM EGTA (to remove extracellularly bound 45Ca²⁺). Cells were lysed with 250 µl of 1% SDS (w/v). The radioactivity of the lysate was measured by scintillation counting (Packard 460C, Zurich, Switzerland). Values are expressed as mean ± S.E.

**Data Analysis—**Data analysis was performed using the software GraphPad Prism (GraphPad Software, San Diego, CA) and Matlab (The MathWorks Inc, Natick, MA). Results are expressed as means ± S.E. Statistical significance of the different values was assessed with the unpaired Student’s t test or one-way analysis of variance test followed by a Dunnett test. p > 0.05 was considered not significant; p values ≤ 0.05 were considered significant.

**RESULTS**

As the removal of intracellular Ca²⁺ is required for the complete reconstitution of aequorin (41), SNAP-25 aequorin—expressing cells were preincubated in Ca²⁺-free PSS containing 0.1 mM EGTA for 1 h before performing the experiment. For each experiment the basal level of luminescence was recorded for 1 min before performing the experiment. The stimulus applied was PSS containing 1.2 mM CaCl₂ and the test compounds.

**SNAP Aequorin Localization—**To investigate the localization of the SNAP-25 aequorin, cells were transfected with cDNA encoding the GFP fused with the SNAP-25 targeting sequence. Fig. 1 shows a representative confocal section of a transfected living mdx myotube after 3 days of differentiation.
SNAP-25-GFP-mediated fluorescence (Fig. 1A) appeared to be mainly localized at the sarcolemma when compared with the localization assessed with differential interference contrast imaging (Fig. 1, B and C). However, some SNAP-25-GFP fluorescence was detected in the cytosol, which could correspond to a non-palmitoylated protein (35). Similar results were obtained with control C57 myotubes (data not shown).

To further investigate the localization of the SNAP-25 aequorin, cells were incubated for 10 min with the high affinity Ca\(^{2+}\)/H\(^{+}\) chelator BAPTA-AM to buffer cytosolic Ca\(^{2+}\)/H\(^{+}\). Cytosolic Ca\(^{2+}\)/H\(^{+}\) responses assessed with cytosolic aequorin triggered by CCh and Ca\(^{2+}\)/H\(^{+}\) readdition were completely inhibited in cells loaded with BAPTA-AM (Fig. 2A). In contrast, in cells transfected with SNAP-25 aequorin the maximal Ca\(^{2+}\) responses and time to peak values (17.50 ± 2.0 s and 20.17 ± 2.7 s) were not significantly reduced by BAPTA-AM (Fig. 2B). These results indicate that Ca\(^{2+}\) responses measured with SNAP-25 aequorin were due to Ca\(^{2+}\) increases in a restricted area under the plasma membrane, presumably where BAPTA-AM had very limited access. Moreover, as shown in Fig. 2C, the Ca\(^{2+}\) peaks triggered by these stimuli were 10 times larger when measured with SNAP-25 aequorin than with cytosolic aequorin (4.97 ± 0.66 and 0.53 ± 0.04 μM, respectively). Altogether, these results indicate that SNAP-25 aequorin was mainly localized at the plasma membrane. Therefore the probe appears to be a reliable tool for measuring near-plasma membrane Ca\(^{2+}\) concentrations.

CCh-induced Ca\(^{2+}\) Responses in Control and Dystrophic Cells—To test the hypothesis that the IP\(_3\) receptor pathway could be involved in acetylcholine receptor activation, we measured the effects of the nicotinic agonist carbachol (100 μM) and the effects of the potent IP\(_3\) receptor inhibitor, 2-aminoethoxydiphenyl borate (2-APB) (42), on subsarcolemmal Ca\(^{2+}\) transients in both control and dystrophic myotubes. As shown in Fig. 3, 100 μM CCh together with 1.2 mM Ca\(^{2+}\) triggered peak pm[Ca\(^{2+}\)] increases of 1.06 ± 0.11 μM for control cells and 4.97 ± 0.66 μM for dystrophic cells, representing a 4.5-fold increase. When cells were incubated for 10 min with 75 μM 2-APB, the CCh-induced Ca\(^{2+}\) response was not significantly changed in control myotubes but was significantly decreased in dystrophic myotubes (from 4.97 ± 0.66 to 1.63 ± 0.21 μM). These results therefore suggest that the Ca\(^{2+}\)-release pathways involved in CCh responses are different between control and dystrophic myotubes and that the IP\(_3\) pathway may be involved in CCh-induced Ca\(^{2+}\) responses. We then investigated the pathway involved in dystrophic myotubes.

Ca\(^{2+}\) Increases Triggered by CCh Are Mediated by Nicotinic Receptors—The application of CCh together with Ca\(^{2+}\) trig-
gated pmCa\(^{2+}\) increases of a maximal amplitude of 4.97 \pm 0.66 \mu M. As shown in Fig. 4, C and E, preincubation of cells for 10 min with atropine (100 \mu M), a selective muscarinic receptor antagonist, did not affect the maximal amplitude of the Ca\(^{2+}\)-induced Ca\(^{2+}\) peak. On the other hand, 10 min of incubation with the highly selective nicotinic receptor antagonist d-tubocurarine (10 \mu M) decreased CCh-induced Ca\(^{2+}\) responses from 4.97 \pm 0.66 \mu M in non-treated cells to 1.47 \pm 0.03 \mu M in the presence of the compound (Fig. 4, D and E). Average CCh responses in the presence of d-tubocurarine were not statistically different from the Ca\(^{2+}\) responses triggered by Ca\(^{2+}\) readdition alone. Altogether, these results indicate that CCh-induced Ca\(^{2+}\) increases were mediated by the activation of nicotinic receptors.

**Initial Ca\(^{2+}\) Influx Induced by CCh—Ca\(^{2+}\) increases triggered by CCh may rely on Ca\(^{2+}\) influx through plasma membrane channels.** Fig. 5A shows that CCh-induced Ca\(^{2+}\) increases were triggered by an influx through plasma membrane Ca\(^{2+}\) channels. Indeed, incubation for 10 min with 1 mM cadmium, a non-selective Ca\(^{2+}\) channel inhibitor (43), drastically decreased the CCh-induced Ca\(^{2+}\) response (from 4.97 \pm 0.66 \mu M for non-treated cells to 0.46 \pm 0.02 \mu M for treated cells). Moreover, CCh had no effect on the pmCa\(^{2+}\) when added to a Ca\(^{2+}\)-free solution, whereas the addition of CCh together with Ca\(^{2+}\) led to a Ca\(^{2+}\) response (Fig. 5B). These results strongly suggest that the Ca\(^{2+}\) responses were triggered at least in part by Ca\(^{2+}\) influx through plasma membrane Ca\(^{2+}\) channels.

We then tested the effect of nifedipine (1 \mu M), a potent and selective blocker of L-type voltage-gated Ca\(^{2+}\) channels, on the CCh-induced Ca\(^{2+}\) response. Results indicated that L-type voltage-gated Ca\(^{2+}\) channel activation was involved in the response triggered by CCh (Fig. 5C). To confirm that this response was truly due to influx, we used the 45Ca\(^{2+}\) influx technique. As shown in Fig. 5D, Ca\(^{2+}\) influx was increased from 638 \pm 51 cpm/well with Ca\(^{2+}\) alone to 910 \pm 69 cpm/well with 100 \mu M CCh. Moreover, when cells were incubated with 1 \mu M nifedipine and both Ca\(^{2+}\) and CCh, the Ca\(^{2+}\) influx was decreased (591 \pm 13 cpm/well). These results confirm that CCh triggered Ca\(^{2+}\) influx through voltage-gated L-type Ca\(^{2+}\) channels.

**Quick Sarcoplasmic Reticulum Ca\(^{2+}\) Refilling and Ca\(^{2+}\) Release—**Results from experiments described above have shown that in dystrophic myotubes CCh-induced Ca\(^{2+}\) responses were at least in part due to Ca\(^{2+}\) influx. To go further, we tested the effect of the sarcoplasmic-endoplasmic reticulum Ca\(^{2+}\) ATPase (SERCA) blocker thapsigargin (44). As shown in Fig. 6, incubation for 10 min with 1 \mu M thapsigargin decreased the CCh-induced Ca\(^{2+}\) response from 4.97 \pm 0.66 to 1.92 \pm 0.24 \mu M for the thapsigargin-treated cells, which is very similar to the response triggered by Ca\(^{2+}\) alone (1.84 \pm 4.4 \mu M). Altogether these results show that the
CCh-induced Ca\(^{2+}\) transients are due to both Ca\(^{2+}\) influx and Ca\(^{2+}\) release from the sarcoplasmic reticulum.

Involvement of the IP\(_3\) Pathway in CCh-induced Response—
Results shown in Fig. 3 suggest that IP\(_3\) pathway may be involved in CCh-induced Ca\(^{2+}\) response in dystrophic myotubes. To test the involvement of this pathway, we investigated the effect of U73122, a potent phospholipase C (PLC) inhibitor (45). Indeed, PLC is required for IP\(_3\) formation through phosphatidylinositol 4,5-bisphosphate cleavage. As shown in Fig. 7, incubation of the cells with 20 \(\mu M\) U73122 decreased the CCh-induced Ca\(^{2+}\) peak amplitude from 4.97 ± 0.66 \(\mu M\) for non-treated cells to 2.76 ± 0.19 \(\mu M\) for treated cells. This result

**Fig. 4.** Nicotinic receptors are involved in the CCh-induced response. A–D, representative traces of subsarcolemmal Ca\(^{2+}\) increases in a population of EDL-derived mdx myotubes showing the effect of 1.2 mM Ca\(^{2+}\) readdition (A), 1.2 mM Ca\(^{2+}\) readdition together with 100 \(\mu M\) CCh (B), 100 \(\mu M\) atropine (C), and 10 \(\mu M\) \(d\)-tubocurarine (D). Arrows indicate the beginning of stimulation with CCh. E, summarizing histogram; the bar graphs correspond to mean pm\([Ca^{2+}]\) values ± S.E. (n = 6; ns, not significant (p > 0.05); * and ** correspond to a significant inhibition (p < 0.05 and 0.01 and p < 0.001, respectively) using a one-way analysis of variance test followed by a Dunnett test to compare all of the columns with the 1.2 mM Ca\(^{2+}\) and 100 \(\mu M\) CCh bar).
suggests that the PLC/IP3 pathway may be involved in the CCh-mediated Ca2+ response. Moreover, we tested the effect of xestospongin D, a potent blocker of IP3 receptors, at a very low concentration (0.1 μM) (46). Preincubation of the cells for 10 min with this compound decreased the CCh-induced Ca2+ responses from 4.97 ± 0.66 to 2.44 ± 0.34 μM.

Because 2-APB and xestospongin D may have nonspecific effects (47, 48) we co-transfected cells with both a high affinity IP3 sponge and SNAP-25 aequorin. This IP3 sponge has been shown to behave as an IP3 chelator and to inhibit IP3-mediated Ca2+ responses (36). Again, the CCh-induced Ca2+ peak was strongly decreased in co-transfected cells (from 4.97 ± 0.66 to 0.72 ± 0.13 μM) and was below that of the response due to Ca2+ alone. Altogether, these results clearly show that the IP3 pathway was involved in CCh-induced Ca2+ response in our mdx myotubes.

**DISCUSSION**

In this study we used SNAP-25, a plasma membrane protein, to target the Ca2+ sensitive photoprotein aequorin to the subsarcolemmal area of both control and dystrophic myotubes. Because of the complexity (different degrees of maturation) and density (heavy cytoskeleton, actin, and myosin) of skeletal muscle cells, we first checked the localization of the probe. This was investigated by confocal microscopy using SNAP-25-GFP on living mdx myotubes. Results suggest that this probe and, therefore, also SNAP-25 aequorin was preferentially localized at the sarcolemma. Confocal sections of mdx myotubes also showed some cytosolic fluorescence that corresponds to non-targeted protein or, most probably, non-palmitoylated SNAP-25. Indeed, working with live cells indicates that proteins are continuously expressed, and it has been shown previously that the SNAP-25 protein must be palmitoylated on cysteine resi-
bigger when measured under the plasma membrane rather than in the cytosol. This is very likely due to the fact that SNAP-25 aequorin is very close to Ca\textsuperscript{2+} channels and also that Ca\textsuperscript{2+} increases may occur in a physically restricted area, which prevents diffusion of Ca\textsuperscript{2+} in the bulk cytosol.

CCh-induced pm[Ca\textsuperscript{2+}] increases were 4.5 times bigger in dystrophic myotubes than in control myotubes. This result is in agreement with previous results obtained in muscle fibers that also showed a subsarcolemmal Ca\textsuperscript{2+} overload due to enhanced Ca\textsuperscript{2+} influx in dystrophic fibers as compared with control (14). Moreover, we found that 2-APB, an IP\textsubscript{3} receptor inhibitor, strongly decreased the CCh-induced Ca\textsuperscript{2+} responses in dystrophic but not in control C57 myotubes. These results also suggest that the mechanisms involved in CCh-induced subsarcolemmal increases may be different between control and dystrophic myotubes.

In mdx myotubes, CCh was able to trigger Ca\textsuperscript{2+} increases only when Ca\textsuperscript{2+} was added and CCh-induced Ca\textsuperscript{2+} responses were completely blocked by cadmium ion, a non-selective Ca\textsuperscript{2+} channel blocker. This result indicates that CCh-induced Ca\textsuperscript{2+} increases after preincubation in a Ca\textsuperscript{2+}-free solution are triggered by Ca\textsuperscript{2+} influx through plasma membrane Ca\textsuperscript{2+} channels. Moreover, CCh-induced Ca\textsuperscript{2+} responses were partially inhibited by nifedipine, showing that t-type voltage-gated Ca\textsuperscript{2+} channel activation is involved in CCh-induced Ca\textsuperscript{2+} responses. When cells were incubated with the sarcoplasmic-endoplasmic reticulum Ca\textsuperscript{2+} ATPase blocker thapsigargin, the CCh-induced Ca\textsuperscript{2+} responses were significantly decreased and reached the same amplitude as the response triggered by Ca\textsuperscript{2+} readdition. Altogether, these results suggest that CCh-induced Ca\textsuperscript{2+} responses are due to Ca\textsuperscript{2+} influx through plasma membrane Ca\textsuperscript{2+} channels followed by a fast Ca\textsuperscript{2+} refilling of the sarcoplasmic reticulum and a secondary Ca\textsuperscript{2+} release from the store.

In mdx myotubes, CCh-induced pm[Ca\textsuperscript{2+}] increases were significantly reduced when cells were incubated with two potent IP\textsubscript{3} receptor inhibitors (2-APB and xestospongion D) or when cells were co-transfected with an IP\textsubscript{3} sponge together with SNAP-25 aequorin. These results demonstrate that IP\textsubscript{3} receptors are involved in CCh-induced Ca\textsuperscript{2+} increases in dystrophic myotubes. Consistently, CCh-induced Ca\textsuperscript{2+} increases were also inhibited by the phospholipase C inhibitor U73122. Altogether, these results indicate that nicotinic receptor activation triggers PLC activation and IP\textsubscript{3} release in our dystrophic myotubes, raising a question about the mechanism of PLC activation. It has been shown recently that the activation of voltage-gated Ca\textsuperscript{2+} channels by potassium chloride depolarization can activate PLC in myotubes by a mechanism involving a G-protein (32). This could indeed be the case in our dystrophic myotubes, as CCh triggers activation of t-type voltage-gated Ca\textsuperscript{2+} channels. PLC may therefore be activated by near-plasma membrane Ca\textsuperscript{2+} increases due to Ca\textsuperscript{2+} influx occurring through nicotinic receptors and/or voltage-gated Ca\textsuperscript{2+} channels (51). Altogether, these results indicate that CCh-induced Ca\textsuperscript{2+} increases rely on IP\textsubscript{3} receptor activation in dystrophic myotubes. On the other hand, 2-APB was without effect on CCh-induced Ca\textsuperscript{2+} responses in control myotubes, indicating that the IP\textsubscript{3} pathway is not involved in nicotinic responses in this cell type.

However, recent studies have shown that 2-APB is also a potent inhibitor of channels activated by the depletion of the Ca\textsuperscript{2+} stores (store-operated channels or SOCs) (52, 53). Indeed, since our cells were incubated for 1 h in a Ca\textsuperscript{2+}-free solution for complete aequorin reconstitution, Ca\textsuperscript{2+} stores have been depleted. These conditions raise the question about the involvement of SOCs in the CCh-induced Ca\textsuperscript{2+} responses. Our results...
with thapsigargin and IP$_3$ pathway inhibitors strongly suggest that IP$_3$ receptor-induced Ca$^{2+}$ release is involved in CCh-induced Ca$^{2+}$ response. However, we cannot exclude the involvement of SOCs in CCh responses. Indeed, IP$_3$ receptor activation could trigger SOCs opening, as has already been shown in numerous types of cells including skeletal muscle cells (24, 26, 54). Moreover it has been shown that SOCs, which belong to the family of the transient receptor potential channels (TRPC) (55), could be involved in the increased Ca$^{2+}$ influx in dystrophic fibers (56).

In conclusion, our results indicate that IP$_3$ receptors are involved in near-plasma membrane Ca$^{2+}$ increases triggered by the activation of nicotinic receptors in dystrophic myotubes. The involvement of IP$_3$ receptors represents a new and unexpected pathway for the excitation of dystrophic skeletal muscle cells. Indeed, our control myotubes do not display any IP$_3$
inhibitor sensitivity. It has been shown in muscle cells that the main pathway for Ca\(^{2+}\) release from the sarcoplasmic reticulum and muscle contraction is the ryanodine receptor (19). Moreover, increases in IP\(_3\) receptor number and a 2- to 3-fold increase in IP\(_3\) levels have been reported in mdx myotubes (21).

Altogether, these results suggest that IP\(_3\) receptors may be involved in altered Ca\(^{2+}\) homeostasis and the subsequent muscle degeneration that occurs in dystrophic muscle cells.

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Involvement of Inositol 1,4,5-Trisphosphate in Nicotinic Calcium Responses in Dystrophic Myotubes Assessed by Near-plasma Membrane Calcium Measurement

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